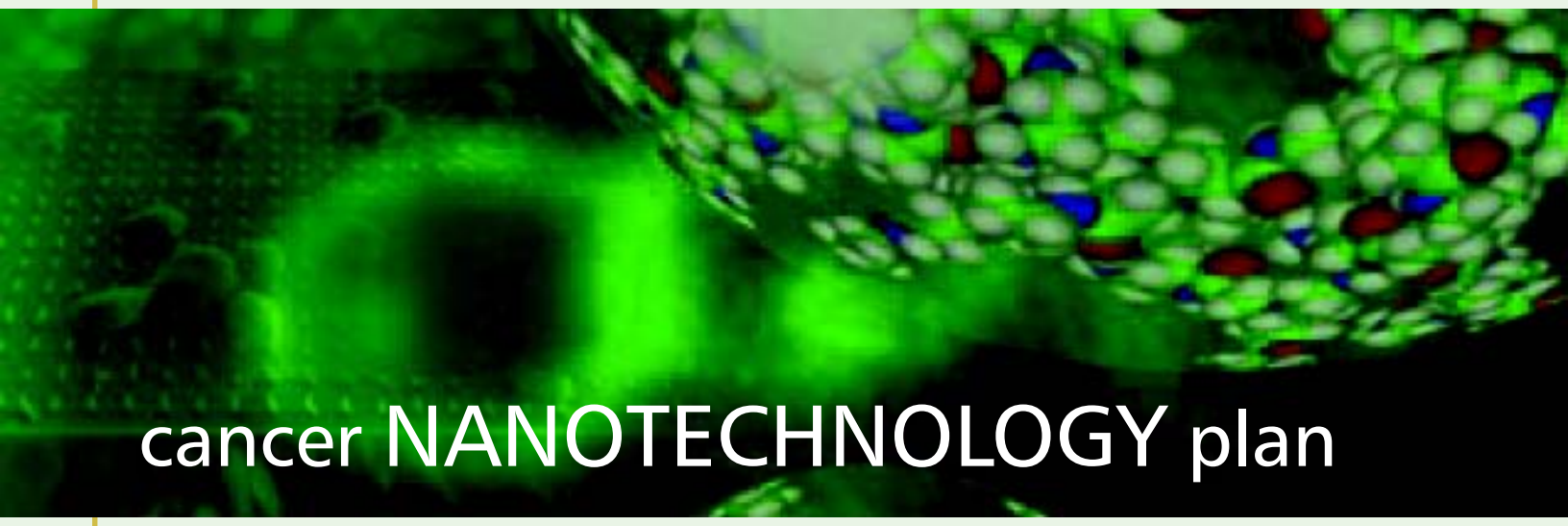

cancer NANOTECHNOLOGY plan

A Strategic Initiative To Transform Clinical Oncology and Basic Research Through the Directed Application of Nanotechnology

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U.S. DEPARTMENT OF
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National Cancer Institute

Contents

Message From the Director

To help meet the Challenge Goal of eliminating suffering and death from cancer by 2015, the National Cancer Institute (NCI) is engaged in a concerted effort to harness the power of nanotechnology[1] to radically change the way we diagnose, treat, and prevent cancer. Over the past 5 years, the NCI has taken the lead in ***integrating nanotechnology*** into biomedical research through a variety of programs. The results of these initial funding efforts have demonstrated clearly that melding nanotechnology and cancer research and development efforts will have a profound, disruptive effect on how we diagnose, treat, and prevent cancer.

The application of nanotechnology to cancer research could not come at a more opportune time given the recent exponential increase in our understanding of the ***process of how cancer develops***. It is my belief that nanomaterials and nanodevices will play a critical and unique role in turning that knowledge into clinically useful advances that detect and interact with the cancer cell and its surroundings early in this process. By doing so, we will change for the better the way we diagnose, treat, and ultimately prevent cancer.

Thanks to the scientific expertise and translational development capacity concentrated in our Comprehensive Cancer Centers, SPOREs (Specialized Programs of Research Excellence), research networks, and intramural program, the NCI is well positioned to seize this important opportunity. In particular, I believe it is possible that a concerted, ***multidisciplinary*** research effort will quickly yield new technologies that will detect and pinpoint the molecular signatures of cancer at its earliest stages and enable physicians to determine early whether an anticancer therapeutic is working. These advances will change the way we care for cancer patients. Such technological advances will have an even greater impact because of their ability to change the way new cancer therapies will be tested and approved, increasing the speed with which new science is turned into new therapies.

Future developments from nanotechnology also include multifunctional nanoscale devices capable of simultaneously detecting and treating cancer. Also in the offing are novel methods for preventing cancer and ameliorating the symptoms that negatively impact a patient's quality of life. Nanotechnology will also create a host of powerful tools that cancer researchers will use to make the next generation of discoveries that will ultimately lead to clinical advances.

To ensure that we capitalize on this opportunity to make dramatic progress today, the NCI has developed this Cancer Nanotechnology Plan (CNPlan). Over the past year, the NCI has held numerous symposia exploring the intersections of nanotechnology and cancer research, and the NCI staff has solicited input from a broad cross-section of the cancer research and clinical oncology communities. Intramural and extramural research working groups have discussed how best to apply the lessons of the NCI's initial explorations into nanotechnology to a focused and coordinated translational research effort that will have near-term benefits for patients.

Created with input from these experts, the CNPlan lays out a pathway and a set of directed mechanisms through which nanotechnology will be the fundamental driver of advances in oncology and cancer research conducted by multidisciplinary teams. The CNPlan will rely heavily on our substantial investments in our Comprehensive Cancer Centers and SPOREs, but it also calls for the development of as many as five Centers of Cancer Nanotechnology Excellence (CCNEs) that will contribute their expertise in nanotechnology to milestone-driven projects. To avoid duplicating efforts conducted through other Federal programs, including the National Nanotechnology Initiative and the NIH Roadmap for Medical Research, the projects initiated

[1]Nanotechnology refers to the interactions of cellular and molecular components and engineered materials—typically clusters of atoms, molecules, and molecular fragments—at the most elemental level of biology. Such nanoscale objects—typically, though not exclusively, with dimensions smaller than 100 nanometers—can be useful by themselves or as part of larger devices containing multiple nanoscale objects.

under the CNPlan will be integrated, milestone driven, and product oriented, with ***targeted objectives and goals***, and will use a project-management approach to capitalize in relatively short order on today's opportunities to create the tools that both clinicians and cancer researchers need now to eliminate suffering and death from cancer by 2015. Recognizing the importance of bringing expertise from many areas, ***partnership*** opportunities with other Federal agencies and the private sector will be critical, particularly in terms of clinical development activities and in our efforts to ensure that nanoscale devices will not themselves be harmful to cancer patients or the environment.

Ultimately, this is not just a plan for the NCI, but a call to action for the cancer research community. It emphasizes the process of building partnerships between the private and public sectors with the goal of creating teams best equipped to translate today's knowledge about cancer biology and nanotechnology into clinically useful products. By joining together, I am confident that we will continue to make substantial scientific and medical progress to achieve the one goal that matters most: the reduction and elimination of the burden of cancer for all who are in need.

Andrew C. von Eschenbach, M.D.
Director
National Cancer Institute

Nanotechnology offers the unprecedented and paradigm-changing opportunity to study and interact with normal and cancer cells in real time, at the molecular and cellular scales, and during the earliest stages of the cancer process. Through the concerted development of nanoscale devices or devices with nanoscale components spearheaded by the NCI, the Comprehensive Cancer Centers, and the SPOREs, and in collaboration with other Federal agencies, nanotechnology will be the enabling technology for:

- Early imaging agents and diagnostics that will allow clinicians to detect cancer in its earliest, most easily treatable, presymptomatic stage
- Systems that will provide real-time assessments of therapeutic and surgical efficacy for accelerating clinical translation
- Multifunctional, targeted devices capable of bypassing biological barriers to deliver multiple therapeutic agents at high local concentrations, with physiologically appropriate timing, directly to cancer cells and those tissues in the microenvironment that play a critical role in the growth and metastasis of cancer
- Agents capable of monitoring predictive molecular changes and preventing precancerous cells from becoming malignant
- Surveillance systems that will detect mutations that may trigger the cancer process and genetic markers that indicate a predisposition for cancer
- Novel methods for managing the symptoms of cancer that adversely impact quality of life
- Research tools that will enable investigators to quickly identify new targets for clinical development and predict drug resistance

In taking a leadership role, the NCI recognizes that these translational initiatives would benefit greatly from a concerted and coordinated effort to characterize and standardize the wide range of nanoscale devices that are now available for use by the research community and that will undoubtedly be developed in the near future. This role will be filled by the Nanotechnology Characterization Laboratory (NCL), which the NCI will establish at its NCI-Frederick facility. A primary objective of the NCL is to develop data on how nanomaterials and nanodevices interact with biological systems. These research endeavors will chart the common baseline and scientific data that would inform research and development (R&D) as well as future regulatory actions involving nanoscale diagnostics, imaging agents, and therapeutics. Moreover, this information will be linked to the Comprehensive Cancer Centers and related programs through public databases available through the Cancer Biomedical Informatics Grid (CaBIG).

Achieving this vision will also require training a cadre of researchers who are skilled in applying the tools of nanotechnology to critical problems in cancer research and clinical oncology. And given the complex nature of this endeavor, building multidisciplinary teams will be essential to realizing this vision.[2] Thus, the NCI must take a leadership role by providing the necessary funds and opportunities for the cross-disciplinary training and collaboration that will be needed to maximize the impact that nanotechnology can have on meeting the Challenge Goal of eliminating the suffering and death from cancer by 2015.

The CNPlan lays out the pathway and directed programmatic mechanisms through which nanotechnology will become a fundamental driver of advances in oncology and cancer research. The CNPlan reflects a consensus among the entire cancer community that four significant obstacles impede the revolutionary changes that must occur to meet the 2015 Challenge Goal[3]:

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[2]National Institutes of Health. *Catalyzing Team Science: Report from the 2003 BECON Symposium*. http://www.becon2.nih.gov/symposia_2003/becon2003_symposium_final.pdf.

[3]National Cancer Institute. *Leveraging Multi-Sector Technology Development Resources and Capabilities to Accelerate Progress Against Cancer: A National Cancer Institute Roundtable*. 2004.

- The need for cross-disciplinary collaborations
- The widening "gap" between late discovery and early development of diagnostics and therapeutics
- The critical lack of available standards
- The requirement for cross-cutting technology platforms

By taking the pathway and utilizing the mechanisms detailed in the CNPlan, which rely heavily on capacity already developed by the NCI through its national infrastructure, the CNPlan will lower the barriers for developing technology that will become integrated in clinical, basic, and applied research. Nanotechnology will thereby become a core component in the training and translational programs at all leading cancer research institutions and a significant part of comprehensive cancer care. Thus, the focus will be achieving product-driven goals with demanding timelines, realizing that such an approach is necessary to meet the 2015 Challenge Goal.

On the basis of discussions with a wide range of clinicians, cancer researchers, and technologists, it is clear that nanotechnology is ready today to solve mission-critical problems in cancer research. Indeed, one of the goals of the CNPlan is to increase the visibility and availability of nanomaterials and nanoscale devices technology within the cancer research and development community to allow investigators the opportunity to do what they do best—discover and invent using new tools, just as they are doing with other disruptive technologies such as DNA microarrays and proteomic analysis.

But the NCI's major goal for the CNPlan is to catalyze targeted discovery and development efforts that offer the greatest opportunity for advances in the near and medium terms and to lower the barriers for those advances to be handed off to the private sector for commercial development. The CNPlan focuses on translational research and development work in the following six major challenge areas, where nanotechnology can have the biggest and fastest impact.

Molecular Imaging and Early Detection

Nanotechnology can have an early, paradigm-changing impact on how clinicians will detect cancer in its earliest stages. Exquisitely sensitive devices constructed of nanoscale components—such as nanocantilevers, nanowires, and nanochannels—offer the potential for detecting even the rarest molecular signals associated with malignancy. Collecting those signals for analysis could fall to nanoscale harvesters, already under development, that selectively isolate cancer-related molecules such as proteins and peptides present in minute amounts from the bloodstream or lymphatic system. Investigators have already demonstrated the feasibility of this approach using the serum protein albumin (a naturally existing nanoparticle), which happens to collect proteins that can signal the presence of malignant ovarian tissue.

Another area with near-term potential is detecting mutations and genome instability *in situ*. Already, investigators have developed novel nanoscale *in vitro* techniques that can analyze genomic variations across different tumor types and distinguish normal from malignant cells. Nanopores are finding use as real-time DNA sequencers, and nanotubes are showing promise in detecting mutations using a scanning electron microscope. Further work could result in a nanoscale system capable of differentiating among different types of tumors accurately and quickly, information that would be invaluable to clinicians and researchers alike. Along similar lines, other investigators have developed nanoscale technologies capable of determining protein expression patterns directly from tissue using mass spectroscopy. This technique has already shown that it can identify different types of cancer and provide data that correlate with clinical prognosis.

In addition, nanoscale devices can enable new approaches for real-time monitoring of exposures to environmental and lifestyle cancer risk factors. Such information would be important not only for identifying individuals who may be at risk for developing cancer, but also for opening the door to complex studies of gene-environment interactions as they relate to the development of or resistance to cancer.

In Vivo Imaging

One of the most pressing needs in clinical oncology is for imaging agents that can identify tumors that are far smaller than those detectable with today's technology, at a scale of 100,000 cells rather than 1,000,000,000 cells. Achieving this level of sensitivity requires better targeting of imaging agents and generation of a bigger imaging signal, both of which nanoscale devices are capable of accomplishing. When attached to a dendrimer, for example, the magnetic resonance imaging (MRI) contrast agent gadolinium generates a 50-fold stronger signal than in its usual form, and given that nanoscale particles can host multiple gadolinium ions, affords an opportunity to create a powerful contrast agent. When linked to one of the increasing number of targeting agents, such a construct would have the potential of meeting the 100,000 cell detection level.

First-generation nanoscale imaging contrast agents are already pointing the way to new methods for spotting tumors and metastatic lesions much earlier in their development, before they are even visible to the eye. In the future, implantable nanoscale biomolecular sensors may enable clinicians to more carefully monitor the disease-free status of patients who have undergone treatment or individuals susceptible to cancer because of various risk factors.

Imaging agents should also be targeted to changes that occur in the environment surrounding a tumor, such as angiogenesis, that are now beyond our capability to detect in the human body. Already, various nanoparticles are being targeted to integrins expressed by growing capillaries. Given that angiogenesis occurs in distinct stages and that antiangiogenic therapies will need to be specific for a given angiogenic state, angiogenesis imaging agents that can distinguish among these stages will be invaluable for obtaining optimal benefit from therapeutics that target angiogenesis.

Reporters of Efficacy

Today, clinicians and patients must often wait months for signs that a given therapy is working. In many instances, this delay means that should the initial therapy fail, subsequent treatments may have a reduced chance of success. This lag also adversely impacts how new therapies undergo clinical testing, since it leaves regulatory agencies reluctant to allow new cancer therapies to be tested on anyone but those patients who have exhausted all other therapeutic possibilities. Unfortunately, this set of patients is far less likely to respond to any therapy, particularly to those molecularly targeted therapies that aim to stop cancer early in its progression, an approach that virtually all of our knowledge says is the best approach for treating cancer.

Nanotechnology offers the potential for developing highly sensitive imaging agents and *ex vivo* diagnostics that can determine whether a therapeutic agent is reaching its intended target and whether that agent is killing malignant or support cells, such as growing blood vessels. Targeted nanoscale devices may also enable surgeons to more readily detect the margins of a tumor before resection or to detect micrometastases in lymph nodes or tissues distant from the primary tumor, information that would inform therapeutic decisions and have a positive impact on patient quality-of-life issues.

The greatest potential for immediate results in this area would focus on detecting apoptosis following cancer therapy. Such systems could be constructed using nanoparticles containing an imaging contrast agent and a targeting molecule that recognizes a biochemical signal seen only when cells undergo apoptosis. Using the molecule annexin V as the targeting ligand attached to nanoscale iron oxide particles, which act as a powerful MRI contrast agent, investigators have shown that they can detect apoptosis in isolated cells and in tumor-bearing mice undergoing successful chemotherapy. Further development of this type of system could provide clinicians with a way of determining therapeutic efficacy in a matter of days after treatment. Other systems could be designed to detect when the p53 system is reactivated or when a therapeutic agent turns on or off the biochemical system that it targets in a cancer cell, such as angiogenesis.

Another approach may be to use targeted nanoparticles that would bind avidly, or perhaps even irreversibly, to a tumor and then be released back into the bloodstream as cells in the tumor under apoptosis following therapy. If labeled with a fluorescent probe, these particles could be easily detected in a patient's urine. If also labeled with an imaging contrast agent, such a construct could double as a diagnostic imaging probe.

Multifunctional Therapeutics

Because of their multifunctional capabilities, nanoscale devices can contain both targeting agents and therapeutic payloads at levels that can produce high local levels of a given anticancer drug, particularly in areas of the body that are difficult to access because of a variety of biological barriers, including those developed by tumors. Multifunctional nanoscale devices also offer the opportunity to utilize new approaches to therapy, such as localized heating or reactive oxygen generation, and to combine a diagnostic or imaging agent with a therapeutic and even a reporter of therapeutic efficacy in the same package. "Smart"

nanotherapeutics may provide clinicians with the ability to time the release of an anticancer drug or deliver multiple drugs sequentially in a timed manner or at several locations in the body. Smart nanotherapeutics may also usher in an era of sustained therapy for those cancers that must be treated chronically or to control the quality-of-life symptoms resulting from cancers that cannot be treated successfully. Smart nanotherapeutics could also be used to house engineered cellular "factories" that would make and secrete multiple proteins and other antigrowth factors that would impact both a tumor and its immediate environment.

The list of potential multifunctional nanoscale therapeutics grows with each new targeting ligand discovered through the use of tools such as proteomics. Nanoscale devices containing a given therapeutic agent would be "decorated" with a targeting agent, be it a monoclonal antibody or F_v fragment to a tumor surface molecule, a ligand for a tumor-associated receptor, or other tumor-specific marker. In most cases, such nanotherapeutics could double as imaging agents.

Many nanoparticles will respond to an externally applied field, be it magnetic, focused heat, or light, in ways that might make them ideal therapeutics or therapeutic delivery vehicles. For example, nanoparticulate hydrogels can be targeted to sites of angiogenesis, and, once they have bound to vessels undergoing angiogenesis, it should be possible to apply localized heat to "melt" the hydrogel and release an antiangiogenic drug. Similarly, iron oxide nanoparticles, which can serve as the foundation for targeted MRI contrast agents, can be heated to temperatures lethal to a cancer cell merely by increasing the magnetic field at the very location where these nanoparticles are bound to tumor cells.

In some instances, nanoscale particles will target certain tissue strictly because of their size. Nanoscale dendrimers and iron oxide particles of a specific size will target lymph nodes without any molecular targeting. Nanoscale particles can also be designed to be taken up by cells of the reticuloendothelial system, which raises the possibility of delivering potent chemotherapeutics to the liver, for example.

Nanoscale devices should also find use in creating immunoprotected cellular factories capable of synthesizing and secreting multiple therapeutic compounds. Early-stage research has already demonstrated the value of such cellular factories, and a concerted effort could turn this research into a powerful multivalent therapeutic capable of responding to local conditions in a physiologically relevant manner.

Prevention and Control

Many of the advances that nanotechnology will enable in each of the four preceding challenge areas will also find widespread applicability in efforts to prevent and control cancer. Advances driven by the NCI's initiatives in proteomics and bioinformatics will enable researchers to identify markers of cancer susceptibility and precancerous lesions, and nanotechnology will then be used to develop devices capable of signaling when those markers appear in the body and deliver agents that would reverse premalignant changes or kill those cells that have the potential for becoming malignant. Nanoscale devices may also prove valuable for delivering polyepitope cancer vaccines that would engage the body's immune system or for delivering cancer-preventing nutraceuticals or other chemopreventive agents in a sustained, time-release and targeted manner.

One intriguing idea for preventing breast cancer comes from work suggesting that breast malignancies may derive from a limited population of pluripotent stem cells in breast tissue. Should this prove true, it may be possible to develop a nanoscale device that could be injected into the ductal system of the breast, bind only to those stem cells, and deliver an agent capable of killing those cells. Such an agent could then be administered to women who are at an increased risk of breast cancer as a preventive therapy.

Research Enablers

Nanotechnology offers a wide range of tools, from chip-based nanolabs capable of monitoring and manipulating individual cells to nanoscale probes that can track the movements of cells, and even individual

molecules, as they move about in their environment. Using such tools will enable cancer biologists to study, monitor, and alter the multiple systems that go awry in the cancer process and identify key biochemical and genetic "choke points" at which the coming wave of molecular therapies might best be directed. As such, nanotechnology can serve as the perfect complement to other technology platforms, such as proteomics and bioinformatics, that the NCI is emphasizing in its research initiatives as critical components of the discovery and development engine that will power both near-term and long-term advances in cancer diagnosis, treatment, and prevention.

The discussion above has already highlighted the potential for nanoscale devices to act as molecular harvesting agents. Such a tool would be invaluable to proteomics efforts aimed at identifying tumor-specific indicators. Similarly, nanoscale devices that can detect the biological changes associated with therapeutic efficacy should also find widespread use as a tool for understanding how cells respond to a variety of perturbations. One of the most powerful near-term uses of nanotechnology to accelerate basic research will come from using molecular-size nanoparticles with a wide range of optical properties, such as quantum dots, to track individual molecules as they move through a cell or individual cells as they move through the body. In combination with the new generation of mouse models that more accurately reproduce the genetic, biochemical, and physiological properties of human cancers, these nanolabels will prove invaluable for systems-scale research. Increased focus on the development of nanoscale devices for making simultaneous biochemical measurements on multiple cells, particularly those grown in such a way as to mimic tissue development *in vivo*, will also have a significant impact on basic cancer research.

Nanoscale devices should also enable direct analysis of single nucleotide polymorphisms (SNPs) and large-scale mutational screening for cancer susceptibility genes. Real-time methylation analysis should also benefit from various nanoscale tools and devices. Indeed, nanotechnology should prove to be a valuable technology platform for the burgeoning field of cancer molecular epidemiology.

Funding activities conducted within the framework of the CNPlan will occur in four areas as detailed below. The first will be to develop three to five CCNEs that will provide engineering and physical science expertise to leverage the cancer biology expertise and access to cancer patients at the Nation's Comprehensive Cancer Centers, SPOREs, and large population infrastructures, such as the Breast and Colon Cancer Family Registries. Second, the CNPlan will fund cross-disciplinary training programs as a means of fostering the creation of the multidisciplinary teams needed to integrate nanotechnology and cancer biology. Third, the CNPlan will fund focused nanotechnology development initiatives that will be milestone driven and product oriented, with an emphasis on commercialization through small-business and larger private-sector project team members. Fourth, the CNPlan will fund projects that apply nanotechnology in cancer biology and translational research, through basic research project grants and other mechanisms. Since the R01 mechanism has historically not been the best mechanism to fund individual investigator-initiated technology development and application projects, the NCI will also make use of program announcements, requests for applications, and request for proposals, as well as a variety of program management and funding mechanisms that have been shown to be successful in prior technology development programs. The NCI will also examine opportunities through the Small Business Innovation Research/Small Business Technology Transfer Research (SBIR/STTR) programs as well as administrative supplements to existing awards to accelerate the integration of nanotechnology into the NCI research program.

In addition to the largely extramural focus of the CNPlan, a variety of demonstration projects in the NCI intramural program will add to this overall effort by acting as developmental catalysts. For example, the NCI has contracted with a nanotechnology foundry to fabricate materials and provide engineering expertise to aid *in vivo* projects using nanoscale devices. The NCI's intramural expertise, when used in this type of synergistic manner, will accelerate the development of new nanotechnology-driven advances in oncology.

Helping guide these programmatic activities will be the Cancer Nanotechnology Working Group (CNWG), which was recently formed from the Cancer Nanotechnology Intramural Working Group and the Cancer Nanotechnology Extramural Intramural Working Group. The CNWG will have a tracking function and will continue (as the two subgroups have for the past year) to act in an advisory capacity as the CNPlan moves forward. The CNWG is playing a key role in planning an NCI-sponsored intramural nanotechnology seminar series scheduled for fall 2004 and coordinating symposia held at regional cancer and advanced technology centers.

The CNPlan will also include development of program evaluation tools related to the programmatic milestones proposed in this plan as well as mechanisms for conducting annual evaluations. The evaluation processes will involve independent, outside review teams and will assess how program activities conducted as part of the CNPlan meet the goals and milestones set forth in this plan. Feedback from these evaluations will facilitate appropriate milestone adjustment course corrections in the implementation of the plan.

Centers of Cancer Nanotechnology Excellence (CCNEs)

The primary goal of the CCNEs is to integrate nanotechnology development into basic and applied cancer research that is necessary to rapidly facilitate the application of this science to clinical research. The critical requirements for each CCNE will be:

- Integration with a Comprehensive Cancer Center/SPORE program
- Affiliation with university or research centers of engineering and physical sciences (e.g., mathematics, chemistry, physics, and material sciences)
- Advanced biocomputing capabilities
- Required existing not-for-profit/private technology development partnerships

Outcomes objectives (performance measures) represent technologies that are developed and effectively utilized to overcome cancer processes. A steering committee will coordinate efforts across all the CCNEs, to facilitate data and technology transfer across centers, interconnecting and leveraging the strengths and advances of each.

Nanotechnology Characterization Laboratory (NCL)

Nanoscale particles and devices are similar in size to biomolecules and can easily enter most cells. Our ability to manipulate the physical, chemical, and biological properties of these particles affords researchers the ability to engineer and use nanoparticles for drug delivery, as image contrast agents, and for diagnostic purposes. NCI is establishing the Nanotechnology Characterization Laboratory (NCL) at its NCI-Frederick facility to provide critical infrastructure support to this rapidly developing field. The intent of the NCL is to accelerate the transition of basic nano-biotech research into clinical applications. (See page 23 for more information on the NCL.)

Building Research Teams

The NCI will create the incentives necessary to integrate nanotechnology into the mainstream of basic and applied cancer research. The CNPlan's approach is centered on supporting training and career development initiatives to establish integrated teams of cancer researchers, including epidemiologists, and engineers with the cancer biology and physical science skills and knowledge base of nanotechnology to approach the fundamental challenges of cancer. One policy consideration is to investigate opportunities for naming multiple principal investigators per project as an incentive for conducting team science.

Under the CNPlan, the NCI will initially use existing training and career development mechanisms to direct talent to this area as quickly as possible. The NCI recognizes, however, that new mechanisms for developing multidisciplinary teams may be needed. The NCI will also encourage programs to be developed with interfaces to the training programs of other Federal agencies as components of the National Nanotechnology Initiative (NNI). The advantages are to rapidly translate knowledge from fundamental nanotechnology sciences to directed application in cancer biology.

Other possible mechanisms for fostering team-building include the Bioengineering Research Partnerships (BRPs) and Bioengineering Research Grants (BRGs). The BRPs are designed to fund basic, applied, and translational multidisciplinary research that addresses important biological or medical research problems. In the context of this program, a partnership is a multidisciplinary research team that applies an integrative, systems approach to developing knowledge and/or methods to prevent, detect, diagnose, or treat disease or to understand health and behavior. The partnership must include appropriate bioengineering or allied quantitative sciences in combination with biomedical and/or clinical components. The smaller BRG awards support multidisciplinary research performed in a single laboratory or by a small number of investigators that applies an integrative, systems approach to developing knowledge and/or methods to prevent, detect, diagnose, or treat disease or to understand health and behavior. A BRG application may propose hypothesis-driven, discovery-driven, developmental, or design-directed research at universities, national laboratories, medical schools, large or small businesses, or other public and private entities.

Outcome objectives (performance measures) represent institutions with training programs and scientists and engineers who are trained in cancer nanotechnology. A 3- to 5-year benchmark is to support the entry of 30 scientists with formal training experiences in nanotechnology applied to cancer biology. Recommended mechanisms include the following:

- **F33 NIH National Research Service Awards for Senior Fellows.** This approach would enable experienced cancer researchers and engineers/physical scientists with directed programs of training to be independent researchers and to provide the future building of training programs.

- **F32 NIH National Research Service Awards for Individual Postdoctoral Fellows.** This approach would provide cross-disciplinary research training opportunities for postdoctoral fellows with training in either cancer or technology to gain experience in the other discipline.
- **K08 and K25 Mentored Clinical Scientist Development Awards.** This approach begins to develop research teams with clinical applications of nanotechnology to allow integration of nanotechnology into the clinical assessment phase. At present, there are no programs that support technology development and applications training for clinical researchers. This gap will be an important one to facilitate the clinical testing of nanotechnologies. In these programs, clinical researchers will be offered opportunities in developing clinical assessment paradigms for diagnosis, treatment, and prevention using nanotechnologies.
- **T32 Institutional Training Grant Program.** This approach enables eligible institutions to develop or enhance research training opportunities for predoctoral or postdoctoral trainees, who are training for careers in specified areas of biomedical and clinical research.
- **R25 Cancer Education Grant Program.** This mechanism will be used to develop critical educational programs for cancer biologists, engineers/physical scientists, and trainees. The focus will be on developing programmatic activities at CCNEs to develop curricula, educational programs/seminars, and national forums focused on cancer nanotechnology.

Planning for future training and career development needs will be developed on the basis of the initial success of the above strategies and the assessment of program needs. The NCI recognizes, for example, that there will likely be a need to foster curriculum development for undergraduate and graduate programs that would cross-fertilize training in the biological sciences with engineering, chemistry, and other physical sciences and vice versa.

Creating Cancer Nanotechnology Platforms Through Directed Research Programs

Using Broad Agency Announcements (BAAs), NCI will identify to the R&D community three to five critical technology platform needs for cancer, such as *in vivo* nanotechnology imaging systems and nanotechnology-enabled systems for rapidly assessing therapeutic efficacy and addressing cancer biology processes. The program will fund 3-year technology projects through a contract mechanism that is overseen by project specialists. The project will target cancer centers, small businesses, and Federal laboratories that prepare and submit concepts and project objectives. Upon review of initial submissions, full solicitations will be sought from those of highest value. Technology programs will create platforms that are aimed at deployment for clinical application in cancer research. Applicants will be required to team with the Comprehensive Cancer Centers or SPOREs with a plan for dissemination of the technology.

Basic and Applied Initiatives for Nanotechnology in Cancer

Requests for Application (RFAs) and Program Announcements (PAs) will be issued to solicit applications for projects that apply nanotechnology for specific opportunities in cancer biology and translational research. These may focus on investigator-initiated proposals that address specific biology processes, diagnostic technologies, or drug development methods. Research projects that address the fundamental biology questions identified in the CNPlan will be considered.

Mechanisms for funding would consider R21/R33 approaches for phased innovation with programmatic review of attainment of project milestones. The small-business community would be targeted for use of R41 and R43 mechanisms in this area.

Timeline and Programmatic Milestones

A defining element of the CNPlan is that it calls for the NCI to mark progress in six key areas (see Key Opportunities for Cancer Nanotechnology) over two time periods. During the initial 1 to 3 years, the CNPlan will accelerate selected projects that are already under way and catalyze the development of products that are primed for near-term clinical application. The second period, 3 to 5 years, will see projects come to fruition that reflect solving more difficult technological and biological problems or that require the integration of multiple technological components but have the potential for making paradigm-changing impacts on the detection, treatment, and prevention of cancer. Milestones reached during this latter period will also reflect the growth of the investigator pool that will be catalyzed by the CNPlan. By the end of 5 years, we expect that most of these efforts will generate products in clinical trials or even in clinical use.

The CNPlan represents an integrated program of activities to use a disruptive technology—nanotechnology—as an enabler of rapid clinical and research advances and as a means of lowering the barriers to technology development and commercialization by the private sector, particularly among small businesses. Over the next 5 years, a timeframe merited by the urgency of meeting the NCI's 2015 Challenge Goal and supported by the solid foundation of promising advances from the NCI's basic research portfolio, the CNPlan calls for the use of targeted contract funding with project management oversight to meet the following milestones:

Key Opportunity	1-3 Years	3-5 Years
Molecular Imaging and Early Detection	• Begin clinical trials of nanotechnology-assisted automated assay for rapid detection of genetic abnormalities. • Refine *in vitro* nanotechnology systems (cantilevers, nanowires, nanochannels) for rapid, sensitive analysis of cancer biomarkers. Such systems will be easily expanded as new markers are identified.	• Disseminate nanoscale devices for routine validation of cancer biomarkers. • Develop rapid multifactorial genomic and proteomic diagnostic system for tumor identification and staging. • Begin clinical trials with multicomponent nanotechnology platform early diagnosis and therapeutic monitoring.
***In Vivo* Imaging**	• File Investigational New Drug (IND) application to begin clinical trials of nanoscale MRI contrast agents capable of identifying fewer than 100,000 actively aggressive cancer cells. • Conduct clinical trials for three targeted nanoscale imaging agents using a variety of imaging modalities, including MRI, ultrasound, and near-infrared optical imaging.	• Complete clinical trials and file New Drug Application (NDA) for first nanoscale imaging agent capable of detecting <100,000 actively aggressive tumor cells. • Begin clinical trials with multiple nanoscale imaging agents. • Develop capabilities for monitoring active cellular processes as they change over time.

Key Opportunity	1-3 Years	3-5 Years
Reporters of Efficacy	• Begin clinical trials for nanoscale device (imaging-based or *ex vivo*) that can rapidly assess apoptosis in clinical trials. • Develop capabilities for monitoring disruption of vascular networks associated with primary solid tumors and metastatic lesions. • Develop nanoscale devices to identify and quantify biological and chemical changes (other than apoptosis) resulting from therapeutic treatment. • Demonstrate proof of concept for nanoscale devices (imaging-based or *ex vivo*) that can be used with a variety of therapeutics to determine biodistribution *in vivo*. • Begin clinical trials with one optical imaging device capable of showing surgical margins using nanoscale agents.	• Demonstrate multiple systems (imaging-based or *ex vivo*) that can rapidly assess therapeutic efficacy in terms of apoptosis, angiogenesis regression, and other markers. • Demonstrate multiple systems for monitoring real-time drug distribution. • Promote routine use of nanoscale efficacy reporters for surrogate end point measurements in clinical trials.
Multifunctional Therapeutics	• File IND to begin clinical trials of one targeted sensitizer (radiation, light, magnetic field). • File IND to begin clinical trials of one multifunctional therapeutic complete with accompanying therapeutic assessment tool. • Develop nanoscale devices capable of multivariate targeting and intervention. • File IND application to begin clinical trials of one nanoscale therapeutic targeting reticuloendothelial system.	• Conduct multiple clinical trials with targeted sensitizers (radiation, light, magnetic field). • File INDs to begin clinical trials of multiple targeted therapeutics, complete with accompanying therapeutic assessment tool. • File IND to begin clinical trials of one multifactorial targeted therapeutic agent at IND stage. • Demonstrate five "failed" drugs reconstituted in targeted, "smart" nanoscale devices for retesting in new generation of preclinical models.
Prevention and Control	• Demonstrate proof of concept for nanoscale device capable of monitoring genetic changes associated with early cancer processes and hyperplasia with the aim of preventing subsequent development of cancer.	• File IND to begin clinical trials of a nanoscale device capable of identifying markers of early cancer processes. • Demonstrate proof of concept for nanoscale device capable of metastasis detection.

Key Opportunity	1-3 Years	3-5 Years
Research Enablers	• Develop nanoscale harvesting devices for proteomics analysis and biomarker identification. • Create prototype for real-time, *in situ* genome sequencing of malignant and pre-malignant cells. • Develop instrumented cell coculture systems biology research. • Refine cell and cell-component labeling with nanoparticulates such as quantum dots for application to studies of integrated pathways and processes in cancer. • Develop toxicology database for nanoscale devices and nanoparticulates. • Create a scientific framework for regulatory approval of nanoscale diagnostics, therapies, and preventive agents.	• Develop nanoscale analytical devices to study DNA methylation and protein phosphorylation. • Promote routine use of nanoscale technology to characterize tumor heterogeneity. • Demonstrate nanoscale technology for detecting multiple mutations i*n vivo.* • Promote routine use of nanoscale analytical tools for studying cellular signaling pathways.

To rapidly harness the potential of nanotechnology to meet our 2015 Challenge Goal of eliminating suffering and death from cancer, the NCI has crafted the CNPlan. Over the past year, the NCI has held several workshops and symposia exploring the intersections of nanotechnology and various areas of cancer research, and the NCI staff has solicited input from a broad cross-section of the cancer research and clinical oncology communities. Intramural and extramural research working groups have discussed how best to apply the lessons of the NCI's initial forays into nanotechnology to a concerted translational research effort that will have near-term benefits for patients. During this time, the NCI also convened a roundtable of leaders from the private sector, foundations, patient advocacy groups, the Comprehensive Cancer Centers, academia, and other government agencies to identify new ways of leveraging technology to aid in our battle against cancer.[3]

During the course of these fact-finding discussions, it became clear that nanotechnology offers tremendous opportunities, the most promising of which are presented in this report and represent the major focus of the CNPlan. However, these discussions also increased the NCI's awareness that there are a number of nonscientific barriers that could impede the rapid translation of cancer nanotechnology research into clinically useful, paradigm-changing advances in diagnosing, treating, and preventing cancer. Though numerous in detail, these potential barriers followed several themes:

- **Cross-Disciplinary Collaborations.** For cancer nanotechnology to have its biggest impact, barriers to multidisciplinary and multiple partner collaborations must fall. Though there are many institutional barriers to such research collaborations over which the NCI has no direct control, the NCI can use alternative funding mechanisms to encourage and facilitate such collaborations. In particular, the NCI can use these funding mechanisms to promote increased collaborations among the public, private, and nonprofit sectors that reduce overall development risk.
- **"Gap" Between Late Discovery and Early Development of Diagnostics and Therapeutics.** Too many potential products that reach clinical development fail as they move forward because of a lack of solid science to back up regulatory filings. Moreover, to conduct clinical trials, there is insufficient financial and intellectual support for smaller companies to move novel products through the testing and regulatory approval process and, ultimately, failure to match development goals with clinical and patient needs.
- **Regulatory Uncertainty.** There is no clear regulatory pathway for approval of nanoscale devices, increasing the risk for private-sector development of promising new diagnostics, therapies, and preventive agents. In particular, there is a concern that each new use of a given nanoscale device, such as a particular type of particle, will require full-scale preclinical and clinical testing, a requirement that would dramatically drive up development costs. There is also concern about the difficulty of gaining regulatory approval for nanoscale devices that combine diagnostic and therapeutic modalities or multiple therapeutic agents in the same construct.
- **Standardization and Characterization.** Because nanotechnology is such a new field, there are few standards and little reference physical and biological characterization data that researchers can use to choose which nanodevices might be most suitable for a given clinical or research application. A lack of standard assay and characterization methods also makes it difficult to compare results from different laboratories.
- ***In Vivo* Behavior.** There is good reason to expect that critical *in vivo* properties of nanoscale devices, such as pharmacokinetics, pharmacodynamics, and biodistribution, will differ markedly from that of current imaging and therapeutic agents; yet there is a marked lack of data on these base characteristics. There is also, however, little ongoing research that will generate these essential data.
- **Technology Transfer and Knowledge Exchange.** Cancer nanotechnology is inherently a discipline that will succeed because of its combinatorial nature—Any given nanoscale technology or device may be combined with any number of diagnostic, imaging, therapeutic, or preventive agents. As a result, there is a need for new mechanisms for sharing and cross-licensing intellectual property to facilitate technology

transfer and knowledge exchange. Though the NCI cannot by itself create such a system, it can work with other Federal agencies to act as a facilitator among the multiple interest groups by convening roundtable events for discussion and problem-solving.

Awareness of these overarching concerns had a great impact on the development of the CNPlan. A major role of the NCL, for example, will be to eliminate barriers resulting from the current lack of standards and characterization data. The CNPlan addresses potential barriers by making the U.S. Food and Drug Administration (FDA) an important partner in this endeavor. The CNPlan's emphasis on contract-based funding will place a premium on collaborations, particularly between the public and private sectors.

Although the NCI is a strong supporter of investigator-initiated, R01-supported research, the Institute also recognizes that this funding mechanism is not universally applicable to all its research initiatives. In particular, the NCI believes that to be effective, the CNPlan must utilize funding mechanisms that place a premium on meeting project goals on a timely basis, produce a desired deliverable at the end of the project's lifetime, and integrate with other planning initiatives within the NCI. Through its experience with existing technology development programs and with input from the research community and from other government agencies—specifically the Defense Advanced Research Projects Agency (DARPA) and the Homeland Security Advanced Research Projects Agency (HSARPA)—the NCI recognizes that project-management style contracts with specified goals, timelines, and deliverables must be the central funding mechanism used in conjunction with the CNPlan if this initiative is to achieve its admittedly aggressive vision and associated goals and milestones.

Utilizing contract-based, project-management style funding will require that NCI program officers work closely with potential contracting groups, with an emphasis on helping prospective participants put together the multidisciplinary teams that the NCI envisions will be needed to accomplish the aggressive goals of the CNPlan. Such teams, which will preferentially include private-sector partners and small-business participation, will form the core element of CNPlan-related contracts.

Coordination with other NCI initiatives will be monitored by both the program officers and the planning coordinator in the Office of the NCI Director. Though the NCI has been funding nanotechnology research for a number of years now, nanotechnology, as part of the new NIH Roadmap initiative, has emerged as an area of interest across the entire NIH. The current goals of the NIH nanomedicine initiative are much more basic and obviously less focused than those laid out in the CNPlan. The nanomedicine roadmap group has just released a solicitation that will lead by the end of 2005 to the funding of planning awards for nanomedicine center development. The goal of this initiative is to fund centers using and developing nanotechnology to examine biological processes compatible with the missions of the various NIH institutes. This supports a long-term goal of the NIH to support infrastructure development in nanomedicine. In contrast, the CNPlan is a focused plan to capitalize on past NCI investment in nanotechnology and focus those and new efforts on the immediate mission of the NCI. The plan carries a shorter timeline and specific milestones to achieve the NCI goals. The NCI plans on continued support and participation with the NIH nanomedicine as well as all of the roadmap working groups where appropriate.

Discussions with leaders in academia, at the NCI Comprehensive Cancer Centers and SPOREs, and in the private sector indicate that this type of managed, targeted, milestone-driven, team-based funding mechanism, though admittedly novel for most researchers in the public sector, will be embraced by those members of the cancer research community who want to see their work turned rapidly into advances that help cancer patients. Furthermore, the consensus among the entire cancer community is that this type of project-management structure is critically needed at this very moment in order to most efficiently and rapidly translate 21st century science and technology into the tools and products that will revolutionize the detection, treatment, and prevention of cancer.

Reflecting the recommendations of the NIH Bioengineering Consortium report on promoting team science, the CNPlan places a premium on supporting cross-disciplinary teams that partner with the Comprehensive Cancer Centers, SPOREs, CCNEs, large existing population infrastructures such as the Breast and Colon Cancer Family Registries, and the private sector. Such partnerships, operating in a project-management environment, present an opportunity to leverage existing skills in a way that enables such teams to meet the milestones and deliverables that will be called for under CNPlan contracts and grants. By placing a premium on building cross-disciplinary teams, the CNPlan will also bring in expertise, such as in population genetics and epidemiology, that is often overlooked in terms of potential contributions to research and development efforts.

In addition, the CNPlan will initially utilize existing F33, K08, and K25 training grant programs to incentivize cross-disciplinary research through training. F33 awards go to experienced scientists who wish to make major changes in the direction of their research careers or wish to broaden their scientific background by acquiring new research capabilities. These awards will enable current established cancer investigators to train in the labs of leading nanotechnologists to facilitate bringing the technology back to their own labs to be applied toward future research activities. Alternatively, nanotechnologists could be funded to spend a year gaining insight into cancer research so that these problems could be addressed when returning to the nanotechnologist's lab. In both cases, the spillover of ideas from the trainee to the mentor's lab will continue to cross-pollinate the cancer and nanotechnology fields. The K08 and K25 mechanisms provide for specialized postdoctoral study for individuals with a health professional doctoral degree committed to a career in laboratory or field-based research. These awards will bring clinicians into nanotechnology-focused laboratories as a means of providing clinical expertise to nanotechnology-driven development programs. After 3 years, the NCI will evaluate the success of these programs to increase cross-disciplinary activities and determine whether new programs are necessary. For now, however, these existing mechanisms will provide a needed boost to such efforts. (For additional recommendations on how training can be used to incentivize cross-disciplinary activities, see Appendix A.)

Today, thanks in part to the growing acceptance of the 2015 Challenge Goal by the cancer community, the NCI believes that the majority of cancer researchers now appreciate the need to pick the most promising areas of research and focus on conducting the translational work needed to turn promise into clinical benefit. Indeed, there is a realization within the broad cancer community that while R01-style research efforts are key to generating the stream of discoveries upon which the CNPlan will capitalize, the time is ripe to select the most promising projects for focused development. The CNPlan represents the NCI's effort to capitalize on the gathering momentum within the field to do something different.

Interagency collaborations will also play a critical role in realizing the CNPlan's vision, achieving its goals, and meeting its milestones, and the NCI is already in discussions with multiple Federal agencies and other NIH Institutes to develop such cooperative efforts. In particular, a potential joint collaboration with the National Institute of Standards and Technology (NIST) and the FDA is a high priority. This collaboration will focus on developing standards for nanoscale devices and both *in vitro* and *in vivo* characterization assays that could serve as a starting point for regulatory filings. The NCI-FDA Interagency Oncology Task Force, which facilitates dialogue between the two agencies on research and policy issues, will also be addressing nanotechnology programs. The U.S. Department of Defense, which has its own cancer research programs and appreciates the growing burden that cancer represents for current and former members of the Armed Forces, is also a potential collaborator. Both the DARPA and the HSARPA, which have extensive, successful experience using project-management, product-focused research contracts, are providing guidance to the NCI as it develops new funding mechanisms. The NCI and the U.S. Department of Energy, which has a significant biomedical research initiative, are also discussing areas of joint interest in the nanotechnology field. The NCI recognizes the importance of science that supports safe use of nanomaterials in humans and will work with other institutes and centers as well as other programs, such as the National Institute of Environmental Health Sciences and the National Toxicology Program, to characterize any potential health and environmental issues with biomedical nanoscale devices.

During the course of the NCI's activities to develop the CNPlan, it became clear that the lack of standards and characterization data for the many nanoscale devices being developed could become a significant obstacle on the development and regulatory approval pathways. On the basis of input from the academic and private sectors, the NCI believes that the most effective manner for removing this potential obstacle is to establish and fund a national Nanotechnology Characterization Laboratory (NCL), which would work in concert with the NIST and the FDA to perform and standardize the preclinical characterization of nanoscale devices in a way that will facilitate the accelerated regulatory review and translation of these devices into the clinical realm.

The NCL, which will be operated under a contract with SAIC-Frederick, will have the following goals:

- Standardize the preclinical testing and characterization of nanoscale devices to speed the regulatory review of novel diagnostics, therapeutics, and prevention strategies that use nanoscale devices.
- Perform preclinical toxicology, pharmacology, and efficacy testing of nanoscale devices created by both NCI intramural and extramural efforts as well as the private sector.
- Facilitate collaborations between the NCI, academia, and the private sector to accelerate the translation of basic nanotechnology research into clinical advances.
- Serve as a nexus for multidisciplinary research, development, and clinical applications of nanotechnology; provide resources, knowledge, tools, and methods for intramural and extramural cancer researchers.
- Collaborate with other government agencies to leverage resources and expertise in pursuit of common goals in the acceleration of the use of nanotechnology for critical national applications, and team with industry to bring those applications to market.

A key activity of the NCL will be to work together with FDA scientists to develop an assay cascade that can serve as the standard protocol for preclinical toxicology, pharmacology, and efficacy testing of nanoscale devices. This assay cascade will characterize a nanoscale device's physical attributes, its *in vitro* biological properties, and its *in vivo* compatibility.

In carrying out these functions, the NCL will provide a comprehensive set of baseline characterization parameters that will enable cancer biologists, drug and diagnostic developers, and clinical oncologists to concentrate on what they do best—applying these tools to solving problems that most affect cancer patients. This work will also lay a scientific foundation that will enable the FDA to make sound decisions concerning testing and approval of nanoscale cancer diagnostics, imaging agents, and therapeutics.

From its discussions with experts in academia and the private sector, the NCI believes that the NCL's activities will markedly speed the development of nanotechnology-based products for cancer patients, reduce the risk of doing so, and encourage private-sector investment in this promising area of technology development. By taking on this role, the NCL will greatly accelerate the development of the paradigm-changing advances needed to meet the goal of eliminating suffering and death from cancer by 2015.

Interfacing With the Cancer Research Community

A central goal of the NCL is to leverage the existing resources in science and technology which are needed to accelerate the translation of basic research into clinical advances, whether in the public or the private sector. Substantial investments have been made and continue to be made in nanoscience and nanotechnology:

- Through funding from the National Nanotechnology Initiative to support fundamental and applied research, the establishment of multidisciplinary centers of excellence, and the development of infrastructure,
- Through NCI-funded intramural and extramural projects, such as those funded by the Unconventional Innovations Programs (UIP), to support development of novel technologies for noninvasive detection, diagnosis, and treatment of cancer,

- Through other government agency investment, such as the Nanomedicine Roadmap Initiative at NIH to understand molecular pathways and networks and to use that knowledge to design and develop new technologies and devices to improve human health, and
- Through private investment across industry, but primarily through the increasing investment in small businesses to bring new nanomaterials and nanotechnology products to market.

There has also been large investment in technology areas that are critical to the rapid development and application of nanotechnology, such as:

- Investments in microfluidics, MEMS, biotechnology, and bioinformatics, and
- Development of new and advanced measurement technologies and devices, such as the atomic force microscope and MALDI-TOF spectroscopy, which are capable of providing measurements with unprecedented detail and precision.

The new Advanced Measurement Laboratory at NIST, created to respond to the need for advanced measurement methods and standardization in research and development, is another example of the type of facilities that can be leveraged to achieve the NCI's 2015 Challenge Goal.

In order to accelerate the transition of nanotechnology to clinical applications, the NCL must also work closely with regulatory bodies, primarily the FDA, in providing a much closer relationship with industry throughout the pre-clinical tests and clinical trials. The mechanism for this enhanced relationship is already in place in the NCI/FDA Oncology Task Force, an interagency agreement between NCI and FDA to share knowledge and resources to facilitate the development of new cancer drugs and speed their delivery to patients. The NCL can play a significant role in accelerating the transition of nanomaterials and nanodevices to aid in delivering and targeting new cancer drugs as well as contrast agents and reporters to aid in cancer detection and diagnosis.

This relationship with the FDA is crucial in the NCL's interaction with industry. Industry presently assumes significant risk in nanoparticles R&D for clinical applications; the regulatory guidelines are presently undefined. A standardized assay cascade, developed in collaboration with the FDA, will "incentivize" industry to submit nanomaterials to the NCL for characterization, thereby reducing the high risks associated with regulatory approval.

The lack of knowledge concerning the health and safety of nanomaterials may also become an obstacle to the rapid implementation of nanotechnology. Although industry has long manufactured fine and ultra-fine particles for use in a variety of applications, the effects of those particles on human health has been studied only for a small number of materials and applications. In addition, the waste streams generated by the manufacturing and assembly processes for nanomaterials and by their disposal have generally not been subjected to detailed examination and analysis. The assay cascades developed by the NCL to characterize the effect of nanomaterials and platforms in *in vitro* and *in vivo* tests can also provide standardized measures of the effect of these materials, devices, and waste products on human safety—especially the carcinogenic properties of nanomaterials. This additional NCL service will require close collaboration with nanotechnology research institutions and product developers and manufacturers to develop the appropriate standard assays and protocols in response to this public need.

It is precisely this sharp focus on the many facets of cancer research that enables the NCL to serve as a nexus for trans-disciplinary research, development, and clinical applications of nanotechnology. The NCL seeks to provide resources, knowledge, tools, and methods for cancer researchers. It does not seek to duplicate the efforts of established and emerging programs by academia, industry, or government in nanotechnology or to intrude on the domain of other programs. Rather it seeks to partner with these programs. To this end the NCL will collaborate wherever possible with other government agencies, academia, and industry to leverage their resources and expertise in pursuit of common goals and to accelerate the use of nanotechnology in critical national applications to cancer.

What Is Nanotechnology?

Nanotechnology refers to the interactions of cellular and molecular components and engineered materials—typically clusters of atoms, molecules, and molecular fragments—at the most elemental level of biology. Such nanoscale objects—typically, though not exclusively, with dimensions smaller than 100 nanometers—can be useful by themselves or as part of larger devices containing multiple nanoscale objects. At the nanoscale, the physical, chemical, and biological properties of materials differ fundamentally and often unexpectedly from those of the corresponding bulk material because the quantum mechanical properties of atomic interactions are influenced by material variations on the nanometer scale.

Nanoscale devices and nanoscale components of larger devices are of the same size as biological entities. They are smaller than human cells (10,000 to 20,000 nanometers in diameter) and organelles and similar in size to large biological macromolecules such as enzymes and receptors—hemoglobin, for example, is approximately 5 nanometers in diameter, while the lipid bilayer surrounding cells is on the order of 6 nanometers thick. Nanoscale devices smaller than 50 nanometers can easily enter most cells, while those smaller than 20 nanometers can transit out of blood vessels, offering the possibility that nanoscale devices will be able to penetrate biological barriers such as the blood-brain barrier or the stomach epithelium that can make it difficult for therapeutic and imaging agents to reach certain tumors. And because of their size, nanoscale devices can readily interact with biomolecules on both the cell surface and within the cell, often in ways that do not alter the behavior and biochemical properties of those molecules.

Such ready, noninvasive access to the interior of a living cell affords the opportunity for unprecedented gains on both the clinical and basic research frontiers. The ability to simultaneously interact with multiple critical proteins and nucleic acids at their own molecular scales should provide the data needed to better understand the complex regulatory and signaling networks that govern the behavior of cells in their normal state and as they undergo the changes that transform them into malignant cells. In particular, nanotechnology will provide an important platform for integrating efforts in proteomics with other scientific investigations into the molecular nature of cancer. Similarly, nanoscale devices are already proving that they can deliver therapeutic agents that can act where they are likely to be most effective, that is, within the cell or even within specific organelles. Yet despite their small size, nanoscale devices can also hold tens of thousands of small molecules, such as an MRI contrast agent or a multicomponent diagnostic system capable of assaying a cell's metabolic state, creating the opportunity for unmatched detection sensitivity of cancer in its earliest stages.

In some instances, nanotechnology will take advantage of years of clinically relevant technological developments at larger scales. A good example of this approach will capitalize on existing "lab-on-a-chip" and microarray technologies developed at the micron scale. Widely used in biomedical research and clinical diagnostic applications today, these technologies will find new uses when shrunk to the nanoscale. There, they will be able to interact with an individual cell in real time and in that cell's native environment. The CNPlan, with its targeted approach to development, will take advantage of such synergies through several projects directed toward developing real-time diagnostics, reporter systems, and new tools for studying cancer cell and molecular biology.

Current Progress in Cancer Nanotechnology

Today, clinical, cancer-related nanotechnology research is proceeding on two main fronts: laboratory-based diagnostics and *in vivo* diagnostics imaging and therapeutics. Here are just a few of the illustrative highlights of progress in these areas, as well as with the use of nanotechnology to extend our understanding of cancer cellular and molecular biology.

Nanotechnology and Molecular Imaging

- 1-2 nanometer-wide wires built on a micron-scale silicon grid can be coated with monoclonal antibodies directed against various tumor markers, leading to a hundredfold increase in sensitivity over current diagnostic techniques with minimal sample preparation.
- Nanoscale "lab-on-a-chip" applications are now capable of conducting real-time analysis of single biochemical markers.
- Quantum dots have been used to tag and follow multiple individual molecules within cells, providing an opportunity to study the biochemical and genetic systems that go awry in cancer.
- Nanoscale "harvesting" devices have collected proteins capable of distinguishing cancerous tissue from normal tissue.

Nanotechnology and *In Vivo* Imaging

- Nanoscale MRI contrast agents, containing paramagnetic iron nanoparticles, dramatically improve the ability to detect metastatic lesions in lymph nodes associated with breast and prostate cancer.
- Gold nanoparticles demonstrate usefulness contrast agents for *in vivo* endoscopic optical imaging of specific molecular cancer markers.
- Gas-filled lipid nanoparticles have shown promise for use as acoustically activated imaging agents, and perhaps targeted drug delivery systems, for tumors with a spatial resolution of 0.5 to 1.0 millimeters and a temporal timeframe of several images per second.
- Her-2 conjugated, gold-coated nanoparticles with a dielectric silicon core can identify breast carcinoma cells *in vivo*. Once bound to their target cells, these nanoparticles were subjected to increased optical power, turning them into nanoscale thermal scalpels that attain cell-killing temperatures.

Nanotechnology and Cancer Therapy

- A wide variety of synthetic nanoscale particles are shown to target tumor cells, enter cancer cells, and release therapeutic agents.
- Engineered virus particles can serve as multifunctional, targeted non-immunogenic nanoscale devices with potential for a broad range of *in vivo* uses.
- Photosensitizers used in photodynamic therapy, in which light is used to generate reactive oxygen locally within tumors, have also been entrapped in targeted nanoscale devices. The next step in this work is to also entrap a light-generating system, such as the luciferin-luciferase pair, in such a way as to trigger light production only after the nanoparticles have been taken up by a targeted cell. If successful, such an approach would greatly extend the usefulness of photodynamic therapy to include treatment of tumors deep within the body.

Nanotechnology as a Research Enabler

- Construction and testing of nanoplatforms can consolidate cell biology lab tests on a chip. These nanoplatforms can be constructed to accurately mimic the microenvironment in which a particular cell normally grows, producing a system capable of both perturbing cells and recording their responses in a manner more representative of how those cells would behave in the body than is observed in cells grown in standard tissue culture systems.
- A nanoscale device analyzes genome complexity and shows that early-stage tumors expressing similar phenotypes can be distinguished on the basis of how each tumor selects a slightly different approach to derange its genome.

Opportunities From the Fundamental Understanding of Cancer Processes

Nanotechnology offers a wide range of tools, from chip-based nanolabs capable of monitoring and manipulating individual cells to nanoscale probes that can track the movements of cells, and even individual molecules, as they move about in their environment. Using such tools will enable cancer biologists to study, monitor, and alter the multiple systems that go awry in the cancer process and identify key biochemical and genetic "choke points" at which the coming wave of molecular therapies might best be directed. As such,

nanotechnology can serve as the perfect complement to other technology platforms, such as proteomics and bioinformatics, that the NCI is emphasizing in its research initiatives as critical components of the discovery and development engine that will power both near-term and long-term advances in cancer diagnosis, treatment, and prevention. More importantly, however, nanotechnology will serve as a versatile development platform that will be able to quickly turn biological insights into clinically useful products.

Thirty years ago, cancer was a poorly understood and usually deadly disease. This is no longer the case. Today, we know that a cell becomes malignant as a result of changes to its genetic material and that accompanying biological characteristics of the cell also change over a progression of steps that can take years to reach the stage at which a cell becomes malignant and develops into a tumor. These changes are unique molecular "signatures" and serve as signals of the presence of cancer and of the cellular states that precede cancer. This more robust understanding of the genetic alterations that occur within a cancer cell has changed the course of cancer research and has fueled new approaches to prevention, detection, diagnosis, and treatment. One goal of the CNPlan is to foster the development of nanoscale devices that can identify the early molecular signatures of cancer and deliver therapeutic or preventive agents that can intervene in the cancer process at this early stage.

However, the cancer cell is only part of the story in cancer development. As a cancer cell grows within the elaborate architecture of the body's tissues and organs, it interacts with its surrounding environment. Mounting evidence now suggests that a dynamic interaction occurs between the cancer cell and its local and systemic microenvironment, with each profoundly influencing the behavior of the other. This "tumor microenvironment" is populated with a variety of different cell types, is rich in growth factors and enzymes, and includes parts of the blood and lymphatic systems. It promotes some of the most destructive characteristics of cancer cells and permits the tumor to grow and spread. Nanoscale devices, because of their designed multifunctionality, offer the opportunity to manage this complex interaction in ways that could stop the growth and spread of cancer.

The microenvironment can also influence the access of therapeutic agents to tumor cells, the body's processing of treatment agents, and the development of resistance to cancer treatments. Again, these are problems that nanoscale devices should be able to address. Although the cells in the microenvironment may not be genetically altered, their behavior can be changed through interactions with tumor cells. Physicians now realize that they confront a tumor entity that consists of malignant cells combined with their host tumor environment when treating a cancer patient. The tumor cells and their surrounding environment both need to be fully characterized to understand how cancer grows in the body, and both need to be considered when developing new interventions to fight it.

We now understand that cancer is the culmination of many biochemical and genetic processes going awry in the malignant cell and its microenvironment and that no one change will cause a cell to become cancerous. Thus, we now view cancer as a "systems" disease, one that involves the interactions of many cellular processes. The changes that affect these processes fall into seven broad categories, which can be characterized as follows:

Cancer Cells Attain Self-Sufficiency in Growth Signals

Cells grow and multiply in response to a wide variety of growth signals that trigger a series of orchestrated biochemical and genetic events. The production of these growth signals is tightly controlled in a normal cellular environment, but malignant cells have developed numerous ways of either producing their own growth signals or short-circuiting the control mechanisms associated with these growth signals. Many of the oncogenes discovered to date give cancer cells the ability to mimic normal growth signaling processes. Because of the multifactor nature of growth factor activity, it may be necessary to deliver several molecularly targeted agents to a tumor to control its growth, a task for which multifunctional nanoscale devices are ideally suited.

Cancer Cells Become Insensitive to Antigrowth Signals

The normal cellular environment also provides multiple antigrowth signals that act as a check to unregulated cellular reproduction. These growth-controlling signals come mainly from neighboring cells and the extracellular matrix, and they too trigger a series of orchestrated biochemical and genetic events that regulate the cell cycle. Our current understanding of these systems suggests that these signals come through three closely related receptors on the cell surface and that cancer cells are able to disrupt these receptors or the systems that these receptors control. Again, the multifunctional nature of nanoscale devices offers the potential for interacting with more than one of these receptors simultaneously.

Cancer Cells Escape Apoptosis

A third mechanism for regulating improper cell growth involves apoptosis, a set of programmed cellular processes that result in cell death. It is clear from a variety of studies that cancer cells acquire the ability to avoid apoptosis and that effective cancer therapies are able to trigger reactive apoptosis in malignant cells. A cell's apoptotic machinery consists of sensors that monitor the internal and external state of a cell and its environment and effectors that trigger apoptosis when the sensors detect abnormal conditions. The loss of the p53 protein, characteristic of over half of all cancers, allows cells to avoid apoptosis. It appears, however, that cancer cells with damaged apoptotic systems may possess redundant, though inactive, mechanisms for triggering apoptosis. Nanoscale devices will be critical to detecting the reappearance of apoptosis as a sign that cancer therapy is working.

Cancer Cells Gain Limitless Potential for Replication

Telomeres, a stretch of repeat sequences located at the ends of chromosomes, represent a fourth mechanism for controlling the unlimited cellular growth that characterizes cancer. Each time a cell reproduces normally, its chromosomes fail to fully replicate the telomeres, and when the telomeres reach a defined, shortened length, the chromosomes begin to fuse, triggering apoptosis. Thus, telomeres act as a "reproduction counter" that limits a cell's potential for immortality. Some 85 to 90 percent of all cancer cells develop the ability to turn on expression of telomerase, an enzyme that can maintain normal telomere length and that is strongly suppressed in almost all normal cells. The remaining 10 to 15 percent develop a mechanism that maintains telomere length through chromosome-to-chromosome sequence exchange. Nanoparticles, because of their ability to deliver substances to specific cells, and perhaps compartments within a cell, may be the technological platform needed for therapeutic and preventive agents that would intervene in this process.

Cancer Cells Trigger Sustained Angiogenesis

All solid tumors develop the ability to trigger angiogenesis in order to provide oxygen and nutrients. Incipient tumors do not immediately trigger angiogenesis, but at some point tumors are able to alter the balance between angiogenic and antiangiogenic factors in favor of capillary growth in a multi-step process that can be reversed. Recent work with mouse models has shown that different antiangiogenic factors are effective at turning off angiogenesis and starving tumors at specific stages of the angiogenesis and tumor growth. It is also clear that different types of tumor cells use distinct molecular strategies to trigger angiogenesis. Nanoscale devices capable of imaging angiogenesis could provide a new early detection technology; multifunctional nanoscale devices will be able to deliver multiple angiogenesis inhibitors simultaneously.

Cancer Cells Metastasize and Invade Other Tissues

Approximately 90 percent of all cancer deaths result from metastatic spread of the primary tumor. At some point in their development, some number of malignant cells develop an ability to dissociate themselves from the primary tumor mass, invade adjacent tissues, and spread to sites throughout the body. It is clear that invasion and metastatic spread result from a complex series of biochemical and genetic events that affect numerous systems, both in the metastatic cell and in the tissues that it invades. Though most of these events are still poorly characterized, recent work has established that the molecular systems involved in maintaining the normal contact between neighboring cells become altered prior to metastasis. In addition, metastatic cells turn on the expression of proteases capable of degrading the extracellular matrix. Nanoscale analytical devices may be able to detect the early molecular signatures of metastasis before secondary tumors are detectable by other means.

Cancer Cell Genomes Become Unstable

There is little doubt that most of the six molecular characteristics of cancer cells listed above result from genetic changes in a cancer cell, but acquiring multiple mutations through random processes is unlikely given the enormous effort that cells put into maintaining the integrity of their genomes. Yet, cancer cells do accumulate the necessary mutations needed to change from normal to pre-malignant to malignant, suggesting that cancer cells must also have genomes that are unnaturally unstable; indeed, recent research has shown that malignant cells do have grossly rearranged genomes, including multiple copies of specific chromosomes. Furthermore, this research has shown that cells can acquire one or more of the above traits, but they will not become cancerous until their genomes exhibit such instability. Already, nanoscale devices are being developed that can detect genetic mutation and genome instability.

Some of the unanswered questions concerning nanoscale devices relate to their potential toxicity or their fate in the environment, neither of which has yet to be studied in any concerted manner. To date, the few published studies in these areas have concentrated on the potential toxicity of inhaled nanoscale particles, specifically various forms of C60, including "buckyballs" and single-walled carbon nanotubes. That such nanoparticles, when inhaled, might have the potential to damage lung tissue is no surprise given the well-documented hazardous nature of nanoscale diesel exhaust particles. However, such particles are not currently envisioned as having use in the clinical setting. Nevertheless, these studies reinforce the recognized need to conduct thorough toxicology studies on nanoscale devices. Of course, given that any material envisioned for use in humans must undergo rigorous toxicology studies as part of the regulatory approval process, this requirement is neither unexpected nor onerous.

To help address such safety issues, the NCI plans several approaches to supplement the standard complement of toxicology studies that the private sector or any public-private partnerships will conduct as part of the preclinical development process. Under the aegis of the CNPlan, the NCL, in close collaboration with the FDA, will develop a battery of toxicology and safety tests as part of its assay cascade. The NCL will then make these assays available to the field at large as well as use them to develop baseline toxicology data for a wide range of nanoscale particles and devices. The NCI will be evaluating future collaborations and partnerships with the National Toxicology Program and the National Institute of Environmental Health Sciences for these important areas of science.

The NCI's CNPlan dovetails perfectly with the Institute's Action Plan for 2005 and various initiatives aimed at meeting the Challenge Goal of eliminating suffering and death due to cancer by 2015. In particular, the CNPlan stresses work that strengthens the Institute's core multidisciplinary scientific areas of emphasis, including:

- Elucidating the *Signatures of the Cancer Cell and Its Microenvironment*
- Validating and developing effective agents aimed at *Molecular Targets of Prevention, Diagnosis, and Treatment*
- Optimizing *Cancer Imaging and Molecular Sensing* technologies

The CNPlan's heavy emphasis on development and delivery are consistent with goals that the NCI has laid out in its Plan and Budget Proposal for FY 2005. In addition, the CNPlan's activities fit with the Institute's high-profile initiatives in developing new platforms for and enablers of discovery, development, and delivery.

The CNPlan's use of novel, team-oriented funding mechanisms will continue the NCI's work on building capacity through large-scale collaborations. These funding mechanisms also build on efforts to increase translational research involving public-private partnerships.

Appendix A: Training and Cross-Disciplinary Collaboration

One important challenge to reaching the goals of the CNPlan involves bridging the gulf between those who are experts in nanotechnology and those who possess the vision and knowledge to apply this technology to the task of eliminating suffering and death due to cancer. To develop a well-trained cadre of cancer researchers who can bring nanotechnology to the fight against cancer, the NCI anticipates taking a multi-pronged approach. Current, technologically nonspecific funding mechanisms exist that would facilitate building a cancer nanotechnology research program. These can be viewed in terms of immediate impact and future impact. The mechanisms are broken down into categories based on their anticipated effect on the field of cancer nanotechnology.

Immediate Impact Mechanisms

In the short term, the NCI must bring together nanotechnology specialists and cancer specialists for the exchange of ideas, focused educational opportunities, and short-term training and mentoring. Possible mechanisms for accomplishing this goal include:

F33 Awards

- ***F33 NIH National Research Service Awards for Senior Fellows.*** "(T)he National Institutes of Health (NIH) awards NRSA senior fellowships (F33) to experienced scientists who wish to make major changes in the direction of their research careers or who wish to broaden their scientific background by acquiring new research capabilities. These awards will enable individuals with at least seven years of research experience beyond the doctorate, and who have progressed to the stage of independent investigator, to take time from regular professional responsibilities for the purpose of receiving training to increase their scientific capabilities. In most cases, this award is used to support sabbatical experiences for established independent scientists."

  This mechanism would allow current established cancer investigators to train in the labs of leading nanotechnologists to facilitate bringing the technology back to their own labs to be applied toward future research activities. Alternatively, nanotechnologists could be funded to spend a year gaining insight into cancer research so that these problems could be addressed when returning to the nanotechnologist's lab. In both cases, the spillover of ideas from the trainee to the mentor's lab will continue to cross-pollinate the cancer and nanotechnology fields.

K05 Awards

- ***K05 Established Investigator Award in Cancer Prevention, Control, Behavioral, and Population Sciences.*** "The purpose of the NCI Established Investigator Award in Cancer Prevention, Control, Behavioral and Population Research (K05) is to provide established investigators protected time to devote to research and to act as mentors for new investigators and junior faculty members. The target candidates are outstanding established scientists who have demonstrated a sustained, high level of research productivity and significant contributions to cancer prevention, control, behavioral and/or population cancer research. They must demonstrate the need to develop and enhance their own research and a commitment to serve as mentors to new scientists."

  This mechanism can be used by established scientists to free themselves of some administrative responsibilities so that they may mentor recipients of training and career awards in cancer nanotechnology.

R25 Awards

- ***R25E Cancer Education Grant Program.*** "The Cancer Education Grant Program (CEGP) of the National Cancer Institute is a flexible, curriculum-driven program aimed at developing and sustaining innovative educational approaches that ultimately will have an impact on reducing cancer incidence,

mortality and morbidity, as well as on improving the quality of life of cancer patients. The CEGP invites investigator-initiated R25 Grant applications that pursue a wide range of objectives from short courses, national forums, seminars, and/or hands-on workshops designed to educate scientists, health care professionals and the lay community; to the design, development and evaluation of new curricula of special significance to cancer in educational institutions; to structured short-term didactic and research experiences designed to motivate high school; college; and medical, dental and other health professional students to pursue careers in cancer research; to the development and evaluation of new educational methods and tools directed at different audiences with the intent of having an impact on reducing cancer incidence and mortality. The R25 can also be used to fund symposia and support rapidly evolving areas (e.g., courses in innovative screening).

Education Grants such as the R25 can focus on education activities before, during and after the completion of a doctoral level degree (e.g., Ph.D., M.D., D.P.H., D.D.S., and D.N.S.) as long as they address a need that is not fulfilled adequately by any other grant mechanism available at the National Institutes of Health and are dedicated to areas of particular concern to the National Cancer Institute. The CEGP encourages innovative uses of the R25 grant to explore educational approaches that will help promote progress in preventing and curing cancer."

This mechanism can be an integral part of the ability to rapidly adjust to changes in science technology. Nanotechnology workshops, short-term courses, training seminars, and so forth can be developed and funded through this mechanism to quickly bridge the gap and bring nanotechnology to cancer research.

Future Impact Mechanisms

The core of the future cancer nanotechnology cadre will be based not on current established investigators who have adopted a new technology or a new application for their technology but on those who have extensive training in both nanotechnology and cancer research. This core will come from those postdoctoral fellows and junior investigators who, over a 3- to 5-year period, train extensively outside their discipline. Ultimately, the field of cancer nanotechnology will be populated by scientists who have received training that has integrated nanotechnology into the research curriculum. The development of these curricula and the implementation and evaluation of these programs will take time but result in cancer researchers who are as versant in nanotechnology as they are in molecular biology, imaging, or any other technology.

K25 Awards

- ***K25 Mentored Quantitative Research Career Development Award.*** "The K25 mechanism is meant to attract to NIH-relevant research those investigators whose quantitative science and engineering research has thus far not been focused primarily on questions of health and disease. Examples of quantitative scientific and technical backgrounds considered appropriate for this award include, but are not limited to: mathematics, statistics, economics, computer science, imaging science, informatics, physics, chemistry, and engineering. This award provides support for a period of supervised study and research for productive professionals with quantitative backgrounds who have the potential to integrate their expertise with NIH-relevant research and develop into productive investigators. It is intended for research-oriented investigators from the postdoctoral level to the level of senior faculty."

  This mechanism is already bringing in scientists with quantitative and engineering backgrounds to apply different technologies and backgrounds to cancer research. Although certain areas were specifically mentioned, nanotechnology was not. We have begun to specifically mention nanotechnology in these announcements to attract this group to cancer research. This mechanism, in conjunction with mechanisms facilitating mentoring opportunities can, within 5 years, bring about a small cadre of nanotechnology-based, independent cancer researchers.

K01 Awards

- ***K01 Howard Temin Award.*** "The goal of the National Cancer Institute's (NCI) Howard Temin Award is to bridge the transition from a mentored research environment to an independent basic cancer research career for scientists who have demonstrated unusually high potential during their initial stages of training and development. This special award is aimed at fostering the research careers of outstanding junior scientists in basic research who are committed to developing research programs directly relevant to the understanding of human biology and human disease as it relates to the etiology, pathogenesis, prevention, diagnosis, and treatment of human cancer. The major objective of the award is to sustain and advance the early research careers of the most promising M.D.s and Ph.D.s while they consolidate and focus their independent research programs and obtain their own research grant support. To achieve this objective, the Howard Temin Award offers candidates up to five years to gain additional skills and knowledge in human cancer research during a period of one to three years in a mentored environment, followed by transition to the equivalent of a junior faculty position to develop an independent research program."

  The Temin Award offers the opportunity for junior scientists, on the cusp of independence, to receive 1 to 3 years of mentoring before setting out as independent investigators. The 1- to 3-year mentoring period is well suited to applying a new technology to a cancer project. These grants, along with the K25, have the ability to build a cadre of young cancer nanotechnologists who can become the nuclei of cancer nanotechnology programs.

K07 Awards

- ***K07 Cancer Prevention, Control, Behavioral and Population Sciences Career Development Award.*** "The purpose of the Cancer Prevention, Control, Behavioral and Population Sciences Career Development Award (K07) is to support the career development of investigators who have made a commitment to focus their research endeavors on cancer prevention, control, behavioral and the population sciences. This is achieved by providing protected time through salary and research support for up to 5 years to individuals with a health professional or science doctoral degree who are 1) already proficient in general epidemiology, behavioral sciences, or other relevant disciplines, and now want to make use of these proficiencies in cancer-focused research careers in prevention, control, population and/or the behavioral sciences, or 2) already trained in cancer epidemiology, etiology, prevention, control and the behavioral and population sciences but are not yet fully independent investigators. Examples of relevant disciplines for this Program Announcement (PA) include any aspect of human cancer prevention (modifiable risk factors, new animal models and extrapolation of these models to human cancer, genetic predisposition to cancer and detection of precursor lesions, patient-oriented research focused on cancer prevention, and behavioral research and behavioral intervention trials in cancer prevention), epidemiology (biochemical, genetic, molecular), biostatistics, human cancer genetics, clinical oncology, human nutrition, behavioral and social sciences, health promotion, health services and health policy research; and medical decision analysis, survivorship and quality of life as they relate to cancer."

  The K07 is a mentored award designed for researchers in the area of prevention, control, behavioral, and population sciences. K07 recipients often progress to the K22 Transition Career Development Award as they begin their independent research career.

K08 Awards

- ***K08 Mentored Clinical Scientist Development Award.*** "The purpose of the Mentored Clinical Scientist Development Award (K08) is to support the development of outstanding clinician research scientists. This mechanism provides specialized study for individuals with a health professional doctoral degree committed to a career in laboratory or field-based research. Candidates must have the potential to develop into independent investigators. The K08 supports a three, four, or five year period of supervised research experience that may integrate didactic studies with laboratory or clinically based research. The proposed research must have intrinsic research importance as well as serving as a suitable vehicle for learning the methodology, theories, and conceptualizations necessary for a well trained independent researcher."

The K08 mechanism provides a postdoctoral experience for clinically degreed individuals. It is anticipated that this mechanism will be used in a similar manner as the F32 with the added possibility that research produced under the K08 mechanism may have an increased capacity to be translated into the clinic due to the clinical degrees of the applicants. Many K08 recipients progress to the K22 mechanism as they transition to independence.

K22 Awards

- ***K22 NCI Transition Career Development Award.*** "This K22 award is intended to facilitate the transition of investigators from the mentored to the independent stage of their careers in cancer research, by providing 'protected time' for newly independent investigators to develop and receive support for their initial cancer research programs. The award applies to clinicians who are pursuing basic science careers; clinicians who are pursuing careers in patient-oriented research; and to individuals pursuing careers in the prevention, control and population sciences. To apply, a candidate must have completed two years of postdoctoral, mentored research or have been in an independent position for less than two years at the time the application is submitted. The unique feature of this award is that individuals may apply without a sponsoring institution while they are still in a 'mentored' position. Successful postdoctoral applicants will be given up to 12 months to identify an independent, preferably tenure-track, position at a sponsoring institution before an award can be activated. For postdoctoral applicants, the sponsoring institution for a K22 award can be their current institution or a new institution."

  As our career awardees develop and are ready to achieve independence, it will be critical to provide them with the protected time to establish their own labs and with the preliminary data required to successfully compete for research grants. The K22 award is designed to bridge the time between mentored status and independently funded investigator.

R25T, K12, and T32 Awards

- ***R25T Cancer Education and Career Development Program.*** "The purpose of NCI Cancer Education and Career Development Program (R25) is to train predoctoral and postdoctoral candidates in cancer research settings that are highly interdisciplinary and collaborative. This Program requires sustained leadership, dedicated faculty time, specialized curriculum development and implementation, interdisciplinary research environments, and more than one mentor per trainee to achieve career development research and education objectives. Areas of research particularly applicable but not all inclusive to interdisciplinary training are cancer prevention and control, nutrition, population sciences, behavioral sciences, imaging and molecular diagnostics."

- ***K12 Institutional Clinical Oncology Research Career Development Program.*** "The purpose of the National Cancer Institute (NCI) Institutional Clinical Oncology Career Development Program is to increase the number of medical doctors and doctorally degreed Oncology Registered Nurses who are motivated and properly trained to: (1) communicate and collaborate with basic/behavioral research scientists in order to expedite the translation of basic/behavioral research information into patient-oriented cancer research; (2) perform independent clinical oncology research that develops and tests rational scientific hypotheses based on fundamental and clinical research findings with the potential for improving the medical care of cancer patients; and (3) design and test innovative clinical protocols and manage all phases (i.e., pilot/Phase I, Phase II, and Phase III) of clinical trials research. To achieve this purpose, awards are made to institutions for up to five years for the development and implementation of training programs providing clinicians with all of the necessary information and training that will enable them to design, implement and manage all phases of cancer clinical trials research. The distinguishing features of this career development Program are that a Program Leader in the institution together with an Advisory Committee selects the candidates and oversees the course of their training, and that candidates are likely to have more than one mentor as they are exposed to the basic sciences and to the many disciplines critical to the clinical sciences."

- ***T32 NIH National Research Service Award Institutional Research Training Grants.*** "The National Institutes of Health (NIH) will award National Research Service Award (NRSA) Institutional Training Grants (T32) to eligible institutions to develop or enhance research training opportunities for individuals, selected by the institution, who are training for careers in specified areas of biomedical, behavioral, and clinical research. The purpose of the NRSA program is to help ensure that a diverse and highly trained workforce is available to assume leadership roles related to the Nation's biomedical and behavioral research agenda. Accordingly, the NRSA program supports predoctoral, postdoctoral, and short-term research training experiences."

All three mechanisms are designed to create a training environment within the institutions. Through these mechanisms, cancer nanotechnology training programs can be created in basic research (T32), prevention, control, behavioral, and population sciences (including screening, diagnostic, and imaging) (R25T) and, as the field matures and products are ready to enter the clinic, clinical oncology (K12).

F32 Awards

- ***F32 Ruth L. Kirschstein National Research Service Awards for Individual Postdoctoral Fellows.*** "The Congress of the United States enacted the National Research Service Award (NRSA) Program in 1974 to help ensure that highly trained scientists will be available in adequate numbers and in appropriate research areas to carry out the Nation's biomedical and behavioral research agenda. Under this congressional authority, the National Institutes of Health (NIH) awards NRSA individual postdoctoral fellowships (F32) to promising applicants with the potential to become productive, independent investigators in fields related to the mission of the NIH constituent institutes and centers."

  This is the basic postdoctoral funding mechanism and will undoubtedly provide the bulk of the future nanotechnology-focused cancer researchers. In the short term, it is anticipated that Ph.D.s with training in either cancer or technology will use the F32 to gain postdoctoral experience in the other discipline. Eventually, once a cadre of cancer nanotechnology researchers has been established, graduates will be able to obtain research experience specifically in cancer nanotechnology.

K23 and K24 Awards

- ***K23 Mentored Patient-Oriented Research Career Development Award.*** "The purpose of the Mentored Patient-oriented Research Career Development Award (K23) is to support the career development of investigators who have made a commitment to focus their research endeavors on patient-oriented research. This mechanism provides support for three to five years of supervised study and research for clinically trained professionals who have the potential to develop into productive, clinical investigators focusing on patient-oriented research."

- ***K24 Midcareer Investigator Award in Patient-Oriented Research.*** "The purpose of the Midcareer Investigator Award in Patient-Oriented Research (K24) is to provide support for clinicians to allow them protected time to devote to patient-oriented research and to act as mentors for beginning clinical investigators. The target candidates are outstanding clinical scientists who are actively engaged in patient-oriented research. Candidates are generally within 15 years of their specialty training. Candidates must be able to demonstrate the need for a period of intensive research focus as a means of enhancing their clinical research careers and must be committed to mentoring the next generation of patient-oriented researchers. The award is intended to further both the research and mentoring endeavors of outstanding patient-oriented investigators, to enable them to expand their potential for significant contributions to their field, and to act as mentors for beginning clinician researchers."

As the cancer nanotechnology field matures and products begin to make their way to the clinics, it will be important to develop cancer nanotechnology researchers who are involved in patient-oriented research. The K23 mechanism provides a mentored experience for patient-oriented researchers and the K24 provides the mentors with the protected time to do patient-oriented research and act as mentors for K23 fellows.

Cowpea mosaic virus. From Marianne Manchester and M.G. Finn, Scripps Research Institute

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Cancer Nanotechnology Plan

Office of Cancer Nanotechnology Research
Center for Strategic Scientific Initiatives (CSSI)

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Table of Content

Foreword

The NCI Alliance for Nanotechnology in Cancer (ANC) was launched on the premise that nanotechnology based materials and devices can strongly benefit cancer research and clinical oncology. They can also contribute to new solutions in molecular imaging and early detection, *in vivo* imaging, and multi-functional therapeutics for effective cancer treatment. The direction and strategy behind Phase I (funding period of 2005 to 2010) of the Alliance were derived from the Cancer Nanotechnology Plan (CaNanoPlan) published in 2004.

The new CaNanoPlan 2010 summarizes the present state of significant areas in the field and builds upon recent discoveries. We asked several investigators participating in Phase I of the program to contribute a chapter; we also drew on the opinions voiced at the series of Strategic meetings held at NCI. Each chapter presents the current status of development and also highlights avenues for growth and opportunity, elucidates clinical applications for the technologies, and forecasts what goals might be achieved in the next 3-10 years.

We, the NCI Office of Cancer Nanotechnology Research, would like to thank all who contributed to CaNanoPlan 2010. Establishing forward strategy is important – there are always multiple paths to take and optimizing the ones we do take will bring us all closer to the goal of achieving new and more effective ways of diagnosing, treating, and preventing cancer. These efforts will ultimately change the lives of cancer patients.

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Office of Cancer Nanotechnology Research/ Center for Strategic Scientific Initiatives
National Cancer Institute/ NIH

Piotr Grodzinski

Dorothy Farrell, George Hinkal, Sara S. Hook, Nicholas Panaro, Krzysztof Ptak

Introduction

Sara S. Hook, Krzysztof Ptak, Dorothy Farrell, George Hinkal, Nicholas Panaro, and Piotr Grodzinski

Office of Cancer Nanotechnology Research, CSSI, National Cancer Institute, NIH, Bethesda, MD

The complexity of cancer as a disease

Cancer remains one of the most complex diseases affecting humans and, despite the impressive advances that have been made in molecular and cell biology, how cancer cells progress through carcinogenesis and acquire their metastatic ability is still widely debated. The idea that cancer might be attributed to inherent changes within the organism's own genome did not arise until after the discovery that retroviruses could transform host cells and often they contain variants of cellular genes which are necessary for oncogenic transformation. Consequently, for perhaps nearly twenty years, the field of oncology was synonymous with virology and a major focus was on identifying these proto-oncogenes or genes that could be turned into cancer-causing genes. Today, cancer is recognized as a highly heterogeneous disease and over 100 distinct types have been described with various tumor subtypes found within specific organs. It is now also recognized that genetic and phenotypical variability primarily determines the self-progressive growth, invasiveness, and metastatic potential of neoplastic disease and its response or resistance to therapy. It seems that this multi-level complexity of cancer explains the clinical diversity of histologically similar neoplasias.

Recent advances in other disciplines have uncovered that in addition to virus infection, disregulation of many normal cellular processes such as gene regulation, cell cycle control, DNA repair and replication, checkpoint signaling, differentiation, and apoptosis, etc. can lead to cancer. The mechanisms of transformation can be complex with multiple pathways affected. For example, genetic changes in the p53 gene resulting in loss of heterozygosity are known to affect the pattern of gene activation and repression, dampen cell cycle checkpoints, and incapacitate the induction of apoptosis (Farnebo *et al.*, 2010). In addition to multiple pathways being compromised in tumor cells, tumors can arise in a cell- or tissue-specific manner. For instance, mutations in the breast cancer susceptibility gene, *BRCA1*, are associated with approximately half of the inherited forms of breast and ovarian cancer, but they do not predispose carriers to most other forms of cancer even though the gene is ubiquitously expressed and is involved in the fundamental processes of transcriptional regulation and DNA repair (Linger and Kruk, 2010). While some times there are common mutations frequently associated with many cancers, the majority of cancers arise from a diverse array of malfunctions that result in a tumor that is unique to that patient. The complexity of cancer combined with an avalanche of basic science research uncovering the plethora of pathways that feed into cellular growth control reveals many potential therapeutic targets. As such, there is a critical need for cancer biologists with a broad knowledge of the mechanisms of tumorigenesis to team up with clinical oncologists to address just how this information can be utilized to advance clinical therapies.

The need to advance cancer clinical therapies

To this day, the mainstay of cancer treatment has been the same for nearly 40 years and consists of surgical resection, radiation, and/or chemotherapy. This approach involves physically removing as much of the tumor bulk as possible then subjecting the entire body to agents that kill cells by non-selectively damaging the DNA of both cycling tumor and healthy cells. These therapies have limited effectiveness, high cytotoxicity, and untoward side effects. Additionally, the nature of the disease is such that unless all tumor cells are destroyed the cancer will eventually return, often in a form more aggressive and more refractory to treatment. There is a distinct paucity of effective therapies for cancers such as pancreatic and ovarian, which have relatively lower survival rates compared with other types of cancers and where most patients present with advanced stages of the disease at the time of diagnosis. Thus, there is a critical need for not only specific, effective therapies without side effects, but also mechanisms for early detection to ensure that therapies have the best opportunity to be timely and effective.

Nanotechnology approaches for cancer

The National Cancer Institute (NCI) has recognized these critical clinical deficiencies and has been on the forefront of identifying and developing new and innovative ways to approach cancer diagnosis, treatment, and management. Having witnessed substantial technological advances in the field of nanotechnology in various disciplines including physical sciences, engineering, physics, and chemistry in developing new materials and devices to be used in electronics and energy conservation, the NCI recognizes nanotechnology as an exciting and promising approach to address cancer applications as well.

Nanotechnology involves research and technology development at the atomic, molecular, or macromolecular levels and allows the creation and use of functionalized structures, devices, and systems that take advantage of specific properties of matter that exist at the nanoscale. Nanoscale structures can be manipulated on the atomic scale and integrated into larger material components, systems, and architectures. The potential for using nanotechnology in medicine and especially in the area of cancer is vast. For example, nanoparticles targeting tumor cells, using the knowledge we have about cellular biology, will enable clinicians to deliver therapy specifically to the tumor while reducing unwanted side effects. In addition, increased capacity to image tumor cells will enable earlier diagnosis, confer increased accuracy for surgical resection, offer real-time assessment of treatment effectiveness, and enhance monitoring for metastasis or primary tumor re-growth. Furthermore, powerful chemotherapeutic agents that were abandoned due to toxic side effects can be resurrected using nanotechnology enabled delivery systems thus enabling them to become viable treatment options.

Establishment of the Alliance for Nanotechnology in Cancer (Phase I)

In the late 1990s, the NCI established the Unconventional Innovations Program (UIP) to work with university research groups and small companies to evaluate potential nanotechnology applications in cancer. Building upon the productive experience of the UIP program, NCI established the Alliance for Nanotechnology in Cancer (ANC) program in September 2004. The overarching goal of this program has been to discover and develop nanotechnologies for applications ranging from discovery through translation and delivery of innovative, clinically relevant technologies for cancer prevention, diagnosis, and treatment. The Alliance's development model calls for the most promising strategies discovered and developed by Alliance grantees to be handed off to private sector partners for clinical translation and commercial development. In its first five years, the program focused on basic research and developmental efforts in six major challenge areas: molecular imaging and early detection, *in vivo* nanotechnology imaging systems, reporters of efficacy, multi-functional therapeutics, prevention and control, and research enablers.

The Phase I funding period (2005-2010) involved funding a constellation of eight Centers for Cancer Nanotechnology Excellence (CCNEs) and twelve Cancer Nanotechnology Platform Partnerships (CNPPs), together with eleven Multi-disciplinary Research Training and Team Development awards. CCNE teams were focused on developing integrated nanotechnology solutions with future potential for clinical applications. The CCNEs evolved into research organisms having distinct area(s) of technical excellence and core resources (e.g. fabrication and materials development, diagnostic assays, toxicology, drug delivery, *in vivo* technology validation, informatics). The CNPPs were individual research projects. The CCNEs provided infrastructure and translational support to the CNPPs where appropriate. The Multi-disciplinary Research Training and Team Development program was dedicated to training graduate students and post-doctoral fellows. The NCI also formed an intramural laboratory, the Nanotechnology Characterization Laboratory (NCL), to serve as a centralized facility to characterize nanomaterials. The NCL is a formal collaboration with the National Institute of Standards and Technology (NIST) and U.S. Food and Drug Administration (FDA). The NCL's role in the Alliance was to perform standardized characterizations and safety evaluation of nanoscale materials developed by researchers from academia, government, and industry. The NCL will have a more integral role in the next funding phase (Phase II) of the program as more technologies advance towards clinical development. In addition, there are some slight shifts in the programmatic focus as well as additional funding mechanisms that will strengthen training and collaborative efforts.

Challenges to Developing New Nanomaterials

*Joseph M. DeSimone and Robert Petros**

Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill and North Carolina State University, Raleigh, NC; *Currently at University of Texas, Denton, TX

Engineered nanoparticles have the potential to revolutionize the diagnosis and treatment of many diseases; for example, by allowing the targeted delivery of a drug to particular subsets of cells. However, so far, such nanoparticles have not proven capable of surmounting all of the biological barriers required to achieve this goal. Nevertheless, advances in nanoparticle engineering, as well as the understanding of the importance of nanoparticle characteristics such as size, shape and surface properties for biological interactions, have created new opportunities for the development of nanoparticles for therapeutic applications. In the past two decades, several therapeutics-based on nanoparticles have been successfully introduced for the treatment of cancer, pain, and infectious diseases (Davis *et al*., 2008; Petros and DeSimone, 2010; Zhang *et al*., 2008). These therapeutics harness the opportunities provided by nanomaterials to target the delivery of drugs more specifically, improve solubility, extend half-life, improve therapeutic index, and reduce immunogenicity.

General nanoparticle characteristics

The size, surface characteristics and shape of a nanoparticle play a key role in its biodistribution *in vivo*. Spherically shaped, passively targeted, nanoparticles less than 5 nm in diameter are rapidly cleared from circulation via extravasation or renal clearance, and as particle size increases from the nanometer range to ~15 micrometers, accumulation occurs primarily in the liver, spleen and bone marrow. Nanoparticle behavior in the size range ~10 nm to ~15 micrometers varies widely in terms of biodistribution and cellular uptake of nanoparticles in this range is heavily dependent on cell type. Under normal circumstances, nanoparticles are mechanically filtered by sinusoids in the spleen and removed from circulation via cells of the reticuloendothelial system (RES). In addition, Kuppfer cells in the liver, also part of the RES, play a key role in particle removal (Petros and DeSimone, 2010).

The propensity for accumulation of nanoparticles in cells of the RES is dictated by specific proteins adsorbed *in vivo* to the particle surface, which can be influenced through modifications of surface characteristics. This process of protein adsorption, known as opsonization, begins immediately after particles come in contact with plasma. The exact nature of the types and quantities of proteins and their conformations dictate the body's reaction. The mechanisms involved in this process are not well understood; however, the major opsonins are known. Immunoglobulin (Ig) and complement proteins are the predominant contributors to the recognition of foreign particles by the cells of the RES (that is, macrophages). Complement activation can further complicate targeted drug delivery by inducing hypersensitivity reactions. Finally, particulate matter larger than ~15 micrometers is removed from circulation via mechanical filtration in capillaries and can be lethal depending on dose.

Current methods for addressing the negative attributes associated with opsonization have focused almost exclusively on slowing the process by rendering the particle surface more hydrophilic or by neutralizing surface charge. The predominant strategy has been to adsorb or graft a hydrophilic polymeric coating, such as polyethylene glycol (PEG) to the surface of the particle. These polymer chains, depending on density, act as a steric brush that imparts resistance to protein adsorption. However, the PEG effect is transient, so eventual opsonization and macrophage clearance still occur (Howard *et al*., 2008).

Although studies have demonstrated the positive effects that can be achieved by dictating which proteins adsorb to the surface of nanoparticles, methods that have been employed in the design of potential nanoparticle therapeutics to date are limited in scope (Petros and DeSimone, 2010). Particle size is also known to influence the mechanism of cellular internalization — that is, macropinocytosis, clathrin-mediated endocytosis, or caveolin-mediated endocytosis — which in turn dictates the microenvironments an engineered nanoparticle experiences upon internalization (Figure 1). Detailed knowledge of the mode of entry into the cell is invaluable because it could be used to design an engineered nanoparticle targeted to specific intracellular microenvironments, as discussed in more depth later. As noted above, so far, the impact of size on biodistribution

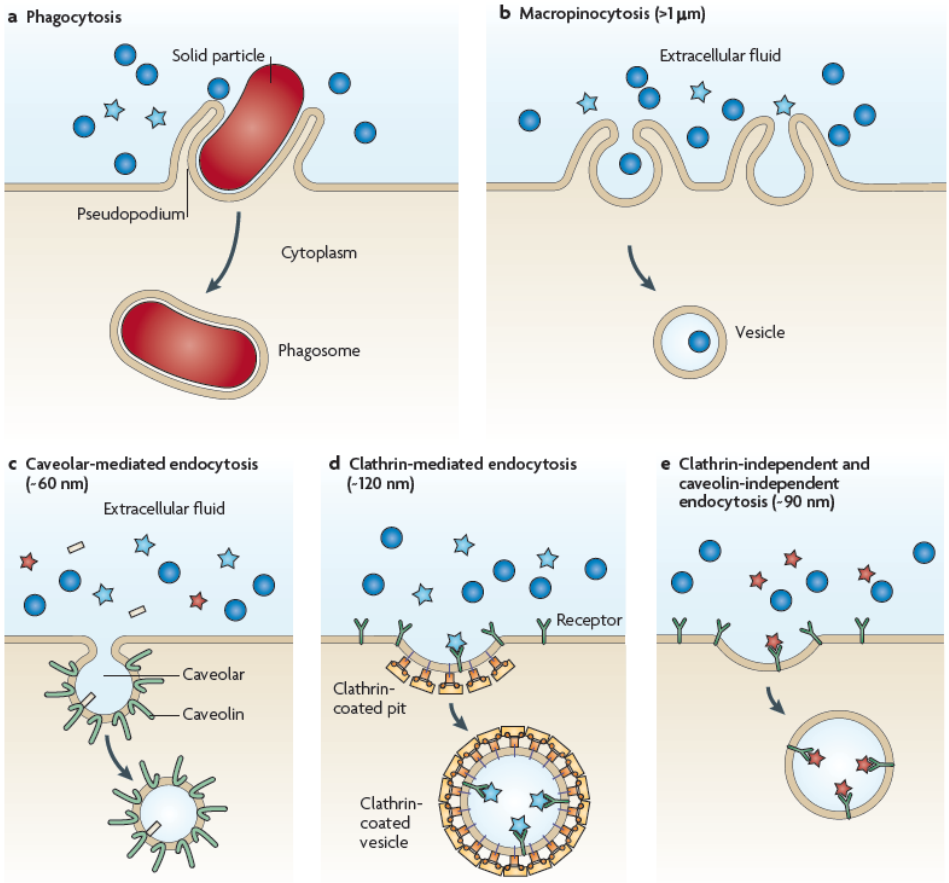

Figure 1 **Modes of cellular internalization of nanoparticles and respective size limitations. (a) Internalization of large particles is facilitated by phagocytosis. (b) Nonspecific internalization of smaller particles (>1 µm) can occur through macropinocytosis. (c) Smaller nanoparticles can be internalized through several pathways, including caveolar-mediated endocytosis, (d) clathrin-mediated endocytosis and (e) clathrin-independent and caveolin-independent endocytosis , with each being subject to slightly different size constraints. Nanoparticles are represented by blue circles (> 1 µm), blue stars (about 120 nm), red stars (about 90 nm) and yellow rods (about 60 nm) (reprinted with permission from Petros and DeSimone, 2010, Copyright, Nature Publishing Group).**

and cellular internalization has largely been elucidated using spherically-shaped particles. However, recent findings (Champion and Mitragotri, 2006; Decuzzi *et al*., 2010; Geng *et al*., 2007; Gratton *et al*., 2008) indicate that particle shape is as important, if not more so, than size in controlling key aspects of both these phenomena. For example, in HeLa cells there is a clear correlation between the rate of internalization and the shape and size of the particles (Gratton *et al*., 2008). Interestingly, they also showed that particles with similar volumes but different shapes were internalized at drastically different rates. In addition, the geometry of interaction between a cell and particle can induce or inhibit internalization (Champion and Mitragotri, 2006) and the shape has a significant impact on biodistribution (Geng *et al*., 2007) with filamentous engineered nanoparticles having single dimensions as long as 18 µm exhibiting circulation half-lives of ~5 days, which was much longer than even "stealth" liposomes.

Methods for incorporating cargo into engineered nanoparticles can be classified into two broad categories. In one category, the cargo is physically entrapped in or absorbed onto the nanoparticle through non-covalent interactions. The second category includes examples where the cargo has been directly attached to the nanoparticle matrix via degradable or non-degradable covalent bonds. The use of stimuli-responsive materials allows for release of cargo once the engineered nanoparticle reaches its intended location *in vivo*. The bulk composition of the engineered nanoparticle must be carefully chosen based on its biocompatibility, immunotoxicity (Dobrovolskaia and McNeil, 2007), and its ability to solubilize or sequester the cargo of interest. Beyond these basic features of nanoparticle design, a multitude of approaches for targeting specific cellular populations or altering the biodistribution of engineered nanoparticles *in vivo* are being developed. Targeting has been achieved using three predominant strategies that rely on either active or passive modes of

action, which can be further characterized as selective or non-selective.

General biological barriers

To achieve intracellular drug delivery, strategies for overcoming a variety of biological barriers — from the system level, to the organ level, to the cellular level — are needed. The initial barriers encountered depend on the mode of administration (that is, inhalation, oral, intravenous, or intraperitoneal injection). The degree of success in utilizing each of these modes of entry can be strongly influenced by attributes of the nanoparticles themselves. For example, size can be a major determinant for effective pulmonary delivery, whereas successful strategies for oral administration must address carrier stability during the harsh conditions in the gastrointestinal tract, while simultaneously targeting a specific site for entry. Intravenous injections must overcome the RES if prolonged circulation is to be attained and a method for escaping the endothelium is required in order to exit circulation into the desired tissue. Intraperitoneal injection allows tissue-specific delivery; however, nanoparticles can be rapidly cleared via the lymphatic system unless special steps are taken to avoid this.

Organ level: For intravenously injected engineered nanoparticles, avoidance of multiple organ-level clearance mechanisms, such as those operating in the spleen and liver, must be compensated for if the carrier is to reach its intended destination (Petros and DeSimone, 2010). Fenestrations in the spleen typically do not exceed 200-500 nm in width so particles larger than ~200 nm must be engineered to have some degree of deformability in order to remain in circulation. A method for attenuating the activity of cells of the RES is also usually necessary to prolong circulation times.

Several strategies can be employed to circumvent carrier removal by macrophages. First, decoy carriers can be pre-injected to saturate the phagocytic capacity of the RES, followed by injection of carriers containing the active ingredient. Second, altering the hydrophilicity of the carrier surface has been shown to reduce the rate of protein opsonization, which ultimately marks carriers for sequestration and removal. Third, specific proteins can be adsorbed or covalently linked onto the surface of the carrier that help minimize or avoid complement activation. Finally, markers-of-self can be attached to the surface of the carrier.

In view of these desired characteristics of engineered nanoparticles, red blood cells (RBCs) could be considered as a prototypical model (Petros and DeSimone, 2010). First, they are capable of traversing biological barriers that are impenetrable to objects less than one tenth their size and manage to avoid clearance by macrophages for up to three months. A number of factors are believed to contribute to their extended circulation, including their shape, deformability (which allows them to navigate through much smaller sinusoids in the spleen), and the presence of ligands, such as CD47 and CD200 that bind to inhibitory receptors expressed by macrophages (absence of these markers leads to immediate removal of RBCs by macrophages).

Cellular level: There are several biological barriers at the cellular level that an engineered nanoparticle must overcome. The cell membrane blocks diffusion of complexes larger than ~1 kDa. Several endocytic mechanisms can be engaged to facilitate internalization of a carrier. The details of the exact mode of endocytosis are important because they dictate the path of trafficking through various possible subcellular compartments. For example, engineered nanoparticles internalized via clathrin-mediated endocytosis are destined for lysosomal compartments, whereas those internalized via a caveolin-mediated process are not. In the former, endosomal escape must occur prior to fusion with a lysosome to prevent degradation of the cargo under harsh lysosomal conditions. In either case, endosomal escape is usually necessary to allow access of the carrier to the desired subcellular compartment whether it is the cytosol, mitochondria, or nucleus.

Ligands conjugated to the surface of engineered nanoparticles can influence the mode of cellular internalization. Ligands such as folic acid, albumin, and cholesterol have been shown to facilitate uptake via caveolin-mediated endocytosis whereas ligands for glycoreceptors promote clathrin-mediated endocytosis (Figure 1). Alternatively, macropinocytosis, a non-caveolin, non-clathrin-mediated process, can be engaged by incorporating cell-penetrating peptides, such as a TaT peptide (*trans*-activating transcriptional activator) into the design of engineered nanoparticles. What is not well understood is the interdependent role(s) of particle size, shape and flexibility with ligand type, density, multiplexing, and regio-specific labeling on the particles. The nuclear membrane is the final barrier for many engineered nanoparticles although recent advances have been made in the ability to target specific organelles (Petros and DeSimone, 2010).

Conclusions

Several particle characteristics have emerged as central to the function of engineered nanoparticles and should therefore be used to guide future design efforts.

Particle size: For rigid, spherical particles, the 100-200 nm size range has the highest potential for prolonged circulation because they are large enough to avoid uptake in the liver, but small enough to avoid filtration in the spleen. The design of non-spherical and/or flexible particles can, however, dramatically extend the particle's circulation time *in vivo*. The same general principles govern the biodistribution profile of these particles: for long-circulating particles, uptake by the liver and spleen must be avoided. This can be accomplished practically by engineering deformability into particles >300 nm or by keeping at least one dimension of the particle on a length scale >100 nm to prevent accumulation in the liver while maintaining at least two dimensions at <200 nm, thereby allowing the particle to navigate the sinusoids of the spleen.

Particle shape: In some instances, the effects of particle shape can be intimately coupled to particle size, as described for long-circulating non-spherical particles. Particle geometry also plays a key role in particle internalization. Although preliminary data exist demonstrating the marked effects of particle shape, the optimum parameters for engineered nanoparticles have yet to be determined.

Surface characteristics: This particle attribute has three vital roles in the function of engineered nanoparticles. First, surface chemistry is known to heavily influence the process of opsonization, which ultimately dictates RES response. Several methods designed to circumvent the activation of the immune system are described above. Second, to achieve cellular targeting, ligands known to bind cell-surface receptors of selected cells should be included in the design of engineered nanoparticles. Third, if organelle targeting is also required, those ligands must also be incorporated into surface design.

Release of therapeutics: Achieving tailored, activated release still represents a major barrier in the field of engineered nanoparticles. The predominant strategies to date incorporate materials that are enzymatically degradable, pH-sensitive, or reductively labile. The latter category facilitates either bond-breaking between drug and carrier or destabilization of the carrier upon reaching the intended site of action.

In summary, great strides have been made in the design and application of engineered nanoparticles over the last 50 years. However, significant challenges remain. Our ability to shepherd cargo to sites in the body to achieve precisely defined therapeutic effects is still in its infancy. Development of the requisite tools to dictate events occurring at the biotic/abiotic interface requires a highly interdisciplinary approach, which is benefiting tremendously from the increasing collaborations amongst scientists from the physical and life sciences. As this trend continues, the potential of appropriately engineered nanoparticles of increasing complexity and efficacy will be realized.

Milestones

3-year:

- Adopt standardized techniques for the characterization of nanoparticles both *in vitro* and *in vivo*.
- Design nanoparticle compositions with reproducible, activated, release properties *in vivo*.
- Conduct clinical trials of a variety of nanoparticles.

5-year:

- Determine the effects of surface regiochemistry on nanoparticle internalization and biodistribution.
- Expect the first polymer-based, nanoparticle therapeutic to be approved by the FDA.

10-year:

- Complete a map of nanoparticle biodistribution as a function of size, shape, deformability, zeta potential, and surface chemistry.
- Develop several cancer vaccines.
- Create long-circulating nanostructures via active strategies. Next generation methods should focus on engineering particle shape and modulus and the tailoring of particle surface chemistry to actively interact with the immune system.

In Vitro Multiplex Protein Assays and Sensors for Cancer Research and Clinical Applications

James R. Heath

Nanosystems Biology Cancer Center and Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA

Traditional *in vitro* measurements for cancer diagnostics have been single-parameter based. Examples include the measurement of prostate specific antigen (PSA) for prostate cancer, or measurement of Cancer Antigen 125 (CA125) for detecting the recurrence of ovarian cancer. However, a recent and growing trend has been to assess the levels of increasingly large panels of molecular biomarkers from ever smaller blood samples or tissue specimens. In this context, genome (DNA) and transcriptome (mRNA) measurements are playing important roles. However, for monitoring evolving health conditions, such as the response of a patient to a drug, assessing immune system status, or for monitoring evolving disease within a patient, measurements of protein biomarkers are the most informative.

In contrast with genome sequencing or mRNA profiling, the cost of protein biomarker measurements has remained relatively stagnant over time. This is for multiple reasons. First, the only reliable and broadly translatable assays for sensitively quantifying protein levels are based upon the use of affinity agents (antibodies). In fact, the gold standard, which is the Enzyme Linked Immunosorbent Assay (ELISA), requires two antibodies per detected protein. Antibodies are expensive, unstable, and often unavailable against their target proteins. The instability of antibodies, and the cross-reactivity of antibodies for non-cognate proteins can, in turn, make it difficult to reliably assess a large panel of proteins. In addition, the cost and time gains that are often achieved via miniaturization are non-trivial to realize for protein assays. For example, the use of microfluidics platforms within modern sequencing machines permits more sequencing more quickly and with less sample. However, antibody arrays are difficult to construct and maintain within microfluidics environments, since the fabrication of such platforms usually requires elevated thermal processing. As a result, even as sequencing technologies march towards (and beyond) sequencing a genome for under $1000, the cost of a single protein assay has remained around $50 per protein. However, there are a number of technology advances, many of them supported within the existing NCI-funded nanotechnology programs that have the potential to increase the flexibility of multiplex protein diagnostic measurements and dramatically decrease cost and performance time. These include (1) approaches that integrate blood and/or tissue handling onto the assay platform; (2) surface chemistries that permit antibody integration into microfluidics chips and that reduce non-selective protein adsorption; (3) miniaturized, multiplex and quantitative measurement platforms; and, perhaps most critical, (4) chemical technologies for the production of physically and chemically robust protein capture agents.

There are many benefits of multiplexed, integrated (blood/tissue handling are integrated onto the assay platform), and miniaturized diagnostic assays. An appropriately designed platform for clinical use can potentially serve as a point-of-care (POC) diagnostic tool, implying that the assay results are available to the patient during the same office visit. Most existing POC devices (pregnancy tests, developing world HIV and Hepatitis tests, etc.) are neither quantitative nor multiplex but they do yield a rapid and often reliable answer to a clinically relevant question.

Integrated assay devices

An integrated, multiplex diagnostic platform can minimize two of the key variables that most detrimentally impact biospecimen quality – handling by laboratory and clinical personnel, and the time between specimen collection and assay completion. Multiplex assays on small volume blood (*e.g.* pinprick) or tissue (*e.g.* skinny needle biopsy) samples can enable higher throughput of patient samples. When coupled with the right biomarkers, such approaches have the potential to accelerate clinical decision making regarding continuation of a therapy, adjusting dosing levels, etc. In addition, such assays can enable more information to be extracted from precious samples, such as circulating tumor cells, tumor infiltrating lymphocytes, cancer stem cells, small biopsy samples from tumor margins, etc. (Figure 2). Finally, highly multiplex assays can assist with the biomarker discovery process,

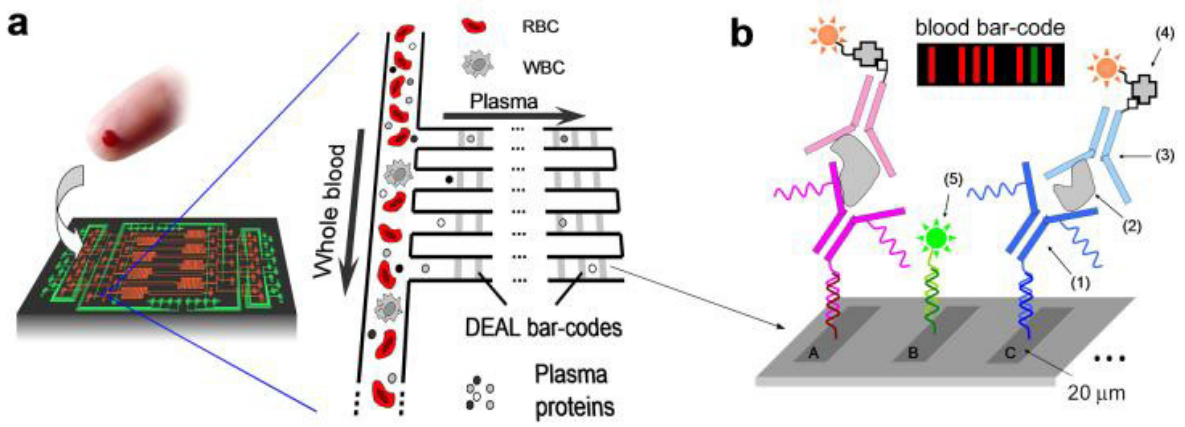

Figure 2 **Design of an integrated blood barcode chip (IBBC). (a) Scheme depicting plasma separation from a fingerprick of blood by harnessing the Zweifach-Fung effect. Multiple DNA-encoded antibody barcode arrays are patterned within the plasma skimming channels for *in situ* protein measurements. (b) Illustration of DEAL barcode arrays patterned in plasma channels for *in situ* protein measurement. A, B, C indicate different DNA codes. (1)-(5) denote DNA-antibody conjugate, plasma protein, biotin-labeled detection antibody, streptavidin-Cy5 fluorescence probe, and complementary DNA-Cy3 reference probe, respectively. The inset represents a barcode of protein biomarkers, which is read out using fluorescence detection. The green bar represents an alignment marker (reprinted with permission from Fan *et al*., 2008, Copyright, Nature Publishing Group).**

since they can permit many potential biomarkers to be assayed at a cost that is only incrementally greater than measuring a single assay. A number of relevant technology advances for multiplex protein cancer diagnostics have occurred over the past 5-10 years and, equally important, the goals of the technology developers have become increasingly aligned with the needs of the cancer biologists and clinical oncologists. Over this same period, certain technologies, such as nanotube (Chen *et al*., 2001; Besteman *et al*., 2003), nanowire or nanocantilever sensors, that were initially viewed as promising have failed to deliver for reasons of robustness, cost, or other practical considerations, although those technologies may still find non-clinical applications (Heath and Davis, 2008; Giljohann and Mirkin, 2009). By contrast, blood and tissue handling on chip (Heath and Davis, 2008) is becoming increasingly sophisticated and effective, even as the platforms have decreased in complexity (Qin *et al*., 2009; Nie *et al*., 2010) and likewise increased in robustness. Multiplexing via spatial (Fan *et al*., 2008) or colorimetric (Giljohann and Mirkin, 2009) encoding has been enabled by various nano- and micro- technologies. Quantitative protein assays with sensitivities far exceeding what was possible a decade ago have been developed (Armani *et al*., 2007; Heath and Davis, 2008), with some already in the clinic. Platforms that can execute multiplex protein assays from a variety of body fluids (Osterfeld *et al*., 2008; Gaster *et al*., 2009) and chip-based rare cell capture and analysis have been reported (Nagrath *et al*., 2007; Kwong *et al*., 2009). Microfluidics strategies that integrate highly multiplex protein assays (Bailey *et al*., 2007) and plasma separation from whole blood have also made it into human trials. In fact, it is likely that platforms that combine microfluidics, surface chemistry, and nanotechnology will dominate multiplex clinical protein biomarker measurements by the end of this decade.

Future developments

The biology of cancer, as well as the demands of clinical oncology, will likely serve as drivers for the further development of micro/nano technologies. As representative examples, drivers include protein biomarker development, understanding the tumor microenvironment, interrogating the functional status of the immune system of cancer patients, interrogating the interrelationship between the immune system and cancer, and stratifying patients and patient responses for molecularly targeted therapies. The best technology solutions will be cost effective, rapid, highly multiplex, and, of course, robust. It is likely that many of those technology solutions are at least already partially in hand. Some associated technology challenges have, as yet, no clear solution.

Practically all of the new nano/micro technologies that have emerged for quantitative, multiplex protein assays for clinical applications rely upon antibodies as the basic protein detection approach. This is a major limitation. The replacement of antibodies with alternative protein capture agents that exhibit the selectivity and affinity of good monoclonal antibodies, and yet are chemically and physically robust, is probably the toughest technology challenge today for multiplex protein diagnostics. Several approaches have emerged, ranging from nucleic acid aptamers (Proske *et al*., 2005) to peptides (Lam *et al*., 1993) to peptide multi-ligands (Agnew *et al*., 2009) assembled via *in situ* click chemistry. None of the approaches, however, has yet been demonstrated to compete effectively with monoclonal antibodies in terms of the combination of cost, ease of production, and selectivity/affinity for the cognate protein. If a solution to this problem does emerge, it will accelerate the development and deployment of many of the micro/nano technologies alluded to above.

Milestones

3-year:

- Develop and refine non-antibody-based methods to detect protein biomarkers.
- Devise mechanisms to incorporate antibodies into microfluidics chips.
- Increase the focus on developing and refining methods for blood and tissue processing within the assay platform.

5-year:

- Incorporate the methodologies developed above into multiplexed, integrated, miniaturized diagnostic assays. Hopefully these will be point-of-care tests.
- Conduct clinical trials on emerging diagnostic tests.
- Gain FDA approval for the first cancer nanotechnology-based diagnostic test.

10-year:

- Increase the use of multiplexed assays applicable to biomarker discovery research.
- FDA approval of various next generation diagnostic tests.

Nanotechnology in Tumor MicroRNA Profiling and Validation

Shanthi Ganesh and Mansoor Amiji

Northeastern University, Boston, MA

Tumor microRNA

MicroRNAs (miRNA) are a class of endogenous small, single stranded non-coding RNA molecules (about 22 nucleotides long) that play key roles in a variety of biological processes such as development, differentiation, proliferation, and cellular apoptosis. They generally function by blocking messenger RNA translation and/or affecting endogenous mRNA degradation (Figure 3). Accumulating evidence indicates that miRNAs are mechanistically involved in the development of various human malignancies, an observation which suggests these molecules represent a promising new class of cancer biomarkers and a significant target for cancer prevention and therapy (Paranjape *et al*., 2009). Many miRNAs function as oncogenes or tumor suppressors, hence they are often dysregulated in a variety of cancers (Ventura and Jacks, 2009). Although major advances have been achieved over the last several years in cancer biology and new targeted therapeutics, the development of early diagnostic methods are still inadequate leading to late diagnoses. The evidence that indicates alterations in miRNA expression levels in various tumor cells as compared to normal cells is considered indicative of the correlation with disease initiation and progression (Visone and Croce, 2009).

Current microRNA profiling technologies

Tumor miRNA profiling is one possible application towards establishing a cancer diagnosis. Two of the widely used high throughput techniques used for miRNA profiling are the solid-phase oligo microarray platform (Liu *et al*., 2004) and the bead-based flow cytometric method (Lu *et al*., 2005). The oligo microarray gene expression profiling technique is based on the development of a microchip containing gene specific oligonucleotide probes generated from hundreds of miRNAs. After immobilizing the microchip to the solid support, the sample containing RNA is hybridized to this chip to get the signal (Liu *et al*., 2004). In addition to using large quantities of material, this semi-quantitative method also carries another limitation of cross hybridization among miRNAs of a similar family. The bead-based profiling method involves both amplification and hybridization and requires flow cytometry for analysis (Lu *et al*., 2005). Capture probes for a specific miRNA are synthesized and attached to a bead that is coded by a mixture of two fluorescent dyes for identification. A cDNA library made from the RNA sample is amplified by a PCR reaction using biotinylated primers, which are then enzymatically reacted with streptavidin-phycoerythrin to emit light of a wavelength that can be registered by a flow cytometer. Although this method is technically demanding as it requires both amplification and hybridization steps during sample analysis which introduce sample variability, it has the advantage of increased specificity in differentiating the expression of closely related miRNAs as well as higher sensitivity. Data obtained from both methods need to be validated by a second method such as northern blot or quantitative real-time PCR to confirm the miRNA expression levels. Profiling hundreds of samples using both of these techniques clearly demonstrated aberrant miRNA expression in numerous tumors compared to their normal counterparts suggesting that a link does exist between miRNA and cancer (Iorio *et al*., 2005; Murakami *et al*., 2006; Leidinger *et al*., 2010).

Nanotechnology in microRNA profiling

Nanotechnology is slowly finding its way into the miRNA profiling world in a variety of highly sensitive novel methods. One system involves a combination of surface polyadenylation (polyA) enzyme chemistry and nanoparticle-amplified surface plasmon resonance imaging (SPRI). Briefly, the RNA sample is first hybridized to a complementary, single-stranded locked nucleic acid (LNA) array or capture probes followed by the addition of poly(A) tails to the surface-bound miRNA. Poly(T) coated gold

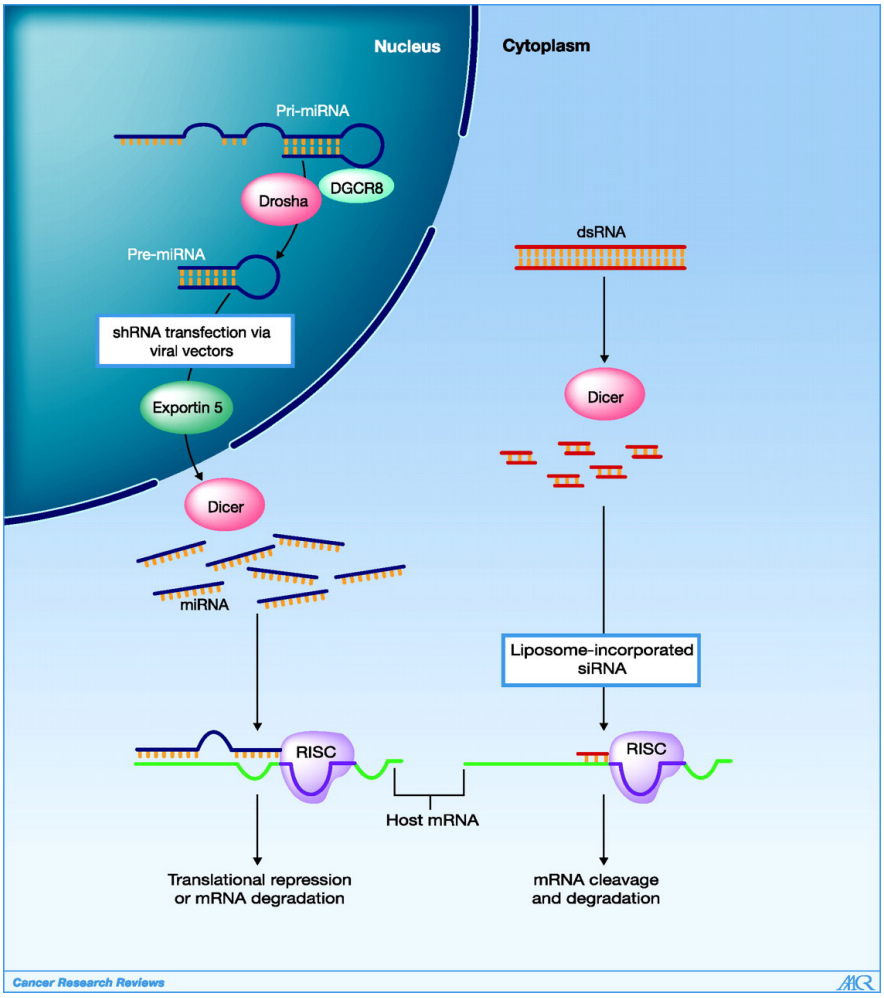

Figure 3 **Multiple components of the RNAi cascade are critical toward maturation of miRNA and siRNA complexes in humans. Altered expression of these entities is associated with poor outcomes and may limit RNAi function in cells. Introduction of exogenous RNAi sequences, such as siRNA, that bypass this machinery, may provide a novel pathway toward drug development in cancer therapeutics (reprinted from Merritt *et al*., 2010, Copyright, American Association for Cancer Research).**

nanoparticles are then hybridized with the poly(A)s present on the surface of bound miRNA for signal amplification and SPRI. A microarray image is obtained from a scanner that detects gold nanoparticles. This novel method is described to be very sensitive and reported to detect miRNAs down to a concentration of 10 fM, detecting a mere 5 attomoles of the miRNA (Fang *et al*., 2006).

Another reported nanotechnology-based method uses a biosensor that has the capacity to detect and quantitate miRNA in the fM range. It uses a microscopic platform made with gold and titanium microelectrodes interspaced with wells containing miRNA capture probes. The miRNA phosphate backbone uses its anionic nature to catalyze the reaction of polyaniline nanowire formation from a solution of cationic aniline particles. This closes an electrical circuit between gapped electrodes and results in an immediate digital readout. The recorded conductance correlates directly to the amount of hybridized miRNA (Fan *et al*., 2007). A method utilizing electrocatalytic nanoparticle tags for microprofiling has also been reported (Gao and Yang, 2006). This involves the generation of isoniazid (an antibiotic) capped OsO_2 nanoparticles and immobilization of oligonucleotide capture probes to an In_2O_3-SnO_2 electrode. After hybridizing the periodate-treated miRNA to the oligonucleotide capture probes, the nanoparticle tags (isoniazid-capped OsO_2 nanoparticles) are brought to the electrode to chemically amplify the signal. The addition of these nanoparticles to the hybridized miRNA molecules leads to the formation of electrocatalytic system generating a measurable current. Although the idea of amplified chemical ligation has been shown with only three miRNAs so far, it could be easily extended to wide range of miRNAs. As reported previously, the methods utilizing nanotechnology also need to be validated by a second method such as northern blot or quantitative PCR to confirm the miRNA expression levels.

These methods have been developed to address the sensitivity and specificity of existing profiling methods. They were also developed to reduce the total RNA required for the assay. Although they are very time consuming,

methods that require hybridization and polymerization steps are reported to be more specific and accurate. These nanotechnology-based procedures have been described to be sensitive to the fM range where previous technologies worked in the picomolar range. In summary, all of the methods used address a variety of specific needs, ranging from cost, sample size, sample quantity, speed, and ability to identify new miRNAs.

miRNA gene profiling, while providing important insights into plant and animal biology, have technical pitfalls associated with the current methodologies that need attention (Nelson *et al*., 2008). For example, various aspects of cellular processing, differential stability of specific miRNAs, and global miRNA expression regulation need special consideration when performing profiling experiments. Additional issues affecting profiling include the impact of pre-clinical variables, the substrate specificity of nucleic acid processing enzymes used in labeling and amplification, and the tissues used in new miRNA discovery and annotation. Another consideration is the cross-comparison between the results of different gene profile platforms. It has been shown previously that different cDNA-based miRNA profiling microarray techniques provide results with lack of reproducible comparability and low accuracy as there is presently no standardized methodology for hybridization-based profiling of miRNA (Yin *et al*., 2008). It is important, therefore, to focus more on technical parameters to increase the validity, reliability, and credibility of the assays.

In summary, a number of key issues need to be addressed to achieve meaningful and reproducible results in miRNA gene expression array studies. These include a well-defined clinical question, a statistically valid experimental design, consideration of tumor heterogeneity, identification of normal controls, and a robust platform using statistical and computational analysis of diagnostic predictors followed by independent validation (Tricoli and Jacobson, 2007). It was also suggested by the experts that accurate miRNA measurements are challenging due to dynamic miRNA expression, high miRNA sequence homology, and the lack of consensus on normalization methods (Tricoli and Jacobson, 2007). One recommendation would be to have probes with control probes with matching melting temperatures. Thus, the usefulness of using miRNA profiles for cancer detection and diagnosis depends on carefully designed translational studies taking into consideration the best methods for sample collection, miRNA isolation, miRNA quantitation, and data analysis.

Milestones

3 year:

- Develop a robust, clinically-relevant multiplexed assay system that can rapidly profile the tumor miRNA in patient samples and aid in early diagnosis of disease.

5 year:

- Complete characterization of tumor miRNA profiles in different types of human solid and hematological cancers as a function of disease progression, aggressiveness, and refractivity.
- Validate and correlate miRNA profiles with other methods of genetic and phenotypic tumor profiling (e.g., histology, western blot, etc.).

10 year:

- Develop a nanotechnology-based platform for rapid characterization of tumor miRNA profiles to allow for patient-specific clinical decision making. Ideally, this device or devices should be multiplexed and allow for small sample analysis such as tumor micro-biopsies.

Targeted Drug Delivery

Dong Moon Shin

Emory University School of Medicine, Atlanta, GA

Targeting tumor cells

The addition of targeting ligands mediates specific interactions between therapeutic nanoparticles (TNPs) and the tumor cell surface. Ligand-targeted therapeutic nanoparticles (TNP) are expected to selectively deliver drugs and especially cytotoxic agents specifically to tumor cells and enhance intracellular drug accumulation. Mechanisms of TNP internalization into target cells via receptor-mediated endocytosis have been well characterized.

Ligands targeting cell surface receptors can be natural molecules like folate or growth factors such as epidermal growth factor (EGF), which have the advantages of lower molecular weights and perhaps lower immunogenicities than antibodies (Figure 4). Modified antibodies can also be used as targeting moieties in an active targeting approach. Monoclonal antibodies (mAb) or antibody fragments, such as antigen binding fragments (Fab') or single chain variable fragments (scFv), are the most frequently used ligands for targeted therapies. Compared with mAbs, antibody fragments can reduce immunogenicity and improve the pharmacokinetic profiles of nanoparticles. In recent years, engineered antibody mimetics called affibodies, such as that against HER2, have been used to conjugate to thermosensitive liposomes (Affisomes) and to poly-(D, L-lactic acid) (PLA)-PEG-maleimide copolymer for delivery of paclitaxel (Alexis *et al*., 2008; Puri *et al*., 2008).

Once active targeting is achieved, the next important question is whether the targeted TNPs can be internalized in the target cells. Drugs released outside the cells can disperse or redistribute to the surrounding normal tissues rather than be delivered exclusively to the cancer

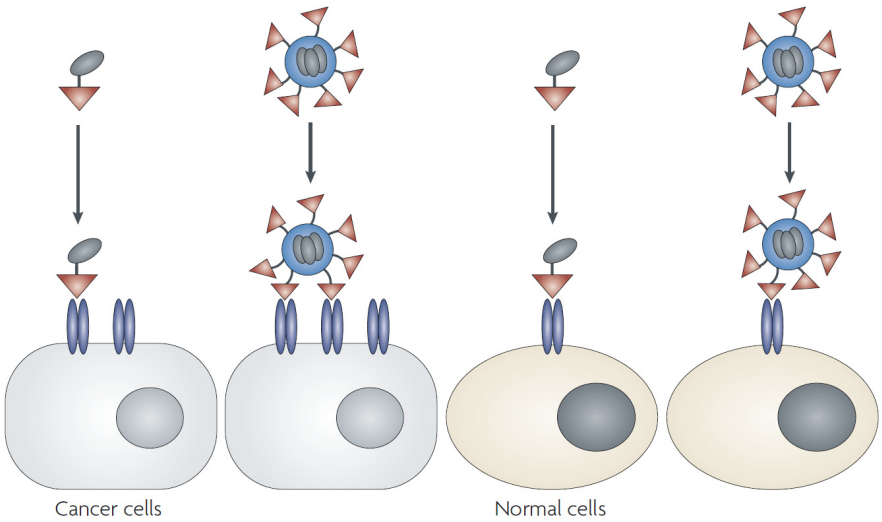

Figure 4 **Nanoparticles with numerous targeting ligands can provide multi-valent binding to the surface of cells with high receptor density. When the surface density of the receptor is low on normal cells, then a molecular conjugate with a single targeting agent and a targeted nanoparticle can compete equally for the receptor as only one ligand–receptor interaction may occur. However, when there is a high surface density of the receptor on cancer cells (for example, the transferrin receptor), then the targeted nanoparticle can engage numerous receptors simultaneously (multi-valency) to provide enhanced interactions over the one ligand–one receptor interaction that would occur with a molecular conjugate (reprinted with permission from Davis *et al*., 2008, Copyright, Nature Publishing Group).**

cells. *In vitro* and *in vivo* comparisons using internalizing or non-internalizing ligands have shown that the intracellular concentration of drug is much higher when the drug is released from TNPs in the cytoplasm after internalization. Several recent studies have demonstrated binding and internalization of targeted TNPs. Transmission electron micrographs have shown a polymer-based TNP containing human transferrin protein targeting agent bound to the cell surface, internalized into the cytoplasm and localized in the endosome. Using N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer-doxorubicin-galactosamine (PK1, FCE28068), which has progressed to a phase II clinical trial, galactosamine moieties bind to the asialoglycoprotein receptor on hepatocytes (Duncan *et al*., 2005). These promising early clinical results suggest the potential of targeted TNPs as effective anti-cancer drug delivery systems. In an *in vivo* animal study, targeted TNP-delivered paclitaxel was mainly located in tumor cells, while non-targeted TNP-delivered paclitaxel was detected intercellularly (Wang *et al*., 2009).

Targeting the tumor microenvironment

There is an ongoing debate as to whether attaching a targeting ligand to a TNP is necessary, because the enhanced permeability and retention (EPR) effect is believed to play a major role in directing TNP accumulation into a cancer tissue area (Figure 5). When tumor vasculature is at a well developed stage, this might be true; however, for small tumors that lack a well-developed vasculature, targeting tumor cells or even the tumor microenvironment could be more effective. For example, the accumulation of Abraxane is in part due to endothelium transcytosis initiated by the binding of albumin to a cell surface glycoprotein gp60 receptor which induces formation of transcytotic vesicles *(*caveolae) (Petrelli *et al*., 2010). These data support the idea that targeting caveolae might provide a universal portal to pump drugs out of the blood and into nearby tissue. The addition of two tumor-homing peptides, LyP-1 and CREKA, selected from phage-display to Abraxane enhances accumulation of this TNP in tumor tissue (Karmali *et al*., 2009). LyP-1-Abraxane inhibits tumor growth in a breast cancer xenograft model significantly better than the nontargeted Abraxane. CREKA can bind to clotted plasma proteins present in tumor vessels and interstitium. As expected, in a xenograft model, the CREKA-conjugated TNPs can block tumor vasculature, reduce blood flow, induce necrosis, and therefore significantly inhibit tumor growth. Other ligands targeting endothelial cells include RGD and urokinase plasminogen activator (uPA). The RGD motif in many proteins has a strong affinity and selectivity for cell surface $\alpha_v\beta_3$ integrins, which are overexpressed on the surface of endothelial cells of neocapillaries and also in some types of tumor cells. Therefore, RGD has been used as a ligand for tumor tissue targeting of TNPs. A tumor-homing iRGD (CRGDK/RGPD/EC) on TNPs achieved binding to tumor vessels and spread into the extravascular tumor parenchyma, while the conventional RGD ligand only delivered nanoparticle to the blood vessels (Sugahara *et al*., 2010).

Targeting metastatic, recurrent, and drug resistant cancers

Cancer metastasis and recurrence are major prognostic factors. Advances in our understanding of the molecular mechanisms by which these aggressive tumor phenotypes develop have provided a solid basis for targeting metastatic cancer using TNPs, which is a new research emphasis in this field. Targeting a specific microenvironment, such as the tumor vasculature to inhibit the colonization of metastatic cancer cells in a new organ site is one application of TNPs in the treatment of metastatic disease. Targeting the extracellular signature of metastatic cancer cells is another task in the field. For example, a PEGylated liposome modified with a fibronectin-mimetic peptide has been developed to target metastatic colon cancer cells which overexpress integrins $\alpha_5\beta_1$, since fibronectin is one of the specific ligands binding to this integrin pair (Garg *et al*., 2009). In addition, as one of the factors contributing to bone metastasis of breast cancer, osteopontin is overexpressed in both osteoclast and breast cancer cells and may be responsible for the interaction between the bone and cancer cells that drives osteolysis. Osteopontin, therefore, serves as a target to prevent bone metastasis. A sustained delivery of polymeric nanoparticles carrying antisense DNA against osteopontin and bone sialoprotein in rats with breast cancer metastasis has shown significant reduction of bone metastasis, establishing this nanoparticle formulation as a promising therapeutic agent (Elazar *et al*., 2010). Currently there are no reports of the specific killing of recurrent cancer cells using targeted TNPs, due to the lack of ligands specific for this population. Similarly, though many studies have illustrated the potential of utilizing TNPs to minimize drug resistance, the lack of specific ligands for drug-resistant cancer cells limits the application of targeted TNPs to these aggressive populations.

Future challenges

These include: (1) Identify appropriate ligands specific to cancer cells from different tissue types and to metastatic, recurrent, and drug-resistant cancer populations. Of particular interest would be to identify ligands that can target both tumor cells and the tumor microenvironment simultaneously; (2) Develop organ-specific orthotopic animal models including those of metastasis and drug resistance, which are essential to evaluate TNPs in the treatment of specific phenotypes; (3) Conduct pre-clinical PD/PK and toxicology studies for Investigational New Drug (IND) filing; and (4) Collaborate with FDA to conduct the relevant clinical trials.

As mentioned, the debate is still ongoing as to the necessity of attaching a targeting ligand to a TNP, since the EPR effect is believed to play a major role in directing TNP accumulation in cancer tissues. To obtain a clear

answer, quantification methods should be developed to address tissue and intracellular drug accumulation when using TNPs for drug delivery. Tumor models representing different types and stages of cancer should then be used to evaluate targeted TNPs as compared with the non-targeted TNPs. Furthermore, catching and killing circulating metastatic cells or cancer stem cells which metastasize or are resistant to conventional cancer treatment by targeted TNPs is another attractive application for the treatment of aggressive cancer types. These studies will also require appropriate animal models.

Clinical potential

Accumulating evidence supports that TNPs, particularly targeted TNPs, have great potential in reducing toxicity and enhancing efficacy of currently used chemotherapeutic agents. In the next few years, more and more clinical trials using targeted TNPs are expected. Furthermore, theranostic nanoparticles will be used in the clinic for early detection and treatment of cancer, particularly metastatic cancers.

Milestones

3 year:

- Develop new targeted TNPs focusing on the tumor, microenvironment as well as metastatic disease.
- Conduct release and biodistribution animal studies for targeted TNPs to provide better insight into how targeted TNPs work *in vivo*.

5 year:

- Conduct phase 0/I/II clinical trials of some new TNPs therapies.

10 year:

- Evaluate the clinical application of TNPs *in vivo* to facilitate better understanding of TNPs in terms of their PK characteristics, tissue distribution, and long-term toxicity assessment.
- Carry out phase III clinical trials and gain FDA approval for TNPs therapies.

Nanotherapeutic Delivery Systems

Dong Moon Shin

Emory University School of Medicine, Atlanta, GA

Current status

Nanotherapeutic delivery systems can be used to deliver therapeutic entities such as small molecule drugs, peptides, proteins and nucleic acids either as single agents or as multiplexed combinations (Gindy and Prud'homme, 2009; Alexis *et al*., 2010; Ruoslahti *et al*., 2010). Increasing evidence indicates that the selective delivery of nanoparticle therapeutic agents into a tumor mass could minimize toxicity to normal tissues and maximize bioavailability and cell killing. These advantages are mainly attributed to changes in drug tissue distribution and pharmacokinetics. Furthermore, it has been demonstrated that nanoparticles can escape from the vasculature through the leaky endothelial tissue that surrounds the tumor and can accumulate in certain solid tumors via the EPR effect. After escaping from the vessel, non-targeted nanoparticles will typically be cleared from the tumor sites due to their lack of cellular uptake. In contrast, tumor-targeted nanoparticles can enter tumor cells from the extracellular space via receptor-mediated internalization (Figure 5). A variety of tumor targeting ligands, such as antibodies, growth factors, and cytokines have been used to facilitate the uptake of carriers into target cells (Dong and Mumper, 2010). Tremendous progress has been made and some tumor-targeted nanotherapeutics are already in clinical trials or have been approved by the FDA.

Diversity of delivery platforms

Many different types of nanoparticles have been widely studied for therapeutic delivery (Portney and Ozkan, 2006). These include polymers (polymeric nanoparticles, micelles, dendrimers), lipids, viruses and nanotubes. These therapeutic delivery carriers have many advantages, such as: 1) water solubility; 2) low or no toxicity; 3) biocompatibility or biodegradability; and 4) amenability of their surface to further modification for related applications (Table I) (Cho *et al*., 2008).

Polymers such as albumin, chitosan, and heparin are ideal carriers for the delivery of nucleic acids, protein and drugs, as demonstrated by nanometer-sized albumin-bound paclitaxel (Abraxane) which is already in clinical use (Fu *et al*., 2009; Kratz, 2008; Petrelli *et al*., 2010). The amphiphilic block copolymers of micelles can form a nano-sized core/shell structure in aqueous media (Venkatraman *et al*., 2010). Hydrophobic drugs can be loaded into the hydrophobic core region, whereas the hydrophilic shell region stabilizes the hydrophobic core and makes the polymers water-soluble. These nanoparticles are appropriate for intravenous administration. Genexol-PM is a cremophor-free polymeric micelle-formulated paclitaxel, which has been studied in a clinical trial in patients with advanced refractory malignancies. In addition, multi-functional polymeric micelles containing targeting ligands with imaging and therapeutic agents are being developed and have the potential to be used in the near future. A dendrimer is a synthetic polymeric macromolecule of nanometer dimensions, composed of multiple highly branched monomers that emerge radially from the central core; their monodisperse size and available hydrophobic internal cavity make them attractive for drug delivery, and the polyamidoamine dendrimer has been used as a cisplatin carrier for tumor therapy. Dendrimer-based multi-functional drug delivery systems consisting of imaging contrast agents, targeting ligands and therapeutic drugs can be engineered due to the modifiable surface characteristics of dendrimers. Liposomes are self-assembling closed colloidal structures composed of lipid bilayers and have a spherical shape in which an outer lipid bilayer surrounds a central aqueous space (Estella-Hermoso de Mendoza *et al*., 2009). Many cancer drugs have been loaded onto such lipid-based systems, including the anthracyclines doxorubicin (Doxil, Myocet) and daunorubicin (DaunoXome), which have been approved for the treatment of metastatic breast cancer and AIDS-related Kaposi's sarcoma. Several types of viruses including cowpea mosaic virus, cowpea chlorotic mottle virus, canine parvovirus, adenovirus, and bacteriophages have been developed for biomedical and nanotechnology applications that include tissue targeting and drug delivery (Farokhzad and Langer, 2009; Singh and Kostarelos, 2009). Additionally, a variety of ligands and antibodies have been conjugated to viruses for specific tumor targeting *in vivo*. Some viruses, such as canine parvovirus, have a natural

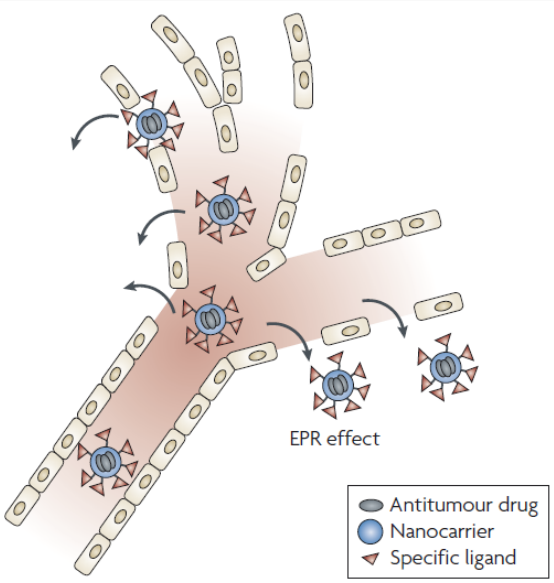

Figure 5 The enhanced permeability and retention (EPR) effect. Nanoparticle agents are designed to utilize the EPR effect to exit blood vessels in the tumour, to target surface receptors on tumour cells, and to enter tumour cells by endocytosis before releasing their drug payloads (reprinted with permission from Davis *et al.*, 2008, Copyright, Nature Publishing Group).

affinity for receptors that are upregulated on a certain tumor type, and thus can be used for targeted drug delivery. Carbon nanotubes are carbon cylinders composed of benzene rings which can be used as carriers to deliver conjugate peptides, proteins, nucleic acids, and therapeutic agents.

Other nanoparticles exploit their own inherent nature for their therapeutic effects. Plasmonic gold nanoparticles are very promising for photothermal cancer therapy because of their strongly enhanced radiative and nonradiative photothermal properties due to surface plasmon resonance; these nanoparticles absorb light $10^{5\text{-}6}$ times more strongly than the most strong light-absorbing dye molecules (Arvizo *et al.*, 2010; Cobley *et al.*, 2010). Thus, when gold nanoparticles are targeted to cancer cells, electromagnetic irradiation with an optical laser will induce heat capable of destroying the surrounding cells. However, most of these diverse nanoparticle carriers do not have inherent imaging properties to enable monitoring of their distribution *in vivo*. Magnetic iron oxide nanoparticles have emerged as a new generation of MRI contrast agents for imaging/guided drug delivery due to their long blood retention time, low toxicity, and biodegradability (Lin *et al.*, 2010; Sokolov *et al.*, 2009). Changes in MRI signals produced by drug-loaded iron oxide nanoparticles may be used to estimate tissue drug levels and facilitate real-time monitoring of the tumor's response to therapy.

There are several strategies to incorporate drugs into nanoparticles - drugs can be linked to the carrier coating, deposited on the surface layer, or trapped within the nanoparticles themselves. After a drug is loaded into the nanoparticle, it can usually be released by (1) diffusion out of the particles; (2) vehicle rupture or dissolution; (3) the process of endocytosis of the formulation; or (4) pH-sensitive or enzyme-sensitive dissociation. Anti-cancer agents such as paclitaxel, doxorubicin, and cisplatin are suitable for nanoparticle delivery, and tumor-targeted nanoparticles are also ideal carriers for systemic delivery of siRNA *in vivo*.

Recently, increasing concerns have focused on the safety of nanotherapeutic delivery systems. Although few studies have shown visible toxicities in animal studies, sub-chronic and chronic toxicity studies have yet to be conducted for most nanoparticles. Little is known about the long term fate of nanoparticles *in vivo*. Most nanotherapeutic delivery systems are non-targeted, thus more intensive studies using tumor-targeted nanoparticles as drug delivery carriers are needed. The precise mechanism by which nanoparticle-loaded drugs are released *in vivo* remains unclear. It will be helpful to label both the nanoparticles and the loaded drugs using special fluorescein dyes to perform real-time monitoring of their biodistribution and intracellular localization *in vivo*. In addition, quantification of nanoparticle and drug levels in different organs must be addressed.

Future challenges

There are still many challenges to overcome when constructing nanoparticles for drug delivery. These include: (1) evaluation and minimization of related toxicities induced by nanoparticles; (2) enhancement of drug loading efficiencies; (3) modification of the surface and control of the size and charge of nanoparticles for adequate delivery; (4) regulation of circulation duration; (5) controlled drug release; (6) nanotherapeutic stability; (7) specific accumulation in the tumor and minimal uptake in normal tissues and organs by selecting ideal tumor-targeted ligands; (8) selection of appropriate nanoparticles for particular drug delivery targets; (9) construction of smart tumor-targeted nanoparticles in which the loaded drug is released only within tumor cells; (10) pre-clinical pharmacokinetic/pharmacodynamic (PK/PD) and toxicity evaluation of nanotherapeutics; and (11) regulatory and approval issues related to nanoparticles.

Clinical potential

A selective increase in tumor tissue uptake of current anti-cancer agents would be of great interest for cancer chemotherapy given the lack of specificity of anti-cancer drugs for cancer cells. Nanotherapeutic delivery systems can be used to carry established drugs that have been widely used in the clinic, and can optimize their therapeutic index by increasing the drug concentration ratio in diseased tissue to normal tissue and by enhancing the anti-tumor effect while reducing side effects. In addition, new anti-tumor macromolecules such as peptides, siRNA, proteins, and small molecule inhibitors can potentially be systemically delivered using these targeted nanoparticle pharmaceuticals, an approach which may be explored in future clinical studies.

Table 1. Types of nanocarriers for drug delivery

System	Structure	Characteristics	Examples of compounds	Ref.
Polymeric nanoparticles (polymer-drug conjugates)	Drugs are conjugated to the side chain of a linear polymer with a linker (cleavable bond)	(*a*) Water-soluble, nontoxic, biodegradable (*b*) Surface modification (pegylation) (*c*) Selective accumulation and retention in tumor tissue (EPR effect) (*d*) Specific targeting of cancer cells while sparing normal cells—receptor-mediated targeting with a ligand	Albumin-Taxol (Abraxane) PGA-Taxol (Xyotax) PGA-Camptothecin (CT-2106) HPMA-DOX (PK1) HPMA-DOX-galactosamine (PK2)	(7) (11) (12) (14) (58)
Polymeric micelles	Amphiphilic block copolymers assemble and form a micelle with a hydrophobic core and hydrophilic shell	(*a*) Suitable carrier for water-insoluble drug (*b*) Biocompatible, self-assembling, biodegradable (*c*) Ease of functional modification (*d*) Targeting potential	PEG-pluronic-DOX PEG-PAA-DOX (NK911) PEG-PLA-Taxol (Genexol-PM)	(16) (17) (18)
Dendrimers	Radially emerging hyperbranched synthetic polymer with regular pattern and repeated units	(*a*) Biodistribution and PK can be tuned (*b*) High structural and chemical homogeneity (*c*) Ease of functionalization, high ligand density (*d*) Controlled degradation (e) Multifunctionality	PAMAM-MTX PAMAM-platinate	(64) (21)
Liposomes	Self-assembling closed colloidal structures composed of lipid bilayers	(*a*) Amphiphilic, biocompatible (*b*) Ease of modification (*c*) Targeting potential	Pegylated liposomal DOX (Doxil) Non-pegylated liposomal DOX (Myocet) Liposomal daunorubicin (DaunoXome)	(22) (23) (24)
Viral nanoparticles	Protein cages, which are multivalent, self-assembled structures	(*a*) Surface modification by mutagenesis or bioconjugation—multivalency (*b*) Specific tumor targeting, multifunctionality (*c*) Defined geometry and remarkable uniformity (*d*) Biological compatibility and inert nature	HSP-DOX CPMV-DOX	(29, 30) (27)
Carbon nanotubes	Carbon cylinders composed of benzene ring	(*a*) Water-soluble and biocompatible through chemical modification (organic functionalization) (*b*) Multifunctionality	CNT-MTX CNT-amphotericin B	(34) (33)

Abbreviations: PGA, poly-(L-glutamate); HPMA, *N*-(2-hydroxypropyl)-methacrylamide copolymer; PEG, polyethylene glycol; PAA, poly-(L-aspartate); PLA, poly-(L-lactide); PAMAM, poly(amidoamine); DOX, doxorubicin; MTX, methotrexate; PK, pharmacokinetics; EPR, enhanced permeability and retention; CNT, carbon nanotube; HSP, heat shock protein; CPMV, cowpea mosaic virus.

(reprinted from Cho *et al.*, 2008, Copyright, American Association for Cancer Research).

Milestones

3 year:

- Synthesize 20-30 tumor-targeted nanotherapeutic delivery systems with high quality and yield for cytotoxic agents such as doxorubicin, paclitaxel, cisplatin, and siRNA as well other small molecules.
- Demonstrate successful delivery of highly potent, toxic therapeutics using nanoparticle platforms. Enable widening of therapeutic window for these compounds through the nanoparticle delivery.

5 year:

- Perform PK/PD studies of the best nanotherapeutic systems in mice and rats (including human tumor xenografts) and in large animals.
- Determine the lowest non-toxic dose using the best nanotherapeutic system in humans. Study nanoparticle biodistribution and toxicity to identify those that are most efficacious and least toxic.
- Extend preclinical toxicology studies of the best nanotherapeutic systems from mice to rats and dogs. Conduct phase O, I, and II clinical trials.
- Gain FDA approval of at least one nanoparticle-based targeted therapeutic.

10 year:

- Gain FDA approval and commercialize several targeted nanotherapeutic delivery systems for cancer applications.

Nanotechnology Theranostics

Demir Akin and Sanjiv Sam Gambhir

Stanford University, Stanford, CA

Theranostic nanoparticles

Theranostics can be classified into two main subgroups based on historical origins: a) Classical theranostics and b) Nanotheranostics. Classical theranostics refers to a treatment platform wherein the therapy is guided by a specific diagnostic test, which stratifies the patients for treatment eligibility. For purposes of this review the focus is on nanotheranostics which will herein be referred to as "theranostics." These are multi-functional nanodevices with capabilities for simultaneous detection and drug delivery in a single device. Theranostics can further be subgrouped into two categories: a) Imaging Theranostics, (ITNs): nanodevices and nanomaterials with diagnostic imaging and therapy functionalities (e.g. optical or electromagnetic nanoparticles, such as drug functionalized Quantum Dots and magnetic nanoparticles) and b) Detection Theranostics (DTNs): theranostics with biodetection and biosensing capabilities and a therapy modality (e.g. polymeric nanomaterials/nanoparticles that sense and respond to their environment and modulate the release of a cargo drug or therapy modality). There are overlapping hybrid, multi-functional theranostics as well, such as the fluorophore-labeled imaging nanoparticles with environment responsive polymeric shells and a therapeutic magnetic core (Figure 6) (Vo-Dinh, 2007).

Theranostic nanoparticles are constructed using a variety of chemistries and come in an array of physical forms. These particles can be composed of metals, non-metals, synthetic polymers, dendrimers, lipids, nucleic acids, biologics (e.g. viral vectors), synthetic peptides, and combinations therein. Their shapes can take the form of solid spheres (e.g. quantum dots, iron oxide nanoparticles, etc.) or non-spherical geometries (e.g. nanorods, nanodiamonds, nanotriangles, nanocages, and hybrids of these forms). Each of these types of nanoparticles has shown to have unique advantages and disadvantages in diagnostic and therapeutic management of various cancers.

There are a number of ITN agents in use today. Encapsulated iron oxide core and polymeric nanoparticles are used for cancer detection via magnetic resonance imaging (MRI) or optical detection (fluorescence, Raman, near-infrared, luminescence) and to directly ablate tumors

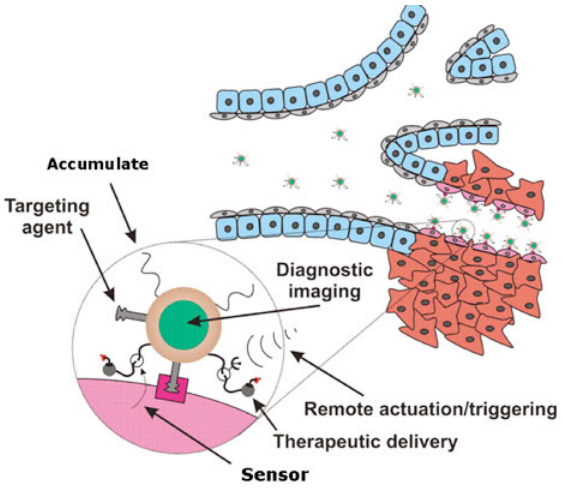

Figure 6 Schematic depicting multi-functional theranostic agents having properties of both the ITN and DTN classes. The nanoparticles interact with tumor cells via a targeting moiety and are capable of imaging, therapy, and sensing the microenvironment (Figure courtesy of Dr. Sangeeta Bhatia, MIT).

via either thermal or non-thermal means. Another available theranostic agent is cancer targeting aptamer-modified Quantum Dots conjugated with Doxorubicin (Ho and Leong, 2010). These agents are typically bio-passivated by incorporating them into liposomes or other polymer-based biocompatible matrices. A different class of theranostic device, such as plasmonic nanobubbles (Lukianova-Hleb *et al*., 2010), uses gold nanoparticles and transient photothermal excitation to create vapor-based nanobubbles for selective non-thermal, mechanical destruction of targeted cancer cells. Due to the photonic nature of the energy source, this theranostic modality is equipped with optical guidance to the desired anatomic location in addition to diagnostics via optical scattering and mechanical therapy.

One example of an up and coming class of DTNs is combining conventional PET imaging with the biomarker F-18 fluorodeoxyglucose (^{18}F-FDG) to monitor

the increased glucose metabolism common to many tumors. Response to imatinib treatment as well as recurrence can be assessed in patients with gastrointestinal stromal tumors using the high sensitivity and resolution capability of a PET camera (Goldstein *et al*., 2005).

Monoclonal antibodies (mAb) as well as engineered antibodies are being used to provide specific diagnostic information in conjunction with PET and other clinical imaging modalities with targeted-therapy for cancers (Wei *et al*., 2008). In a recent study, tumor targeting of radiolabeled-anti-CD20 diabodies, engineered antibody analogs of Rituximab, could detect low-grade B-cell lymphomas (Olafsen *et al*., 2010). The availability of good positron emitters, improvements in radiochemical labeling, and the development of scanners for advanced PET-computed tomography (PET-CT) are the crucial drivers of this theranostic imaging development. It is highly anticipated that immuno-PET will be playing an important role in the future improvements and tailoring of therapy and also in the expansion of the number of this class of theranostics.

Future challenges and clinical aspects

Despite the fact that many nanomedical tools have found great utility and application in *in vitro* studies, pre-clinical cancer models, and/or intra-operative investigational use, to date very few of these technologies have reached the clinical trial stage. Only a few of these platforms, such as the gold or iron oxide-based theranostics and the multi-functional-dendrimeric nanoparticles, are amenable for rapid translation into the clinical development cycle for in-patient use. Some of the issues impeding the progression of the theranostics into the clinic are centered on the lack of acceptable specificity of these theranostic modalities for the cancer target sites and the toxicity associated with these technologies. Our lack of adequate *in vivo* predictive capabilities for the ADME-Tox of these nanomedical tools are the major source of failure in the progression from the research and development phase to clinical use.

Currently, the efficacy of an anticancer treatment is evaluated by gross physical endpoint changes that occur in tumors following the therapy such as tumor volume changes, density/opacity changes, differential distribution pattern of a contrast reagent, and vascularization. Other indicators, such as cell death and apoptosis, occur on a cellular level and can instead provide a faster means of assessment of response to therapy via theranostic imaging using multi-modal nanoparticles equipped with treatment capabilities. This would change the timeframe of verifying the efficacy of a treatment from months to days. Nanotechnology offers the potential to develop highly sensitive imaging agents and *ex vivo* diagnostics that can determine whether a therapeutic agent is reaching its intended target and whether that agent is killing malignant or support cells, such as growing blood vessels. Such systems could be constructed using nanoparticles containing an imaging contrast agent and a targeting molecule that recognizes a biochemical signal only seen when cells undergo apoptosis. Further improvements of this type of system could provide clinicians with a way of determining therapeutic efficacy in a matter of days after treatment, rather than months. Targeted nanoscale devices may also enable surgeons to more readily detect the margins of a tumor prior to resection or to detect micrometastases in lymph nodes or tissues distant from the primary tumor. This information would inform therapeutic decisions and have a positive impact on patient quality-of-life issues.

Tumor and cancer cell phenotype heterogeneity and adaptive anti-cancer drug resistance are complex challenges in cancer necessitating our diagnostic and therapeutic response to be diverse and comprehensive. Future nanomedical interventions have to be safe, specific, affordable, and rapidly adaptive from the perspectives of both targeting as well as choice of therapy in order to tackle the formidable challenge presented by the fast developing drug resistance during the course of an anti-cancer treatment regime. These needs necessitate continued improvements in understanding cancer biology, clinical oncology, drug targeting and delivery, nanotechnology, biologically relevant engineering, and materials science.

Milestones

3 year:

- Accelerate the development of theranostics with improved targeting and biocompatibility, imaging contrast capability, controlled drug release, biobarrier breaching ability, ease of preparation, favorable cancer cell uptake, tumor distribution, reduced toxicity, and controllable clearance from body.
- Demonstrate several examples of preclinical to clinical stage nanoscale devices capable of reliable and validated earlier cancer signature and/or metastasis detection and simultaneous therapy by appropriate multi-faceted approaches. These theranostic devices will be able to interrogate and therapeutically target multiple (≥four) signaling pathways concurrently.

5 year:

- Work closely with the FDA and pertinent entities to facilitate the establishment of scientific framework and guidelines for a timely but properly regulated approval of nanoscale diagnostics, therapeutics, theranostics, and preventive agents.
- Submit at least three to five INDs in the area of multi-functional (≥four functions) nanotheranostics.

10 year:

- Demonstrate proof of concept intelligent nanomedical devices or integrated nanoscale comprehensive device systems that can simultaneously assess different types of genomic, transcriptomic, and proteomic level events involved in cancer predisposition, initiation, progression and metastasis in order to offer multi-faceted targeted therapy for these detected events. Ideally, these active nanomedical devices will be administered for a predetermined duration and operate

in vivo or embedded within the vicinity of target tissues and organs.

- Develop high impact molecular imaging approaches capable of detecting and imaging specific molecular activities that have the potential for clinical applications *in vivo*. These novel molecular imaging developments will focus on both of the following long-term translational goals: (1) imaging the characteristic markers and functions of normal cells in control human subjects and patients and (2) imaging the characteristic markers and biochemical or physiological abnormalities of cancer cells in patients.

siRNA Therapeutics

Sara S. Hook

Office of Cancer Nanotechnology Research, CSSI, National Cancer Institute, Bethesda, MD

Introduction

Often cancers arise due to overexpression of oncogenes or expression of inappropriate protein products produced by gene translocations, insertions, or rearrangements. For example, some types or chronic myelogenous leukemia, acute myelogenous leukemia, or acute lymphoblastic leukemia are caused by chromosomal translocations that fuse together portions of the BCR serine/threonine kinase and the ABL tyrosine kinase (Perrotti *et al*., 2010). The phenotypic effect is that the ABL kinase activity is uncontrolled due to the loss of regulatory protein sequences and addition of non-catalytic sequences from BCR. One approach to treating cancers that arise by these types of mechanisms would be to silence the incorrect gene and/or replace it with a normal copy. The later strategy would only be needed in cases of haplo-insufficiency, where one copy of the normal gene would not suffice and an additional copy is needed. A critical barrier, however, for gene silencing or gene replacement is efficient delivery mechanisms. The promise of nanoparticle-mediated delivery is well recognized and early clinical trials have already shown that double-stranded silencing RNAs or "siRNAs" are a feasible strategy for use in humans in the clinic (Davis *et al*., 2010).

The mechanisms for cellular siRNA processing (as well as for short-hairpin (sh) RNA) have been reviewed elsewhere and will only be briefly addressed here. These RNAs can be taken up by cells "as is" but most efficiently when packaged in either liposomes (siRNAs) or viral vectors (shRNAs). They are processed by the dicer family of enzymes to remove the hairpin sequences (if needed) and then both categories of RNAs are incorporated into the RISC complex which serves to further process them into single-stranded RNAs (Figure 3). According to their sequence homology they bind to endogenous RNAs and either facilitate their degradation or inhibit translation of the RNA into protein, thus effectively silencing gene expression (Morris, 2008). A major advantage to this approach is that once a gene is implicated in cancer initiation, progression, or metastasis, it can be targeted without an intrinsic knowledge of its function, regulation, pathway involvement, etc. In addition, with careful sequence design and validation, the approach can be very specific with little cross reactivity.

Aside from siRNA efficacy and specificity, two physiological factors loom large, those being stability/pharmacokinetics and cell and tissue targeting. There are a number of ongoing clinical trials addressing various diseases that utilize siRNAs and most of these are simple saline-based formulations for local or topical delivery for the eye, respiratory tract, and skin. Systemically, however, siRNAs injected intravenously are subject to rather rapid degradation and clearance via renal excretion. Despite this, some of these "naked" siRNAs have been shown useful in decreasing tumor growth and metastasis in a number of animal xenograft models (Vaishnaw *et al*., 2010). Modifications of the phosphodiester backbone, bases, or ribose ring have been reported to increase half lives in addition to chemical conjugation to cholesterol and protein moieties and undoubtedly research in this area will continue (Singh *et al*., 2010). In the area of targeting "naked" siRNAs, researchers have conjugated them to antibodies through a biotin-strepavidin linkage and successfully directed them to glial cells demonstrating the potential to penetrate the blood/brain barrier (Xia *et al*., 2007).

Delivery strategies for siRNA

In order to increase therapeutic benefit, it would be advantageous to protect the siRNA in "packaging" while specifically delivering the cargo to the intended target cell or tissue (Oh and Park, 2009). This goal in particular is where nanotechnology will shine (Figure 7). Due to the anionic, hydrophilic nature of RNAs, they are especially amenable to packaging within the cationic environment of lipid carriers such as liposomes, micelles, lipid-based nanoparticles, and emulsion formulations. Several examples of siRNA delivery via liposomes are entering phase I trials, including ALN-VSP, which simultaneously targets multiple transcripts of each VEGR and KSP (kinesin spindle protein) for liver tumors (Alnylam Pharmaceuticals website), and ATU027, which targets

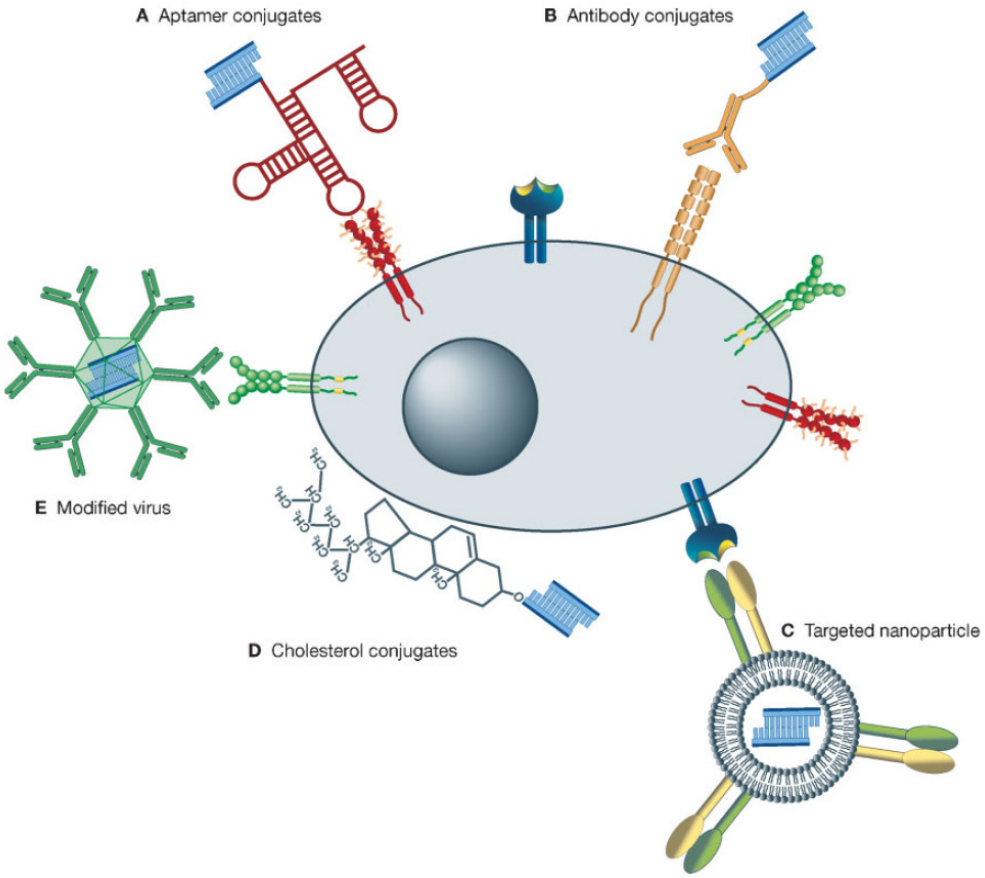

Figure 7 Delivery strategies for RNAi. The cell (grey ellipse) contains a nucleus (dark circle) and a cell membrane (dark ellipse). Cell surface molecules such as receptors are present on the cell surface (shown in color). RNAi therapeutics (mainly siRNA (blue)) can be targeted to the cell surface molecules via different delivery vehicles. They can be conjugated to aptamers (A), which can bind specifically to cell surface molecules and be internalized. siRNAs can also be conjugated to cell specific antibodies (B) and be delivered to the target cells via recognition of cell surface molecules by the specific antibody followed by internalization through endocytosis. Targeted nanoparticles (C) transport RNAi therapeutics to specific cells. The modifications of the nanoparticles (targeting ligand) can interact with receptors on the cell surface and the nanoparticle with its load can be internalized. Cholesterol conjugated siRNAs (D) can be delivered to cells and be internalized by the interaction of the cholesterol with the membrane through hydrophobic interactions, triggering clathrin-dependent endocytosis. Modified viruses (E) can also be used for cell specific delivery of RNAi therapeutics by cell specific cell surface interactions triggering endocytosis (Reprinted from Tiemann and Rossi, 2009, Copyright, Wiley and Sons).

protein kinase N3 (PKN3) and has shown promise in human xenograft tumors of pancreas and prostate in mouse models (Aleku *et al*., 2008). One study from Germany using one patient with CML resistant to both chemotherapy and the abl tyrosine kinase inhibitor imatinib found that siRNA to *BCR-ABL* packaged within liposomes decreased the fusion transcript and resulted in cellular death without adverse side effects (Koldehoff *et al*., 2007). All of these siRNA liposomal formulations, however, while showing promise do not appear to be equipped with a cell specific targeting mechanism. Calando Pharmaceuticals, however, is in the process of phase I trials using the first targeted siRNA for human cancers, CALAA-01 (Davis *et al*., 2010). They silenced the M2 subunit of ribonucleotide reductase (RRM2) by using nanoparticles directed to melanoma cells through a peptide targeting the transferritin receptor. Several lines of evidence indicate that RRM2 mRNA and protein levels are decreased following nanoparticle therapy and that the mechanism is through cellular action of the siRNA. Given the rapid pace in which the signaling pathways of various tumor types are being dissected as well as biomarkers being identified, we can expect to see an increase in this type of targeted, systemic nanoparticle therapy.

Clinical impact

Currently, 14 siRNA-based clinical trials have been initiated (Vaishnaw *et al*., 2010), four of which are for cancer and three of these are in liposomal formulations. Some remarkable features of nanoparticle delivery are the relatively low amount of immune system response (as discussed in a previous section) and decreased drug induced toxicity. Several clinical trials directed at other

diseases utilizing siRNA therapy that are not nanoparticle based have been terminated due to either no overall improvement of the condition (such as visual acuity), or due to non-specific effects of the treatment such as activation of innate immunity (Kleinman *et al*., 2008; Vaishnaw *et al*., 2010). The latter clinical outcome might be circumvented by nanoparticle formulations. Since cancer can arise by a vast array of mechanisms, some of which are more specific to tissue type and others that are integral pathways important for the life of all cells, the therapeutic strategy to combat it would be most advantageous if it were targeted to tumor cells and spared normal cells. This approach can be achieved using nanoparticle formulations.

As the research continues to develop siRNA-based nanotherapeutics, we expect an increasing number of diverse packaging systems for siRNAs (Gao *et al*., 2010). For example, siRNA has recently been incorporated into stimuli-responsive PEGylated nanogels which when subjected to the lower pH of the tumor intracellular environment enhances lysosomal and endosomal release (Oishi and Nagasaki, 2010). In addition, reports have described such concepts as delivering siRNAs via magnetic nanoworms (Agrawal *et al*., 2009), dendrimers (Ravina *et al*., 2010), nanocrystals (Namiki *et al*., 2009), and carbon nanotubes (Menard-Moyon *et al*., 2010). An alternative approach to siRNA but still targeting RNA degradation to decrease gene expression would be to employ DNAzymes. These are short synthetic DNAs with inherent enzymatic activity capable of cleaving target RNAs (Ravina *et al*., 2010). Nanoparticles containing DNAzymes could prove to be a valuable therapeutic approach in the future.

Beyond the potential value of siRNAs in therapy they can also be used for *in vitro* and *in vivo* diagnostics. They have already been used to screen for biological regulators as therapeutic targets and validate them for potential clinical applications. In addition, siRNAs can be useful for assay development and can serve as positive and negative controls to establish the relevant signaling pathways involved in cancer progression, angiogenesis, metastasis, etc. Recently, siRNAs have been tagged with fluorescent markers which can, in theory, be used to track which cells have received the siRNA in a living organism (Oishi and Nagasaki, 2010). In the future, we expect that more and more multi-functional nanoparticles will not only deliver siRNAs to the target tumor types but will also enable real-time imaging, thermal ablation, and/or small molecule drug delivery.

Milestones

3 year:

- Expand the repertoire of chemical modifications to the siRNAs themselves as well conjugation to other carbohydrates, lipids, proteins, etc. to increase stability, bioavailability, and intracellular processing.
- Increase research on catalytic oligonucleotides capable of cleaving the target RNAs.

5 year:

- Test new nanotechnology-based delivery vehicles for siRNA.
- Devlop formulations containing multiple siRNAs to target multiple signal transduction pathways.
- Conduct late stage clinical trials for siRNA delivery.

10 year:

- Increase focus on personalized therapies using tumor sequencing data to direct decisions on nanoformulations using multiple siRNAs specific to the patient's tumor genetic or proteomic profile.
- Gain FDA approval for nanoparticle-based therapies using siRNA delivery.

Nanotechnology to Overcome Tumor Drug Resistance

Lara Milane and Mansoor Amiji

Northeastern University, Boston, MA

Tumor microenvironment, hypoxia, and cancer stem cells

The tumor microenvironment contributes to the development of **m**ulti-**d**rug **r**esistant (MDR) cancer and affects a patient's response to treatment. The microenvironmental selection pressures that contribute to the development of MDR include abnormal tumor vasculature, hypoxia, decreased pH, increased interstitial fluid pressure, and alterations in the expression of tumor suppressors and oncogenes. MDR cells often have increased DNA repair mechanisms, up-regulation of ABC transporters, and a decreased apoptotic response (Figure 8) (Dong and Mumper, 2010; Gottesman *et al*., 2002). Abnormal tumor vasculature is the most defining characteristic of the tumor microenvironment; the vasculature of a tumor is highly disorganized and inefficient relative to normal vasculature. These fluctuating states of vascularization lead to regions of acute and chronic hypoxia. Cancer cells undergo a complex phenotypic transformation under hypoxic conditions. This survival cascade is initiated when the alpha subunit of Hypoxia Inducible Factor (HIF) translocates from the cytoplasm to the nucleus where it complexes with the beta subunit of HIF, forming an active transcription factor. The HIF complex binds to hypoxia responsive elements (HRE's) on target genes, inducing transcription (Harris, 2002; Semenza, 2003; Depping *et al*., 2008). The vast array of HIF targets include genes involved in invasion, proliferation, metabolism, drug resistance, and glycolytic pathways. (Denko, 2008; Semenza, 2010a; Semenza, 2010b). In fact, with less oxygen available for energy acquisition through oxidative phosphorylation, these hypoxic cancer cells revert to aerobic glycolysis for the production of ATP (the Warburg effect) (Guppy, 2002).

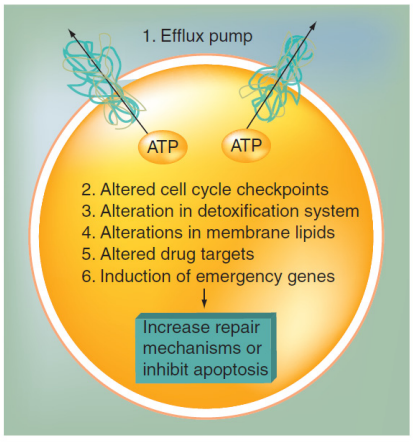

Figure 8 Summary of the mechanisms in which cultured cancer cells have been shown to become resistant to cytotoxic anticancer drugs. The efflux pumps at the plasma membrane include P-glycoprotein, multi-drug resistance protein family members and breast cancer resistance protein (reprinted with permission from Dong and Mumper, 2010, Copyright, Future Medicine Ltd.)

The relationship between MDR, cancer stem cells, and hypoxia is only beginning to be understood (Barnhart and Simon, 2007). There are two primary cancer stem cell theories: (1) cancer stem cells are regular stem cells that have gone awry and cause cancer and (2) cancer stem cells arise from a subpopulation of cancer cells. Probably both of these concepts are correct and vary on the particular tumor. Recently it has been shown that a subpopulation of precancerous cells can acquire stem-like properties, becoming cancer *derived* stem cells (Mani *et al*., 2008; Morel *et al*., 2008). Importantly, many of the mutations that can cause this phenotypic change also facilitate MDR. Different studies have shown that cell stressors such as hypoxia and activation of an epithelial to mesenchymal transition (EMT) are efficient inducers of cancer aggression and MDR phenotypes and induce stem-like properties in cancer cells such as the expression of stem cell factor (SCF) (Jewell *et al*., 2001; Harris, 2002; Kizaka-Kondoh *et al*., 2003; Semenza, 2003; Shannon *et al*., 2003; Brahimi-Horn *et al*., 2007; Cosse and Michiels, 2008; Han *et al*., 2008; Nanduri *et al*., 2008; Semenza, 2008; Ansieau *et al*., 2010). Inhibiting SCF or EMT in MDR cells may increase the effectiveness of treatment by reducing the apoptotic threshold of these putative cancer stem cells, thereby removing the repopulating source of a tumor.

Multi-pronged strategy to overcome MDR – enhancing delivery efficiency and altering cellular phenotype

As our understanding of cancer deepens, one concept that becomes increasingly evident is that cancer is a heterogeneous disease on both the intra- and inter-patient levels. As such, a therapy that treats only one phenotype is not slated for success. For a cancer therapy to be effective the therapy must be multi-faceted, simultaneously treating multiple aspects of the disease.

Nanocarriers serve as ideal delivery solutions for combination therapy which is required for effectively treating MDR cancer. The benefits of nanocarriers include, (1) they can be engineered to achieve multiple effects using one system; (2) nanocarriers improve the therapeutic index of drugs and can alter the pharmacokinetic profile of agents; (3) they preferentially accumulate in the tumor environment thanks to the EPR effect and their capacity to be conjugated to targeting moieties; and (4) nanocarriers avoid drug efflux by preferentially localizing agents in the peri-nuclear region of a cell, away from membrane localized efflux pumps.

The most effective treatment for MDR should address multiple MDR phenotypes which can be facilitated using the multi-functional platforms available through nanotechnolgy. As such, combining a traditional cytotoxic chemotherapeutic agent with one or more of the following strategies could prove effective:

1. inhibiting ABC-transporter mediated drug efflux
   a. small molecule inhibitors such as verapamil
   b. siRNA/shRNA silencing
2. lowering the apoptotic threshold
   a. inhibiting the Warburg effect (aerobic glycolysis)
   b. increasing intracellular ceramide
      i. exogenous delivery
      ii. siRNA silencing of glucosylceramide synthase
   c. stimulating cytochrome c release (mitochondrial permeability transition pore complex)
   d. increasing pro-apoptotic Bcl2 family members
   e. decreasing anti-apoptotic Bcl2 family members
3. increasing tumor suppressor activity (such as p53 gene therapy)
4. decreasing oncogene activity
5. decreasing the stem-like properties of MDR cells (*exploratory, e.g.* silencing stem cell factor)

Tumor-targeted multi-functional nano-delivery systems

Although nanocarriers passively target cancer through the EPR effect, using active targeting can increase the specificity of nanocarriers for MDR cells. It is relatively simple to modify the surface of nanocarriers with targeting residues. Common targeting residues include antibodies for cancer antigens, ligands for over-expressed cell-surface proteins, and lectins for carbohydrate targeting. Active targeting can further improve the therapeutic index of an agent by decreasing off-target accumulation. Common targets include EGFR receptors, transferrin receptors, and folate receptors.

Active targeting also alters the mechanism of uptake of nanocarriers. Non-targeted nanocarriers are taken up by non-specific endocytsosis whereas targeted nanocarriers are internalized via their target-specific mechanism. For example, nanocarriers that target the EGFR receptor are internalized via a flip-flop mechanism, a rapid process compared to endocytosis. Active targeting, therefore, not only decreases the residual toxicity of a system, it can further alter the pharmacokinetic profile of a system. Some nanocarrier systems are designed to target more than one MDR phenotype, further increasing their specificity to MDR cells. However, the *in vivo* effects of active targeting are inconclusive and need to be validated and explored.

Milestones

3 year:

- Develop animal models of refractory disease that recapitulate the human disease in terms of location, genotypic and phenotypic heterogeneity, etc.
- Characterize tumor microenvironmental factors (i.e., soluble and insoluble) on the development of clinically-relevant refractory disease.
- Identify and validate drug targets and strategies to overcome resistance through a multi-factorial approach that utilizes efficiency in drug delivery, residence, and intracellular penetration as well approaches to overcome cellular resistance.

5 year:

- Establish robust pre-clinical programs to develop and test multi-functional nanoparticulate drug delivery systems in appropriate models of refractory diseases.
- Evaluate the toxicological properties of nanoparticulate formulations under GLP conditions.

10 year:

- Establish collaborations with pharmaceutical industries and clinical centers to rapidly facilitate the transfer of technologies from academia to cancer patients.
- Establish a clinical development program for multi-functional nanoparticulate systems using the appropriate guidance from regulatory agencies.

New Contrast Agents with Improved Spatial and Temporal Resolution

Gregory Lanza

Siteman Cancer Center, Washington University, St. Louis, MO

Current status

Molecular imaging agents promise new unprecedented opportunities to assess changes in tumor microanatomical and physiological character with greater spatial and temporal resolution in cancer patients. Until recently, the majority of advancements in cancer imaging have favored improved detectability of minute masses. Today, we can detect minute lesions with high resolution CT and MRI, and the challenge has become deciding whether a lesion is a benign fascinoma or an early malignancy. Early categorization of a pathology as benign stable disease, an inflammatory lesion, or a malignancy has dramatic implications in medical management, but commonly minute tissue anomalies cannot be characterized, necessitating a conservative "wait and see" management approach. Re-evaluation in three to six months is common to assess gross morphological changes that would point to cancer but an aggressive tumor may have already disseminated beyond the original primary site.

Delineation of an unknown pathology suspected of cancer requires biopsy for microscopic and biochemical characterization. Although such procedures are routinely performed, the acquisition of tissue specimens by surgical resection or fine-needle aspiration still presents challenges due to lesion accessibility, tissue sample quality and artifacts, and a patient's willingness to undergo the procedure. Biopsy procedures become particularly troublesome when the lesion is small (< 1 cm) and centrally located. Molecular imaging offers a noninvasive mechanism to assess microanatomical changes, for example the development of a neovasculature, or the expression of important biochemical markers, such as HER-2/neu. These pathological signatures serve not only as an aide in tumor diagnosis and grading, but also as responsive biomarkers to treatment efficacy. Improved noninvasive characterization will lead to definitive diagnoses sooner, and because the lesion is "visualized" *in vivo*, key anatomical and metabolic information destroyed or nonassayable by excising the tissue is retained.

Microanatomical and biochemical measurements of tumors require robust, quantitative techniques with high spatial and temporal resolution, but what constitutes high resolution is often a matter of perspective and dependent on the medical question posed. For example, nuclear "hot spot" imaging with PET or SPECT tracers are detected with very high sensitivity per tracer concentration but low spatial resolution (millimeters) when compared with MRI. PET has high temporal resolution for kinetic studies given adequate nuclear tracer counts, which allows convenient and rapid assessments of probe "wash-in" or "wash out" of a target tissue. Moreover, in some situations, low spatial resolution may be adequate for noninvasive tissue characterization when a boolean answer based on the presence or lack of radioactivity for a pathognomonic receptor or biochemical pathway is sought. Unfortunately, ^{18}FGD is completely nonspecific except for a prevalent accumulation in cells with high metabolic rate and receptor specific ligands are foiled by nature's utilization of the same receptors and pathways for many cell types. For example, radiolabeled RGD peptides (arginine, glycine, aspartate) and antibodies, particularly directed to the $\alpha_v\beta_3$-integrin, have been used to target and characterize tumor angiogenesis by PET (Haubner *et al*., 1999; Beer *et al*., 2007) and SPECT (Liu *et al*., 2007). However, these small molecules, despite exquisite chemistry, readily permeate beyond the tumor and bind many cell types, including macrophages and tumor cells, which diminishes the signal specificity for angiogenesis per se (Zitzmann *et al*., 2002; Liu *et al*., 2007).

Dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) can detect changes in tumor microvasculature permeability to MR blood pool contrast agents and some studies have correlated these kinetic estimates with traditional measures like MVD, but initial clinical trials have yielded inconsistent results either due to insufficient standardization of the endpoints or technique issues (Jayson *et al*., 2002; Liu *et al*., 2005; Schmieder *et al*., 2008). However, MR molecular imaging with paramagnetic nanoparticles facilitates high-resolution 3D mapping of angiogenesis (Schmieder *et al*., 2008; Winter *et al*., 2008). Such *in vivo* studies clearly indicate that angiogenesis is peripherally distributed nonuniformly around a tumor in a heterogeneous pattern associated with

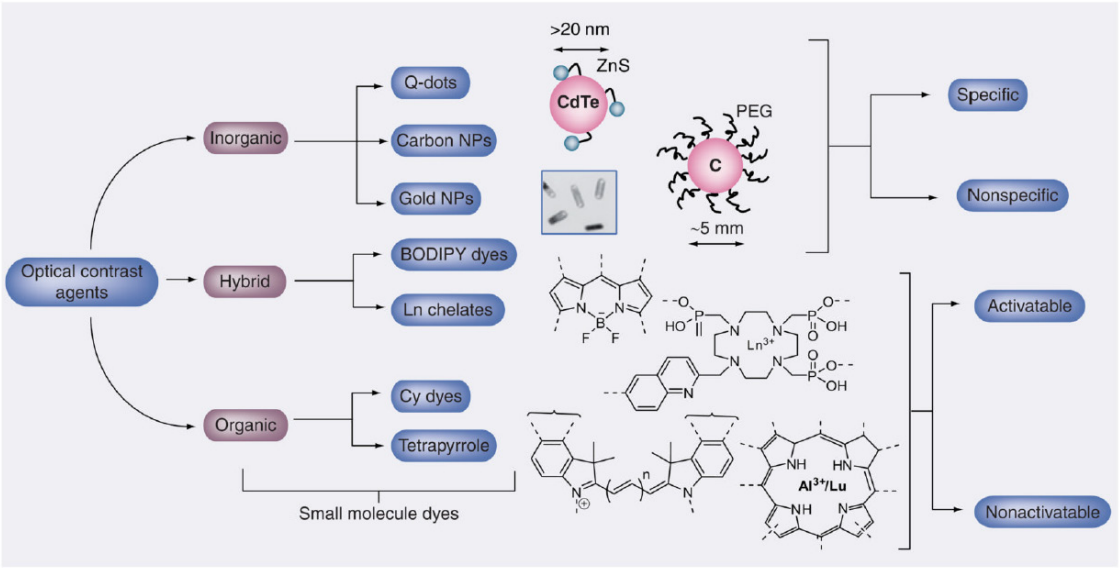

Figure 9 **General classification of optical contrast agents (reprinted from Pan *et al.*, 2010, Copyright, Future Science).**

rapidly proliferating cancer growth fronts. Clearly, neither fine needle aspiration into a tumor core nor routine histology sections randomly oriented on resected tumors are severely prone to sampling error and cannot provide reasonable quantitative estimates of neovascularity, that could be used to risk-stratify patients for anti-angiogenesis treatment.

Like MRI, CT offers tomographic imaging with very high spatial and temporal resolution, which overcomes the issues of motion in many tissues including pulmonary and gut. However, the inherent tissue x-ray contrast is low, necessitating the use of iodinated low molecular weight contrast agents. Although like gadolinium-based DCE, CT can be used for kinetic modeling, the data provide no biochemical and limited pathological prognostic information. New nanoparticle based homing agents have been reported that overcome the marked insensitivity of CT to contrast, but the majority of pre-clinical applications studied to date have been directed toward targets with high epitope density or to passive accumulation in macrophages, liver, or spleen.

Ultrasound is another important clinical imaging modality with moderately high spatial (mm to micron, dependent on frequency) and very high temporal resolution (real-time). Once a planar technique, the advent of 3D ultrasound provides improved spatial registration. Ultrasound is the clear favorite with regard to cost, portability, and ease of use, but it has significant limitations. The most common problems are derived from the limited “acoustic windows” available where bone, gas (bowel or lung), or depth of tissue do not preclude or compromise imaging results. Moreover, achieving high imaging resolution is dependent upon increasing the transducer insonification frequency. While high frequency transducers, 25 MHz and up, offer the best temporal – spatial resolution, but sound penetration decreases with increasing frequency, requiring these targets to be near the skin or accessible with intravascular ultrasound catheters. Ultrasound molecular imaging with microbubbles (Klibanov *et al.*, 1999), echogenic liposomes (Alkan-Onyuksel *et al.*, 1996), and PFC nanoparticles (Lanza *et al.*, 1996) have been demonstrated *in vivo*, but the microbubbles due to their highly amplified ultrasound reflectance, offer the greatest contrast and the greatest noise, even a single bubble, targeted or random, is detectable.

Temporal resolution is becoming an important factor in the clinical use of ligand-targeted molecular imaging agents, particularly with respect to drug delivery with theranostic agents. Initially, molecular imaging will play a role in stratifying patients into optimal treatment plans, but soon thereafter, the effectiveness of treatment, particularly for small tumors in asymptomatic patients, will utilize molecular imaging follow response and manage the pharmacologic strategy. With the advent of theranostic agents (as discussed previously), now demonstrated repeatedly in pre-clinical models, imaging will be used not only to stratify patients to best treatment regimen, but also to confirm dosing of targeted therapy using the coupled imaging feature. While repeat imaging with ultrasound and MRI will pose no known health threats, recurrent use of ionizing radiation (PET and CT) may predispose to unwarranted health side effects. This issue gains significance as younger patients with cancer identified and treated earlier.

A concern of temporal resolution will be inherent in the contrast agent used. For instance, with MRI paramagnetic nanoparticles (and the like), imaging occurs with one to three hours after injection and there is no residual contrast signal at the target site 24 hours after treatment. Repeat imaging can easily occur within two days. In contradistinction, most targeted iron oxide contrast

agents cannot be imaged until 24 or 48 hours after treatment due to blood pool induced magnetic artifacts and the persistence of the iron oxide nanoparticles at the target site can last variably from weeks to months, limiting timely reinterrogation. Ultrasound microbubbles have very short blood half-life and tissue persistence, making them an excellent choice for serial imaging, but the acoustic rupture of microbubbles for perfusion-reperfusion targeting techniques or for acoustically enhanced drug delivery, may alter the presentation of bioepitopes for homing and confound serial imaging results. Both CT and MRI agents dependent upon heavy elements and repeat dosing must address the possibility of toxic accumulation. Metal administered for contrast must be chemically stable *in vivo* and predominantly eliminated from the body in a few days with virtually all of the remaining metal excreted in a few weeks.

Future challenges

The clinical utility of molecular imaging with high spatial and temporal resolution depends on the quantitative reproducibility of signal estimates derived within an individual patient. Contrast imaging must be quantitatively correlated with target expression and be repeatable. To date, the depiction of a tumor hot-spot PET or angiogenic map with MRI are dependent on thresholding techniques, which must be optimized for pathologic correlation and normalized for serial within patient comparison over time. Today's clinical imaging techniques present have 20 to 30% variability related to performance technical issues (e.g., MR coil or nuclear detector placement) and manufacturer provided internal hardware recalibration routines. The current drive to quantitative, reproducible imaging must continue with the institution of more stringent operational standards, development of National Institute of Standards and Technology (NIST) calibration phantoms, and rigorously validated imaging software and hardware capable of absolute measurements. Without meeting this essential challenge, molecular imaging with or without drug delivery will not achieve its potential and could fail to become a proven, clinically relevant and reimbursable procedure to improve cancer management. Fortunately, these goals are more or less engineering accomplishments that can be achieved with determined effort.

Milestones

3 year:

- As nanotechnologies reach the clinic, the potential for early application for molecular imaging will become known as will the challenges of signal detection, reconstruction, and calibration within the human body. This information will be critical as clinical trials proceed. We expect each new agent reaching the clinic will elucidate new problems and uncover unexpected opportunities which will enhance formulation of the global and specific issues governing efficacy, safety, and clinical use compatibility.

5 year:

- The information achieved in clinical trials must drive hardware-software vendors to implement improved validated software to optimize image acquisition and presentation to physicians for clinical interpretation. Molecular imaging literally means detecting, presenting and characterizing nascent cancers, which is akin to finding the proverbial "needle in a haystack" with robust quantitative rigor.
- Concurrently, basic and clinical scientists must work together to devise guidelines for utilizing the imaging information alone and with drug delivery in an effective, cost-responsible manner leading to the improved health care management of cancer patients.
- Because the information developed over the first five years of the clinical molecular imaging revolution will be "first of its kind data in man", dogmatic views and perceptions of the past will need to be revisited, revised and often discarded. Willingness to accept new molecular imaging data and to discard our preconceived notions will be the greatest achievement of this period.

10 year:

- Expanded use of first generation molecular imaging technologies combined with new generation systems, which must robustly overcome the transendothelial barrier to nanoparticle delivery and expand opportunities for direct to cancer cell theranostic medicine. Insight into these pathways and mechanisms to utilize nature's machinery has already been achieved and our understanding is rapidly increasing.
- Next generation product candidates created over the next five years will reach the IND stage for clinical testing in five to eight years with the homing and size specificities needed overcome this targeting obstacle.
- During the last two years of this decade, these new generation nanomedicines should clear phase I safety and proof of concept hurdles and begin focused clinical study toward efficacious cancer applications considered intractable today.

Multi-modal Imaging

Paras N. Prasad

Department of Chemistry, SUNY Buffalo

Introduction

Currently a number of advanced imaging modalities are available in the pre-clinical and clinical setting, including magnetic resonance imaging (MRI), positron emission tomography (PET), computed tomography (CT), and optical imaging (OI) (Willmann *et al*., 2008). However, all these modalities vary in their limits of sensitivity, resolution, and depth profiling. Therefore, it is unlikely that a single imaging modality will provide conclusive evidence of a biological process or therapeutic response. In this regard, a synergistic combination of multiple non-invasive imaging technologies will play a critical role in the early detection of cancer and other diseases. The choice of these imaging techniques is driven by their ability to provide complimentary (structural and functional) information and enable cross-validation of imaging signals along with differences in resolution, sensitivity and clinical application. For example, OI approaches are valuable for *in vitro* and *in vivo* evaluation in pre-clinical model systems but are not ready for 'prime-time' in the surgical setting. In contrast, advanced imaging techniques such as MRI are widely used in clinical diagnosis and monitoring the response of patients to therapy. Therefore, the combined OI-MRI approach is likely to provide valuable information on the diagnostic/staging potential of our nanoplatform. In addition, combined with a therapeutic modality, such a system will facilitate the monitoring of therapy in real time. Such real-time monitoring would allow patients with 'non-responsive' tumors to avoid the side effects of ineffective treatment by enabling them to be switched in a timely manner to more appropriate therapies that are likely to offer better survival benefit (Prasad, 2004). However, successful realization of these objectives will require the development of novel multi-modal and biocompatible agents, along with multi-imaging instrumentation and software capable of co-registering the signals obtained from the various imaging modalities.

Nanoparticle-based probes have several advantages over traditional molecular agents because: (1) they provide a tunable, optically traceable (fluorescence, NIR and/or bioluminescence) chassis upon which targeting agents (antibodies, peptides, small molecules, etc.) can be added or changed to suit a specific need; (2) they enable multi-modality (e.g., optical, MR and radionuclide) imaging thus permitting concurrent evaluation for the same nanoparticle across different imaging platforms; (3) they enable targeted and sustained delivery of potent chemotherapeutic agents specifically to diseased sites, avoiding normal organs; (4) they can be functionalized with both imaging and therapeutic abilities (i.e., "theranostic" nanoparticles); (5) they are of sufficient size to permit multi-valency and therefore the potential for higher affinity binding than standard molecular agents; and (6) they enable imaging from the molecular level, to single cells, and to the entire, intact organism. This attribute further enables validation of the imaging marker by correlating results obtained *in vitro*, e.g., relying on the optical (fluorescence/near-infrared [NIR]) aspects of the probe, with those obtained *in vivo*, which may also rely on optical, radionuclide or MR imaging. Therefore, targeted multi-modal nanoparticles are expected to play a pivotal role in the development of the "next generation" of clinical agents for cancer diagnosis and treatment, as they will facilitate detection of both structural and functional anomalies which are characteristic of the early stages of cancer. Furthermore, the ability to simultaneously deliver chemotherapeutic agents specifically to tumor sites would greatly improve patient survival and post-treatment quality of life.

Current status

The rapid growth of *in vivo* multi-modal imaging arises from the convergence of established fields of *in vivo* imaging technologies, along with nanotechnology, as well as molecular and cell biology (Caruthers *et al*., 2007). The major hallmark of nanomedicine is the fabrication of multi-modal nanoprobes, which would not only incorporate multiple image-contrast agents, but also therapeutic probes and targeting molecules for site-specific delivery. Multi-modal nanoprobes can provide both structural and metabolic information specifically from diseased sites, thus leading to significantly improved imaging techniques for the detection of a variety of human cancers (e.g. breast,

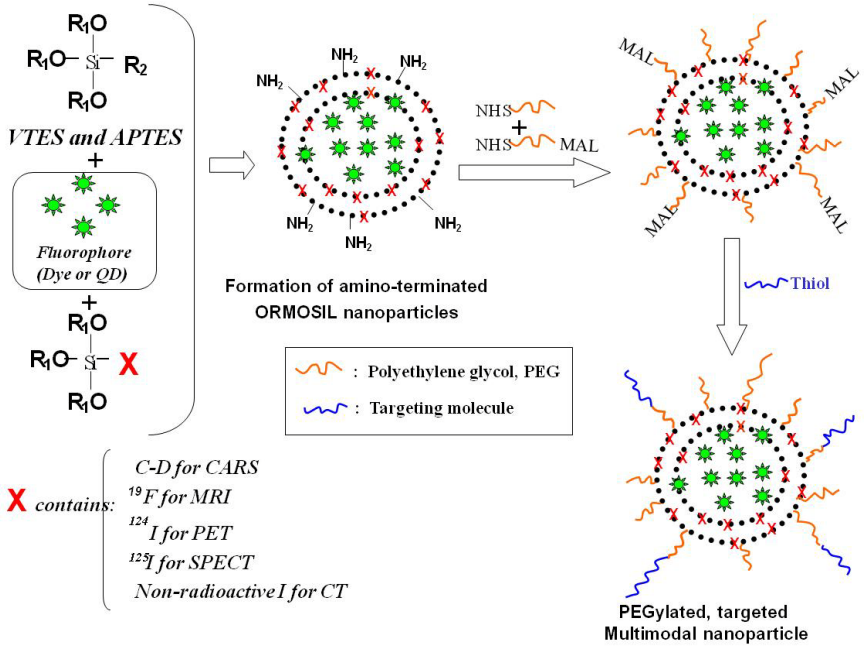

Figure 10 **Synthetic strategies for a ORMOSIL nanoparticle incorporating probes for different imaging modalities like optical, MR, SPEC, CT, and PET (Figure courtesy of P.N. Prasad).**

pancreas, lung, and prostate) including improved staging for occult metastases. In this regard, the combination of optical and MR contrast agents within a nanoparticle have gained much popularity owing to the feasibility of both *in vitro* (mainly using OI) and *in vivo* (mainly using MRI) imaging, without the involvement of any radioisotopes.

Optical imaging further facilitates image-guided surgery, which is an active area of current pre-clinical research. A number of such nanoformulations are currently in active developmental stage in several laboratories. NIR fluorophores have been combined with ultrasmall iron oxide nanoparticles and their effectiveness in imaging of cancer and other diseases, such as atherosclerosis has been shown (McCarthy and Weissleder, 2008). The feasibility of co-encapsulation of iron-oxide nanoparticles and optical probes within a silica shell (multi-functional 'nanoclinics'), which can be targeted specifically to cancer cells has also been demonstrated (Prasad, 2003). In addition, combined optical and MR imaging capability using upconverting nanophosphors with co-incorporated gadolinium has also been developed. Recently, polymeric nanomicelles incorporating optimal amounts of NIR phosphorescent optical probes and gadolinium have demonstrated specific target delivery and combined optical and MR imaging, both *in vitro* and *in vivo* (Kumar *et al*., 2009). All these multi-modal nanoformulations are currently undergoing advanced pre-clinical trials in orthotopic and transgenic cancer models.

In addition to OI and MRI, the rapid evolution of PET-SPECT and PET-CT scanner hybrids in the clinic encourages the fabrication of multi-modal nanoparticles co-incorporating OI, PET and SPECT probes (Nunez *et al*., 2010). In the pre-clinical set-up, there is also a growing interest in building and designing dedicated devices for specific applications, such as high-resolution scanners for imaging small animals in various molecular imaging centers worldwide. Fluorescence and bioluminescence optical imaging will provide a cheaper alternative to the more expensive and specialized microPET, microSPECT, and microMRI scanners. Along with volumetric tomographic imaging technologies such as SPECT and CT, which offer deep tissue penetration and high spatial resolution, noninvasive small animal optical imaging facilities will meet the growing needs of comprehensively imaging specialized animal models. These might include highly metastatic 'transgenic' tumor-model animals as well as larger non-human primates where the pathological anomalies are akin to that observed in humans. In this perspective the versatility of ORMOSIL nanoparticle platform for multi-modal imaging, incorporating a NIR fluorophore and ^{124}I PET imaging probes has been established. In addition, the ease of surface modification of the ORMOSIL based nanoparticles bolstered the conjugation of several imaging probes on the surface of the nanoparticles which includes ^{19}F for MR imaging as well as ^{124}I for SPECT/CT imaging. Figure 10 shows the application of multi-modal ORMOSIL nanoparticles developed for different imaging techniques.

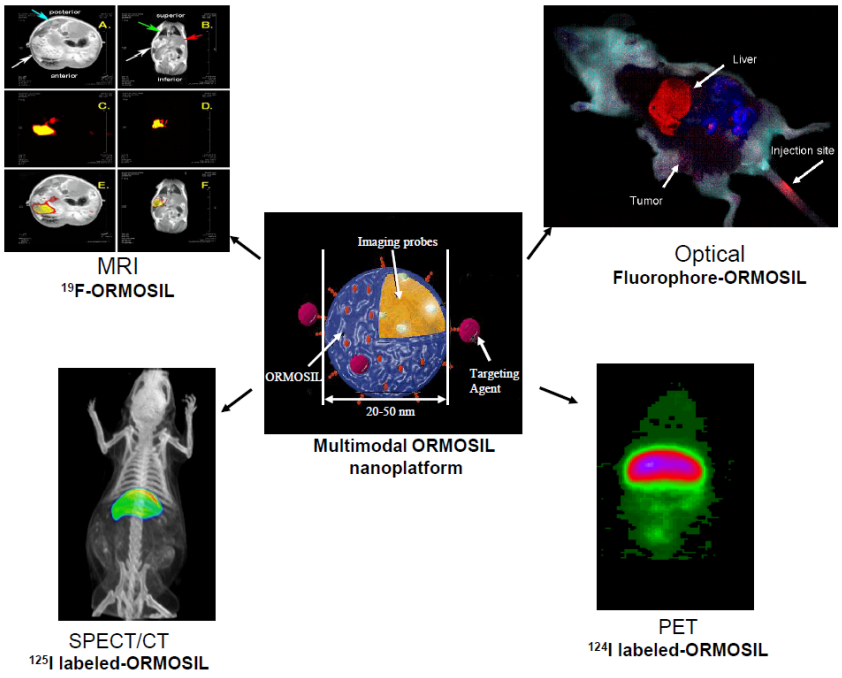

Figure 11 Multi-modal *in vivo* imaging using the ORMOSIL nanoplatform (Figure courtesy of P.N. Prasad).

Future challenges

Multi-modality imaging not only will facilitate the early diagnosis of diseases, but will also have the potential to monitor in real time the progress of a therapeutic intervention. In addition, it also enhances the precision of surgical intervention techniques. For instance, comprehensive surgical removal of cancer necessitates the removal of all cancerous cells surrounding the tumor site. Bioconjugated nanoparticles can be used as sensitive biomarkers to label only the cancerous cells and aid the surgeons in visualizing and safely resecting the tumors while reducing damage to adjacent healthy tissues. The future of multi-modality and nanomedicine would extensively involve efficient packaging of both diagnosis and therapy components within one biocompatible nanoprobe, leading to the fabrication of an ideal 'theranostic' agent.

The biggest challenge that nanotechnology faces at present is meeting all the safety guidelines required for gaining clinical acceptance, particularly those required by the FDA. Over the past decade, several nanoparticles, including polymeric, inorganic, and hybrids have been modified in terms of their size, shape and surface properties in order to meet these guidelines. Remarkable among them are the development of (1) 'stealth' nanoparticles, which can evade capture by the RES, (2) 'target-specific' nanoparticles, which accumulate only in the diseased organs/sites, bypassing normal ones, (3) ultrasmall iron-oxide nanoparticles, as well as cadmium-based quantum dots, which can eliminate themselves from the body through the renal filtration system, and (4) biocompatible nanoparticles, made up of natural polymers/biomolecules, such as chitosan, albumin, and calcium phosphate which are unlikely to evoke an immune response and will be well tolerated by the body. The introduction of Abraxane, the first nanoparticle-based clinical drug delivery system for the treatment of certain human cancers, has strongly mobilized nanotechnology researchers in the pursuit for other, more improved nano-based drug delivery systems (Miele *et al*., 2009). However, despite of all these developments, the non-specific accumulation and long-term persistence of nanoparticles *in vivo* continues to pose serious roadblocks toward their clinical acceptance. This challenge is particularly daunting in regards to multi-modal nanoparticles, where a number of components need to be assembled within a single nanosystem, potentially making the overall nanocomposite cumbersome and large in size. Therefore, the issues that need to be immediately addressed are properly balancing the necessary payloads during the fabrication of a multi-modal nanoparticle.

The significance of nanotechnology in multi-modal imaging relies on the efficient packaging of the different imaging probes and targeting molecules on a single nanoparticle system. There have been several reports mentioned earlier which combine OI as well as MRI efficiently but the foremost challenge still remained unanswered when combining OI and MRI with clinically relevant PET, SPECT/CT imaging. In this context there is speculation that the ORMOSIL nanoparticle which has shown a promise in combining the different modalities together may open a pathway into multi-modal imaging, combining all aspects of the clinically accepted imaging techniques (Figure 11). A systematic titration and assessment of the surface functionalities of the OMROSIL nanoparticles and their conjugation with different imaging

probes will result in a multi-modal nanoparticle platform for efficient *in vivo* imaging.

Clinical potential

There is a dire clinical need for agents that can provide comprehensive diagnostic information, initiate targeted and preferentially externally activated therapy, and assess the progression of therapy in real time. In this regard, multi-modal nanoparticles are ideal candidates that can address all the above challenges comprehensively. However, as stated earlier, meeting the safety requirements for clinical acceptance continues to be a huge challenge. Encouragingly, the incorporation of NIR fluorophores within clinically used iron oxide nanoparticles can potentially pave the way for faster clinical translation of such multi-modal agents. In addition, incorporation of NIR optical imaging probes with radioisotopic imaging probes such as SPECT and PET, within targeted, biocompatible nanoparticles is another attractive approach. Combining multi-modal imaging probes with a clinically acceptable nano-drug delivery system, such as Abraxane, will lead to the development of 'theranostic' agents, where the tumor response to the administered drug can be monitored in real time via non-invasive imaging in the clinic.

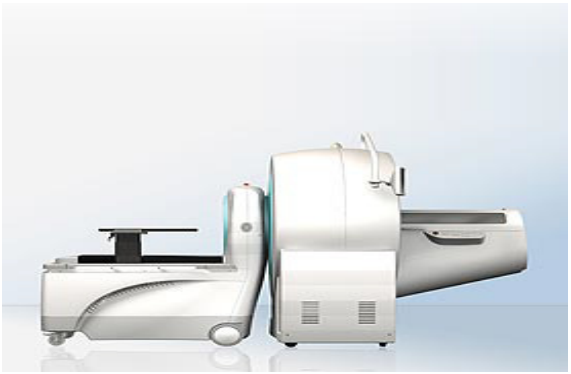

Figure 12 A clinically accepted multi-modal imaging system. The Siemens Inveon Docked PET•SPECT•CT system combines a Docked PET scanner with a SPECT•CT system (Figure courtesy of P.N. Prasad).

The other immediate clinical application of fluorescent nanoparticles is in the intra-operative delineation of the tumor boundary during surgery. Such optical guidance will enable surgeons to accurately resect the tumor mass and any metastatic spread while sparing normal cells/tissues and avoiding the risk of recurrence due to leftover neoplastic cells. The availability of clinically accepted multi-modal imaging systems (Figure 12) has bolstered the need for multi-modal nanoparticle imaging agents. Further development in instrumentation technology combining other modalities like optical and MR in the same instrument will pave the way for additional opportunities in imaging.

Milestones

3 Year:

- Develop multi-imaging scanners for multi-modal imaging of small animals.

5 Year:

- Translate these scanners into human applications in the clinic.
- Complete successful large animal studies such as dogs and non-human primates of at least five formulations of multi-modal nanoparticles.

10 Year:

- Complete successful clinical trials involving at least three formulations of multi-modal nanoparticles. The essential parameters to evaluate will include: (1) low or absent acute and chronic toxicity; (2) early diagnosis of cancer and other diseases, including non-invasive visualization of occult metastases; and (3) non-invasive, real-time monitoring of therapy.

Nanotechnology for Image-Guided Interventions

Shuming Nie

Emory University and Georgia Tech, Atlanta, GA

Overview

There are major opportunities and challenges in developing nanotechnology and advanced instrumentation for image-guided cancer surgery and biopsies (Singhal *et al*., 2010). The ability to visualize tumors in real-time will help the surgeon to delineate tumor margins, to identify residual tumor cells and micrometastases, and to determine if the tumor has been completely removed. This would apply to tumors of many organ sites, especially aggressive lung, pancreatic, ovarian, and metastatic breast cancers. Nanometer-sized particles such as quantum dots, colloidal gold, and biodegradable nanoparticles have functional and structural properties that make them appealing for tumor imaging. When conjugated with targeting ligands such as monoclonal antibodies, peptides or small molecules, these nanoparticles can be used to target malignant tumor cells and the tumor microenvironments (such as tumor stroma and tumor vasculature) with high specificity and affinity. In the "mesoscopic" size range of 10-100 nm diameter, nanoparticles also have large surface areas available for conjugating multiple diagnostic and therapeutic agents, opening up new possibilities for integrated cancer imaging and therapy (Nie *et al*., 2007). Similarly, advanced optical instrumentation provides unique advantages for intraoperative cancer detection that are not available from other imaging modalities. In the visible spectrum, optically labeled tumors are visible to the human eye, and can be seen and resected by the surgeon without any visual aid. In the near-infrared spectrum, standard fiber optics and silicon-based CCD cameras can be used for tumor visualization at high sensitivity and low costs (De Grand and Frangioni, 2003).

Nanotechnology is well suited for image-guided interventions because several problems that are often associated with nanoparticles and optical instrumentation are circumvented under surgical or biopsy conditions. For example, optical methods have relatively limited penetration depths due to tissue scattering and blood absorption, but this is no longer a major limitation during intraoperative cancer detection because the tumors are surgically exposed and are accessible to optical illumination and detection. Another common problem in using nanoparticles and macromolecules for cancer therapy is that they are unable to deeply penetrate solid tumors. This is not an issue as defining the tumor's external margin is the actual goal for surgical resection and internal staining is inconsequential. For intraoperative detection of small and residual tumors, deep penetration is also not required because the small tumors do not have high intra-tumoral pressures or hypoxic/necrotic cores, two main factors in limiting tumor penetration of nanoparticle imaging and therapeutic agents (Lunt *et al*., 2009). Thus, the combined use of nanoparticle contrast agents and imaging technologies is expected to improve the sensitivity and specificity of detecting microscopic tumors and residual tumor cells after resection, with important applications in both image-guided surgery and image-guided biopsy.

Clinical significance

Most human cancers are treated by surgical resection, chemotherapy and/or radiation. Surgery cures approximately 45% of all patients with cancer, and provides a dramatic survival advantage (http://seer.cancer.gov/). To cure a patient with surgery, the surgeon must remove the entire tumor at the time of surgery. A complete resection is the single most important predictor of patient survival for almost all solid tumors. This includes removing the primary tumor and draining lymph nodes that may contain tumor cells and small adjacent satellite nodules. In lung, breast, prostate, colon, and pancreatic cancers, a complete resection has a three to five fold improvement in survival compared to a partial or incomplete resection. Clearly, it is important to maximize the efficacy of surgical procedures because it is the most important method that exists to cure people of their cancer.

Minimally invasive cancer surgery

One of the most important changes in surgical oncology has been the development of minimally invasive surgery, which promises to alter the delivery of cancer care in the U.S. and in the world. Historically, one challenge of cancer surgery has been the loss of six to eight weeks that

occurs following an open procedure. After surgery, there can be a lengthy recovery time during which no adjuvant therapies can be given. Many common set backs including a urinary tract infection, pneumonia or arrhythmia, can delay the start of chemotherapy or radiation an additional month, during which the disease can still progress. Another problem is that many patients do not qualify for open procedures due to frail health and advanced age. The development of minimally invasive surgery has solved these challenges. Lung cancers are now removed by thoracoscopic lobectomy, colon cancers by a laparoscopic colectomy, and prostate cancers by robotic surgical instruments. Consequently, recovery time has dramatically decreased. These surgical techniques have translated well into other realms making rapid diagnoses and specimen retrieval possible with minimal patient duress.

Furthermore, minimally invasive surgery has largely replaced open surgery as an important tool to obtain rapid diagnostic information and specimens. For example, laparoscopic examination of the abdomen is used to evaluate and obtain diagnostic material for ovarian cancer, gastric cancer, and pancreatic cancer. Similarly, thoracoscopic (chest) surgery is used to obtain pleural biopsies in metastatic breast cancer, lymphomas, and mesothelioma. These procedures require only three to four small ports on a patient's chest, and can take place as an outpatient with costs under $5000 (vs. $30,000 for open surgery).

Nanoparticle contrast agents

As advancements in the field of nanoparticle imaging science are made, one of the first theatres for their use will be open and endoscopic conditions. There is considerable evidence indicating that the use of injected contrast agents can improve the detection of tumor margins and small metastases (Sajja *et al*., 2009). New and innovative targeting and contrast agents including small molecules, antibodies, and nanoparticles should be developed for a broad range of tumor types such as breast, brain, pancreatic, and ovarian cancers. At present, a number of organic dye molecules have been approved for human use including (1) indocyanine green (ICG), a near-infrared fluorescent dye; (2) fluorescein, a green fluorescent dye; (3) photofrin, a mixture of fluorescent protoporphyrin oligomers approved for photodynamic therapy, and (4) 5-aminolevulinic acid (ALA), a small molecule that is preferentially taken up by tumor cells leading to biosynthesis and accumulation of protoporphyrin IX, a natural fluorophore with red fluorescence emission. On the other hand, nanoparticles have not received FDA approval for clinical tumor imaging.

A major task is, therefore, to develop biocompatible and nontoxic nanoparticle contrast agents with the potential for FDA approval and human use. Such agents need to show improved sensitivity and specificity for tumor imaging in comparison with small-molecule dyes. In this regard, it is highly promising to develop "smart" or activatable nanoparticles with improved pharmacokinetic, tumor-targeting, and organ clearance properties, based on the use of natural, biodegradable polymers (dextran and heparin). Dextran-based particles are sensitive to pH, and can be rapidly broken down under acidic conditions. Under neutral or slightly basic conditions, on the other hand, the dextran nanoparticles are stable and are able to circulate systemically in blood for 14-15 hours (Gaur *et al*., 2000). In contrast, self-assembled heparin nanoparticles have much shorter blood circulation half lives (about 60-80 min) (Chen *et al*., 2009). For intraoperative use, this short circulation time could be beneficial because the probes will be cleared from the blood quickly, so that surgical operations can start without much delay or waiting. For near-term clinical applications, it is important that both the dextran and heparin particles are able to trap an FDA-approved dye (such as indocyanine green), leading to a new class of imaging contrast agents with improved biodistribution and photophysical properties. Figure 13 shows a class of "nano-ICG" contrast agents that are quenched in their initial state but are activated under *in vivo* conditions (Mohs *et al*., 2010). This class of nanoparticle contrast agents could also be conjugated with tumor targeting ligands such as folate, EGF, or RGD for improved sensitivity and specificity.

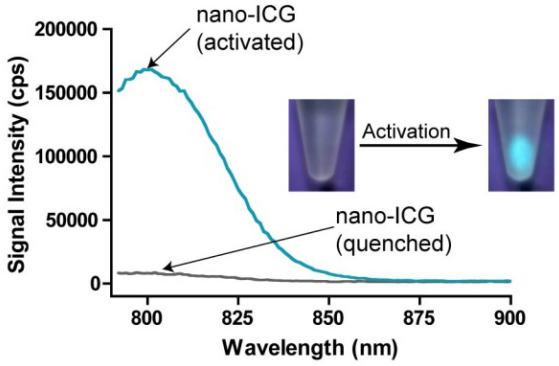

Figure 13 Optical properties of nano-ICG, a new class of biodegradable and self-assembled particles with physically trapped indocyanine green (ICG) molecules. In this type of "nano-ICG" imaging agent, the ICG fluorescence is quenched in the trapped state, and is activated when the dye is released under *in vivo* conditions (reprinted from Mohs *et al*., 2010, Copyright, American Chemical Society).

Milestones

3 year:

- Generate polymer-coated nanoparticles using current FDA-approved fluorescent dyes for residual tumor and metastases labeling. Incorporate tumor-targeting ligands for increased sensitivity and specificity.
- Develop novel nanoparticle imaging dyes that are not subject to photobleaching with targeting moieties to differentiate tumor from normal tissues and precisely delineate tumor margins.

5 year:

- Study *in vivo* toxicity in model organisms.

- Begin clinical trial evaluation of the most successful nanoparticles, coupled with minimally invasive delivery procedures.

10 year:

- Commercialize several targeted nano-imaging particles.

Development of Imaging Hardware Based on Nanotechnology

Otto Zhou
University of North Carolina, Chapel Hill, NC

Introduction

X-ray radiation is widely used today for *in vivo* cancer detection and for radiotherapy. For example, mammography is the most common modality for breast cancer screening and over 50% of the cancer patients in the U.S. undergo radiation therapy. For x-ray based imaging and radiotherapy techniques there is a constant demand to increase resolution to detect tumors at an early stage, minimize the imaging dose to reduce side effects, improve the accuracy of dose delivery during treatment, and minimize normal tissue damage. The new carbon nanotube based x-ray source technology enables the design of new imaging and radiotherapy devices with improved performances in these areas.

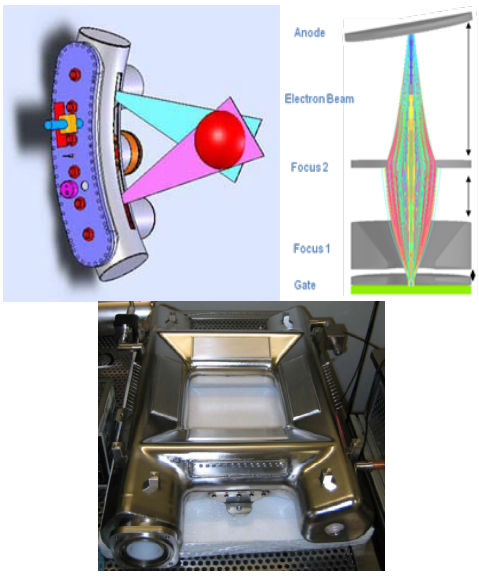

Figure 14 **Schematics showing a nanotube x-ray source array (top) and a square-geometry nanotube x-ray source array with 52 individually controllable x-ray beams (bottom; XinRay Systems).**

Utilizing the recent advances in nanomaterials a new x-ray source technology has been developed. Carbon nanotubes instead of the conventional thermionic filaments are used as the "cold" electron sources for x-ray generation. The technology is capable of generating *temporally* and *spatially* modulated x-ray radiation that can be readily gated and synchronized with physiological signals. The spatially distributed x-ray source array technology opens up new possibilities for designing *in vivo* imaging systems with increased resolution and imaging speed and expanded functionalities. By distributing the x-ray power over a large area, the technology can generate a significantly higher dose rate for certain radiotherapy applications. Since its invention this nanotechnology enabled x-ray source technology has moved from a simple academic curiosity to commercial production (Figure 14). The applications of this new technology for cancer detection and treatment are being actively investigated in academic institutions and in industry. Below are some examples of the *in vivo* imaging systems currently under development with the support of the NCI Alliance for Nanotechnology in Cancer program.

High-resolution micro-CT for *in vivo* imaging of small animal cancer models

Utilizing the electronic programmable capability of the nanotube x-ray source a physiologically gated micro-computed tomography (CT) scanner has been developed for *in vivo* imaging of small animal cancer models (Figure 15) (Cao *et al*., 2009; Cao *et al*., 2010). By synchronizing x-ray exposure and data collection with the non-periodic respiratory and cardiac motions high resolution CT images with minimum motion blurs can be obtained from free-breathing mice. The scanner is used routinely by a large number of cancer researchers at the University of North Carolina-Chapel Hill (UNC) for *in vivo* imaging of their small animals. Additional systems are being constructed and will be installed at UNC and the University of Iowa for cancer research.

"Real-time" tomosynthesis image guidance for radiation therapy

Utilizing the distributed x-ray source array technology, Siemens and XinRay Systems developed a high-speed tomosynthesis scanner to provide real-time image guidance for radiation therapy (Maltz *et al*., 2009). The development won the team the 2010 Sorkin Award from the American Association of Physicists in Medicine. The technology will enable the oncologists to "see" tumors in real time during treatment and will allow more accurate radiation delivery. The scanner has been integrated with the Siemens Artiste treatment system. It is currently under testing at the UNC Cancer Hospital. Clinical tests are scheduled for this year and Institutional Review Board approval has already been obtained.

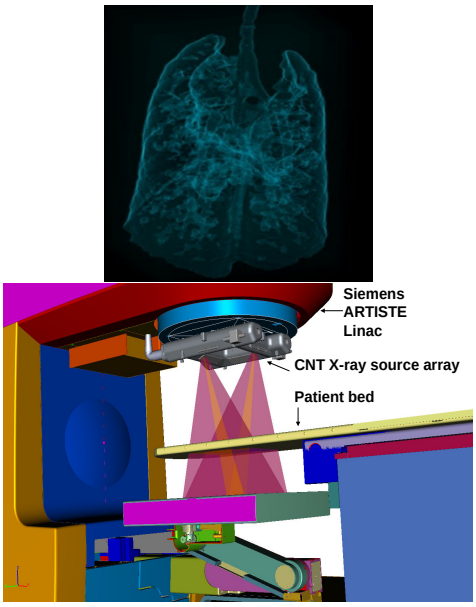

Figure 15 Prospective-gated micro-CT image of a mouse lung tumor model (top; UNC data. Mouse model from Dr. B. Kim). An illustration of CNT x-ray source array mounted on a radiotherapy machine (bottom; image courtesy of J. Maltz of Siemens and P. Lagani of XinRay).

Digital tomosynthesis for early stage detection of human breast tumors

Digital breast tomosynthesis (DBT) has the potential to become the next generation screening tool for breast cancer, replacing the current two-view mammography scanners. This limited-angle tomography technique provides quasi 3D views of the breasts which help radiologists differentiate breast tumor from the surrounding tissues. Utilizing the spatially distributed nanotube x-ray source array technology, a proof-of-concept stationary DBT scanner increases the imaging resolution, improves the detectability of micro-calcification, and reduces the imaging time which reduces the patient discomfort from breast compression, compared to the rotating DBT scanners from commercial vendors that are currently under clinical trials for FDA approval (Qian *et al*., 2009). Encouraged by the initial results a second generation, clinical test ready, scanner is currently under development which will integrate the nanotube x-ray source with a commercial mammography scanner.

Future challenges

From the engineering perspective, the reliability, consistency, and lifetime durability of the devices need to be demonstrated to be comparable or even better than the existing systems before they can be adapted in the clinics. Since imaging and radiotherapy devices are complicated, new device development requires a large multi-disciplinary team with complementary expertise in a wide range of fields as well as close collaborations with industry. The question as to how to organize and finance the research and development effort is always a challenging one.

Clinical potential

Recent research has clearly demonstrated the potentials of the nanotube x-ray based systems for clinical *in vivo* cancer imaging and radiation therapy applications. Some examples include early detection of breast cancer, image guidance for radiation therapy, and novel radiotherapy techniques.

Milestones

3 year:

- Develop stationary tomosynthesis scanners for applications such as breast imaging and image-guided radiation therapy and conduct clinical tests.
- Commercialize imaging systems for small animal models.

5 year:

- Develop microbeam radiation therapy using the nanotube x-ray source array technology for small animal models.
- Conduct studies of their therapeutic effects on small animal brain tumor models.
- Commercialize tomosynthesis imaging systems.

10 year:

- Develop a new generation of CT scanners based on this technology and utilize it in radiotherapy for human patients (for example, microbeam radiation therapy).

Nanotechnology and Cancer Prevention

Sara S. Hook

Office of Cancer Nanotechnology Research, CSSI, National Cancer Institute, NIH, Bethesda, MD

Patient prevention strategies

There are several possible approaches to cancer prevention. Patients can decrease behaviors that put them at risk, be more vigilant in screening and surveillance, opt for surgical pre-intervention, and/or utilize "medicinal" approaches. The latter three areas in particular can benefit from the advances that nanotechnology can offer.

It is well recognized that several factors contribute to and enhance cancer prevention including dietary and lifestyle changes. The field of epidemiology has long been examining what types of risk factors are correlated with certain types of cancers. For instance, probably one of the best documented and most studied behavioral risk factors is that smoking increases the incidence of lung cancer. In fact, smoking also greatly increases the risk of many types of cancers as well as heart attacks (Khan *et al*., 2010). A second well documented example is increased exposure to UVB rays from sunlight clearly damages DNA and can result in an increased risk of various types of skin cancer including the most deadly, melanoma (Cooper and Bowden, 2007).

Patients themselves can also implement mechanisms of surveillance. This would include performing breast self-exams to detect lumps and nodules, monitoring the skin for changes in moles, and seeing a doctor for routine physical exams. For those with a family pre-disposition to cancer, additional monitoring may be in order. For instance, patients who have a primary relative such as a mother or sister with breast cancer might want to undergo genetic testing to determine whether they are carriers of the familial breast cancer susceptibility genes, *BRCA1* and *BRCA2*. Additionally, imaging such as mammography has played an important role in screening at risk women and those over 40 for breast cancer. The areas of diagnostic imaging and molecular *in vitro* screening are areas in which nanotechnology can play a significant role. For example, Dr. Otto Zhou's group at UNC-Chapel Hill is developing a stationery digital breast tomosynthesis scanner using carbon nanotube (CNT) multi-pixel field emission x-ray (MBFEX) technology. This approach will increase image resolution and decrease both patient discomfort and radiation exposure times (Qian *et al*., 2009). The advances and future challenges in cancer imaging have been outlined in several previous sections. Likewise, *in vitro* genomic and proteomic testing strategies based on nanotechnology, such as those outlined earlier in this document, can be more sensitive, more cost effective, more rapid, and possibly more accurate than technologies currently in clinical use. Surgical intervention for "pre-cancerous" lesions detected during routine colonoscopies, or prophylactic breast, ovary or complete hysterectomies for patients at high risk for reproductive cancers likely also play a role in primary and secondary cancer prevention. As previously discussed in other sections, nanotechnology offers the physician increasing ability for image-guided surgical resection of tumors and possibly also pre-cancerous lesions. In fact, one example of this is from Dr. Sanjiv Sam Gambhir's research group where they have used single-walled carbon nanotubes (SWCNTs) combined with Raman imaging to visualize tumors in live small animal models (Keren *et al*., 2008). They are pursuing applications for this technology such as clinical colonoscopy and have already built a flexible endoscope capable of Raman imaging.

"Medicinal" prevention strategies

Many might hope that one day cancer could be prevented using some type of vaccine or pill to ward off the disease. The etiology, however, makes this a huge task due to the myriad of mechanisms by which the disease arises, the ability of cancer cells to escape immune system detection (due to recognition as "self"), the tissue specificity of some tumor types, the altered cellular growth and metabolism pathways, etc. Thus the concept of medical prevention in terms of vaccines and drugs is extremely challenging.

There are strong indications that avenues of medical prevention of cancers may be successful. One approach that is very promising is in the area of human papillomavirus (HPV) vaccines to prevent genital warts and hopefully also cervical, vulvar, and vaginal cancers. Two FDA approved vaccines, Cervarix (GlaxoSmithKline) and Gardasil (Merck), are recombinant versions of virus

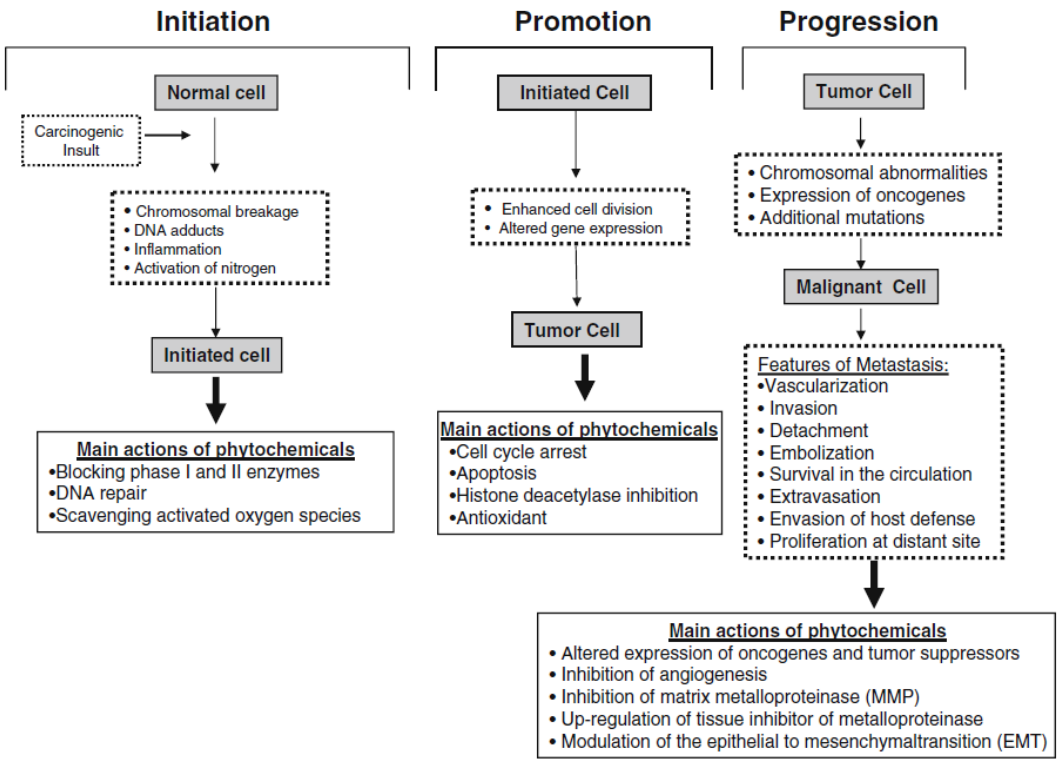

Figure 16 Areas in which neutraceuticals or phytochemicals can intervene in the process of carcinogenesis (reprinted from Mehta *et al*., 2010, Copyright, Springer Science).

like particles of the most common types of HPV, strains 16 and 18. Since up to 75% of cervical cancer cases are caused by HPV-16 and HPV-18 these vaccines might eliminate most of these cases in the future. Additionally, Cervarix has also been shown to decrease infection rates of other cancer causing HPV strains including 31, -33, -45, and -52 (D'Andrilli *et al*., 2010). Clearly these vaccines have shown effective in dramatically decreasing genital HPV infection, but a long term follow-up is needed to establish the efficacy of decreasing cancer incidence and mortality. A randomized, double blind, Phase III trial did show Cervarix decreases the risk of developing CIN2+ pre-cancerous lesions (Paavonen *et al*., 2009). Vaccines, however, for the prevention of cancer might be the exception and not the rule. An alternative approach being pursued by PDS Biotechnology is developing nanoparticles that contain an antigenic peptide to an essential protein component of HPV, E7. These particles target dendritic cells to produce antigens to E7 and promote a cytotoxic response from killer T cells. This approach is unique in that current vaccines only work if the patient is not already infected with strains of HPV, whereas this approach can target patients who are already infected (Chen *et al*., 2008).

Anti-inflammatory drugs directed to the cox-2 (cyclooxygenase-2) family of enzymes responsible for prostaglandin synthesis have shown promise in cancer prevention. Two of these, however, Vioxx (Merck) and Bextra (Pfizer), have been pulled from the market due to side effects related to heart attack, stroke, and gastrointestinal bleeding. Studies initiated well before these drugs were removed from the market have indicated that these drugs significantly reduce the risk of developing cancer of the colon, breast, lung, and prostate (Harris, 2009). The side effects, however, limit their therapeutic value. The remaining cox-2 inhibitor, celecoxib (Celebrex manufactured by Pfizer), has been approved by the FDA for use to prevent colon cancer but only in the extreme case of patients with Familial Adenomatous Polyposis (Half and Arber, 2009). Additionally, Celebrex was found to reduce the growth of basal cell carcinomas by 50% in some patients with a rare genetic condition, Gorlin syndrome, which makes them highly susceptible to tumorigenesis (Tang *et al*., 2010). An additional study suggests that patients who took Celebrex daily for nine months had 60% fewer non-melanoma skin cancers than people who did not take the drug (Elmets, 2009). Numerous studies also suggest that cox-2 inhibitors when given in combination with other therapies can potentiate cancer cell death. In terms of prevention, these types of drugs could be reformulated into targeted nanoparticles to make use of their protective effects in preventing the formation of colon and rectal polyps or skin carcinomas without the unwanted cardiovascular side effects. Although attempts at microemulsion formulations are underway (Margulis-Goshena *et al*., 2010), nanoparticle encapsulation would be most beneficial to circumvent the unwanted side effects of these drugs.

Another avenue that could potentially be exploited for prevention would be the area of anti-inflammatory nutraceuticals (Nair *et al*., 2010). Research

has shown that part of the cancer progression phenotype is chronic inflammation (Grivennikov and Karin, 2010). Quite a number of natural products have been shown to decrease inflammation but in almost all cases, the bioavailability of these compounds is limited. Thus, nanoparticle delivery of such agents as curcumin, green tea polyphenols, coenzyme Q, etc. could be very useful. For example, a catechin, epigallocatechin-3-gallate (EGCG) found in green tea, has chemopreventive potential for human breast, pancreatic, colon, esophageal, and lung cancers, but its oral absorption rate is only 1% (Nair *et al*., 2010). Consequently, more than 5 cups of green tea would need to be consumed for a health benefit (Johnson *et al*., 2010). Nanoparticle delivery of EGCR then would be beneficial. In fact, the formulation of EGCG into PLA-PEG nanoparticles offered a more than 10 fold decrease in the IC50 over free EGCG when monitoring tumor cell viability (Siddiqui *et al*., 2009). EGCG can inhibit tumor cell growth and decrease angiogenesis in mouse xenograft models (Siddiqui *et al*., 2009) on its own but it can also sensitize tumors to growth inhibition by other agents such as interferon-α2b (Nihal *et al*., 2009). In addition to this compound's anti-inflammatory properties, it also decreases signaling of several kinase pathways, insulin-like growth factor, and androgen receptor signaling. In fact, clinical studies in men with prostatic intraepithelial neoplasia (PIN), a pre-cancerous lesion of the prostate, revealed a 90% reduction in the progression to prostate cancer when taking ECGC containing supplements (Bettuzzi *et al*., 2006). Additional studies have, however, indicated that the controlled formulation of nutritional supplements is quite important for biologically efficacious effects (Johnson *et al*., 2010). Green tea catechins are just one example within many that are being evaluated for their chemopreventative potential in similar research studies (Nair *et al*., 2010). Although a great deal of discussion was devoted to natural products, researchers could also build upon these chemical structures using rational drug design approaches to improve upon what nature has given us.

The main focus here has been on prevention meaning before malignant growth has started. Confirmation of whether a compound has this potential is usually through prospective studies where patient cohorts are followed over a long period of time to correlate behavioral risks with cancer development. Neutraceuticals can, however, have an impact with other chemotherapeutic agents even after malignancy has been diagnosed to enhance the effectiveness of these treatment regimes (Mehta *et al*., 2010). As depicted in Figure 16, neutraceuticals can by a vast variety of mechanisms feed into the processes of apoptosis, cell cycle arrest, DNA repair, protection against free radicals, etc., all processed known to be important in preventing cancer formation. Thus, future research will undoubtedly include an increasing focus not only on neutraceutical effects by themselves but also in combination with other therapeutic strategies.

Milestones

3 year:

- Publish more studies on characterizing natural products and their chemopreventive potential.
- Develop nanotechnology delivery systems for neutraceuticals and other chemopreventive agents.
- Carry out more prospective studies to identify genetic, behavioral, and environmental risks for various types of cancers.

5 year:

- Incorporate natural products with more standard therapeutic approaches in an increasing number of clinical trials.
- Conduct rational design experiments to improve on the potential therapeutic effects of existing neutraceuticals.
- Identify other potential targets for cancer vaccine development.

10 year:

- Follow-up studies with patients vaccinated with HPV vaccines will reveal whether they actually decrease the development of cervical, vulvar, and vaginal cancers.
- Develop nanotechnology mechanisms to limit exposure to environmental toxins.

NCI's Nanotechnology Characterization Laboratory

Scott E. McNeil

Nanotechnology Characterization Laboratory, National Cancer Institute, Frederick, MD

Mission

The NCI's Nanotechnology Characterization Laboratory (NCL) provides infrastructure support to NCI's Alliance for Nanotechnology in Cancer. The lab's mission is to provide pre-clinical characterization to its sponsors, and to accelerate the translation of promising nanotechnology-derived cancer treatments into clinical applications. The NCL was founded in 2004 as a formal interagency collaboration among NCI, NIST, and the FDA and is operated through the NCI's Federally Funded Research and Development Center (FFRDC) at SAIC/NCI-Frederick.

NCL has a number of key objectives which include characterizing nanoparticles using standardized methods and conducting structure activity relationship (SAR) studies to identify and delineate critical parameters related to nanomaterial pharmacological properties and toxicology. Additionally they facilitate the regulatory review of nanotechnological constructs and engage in educational and knowledge sharing efforts.

The NCL's services are available for free to researchers developing a nanotechnology cancer therapy or diagnostic. Nanomaterials accepted by NCL are subjected to a three-tiered Assay Cascade of scientific tests, including physic-chemical characterization, *in vitro* assessment, and *in vivo* evaluation for safety and efficacy. The data generated from the NCL characterization are intended for use in support of IND or Investigational Device Exemption (IDE) applications to the FDA. As such, the NCL serves as a bridge to take promising cancer nanotechnology research to regulatory approval.

Achievements

In just six years of operation, the NCL has become a recognized authority in nanotechnology for biomedical applications. The Lab has over 50 collaborations with researchers from academia, industry, and government and has characterized almost 200 different nanomaterial samples – including liposomes, metal colloids, dendrimers, polymers, quantum dots, metal oxides, and fullerene derivatives. Multiple NCL collaborators have now submitted an IND or IDE application and one collaborator has begun Phase II clinical trials.

Lessons learned

One of the ways that the NCL contributes to the Alliance and to the nanotechnology research community in general is by sharing the observations made in its Assay Cascade. Investigators benefit from these "Lessons Learned" thus accelerating the progress of the entire community.

Stability and Scalability. The Lab now has several examples where stability issues negatively impact the rapid development of nanoparticle-based therapies. Particles that release their payload within seconds to minutes of administration offer minimal advantage over traditional small molecule drugs. On the other end of the stability spectrum are nanoparticle formulations that are *too* stable – that is, the drug is not released from the nanoplatform and is generally inefficacious. In the case where drugs are covalently linked to the carrier, it is essential that this linkage is cleavable or otherwise degradable by the intracellular environment. Scale-up is also a common hurdle in the development process. In the case of nanoparticle formulations, early-stage planning can easily circumvent obstacles in this path to commercialization. An obvious example of this pitfall is found in the misunderstanding that academic studies are simply smaller versions of large-scale production.

Sterility. Another problem common to small-scale synthesis is contamination. Academic labs often use glassware not dedicated to aseptic procedures, and generally do not utilize "best practices" to prevent endotoxin contamination. On numerous occasions, investigators have submitted material to the NCL that is rife with endotoxin or other microbial contamination. This type of contamination severely impedes *in vitro* and *in vivo* studies, as it perturbs cell signaling pathways and may induce an immune response.

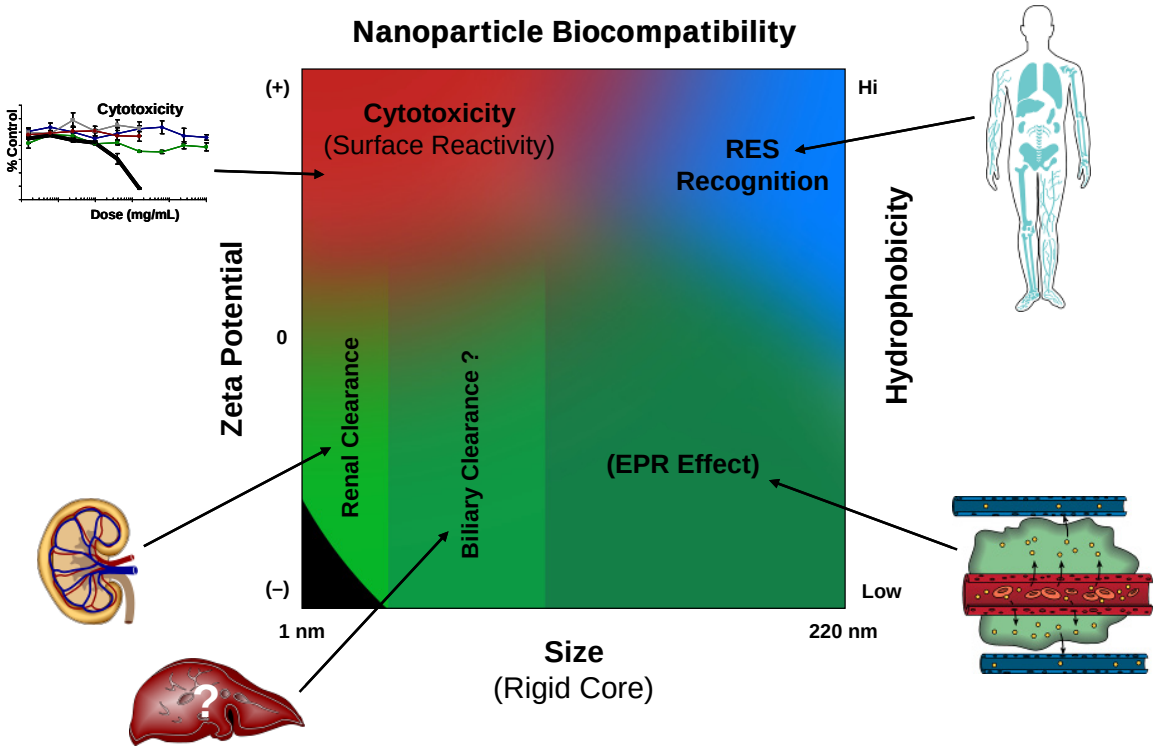

Figure 17 **Nanoparticle Biocompatibility. This plot shows trends the NCL has observed in the relationship between nanoparticle physico-chemical properties and biological responses. The independent variables in this plot are the zeta potential (related to surface charge), size, and hydrophobicity which are plotted versus the dependent variable of biocompatibility, manifested in such biological responses as cytotoxicity, clearance, the EPR effect, and RES recognition. (Adapted from McNeil, 2009).**

Predicting Toxicology Based on Nanoparticle Structure. NCL-generated data has elucidated trends in nanomaterial characteristics and identified critical parameters that influence nanomaterial biocompatibility (Figure 17). This data has contributed substantially to the current understanding of the "nano-bio interface." For example, particles must be smaller than approximately 200 nanometers to transverse the architecture of the liver and spleen. Particles that are hydrophobic (e.g. without a PEGylation layer) will quickly be removed from circulation by macrophages in the RES. With respect to elimination, particles and/or their breakdown products must be less than 10 nm to be excreted through the kidneys, otherwise they may reside in the RES organs for the lifetime of the animal. However, particles as large as 30 nm may be excreted in the bile. Finally, cationic (strongly positively charged) particles are cytotoxic, with or without the chemotherapeutic agent onboard. Investigators should engineer particles with these parameters in mind – exploiting the advantageous particle characteristics to hopefully avoid repeating trial and error studies of the past.

Milestones

3 year:

- Conduct more in-depth structure-activity relationship (SAR) studies. Coordinated efforts to address research gaps are critical to advancing our understanding of the nano-bio interface. In mathematical terms, it is imperative to obtain the *total derivative* (all the parameters that influence biocompatibility) by examining many *partial derivatives* (varying one parameter while holding the others constant). While a few academic labs are attempting this, e.g. varying PEGylation density and observing phagocytosis, a comprehensive study of multiple parameters is desperately needed. Direct resources towards the synthesis of these reagents and testing them in the NCL's assay cascade.
- As the nanotech concepts submitted to the NCL mature, more NCL collaborators are seeking access to IND-enabling pre-clinical characterization resources such as good laboratory practice (GLP)-certified toxicology studies, large animal studies, and good manufacturing process (GMP)-certified manufacturing/synthesis capabilities. Collaboration with other government laboratories (e.g. FDA's National Center for Toxicological Research) will

allow the NCL to leverage such resources without great expense.
- Establish collaborations with industry to reformulate discontinued cancer drugs using nanotechnology.

5 Year:

- Increased interaction with Contract Research Organizations (CROs) will facilitate the scale-up process and transition to GMP manufacturing. NCL will endeavor to make contacts at the CROs that have experience with nanoparticle formulations and to increase our visibility to these organizations.
- Devise analytical methods to differentiate nanoparticle-bound vs. free drug. To support regulatory review of nanoformulations, analytical methods that can determine the free, and therefore "active" drug component of a nanoparticle drug profile are needed.

10 year:

- As NCI's Alliance moves into its second iteration and the nanotech concepts submitted to the NCL continue to mature, the NCL's relationship with the FDA will necessarily evolve as more NCL-characterized concepts enter the regulatory process. Specifically, we expect increased interaction with FDA reviewers. NCL will continue to seek input from the FDA on its assays and to collaborate with the FDA on the regulatory aspects of nanotechnology and SAR studies. In 10 years, NCL aims to facilitate three to five IND filings.

Safety Issues in Pre-clinical and Clinical Evaluation of Nanotechnology-based Products

Subhas Malghan and Carlos Pena

U.S. Food and Drug Administration

Nanotechnology allows scientists to create, explore, and manipulate materials in the nanoscale range. Behavior of such materials in terms of chemical, physical, and biological properties may differ from those of their larger counterparts. A general finding of the "FDA Nanotechnology Task Force Report 2007" is that nanoscale materials present regulatory challenges similar to those posed by products using other emerging technologies. However, distinct challenges may also arise because at the nanoscale, properties of a material might change in ways that could affect the performance, quality, safety and/or effectiveness. While applications of nanoscale materials in cancer treatment are continuing to evolve, one needs to consider the potential unintended health impact of these materials. One reason for this potential is that some of these materials will eventually come into contact with biological structures and processes that frequently occur at the nanoscale.

Understanding interactions of nanoscale materials with biological systems

To assess the interaction of nanoscale material with biological surfaces, reliable and reproducible screening methods are needed. Achieving this goal has become a challenge because of the large variety of new nanoscale materials that are under development, their unique set of novel physico-chemical properties, and uncertainty of how those properties relate to biological outcomes. There is a possibility of a vast number of physico-chemical interactions with biological surfaces when nanoscale materials of different size, composition, shape, surface area, aggregation, crystallinity, surface coating and functionality, and hydrophilic/hydrophobic interactions come in contact with biological fluids, proteins, lipids, DNA, cell membranes, lysosomes, mitochondria, and biological processes (Nel *et al*., 2009). Therefore, a comprehensive physico-chemical characterization as well as pharmacokinetic and biodistribution studies are required to evaluate safety as well as efficacy. Currently, there is considerable discussion on nanomaterial toxicity testing, with the major discussion centering around which toxicological end points to screen for, the adequacy of the screening effort, and the correct balance of *in vitro* (cellular and molecular) versus *in vivo* (animal or whole organism) testing (Oberdorster *et al*., 2005; Borm and Berube, 2008; Nel *et al*., 2009). Attempts to use traditional toxicological assays and models have resulted in conflicting and sometimes irreproducible results.

Additional important questions exist concerning the transport of nanoscale particles in the human body and mechanisms of interaction at the sub-cellular and molecular levels. The unique and diverse physico-chemical properties of engineered nanoscale materials suggest that their toxicological properties may differ from materials of similar composition but larger size. Studies also suggest that particle size, surface area, and surface chemistry of engineered nanoscale materials can impact toxicity equally, if not more so, than chemical composition (Nel *et al*., 2009). Research is in progress to evaluate toxicity of nanoscale materials that represent a cross-section of composition, size, surface coatings, and physico-chemical properties. Many of these studies are designed to investigate fundamental questions concerning how nanoscale materials are absorbed and distributed *in vivo* and whether they can adversely impact biological systems. More studies are needed to detect and quantify nanoscale particles in tissues, mechanisms of nanoscale material absorption, distribution in the body, and subsequent up take by cells. These studies have the potential to develop a better understanding of biological and toxicological interactions.

Different uses may have different requirements with regard to nanoscale material

While biocompatibility and toxicity would be important for devices, absorption, distribution, metabolism, and excretion are relevant in the evaluation of safety of nanoscale materials contained in drugs. Concepts that have been applied in the micron size range may be usefully applied to the nanoscale range, but new challenges are presented based on the small size and possible change in the dissolution-translocation relationship (Nel *et al*., 2009). Solute concentration, surface area, surface morphology, surface energy, dissolution layer properties, adsorbing species, and aggregation are some relevant parameters when considering dissolution at the nanoscale. With regard to the etiopathology caused by nanoscale particles, the metrics of dose (particle number, surface area, mass or shape) is not yet well defined. Analytical procedures for assessing dissolution and translocation include chemical assay and particle characterization. Leaching of components from particle surfaces as well as compartmentalization within the respiratory tract may add another dimension of complexity. Dissolution may be a critical step for some nanoscale materials in determining their fate within the body. An integrated approach combining particle toxicology, material science, and analytical chemistry is required to provide a useful basis for developing relevant dissolution assay(s) for nanoscale particles.

Studies have indicated that various attributes of a particular nanoscale material, including increased specific surface area, morphology, surface features, and charge, can affect the distribution of that material in the body, that material's toxicity, and/or its biocompatibility. In addition, current testing approaches may need to be evaluated and new approaches developed to assess safety, effectiveness, and quality of a product that uses a nanoscale material.

A conclusion of some studies in this area is that current risk assessment methodologies require some modification to address hazards associated with nanoscale materials and in particular that existing toxicological and biocompatibility methods may not be sufficient to address all issues related to nanoscale particles. For exposure evaluation, dose determination requires information on the number of nanoscale particles and/or their surface area in addition to the traditional mass concentration characterization. Equipment for routine measurements in various media for representative exposure to free nanoscale particles is inadequate. In addition, existing assessment methods may not be appropriate to determine the fate of nanoscale particles. While an understanding of general risks of products using nanoscale materials is continuing to evolve, there is greater need for understanding the risks of free or "unconjugated" nanoscale materials because they are likely to behave differently from the same material/compound in a complex nanoparticle which may result in altered biological and toxicological behavior. Nanoscale materials may exhibit unique physico-chemical properties due to surface coatings or other nanotopographical features.

Summary

Inclusion of a nanoscale material in an FDA-regulated product or a change in the nanoscale material(s) used may affect the quality, safety, and effectiveness of that product and may raise questions regarding appropriate testing methods. Accordingly, additional data and testing methods may be needed for assessing the effects of a nanoscale material on a product, whether subject to premarket authorization or not (FDA Nanotechnology Task Force Report 2007). In some cases, the presence of a nanoscale material may also affect the regulatory requirements applicable to a product.

The FDA is available to assist manufacturers and sponsors in identifying and addressing regulatory issues raised by specific uses of particular nanoscale materials, including issues with regard to safety, effectiveness, good manufacturing practices, and possible changes in the regulatory classification or pathway for product approval. Both research and development groups are encouraged to contact the FDA to discuss the proposed use of specific nanoscale materials in an FDA-regulated product even if no legal requirement to notify the Agency applies.

Regulatory Aspects Related to Products Containing Nanoscale Materials

Subhas Malghan and Carlos Pena

U.S. Food and Drug Administration

The FDA regulates a broad range of products under the Federal Food, Drug, and Cosmetic Act (FFDCA) and the Public Health Service Act (PHS Act). The Agency's statutory authorities subject some types of products to premarket authorization requirements, either individually or by category, while permitting other products to be marketed without prior Agency authorization (FDA Nanotechnology Task Force Report 2007). The term "premarket authorization" refers to a number of regulatory actions that the FFDCA, the PHS Act, and agency regulations may refer to by other names, including "approval," "clearance," "licensing," and "listing." Most, if not all, laws and regulations under which the FDA operates are by design general in nature. Therefore, the agency's authorities usually are able to accommodate products made with the use of emerging science, new technologies, or containing new kinds of materials. The use of nanoscale materials in an FDA-regulated product may raise questions regarding which regulatory requirements apply and how they can be satisfied. Nanoscale materials are of particular interest to the FDA, since there is significant potential for their application to a large number of products regulated by the FDA. Nanoscale materials can have physical or biological properties that are different from those of their larger counterparts because of their small size and high specific surface area. Such differences may include altered magnetic properties, altered electrical or optical activity, increased structural integrity, or increased chemical or biological activity. Because of some of their special properties, these materials may present different safety and efficacy issues than their larger counterparts.

Medical products

Drug products (FDA Nanotechnology Task Force Report 2007): New drugs for humans, as well as new animal drugs, are subject to premarket authorization on a product-by-product basis. Information on the identity of products such as the type of product, the size of the components, and the manufacturing protocol is required as part of marketing applications if it is relevant to safety or effectiveness. In the case of replacing a current drug substance or excipient with a nanoscale version, the resulting product may be considered a new product for which a new approval would be needed.

Biological products (FDA Nanotechnology Task Force Report 2007): With regard to human cell and tissue products that might otherwise be subject to regulation only under section 361 of the PHS Act and, therefore, not subject to premarket authorization, we encourage manufacturers to contact the FDA before marketing any version that incorporates nanoscale materials or is otherwise modified at the nanoscale, to confirm whether these features trigger premarket authorization requirements.

Devices (FDA Nanotechnology Task Force Report 2007): Medical devices are regulated according to a tiered classification system that is largely based on the degree of risk posed by the product. Devices that are low risk, for which safety and effectiveness are generally well-established, are designated as Class I devices. These device types are subject to general controls, such as labeling, good manufacturing practices and adverse event reporting. Class II devices are more complex and carry a higher risk than Class I devices. For certain Class I devices and most Class II devices, manufacturers must submit to the FDA a premarket notification to demonstrate that their device is as safe and effective as another legally marketed device in order to obtain FDA clearance before marketing. Class III devices are the most complex, high risk devices and are reviewed under a premarket approval application (PMA). In a PMA, pre-clinical and clinical data, in addition to manufacturing information, are typically used to support the agency's determination that the device provides a reasonable assurance of safety and effectiveness.

Nanoscale material manufacturing issues

Products regulated under the FD&C and PHS Acts must be manufactured to conform with applicable requirements concerning, for example, safety, quality, and purity, and so as to avoid being adulterated. Some are additionally subject to current good manufacturing practice requirements (FDA Nanotechnology Task Force Report 2007). In some cases, the use of nanoscale materials in the development of an FDA regulated product may raise new safety issues that require new or different testing methods. Since there may be some uncertainty in the use of nanoscale materials and its impact upon such products, questions regarding safety may not be specifically addressed in existing guidance. Accordingly, manufacturers may have questions regarding how to ensure sound manufacturing practices for products that use nanoscale materials and they are encouraged to consult with the relevant FDA product center to ensure that new technologies do not present any new safety issues.

Contact FDA

There is a possibility that the presence of certain nanoscale materials used in the manufacture of medical products may affect the safety or effectiveness. Therefore, we encourage applicants to clearly indicate in regulatory submissions the presence of nanoscale materials.

If you are considering using a nanoscale material in your product, contact the FDA to confirm whether the product contains nanoscale material by FDA standards. In addition, the FDA should be contacted to discuss appropriate manufacturing practices and developing testing methods for assessment of product safety, effectiveness, and quality. Communications with the FDA regarding new nano-products will help ensure compliance with all legal obligations and will help the FDA to regulate products effectively and to address regulatory and patient safety issues proactively and efficiently. Following these recommendations will minimize delays to market entry and avoid evoking enforcement authorities to protect the public health.

Clinical Translation of Nanotechnologies: From Academic Laboratory to Start-up Company

Jeff Hrkach

Pharmaceutical Sciences, BIND Biosciences, Cambridge, MA

Developing a successful model of translation

At the highest level, the key elements to successfully translating a technology from academic research into clinical development are: technology, team, innovation and financing. These basic elements hold true for any start-up company, but even more so for the field of cancer nanomedicine given the challenges, complexities, and consequences of optimizing nano-scale technology for the treatment of people suffering from cancer.

When a start-up company is founded based upon academic research, the initial scientific efforts focus on the transfer of the technology from the academic labs into the hands of the company to develop an in-depth understanding of the technology's strengths, weaknesses, and potential when viewed from the very different lens of drug development. From the outset, the regulatory requirements dictated by the FDA for pharmaceutical development of the drug product candidate (as discussed previously) must be taken into consideration along with the pharmaceutical development considerations of product candidate optimization through rigorous pre-clinical evaluation, development of appropriate and robust analytical characterization methods, and of critical importance, manufacturing process development and scale up. The optimization approach requires evaluation of nanoparticle performance using *in vitro* cell-based assays (particle binding interactions, uptake and toxicity, drug activity), *in vivo* pre-clinical evaluation (PK, biodistribution, targeting, tolerability/toxicity, efficacy) as well as several CMC (Chemistry, Manufacturing, and Controls) requirements mandated by current good manufacturing practices (cGMP) and the FDA. These requirements assure among other things batch to batch reproducibility and shelf-life stability based on testing a variety of properties (particle size, drug content and purity, drug release rates, targeting ligand content and activity [if applicable], stability of nanoparticles and drug under storage and in-use conditions). Through the course of pharmaceutical development, the CMC requirements become more stringent; however, it is at this early stage where the company first begins testing these critical parameters.

Innovation and financing are the remaining key elements for successful clinical translation. Not all technologies are created equal, so matching your technology to the right drug and indication and the required technical and clinical innovation to make it happen are critical. A start-up company cannot afford to get it wrong with their first product candidate, as second chances are very difficult to come by. Financing is extremely challenging, with venture capital being the most common funding mechanism for start-up companies. Economic climate has strong impact and over the last few years venture funding has been extremely competitive and sparse making it very challenging to raise the capital required to fund the significant early development costs for pre-clinical testing, GLP pharm/tox studies, process scale-up and GMP clinical drug product manufacturing. Unfortunately, government funding of start-up companies is also quite limited and extremely competitive, often with grant opportunities pitting academic research and start-up early development as competitors in what can be difficult projects to fairly assess against one another given their potentially very different scope and goals. Ironically, venture and government funding are sometimes at odds with each other. If one assumes that venture firms will often fund the most promising companies, then these companies are typically ineligible for SBIR funding, which limits the government from providing additional key funding to reach the clinic.

The two most notable nanotechnology-based drugs are DOXIL® (PEGylated-liposomal doxorubicin, approved in 1995, developed by SEQUUS) for the treatment of ovarian cancer and ABRAXANE® (albumin-bound paclitaxel, approved in 2005) for the treatment of metastatic breast cancer. DOXIL is more potent than doxorubicin and decreases cardiac-related side effects whereas ABRAXANE eliminates the use of the toxic excipient cremophor, allowing a higher dose of paclitaxel. Despite these successes, several nanotechnology start-up

companies have struggled to navigate the clinical translation of their technologies with process scalability and lack of robust analytical characterization leading to some failures, while other companies have appeared to match either the wrong drug or cancer indication with their technology resulting in disappointing clinical outcomes.

Future steps

An exciting opportunity for the future of nanomedicine is the targeting of nanoparticle drugs to specific disease cells through specific binding interactions between ligands on the nanoparticle surface and cell surface receptors present only on or at highly upregulated levels on cancer cells or tumor neovasculature. As is the case with DOXIL, this approach will also require optimization of particle characteristics to take advantage of the enhanced permeability and retention effect to allow for particle circulation in the bloodstream and extravasation through the irregular tumor neovasculature. It is the added impact of the specific nanoparticle binding as well as potential nanoparticle and drug uptake to provide intracellular delivery that offers very exciting possibilities. Early leaders in this area are Calando, which has recently reported early clinical data for their transferrin-receptor targeted siRNA demonstrating dose-dependent accumulation of drug in the melanoma cancer target tissues as well as BIND Biosciences which intends to initiate clinical studies for their prostate specific antigen-targeted docetaxel in multiple solid tumor indications in 2010.

In order to drive these promising nanomedicine technologies and others into clinical development it is essential to build start-up teams that possess the right dynamics. Having the appropriate skills is an obvious requirement, so that the team of scientists, engineers, clinicians and management are equipped to do the job. Early stage drug development presents many obstacles, so recruiting people who have experienced the challenges, failures and successes puts the company in an excellent position. From a culture perspective, individually and collectively, there must be a tremendous work ethic and enthusiasm, a willingness to put the team goals as top priority knowing that if the team wins individuals will win. There also needs to be an understanding that they are facing a marathon and not a sprint with respect to the number of achievements and time required to accomplish the ultimate goal of treating patients with cancer.

Training Programs in Cancer Nanotechnology: Preparing the Next Generation of Researchers and Clinicians

Carolyn J. Anderson

Washington University, St. Louis, MO

Introduction

An important consideration when contemplating the potential that nanotechnology holds to treat cancer and other diseases is how we can best train and educate our young people to meet the challenges of doing research, establishing start-up companies, translating knowledge to the clinic and the like. Harnessing the power of nanomedicine will require scientists and clinicians with inter- and multi-disciplinary training in key aspects of chemistry, physics, biology, medicine, computer science, engineering, and clinical sciences. Interdisciplinary science requires a departure from a parallel-processing model in which individual investigators worked alone. The best scientists in nanomedicine will not be experts in all fields of research, but they will comprehend the role each discipline plays and will competently communicate across fields to achieve better solutions. As most scientists are not trained in an interdisciplinary fashion, it is imperative to develop training programs in nanoscience that fulfill the goals of offering interdisciplinary nanoscience courses and research experiences where trainees will learn many aspects of nanoscience, with a focus on one particular area in this discipline.

The worldwide workforce necessary to support the field of nanotechnology is estimated at two million by 2015 (http://www.nano.gov/html/edu/home_edu.html). Questions arise as to how the U.S. educational system can train technicians, scientists, and clinicians, and how to assure that the students choose the appropriate educational path. Raising awareness and educating K-12 school children hopefully prompts students to study nanoscience at the undergraduate and graduate levels. Formal, didactic degree programs for undergraduate students, as well as strong graduate education and research in nanomedicine are also essential. There are currently many educational programs in nanotechnology at all levels of training, from K-12 to postgraduate experiences. However, the vast majority of the educational programs in place focus on the materials science and engineering aspects of the field. We should encourage programs that combine the physical sciences/engineering aspects with biology and/or medicine to foster the groundbreaking discoveries in the chemistry and materials fields that can be applied towards life-saving cancer treatments.

Current status

The field of nanotechnology has grown exponentially over the past 10 years, in part through government initiatives. The National Nanotechnology Initiative (NNI) was established in 2001 to coordinate Federal nanotechnology research and development. Today the NNI consists of the individual and cooperative nanotechnology-related activities of 25 Federal agencies with a range of research, regulatory roles, and responsibilities (http://www.nano.gov/html/about/home_about.html). The NNI does not fund research; however, it informs and influences the Federal budget and planning processes. One of the key goals of the NNI is to “Develop and sustain educational resources, a skilled workforce, and the supporting infrastructure and tools to advance nanotechnology.” The Education Center on the NNI website provides information on K-12 activities as well as listings of undergraduate and graduate programs in nanotechnology.

Resources for teaching nanotechnology to K-12 children

Several websites have nanoscience resources for classroom teachers and students’ families including the The

National Science Foundation (NSF) (http://www.nsf.gov/news/classroom/nano.jsp), The Nanobiotechnology Center (http://www.nbtc.cornell.edu/), Rice University (http://nanokids.rice.edu/), and the University of Albany (SUNY) College of Nanoscale Science and Engineering (http://cnse.albany.edu/Nano_for_Kids/K_12_links.html).

In addition to web-based resources several other resources for hands-on experience for youth are also available. The Nanobiotechnology Center sponsors such things as field trips for middle school children to learn about scanning electron microscopy and visits to the Strong Museum in Rochester, NY. Likewise the Nanoscale Informal Science Education (NISE) Network has sponsored NanoDays since 2008. NanoDays combine simple hands-on activities for young people with exploration of current research for adults at over 200 science museums, research centers and universities across the country. Through the Program of Excellence in Nanotechnology (PEN) and the Siteman Center of Cancer Nanotechnology Excellence (CCNE) at Washington University, researchers are participating at NanoDays at the St. Louis Science Center by hosting two booths with hands-on activities.

Undergraduate training

Currently, several community colleges working with larger universities offers Associate degrees in Nanotechnology. For instance, the University of Pennsylvania collaborates with Pennsylvania community colleges to offer an Associate degree in Nanobiotechnology. Dakota County Technical College (Rosemount, MN) in conjunction with the University of Minnesota offers an Associate degree in Applied Science in Nanoscience Technology. The North Seattle Community College offers an Associate of Applied Science-T degree in nanotechnology.

At this time, there are no advertised bachelor's degree programs in nanoscience. However, there are several institutions that offer either a minor or a concentration in nanoscience or related discipline. At the University of Texas at Dallas, undergraduates can minor in nanoscience by taking three core NANO-designated courses, the content of which is exclusively related to nanoscience and nanotechnology. Yale University has an undergraduate minor in nanotechnology, where students are required to take an Introduction to Nanotechnology course and five other courses from a selection of engineering and biotechnology electives. Neither of these undergraduate minors require courses related to biology or medicine.

At the University of Wisconsin-Stout, students can obtain a B.S. in Applied Science with a Nanoscience concentration, and a B.S. in Engineering Technology with a concentration in Nanotechnology. Michigan Technological University offers an interdisciplinary minor in Nanotechnology. Several institutions have courses on nanotechnology, targeted towards either undergraduates or graduate students, including Cornell, Florida Institute of Technology, George Mason University, Rice University, University of Central Florida, University of Maryland, University of Texas at Austin, University of Washington, Washington University, and University of Wisconsin.

The majority of these programs emphasize the area of the physical sciences and engineering. There is definitely a need to see more education in nanoscience that incorporates biology and medicine, which will provide a larger pool of trainees for graduate programs, as well as provide a background for students studying medicine to have knowledge of how nanotechnology can be used to treat diseases such as cancer.

Graduate training

There are numerous institutions in the U.S. that train graduate students to do research in the area of nanotechnology, nanoscience, or nanomedicine. There are fewer universities that have formal programs that offer coursework and either a degree, certificate, or specialization. The majority of these programs are focused in the physical sciences and engineering, and there are few that combine the physical sciences and engineering with biology and medicine. One of the more innovative and interdisciplinary programs is at Northeastern University, where they have a Nanomedicine program funded by the NSF IGERT (Integrative Graduate Education and Research Traineeship) initiative and the NCI. There are over 20 faculty involved from Northeastern University, with collaborations with other Boston-area researchers and scientists from neighboring hospitals and industries. Students are enrolled in a Ph.D. program in Biology, Chemistry, Physics, or one of their Engineering programs, and then graduate with a specialization in Nanomedicine Science and Technology. This is one of the best examples of a graduate program that allows students to obtain an interdisciplinary education, learning the science and/or engineering, as well as the biomedical applications.

The University of Michigan has the Michigan Nanotechnology Institute for Medicine and Biological Sciences (http://nano.med.umich.edu/). This program has several talented scientists with expertise in fields ranging from chemistry, biology, medicine, and engineering. Students can earn a Ph.D. in a typical field of study and obtain a certificate in NanoBiology. Coursework is selected from biology, physical sciences, and engineering. The nanoscience courses appear to be explicitly in the areas of the physical sciences and engineering rather than incorporating biology and/or medicine. The University of Texas Health Science Center at Houston opened a Department of NanoMedicine and Biomedical Engineering in 2009 whose mission is "to introduce students to the field of Nanomedicine and the vast opportunities it provides for enhanced therapeutics, personalized medicine, medical diagnostics, imaging, screening, prevention, and regenerative medicine." This program is unique in that it is probably the only one that educates and prepares *medical* students to learn emerging new technologies in biomedical nanotechnology and engineering. Students are required to complete a scholarly research project and present the data at a scientific meeting, as well as prepare a manuscript to

obtain the certificate of completion. There are also journal clubs and other meetings, but at the time of this writing, there were no formal courses described on the website.

Clinical potential

For nanomedicine to reach its full potential, there needs to be more training centers like the ones at Northeastern, University of Michigan, and University of Texas Health Science Center at Houston. Having top-notch researchers in nanomedicine at institutions is obviously important for training the future scientists in the field. However, combining the research with didactic training will provide another level of skill for these future scientists and clinicians. Incorporating nanomedicine into medical student training will also ensure that these students understand how nanomaterials and nanodevices can be applied in medicine, particularly cancer treatments and diagnosis. Additionally, post-graduate training of research residents would also fulfill this role.

Obtaining the support of the NCI cancer centers in promoting nanotechnology education will also be key for future success. Of the Centers for Cancer Nanotechnology Excellence (CCNE) that were funded in 2005, the Siteman at Washington University had outreach and education cores that promoted education to medical specialists, the general public, as well as students at the K-12 through graduate levels. A course in Nanomedicine was offered yearly to graduate and undergraduate students. Outreach events to promote nanomedicine to the public at the St. Louis Science Center were also sponsored by the CCNE.

Future challenges

Federal grants have provided resources for the infrastructure of several educational programs in nanotechnology and have sustained them for the past decade or more. One of the challenges will be to maintain these programs when the funding expires, in particular the K-12 outreach programs. Many of these initiatives are for a limited time, are not renewable, and it is apparent that many programs have ceased over the past few years. Novel ways to maintain K-12 education in nanoscience, possibly through school teachers themselves, as well as alternative funding sources, such as private donors or foundations should be investigated. Encouraging universities and institutions that have strong nanotechnology research and education programs to engage in outreach activities to K-12 school children and the general public would be an inexpensive way to expand the awareness of nanotechnology and nanomedicine and increase the pool of future trainees.

One of the major concerns in undergraduate and graduate education in nanomedicine is that aside from the few programs described above, the vast majority of existing programs offering minors, certificates and/or specializations in nanotechnology are highly focused in the areas of materials science and engineering, with little or no emphasis on combining this with biology and/or medicine. Some of the programs that are focused in the physical sciences and/or engineering are affiliated with strong medical schools and/or cancer centers, and these institutions should be encouraged to collaborate with the cancer biologists and oncologists in educating nano-scientists regarding these medical applications.

As the NNI funding initiatives phase out, funding of research in nanomedicine will likely continue and hopefully expand as the nano grants are submitted to NIH through the traditional mechanisms (e.g. R01, P01, etc.). Unfortunately, requesting funds for educational initiatives through these mechanisms is not allowed. Finding the resources to develop new educational programs in nanomedicine, or even maintenance of existing programs will be a significant challenge. For example, currently only the University of Texas Health Science Center at Houston has a program to train medical students in nanomedicine. Mechanisms for funding the development of similar programs at other institutions should be investigated.

Milestones

3-year:

- Encourage more universities with strong nanotechnology/nanomedicine programs to reach out to the general public and/or K-12 school children and/or their teachers.
- Sponsor a workshop on nanomedicine education, with sessions and panel discussions on education at all levels (general public, K-12 school children, school teachers, undergraduates, graduate students, medical students, and post-graduate education).

5-year:

- Three to five of the existing undergraduate minors/specialities in nanoscience will incorporate biology and medicine into their curriculum.
- An additional two to four graduate programs in nanoscience will add a focus on nanobiology and/or nanomedicine.
- Using the University of Texas Health Science Center at Houston's program for training medical students as a model, there will be one to two more of these programs offered at major universities.

10-year:

- There will be more medical students and graduate students graduating from existing and recently developed programs in nanomedicine, thus increasing the number of qualified scientists working in academia, industry and possibly even private medical practices.
- Due to advances in research and education in nanomedicine, there will be more nano-based agents approved for the diagnosis and/or treatment of cancer as well as other diseases.

Maximizing Research and Technology Development Effectiveness Through a Team Approach

Dorothy Farrell, George Hinkal, Sara S. Hook, Nicholas Panaro, and Krzysztof Ptak, and Piotr Grodzinski

Office of Cancer Nanotechnology Research, CSSI, National Cancer Institute, NIH, Bethesda, MD

In order to develop effective devices and treatments for cancer using nanotechnology, the NCI recognizes that it is imperative for diverse professionals to unite toward this common goal. “Team science is about developing new ideas, forging new partnerships, and collaboratively using new tools to understand cancer as a disease process and a highly complex system," explained Dr. Anna D. Barker, the former deputy director of NCI. "The model includes teams of experts who can not only view the many elements of the cancer process, but can integrate that knowledge and design an innovative and targeted strategy of drugs, biologics, and even devices that can be used at all phases of the cancer process in an integrated fashion. Although the individual investigator will continue to drive innovation, the old model of cancer research taking place in isolated silos is fading away." As an illustration, chemists and engineers have the expertise to design and synthesize the best types of nanoparticles with physical properties that will solubilize drugs, RNAs, and proteins and ensure transport across the blood/brain barrier if needed. Meanwhile, the expertise of biologists and clinicians is imperative to know what tumor type to target, through which molecular mechanism, and which biological read-out to use to monitor the effectiveness of treatment. In 2004 the NCI established the Alliance for Nanotechnology in Cancer to foster this type of interdisciplinary collaboration. One of the avenues they used was to establish CCNEs through an open competition. These centers were lead by multiple program directors (PD) and primary investigators (PI) coming from the areas of medicine, biology, chemistry, physics, and engineering. The power of team science can best be illustrated by stories of the program participants themselves.

Dr. Dennis Carson, the director of the University of California, San Diego’s (UCSD) Moores Cancer Center, had a vision for incorporating aspects of engineering into cancer research, and he knew there was significant talent and interested faculty at UCSD to carry out the large scale multi-disciplinary effort necessary to establish a CCNE. He needed to identify a director at UCSD, however, who could lead this diverse talent to success. In consultation with Dr. Roger Tsien, UCSD’s leading biochemist in the field of nanotechnology, and Dr. Andrew Kummel, a chemist and materials scientist very familiar with the engineering faculty, they quickly reached a bold and unusual decision. Their choice to lead the effort was Dr. Sadik Esener, a professor of Electrical and Computer Engineering and of Materials Sciences at the Jacobs School of Engineering with a strong expertise in electronics and photonics but surprisingly little involvement with cancer or nanoparticle research at UCSD. However, Dr. Esener had the key attributes required for successful leadership of the new center: (1) respect of his colleagues and proven success in running large scientific projects, (2) multiple successes in commercializing medically related chip-based technologies, (3) the ability to work with scientists of different backgrounds and personalities on their ideas, and (4) speed in learning new fields of science.

When Dr. Carson contacted Dr. Esener to ask him if he would agree to serve as the PI, Dr. Esener’s first reaction was there must be a mistake. Dr. Esener was eventually won over and concludes, “Nothing comes close to the fulfillment one feels as a researcher to know that you are wrestling with a problem that if resolved would eliminate so much pain and suffering in the world. Although, I had some doubts before I accepted this position that entails tremendous responsibility, I am now so grateful to have been given this remarkable opportunity to bring a new perspective to this disease as a result of NCI’s bold undertaking and Dennis’ courageous decision. I cannot imagine how I could have been involved with leading edge cancer research without this center and the team science approach.”

Since its inception, the Alliance program has demonstrated that multi-disciplinary teams can synergize to develop clinically translatable technologies and therapies for cancer. The research groups involved in the Alliance have published over 1000 research articles, generated 250

patent applications and disclosures, and started more than 30 companies by which the technologies will be developed and marketed. Currently, 10 clinical trials are ongoing using therapies that have been developed using funds from the program. These innovative technologies and therapies would not have been possible had it not been for the willingness of scientists from divergent fields coming together to lend their expertise, ideas, vision, and passion. With the recent renewal of the Alliance for Nanotechnology in Cancer program, there should be even more outstanding contributions to cancer diagnosis, imaging, treatment, and management in the years to come.

Interdisciplinary collaboration is critical to effectively train young scientists in the area of nanotechnology (as discussed in the previous section). Program efforts to foster a collaborative spirit in the first phase of the Alliance resulted not only in research projects and publications, but in exchanges of personnel and materials. This personnel exchange was particularly important for the program's training components, as numerous graduate students and postdoctoral researchers were able to use network connections formed at investigator meetings to establish their next positions. The next five years of the program, Phase II, has increased research training funding to include Cancer Nanotechnology Training Centers (CNTCs) and Pathway to Independence Awards in Cancer Nanotechnology Research (K99/R00). The funded CNTCs will target graduate student and post-doctoral researchers of broad background (in medicine, biology, and other health sciences as well as in the physical sciences, chemistry, and engineering). The program of multi-disciplinary research education in cancer nanotechnology will primarily focus on mentored training, usually from multiple investigators in different disciplines, through laboratory-based research projects. In addition, centers will offer both short courses and workshops as wells as outreach experiences. Given the challenges more senior post-doctoral fellows face in finishing projects and establishing themselves as independent investigators, the program has invested in funding several Pathway to Independence Awardees. These trainees will benefit not only from their direct mentors but from the more informal mentoring and interaction at PI meetings across the Alliance.

The bread and butter of the program remain the CCNEs and CNPPs. The CCNEs of this new program edition will have a greater focus on clinically-worthy technologies as compared to Phase I. The new program will emphasize more heavily cancers having particularly poor outcomes, including brain, lung, pancreatic, and ovarian cancers. The science will continue to pursue basic discovery and innovation, but will also explore the clinical utility and translation development of the technologies. The collaborative effort then between the physical and basic scientists will be driven by those pressing questions facing clinicians. Collaborations benefit from complimentary skills, experience, perspective, and the use of diverse methodologies, as such the right mix of expertise is crucial for a highly effective interdisciplinary research team. When basic and physical scientists realize, for instance, that one of the important aspects of pancreatic tumorigenesis is the microenvironment, they can begin to address how to develop interventions and therapies to intercede in relevant pathways. The "begin with the end in mind" approach can save valuable time and resources by honing in on the most profitable research direction, foreseeing possible roadblocks, and planning for alternate avenues. Likewise, it is important to consider what data is needed for pre-clinical testing and characterization of various nanoparticles and devices so that the proper experiments can be done early and the process of clearing institutional, legal, and regulatory hurdles may be initiated. It may be wise to seek the advice and guidance of institutional and federal regulatory bodies such as the FDA so that applications for INDs, IDEs, and patents will progress unhampered.

As part of NCI's commitment to clinical translation the NCL will continue to work with investigators as a hub for the pre-clinical characterization of nanomaterials and to assist in the process of bringing nanotechnologies to the stage of IND or IDE submission. The NCL has established protocols for bio-nanoparticle characterization and is currently expanding these protocols as well as working on others pertaining to GMPs such as scale-up process, purity, and batch-to-batch consistency. The lab will continue basic discovery and innovation, but it will also take great care in the evaluation of clinical utility of the technology and put strong emphasis on the translation.

The cross Alliance activity of the investigators can be enhanced by using the Alliance's Cancer Nanotechnology Laboratory (caNanoLab) where researchers and NCL are able to deposit, store, and retrieve nanoparticle characterization data. To date it has primarily been used to house *in vitro* data (physico-chemical properties and biological assays) and protocols but it is expanding to include *in vivo* characterizations of nanoparticles and their functional components. Data relating to the toxicity, pharmacokinetics, and ADME (absoption, distribution, metabolism, and excretion) in vertebrate animals will be collected. Another important aspect of caNanoLab is its contribution to nanotechnology ontology through standardizing vocabulary terms relating to the physical, chemical, and functional characteristics of nanotechnology.

The idea of data sharing usually makes scientific researchers uneasy. After all they have invested huge amounts of time and resources to generating this data. In addition, graduate students and post-doctoral fellows realize the importance to their graduate committees and careers of making an intellectual contribution to a project that results in several high quality, first author publications. However, it is important for trainees to recognize that they can obtain a significant benefit from working with a group of individuals to produce co-authored publications, promote idea exchange, and develop a network of colleagues within their field.

In order for effective data sharing to become a reality, there needs to be trust between all parties involved. First of all, there needs to be trust within each CCNE. Strong committed leadership breeds trust as well as motivation. "Within our own consortium, trusting relationships between people have already been established," noted Dr. Sanjiv Sam Gambhir of the Stanford CCNE. "Indeed, the whole process of building

and applying for the CCNE grant built a great deal of trust between members, and between the university and companies involved." As the leadership development website, http://www.thelearningcenter.net/, states "There are two parts to trust: a feeling part that indicates trust and a performance track record that confirms trust." Many of the investigators within established CCNEs have collaborated and published together thus "confirming" their trust with a previous track record. Trust within new CCNEs and across the Alliance program could be more difficult to establish. Through various programmatic mechanisms, not the least of which is the annual PI meeting, a large number of cross Alliance collaborations have been built. A key to building trust is effective communication. Physical scientists, for instance, know the language and acronyms of their field. Oncologists, however, do not know that specialized language. As Phase II of the Alliance takes shape, sensitivity to communication style, scientific "language," and effective listening strategies becomes crucial for building productive teams and collaborative efforts.

The Alliance has demonstrated that a multi-disciplinary approach to research can catalyze scientific developments and enable clinical translation. Alliance investigators have advanced diagnostic technology, using both *in vitro* assays and novel imaging methods, and offered improved therapies and therapeutic efficacy measures. Many of the technologies developed and clinically translated have applied novel engineering to existing cancer biology strategies. The next stage of cancer nanotechnology research should enable new avenues of cancer care through revolutionary diagnostic tools, imaging techniques, treatment options, and *in situ* tumor characterization.

The scientific strategy for the 2010-2015 segment of the program was formulated based on the lessons learned from Phase I, the evolving strategy of the NNI, and, most importantly, the input of the extramural community. Phase II of the program will promote early diagnosis and better monitoring of therapeutic efficacy using emerging *in vitro* diagnostic techniques and novel imaging technologies such as multiplexed, multi-modal molecular contrast agents. It will be important to correlate outcomes from both approaches. On the therapeutic front, an increasing number of treatments will exploit tumor targeting via cell surface ligands and enhanced formulations for chemotherapeutics that reduce systemic toxicity and improve therapeutic index. Cooperative treatment regimes in which drug delivery is combined with tumor microenviroment engineering to improve treatment response will emerge. In addition, despite early hopes that gene therapy approaches would change the face of medicine, virtually no success has been garnered to date. There are glimpses that silencing genes and hopefully also replacing mutated genes will become routine modalities of treatment due to nanoparticle delivery options. In conclusion, while we do not want to over speculate or promise what we cannot achieve, we feel confident that patients facing this disease will have many more options in their arsenal due to the concerted effort, commitment, dedication, and ingenuity of those in the cancer nanotechnology research field.

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Preface

Nanotechnology offers the capability to unlock new avenues in the patient specific prevention, early diagnosis, control and treatment of cancer. As such, nanotechnology is expected to offer a significant improvement as compared to the current standard of care in oncology. To capitalize on its potential, the U.S. National Cancer Institute (NCI) in 2004 launched the NCI Alliance for Nanotechnology in Cancer. The Alliance is a large multidisciplinary effort involving researchers and clinicians, who have being working tirelessly in developing new nanotechnological approaches to develop new, and improve upon existing, therapeutic modalities, and similarly for diagnostic and detection techniques. The collective focus has remained on one thing; *a decrease in societal cancer-related morbidity/mortality of multiple tumor types via nanotechnology*. In as much, the Alliance has made very significant progress over the last 10 years producing many scientific discoveries and forming multiple companies, which are commercializing the technologies developed in academia.

Since the beginning of the program, the field of cancer nanotechnology has continually evolved and matured. Recognizing this constant evolution, we publish the *Cancer Nanotechnology Plan* (**CaNanoPlan**) to acknowledge these changes and to attempt charting the path forward for this dynamic field. The authors of this book include clinicians and researchers from the academic, industrial and government sectors. Of importance to notice, is that the number of covered topics has grown substantially since the last edition of CaNanoPlan published in 2010—this is a direct result of the ever-expanding number of areas in the cancer research space that nanotechnology solutions are being effectively used for. Our hope is to deliver to you, the reader, a *current and future state of the cancer nanotechnology field*, without bias, and, more importantly, to impart the numerous areas in which nanotechnological discoveries will impact the future of medical approaches to cancer care.

Foreword

Nanomedicines: Are they a platform for drug delivery common to many cancer types or a new approach to design drugs for specific tumor types?

Mark E. Davis, PhD
Department of Chemical Engineering
California Institute of Technology, Pasadena, CA 91125

Simply stated, nanomedicines are both. The NCI Alliance for Nanotechnology in Cancer is now entering the third phase of its existence (Phase I and II funding from 2005-2010 and 2011-2015, respectively), and it is an appropriate time to assess where nanomedicines have been and where they are going. Nanomedicine is the medical application of nanotechnology[1] (specifically for cancer see Chow and Ho[2]), so I consider nanomedicines to be nanoparticle-based therapeutics for the treatment of human disease. At this time, the term nanomedicine is used more liberally in that it is employed to categorize nanoparticle-based, therapeutic entities whether or not they are used for the treatment of humans. Petros and DeSimone[3] provide an excellent historical timeline for the development of nanoparticle-based therapeutic entities, while Davis et al.[4] describe how nanoparticle-based, experimental therapeutics distinguish themselves from previous anticancer therapies. Here, I will address the title question by discussing the transition from the "so called" first generation of nanoparticles (Petros and DeSimone, 2008) to the current application of nanoparticle-based, investigational therapeutics for the treatment of cancer.

First generation nanomedicines such as Doxil® (~ 100 nm nanoparticle - liposome encapsulated doxorubicin; approved in 1995) and Abraxane® (albumin-based nanoparticle formulation (~ 120 nm) containing paclitaxel; approved in 2005) are the most referenced nanomedicines that currently are being used to treat cancer patients. These commercial products have provided benefits to patients. For example, Doxil® greatly assists in mitigating the heart damage that can occur with doxorubicin, and Abraxane® does not have the classic hypersensitivity issues due to the cremophor component of paclitaxel formulations. However, these products do have properties that are undesirable. For example, nanoparticle formulations have the potential to create new toxicities that are not observed with the naked drug molecules,

and this phenomenon is observed with Doxil® (causes a form of skin toxicity that is due to the liposomal formulation, while free doxorubicin does not reveal this side effect). Additionally, Doxil® shows changes in pharmacokinetics (PK) upon multiple-cycle dosing in patients[5]. Abraxane® does not function as a true nanoparticle, and should be called a nanoparticle formulation because it dissolves upon administration due to contact with the blood[6]. As such, the control over drug properties, such as release rates, is not possible with these formulations. While Doxil®, Abraxane® [7,8] and other first generation nanomedicines have certain features that modern nanoparticles strive to eliminate or improve upon, these pioneering therapeutics have provided the field of nanomedicines a legitimate starting point. Additionally, they have generated a baseline of human therapeutic data to learn from and for which modern nanomedicines must strive to exceed[9].

Nanomedicines are evolving platforms for continually improving drug delivery that is common to many cancer types

Nanomedicines can be used to deliver drugs to many cancer types. As the field of nanomedicine has progressed, due in part do to increased knowledge of nanoparticle synthesis (better homogeneity is important[10]) and nanoparticle properties (though improved measurement techniques and methodologies), better understanding of how nanoparticles behave in animals[11,12] and humans[13,14] is occurring. This information is enabling nanomedicines to evolve to the point of providing increased functionality that improves the delivery of drug molecules to cancer patients. Nanomedicines seek to improve PK properties (enhanced solubility of the drug, tunable circulation times, tunable release of the drug, even at the site of active in the tumor) and alter biodistribution; in order to have low amounts of drug in non-target tissues and increased drug in tumors for greatly diminished side effect profiles (and most importantly, no new side effects due to the nanoparticle) in patients. These properties can: (i) enable drug combinations formerly inhibited by toxicity limits, (ii) enable new classes of drug delivery (for example, siRNA), and (iii) provide cell specific targeting within a tumor (all illustrated below).

Liposomal formulations such as those used with products like Doxil® have been improved upon, and now can provide new types of nanomedicines. For example, CPX-351 (Celator Pharmaceuticals) is a liposomal formulation of cytarabine and daunorubin in a 5:1 ratio for the treatment of high-risk AML patients. In

this case, the liposome acts to maintain the two drugs in a ratio that creates a synergistic efficacy of the target cancer. This product showed enhanced efficacy in Phase II clinical trials, and is currently being tested in a Phase III trial (NCT01696084). In addition to delivering drug molecules, lipid-based nanoparticles are now used to deliver small interfering RNA (siRNA)[15,16] and other nucleic acids[17]. Tabernero et al.[15] have published the first-in-human clinical results for simultaneously delivering siRNAs against two different gene targets to cancer patients.

...CRLX101 has now been shown in clinical trials to be combinable with other drugs as well as radiation therapy.

Polymer containing nanoparticles are also being developed as nanomedicines for cancer, and they are showing new and interesting behaviors in animal studies and human clinical trials. For example, Schluep et al.[18] showed that a polymeric nanoparticle containing the tubulysin peptide can be an effective antitumor agent while the tubulysin alone is so toxic that there is no therapeutic window for it, even in mice. These types of data show how nanomedicines can open new opportunities with compounds that are not viable on their own (due to toxicity and/or other issues). Polymeric nanoparticles have also been used to deliver siRNA, and in fact, were the first example of siRNA delivery to cancer patients[19]. Additionally, there are situations where the therapeutic agent need not be delivered to the cancer cells, but rather to other cell types within the tumor (like macrophages or stromal tissue). Ortega et al. recently showed how a polymeric nanoparticle could deliver siRNA to tumor-associated macrophages[20].

Polymer containing nanoparticles are progressing in clinical studies. Examples of this type of nanomedicine are the polymeric micelles Genexol-PM (approved in South Korea) and NK105[21], and the homogeneous polymeric nanoparticles CRLX101[13] and BIND-014[22]. NK105 is currently in Phase III clinical testing (NCT01644890), and both of the polymeric nanoparticles are currently in Phase II clinical studies. Of importance to the field of nanomedicine, CRLX101 has now been shown in clinical trials to be combinable with other drugs as well as radiation therapy. This is an important point, as nanomedicines should produce an efficacious therapy with low side effects that they can be used in typical combination therapy regimens. As it is well understood, that combinations of therapeutic agents are ultimately the desired goal in treating cancer patients, in order to provide efficacy and suppress resistance mechanisms from emerging. Pham et al.[23] recently described how CRLX101 (containing the drug molecule, camptothecin) could be used in combination with bevacizumab in ovarian

(both animal and human results) and kidney (human results) tumors. In refractory, metastatic renal cell carcinoma, the combination therapy significantly outperformed a monotherapy of bevacizumab or topotecan (FDA approved analog of camptothecin). A key point is that in the human clinical trials, the doses of CRLX101 or bevacizumab when used in combination did not have to be lowered from the amounts administered when they are used as monotherapies.

Overall, current investigational nanomedicines are showing interesting behavior in animal and human studies. They are providing new properties that have not previously been available (for example, CRLX101 can provide durable inhibition of HIF-1alpha that can be used in combination with anti-angiogenesis therapeutics[23]), and are enabling new types of therapeutic entities like siRNA.

Nanomedicines are a new approach to design drugs for specific tumor types

In essence, nanomedicines are small chemical systems, so they can consist of several components that are designed to provide multiple functions, such as the targeting of specific tumor types. A clear example of this approach is in the delivery of siRNA. Since siRNA can be used to inhibit essentially any gene, and multiple targets can be simultaneously inhibited, specific tumor types can be targeted and treated using this approach. Recently, Yuan et al. showed that four different siRNAs could be delivered to tumor xenografts using a nanoparticle delivery system[24]. Additionally, improved therapeutic efficacy was observed when simultaneously delivering siRNAs against KRAS and PIK3CA/B. This study nicely demonstrates the power of siRNA therapeutics for cancer by showing that multiple gene targets can be simultaneously inhibited (without increased toxicity like would be the case with combining other therapeutic molecules) to produce greater anti-tumor efficacy. This is the goal for the clinical application of siRNA treatments of cancer, and if achievable, could be a "game changing" way to treat cancer. Information from three finished Phase I trials with siRNA are available to guide future studies[14–16,19]. At this time, all of the clinical trials that have employed siRNA do not attack a specific tumor type. However, it is expected that this approach will be used to treat cancer patients with specific cancer types in the near future.

...four different siRNAs could be delivered to tumor xenografts using a nanoparticle delivery system.

Another approach for creating specific tumor targeting nanomedicines involves the inclusion of a so-called "targeting agent" to the nanoparticle to provide for "active targeting"[25]. These targeting agents engage cell surface receptors to not only provide for active targeting, but also to enable a number of other biological functions. CALAA-01 contains the human transferrin protein (Tf) on its surface to engage transferrin receptors (TfR) that are upregulated on the surface of many cancer cell types[26]. The Tf enhances the amount and rate of nanoparticle uptake into the cancer cells. Thus, in this case and others that target the TfR[27], these nanoparticles are appropriate for treating the limited number of cancer cell types that have upregulated TfR. The targeting agents can have biological functions in addition to providing cancer cell uptake, e.g., antibodies and antibody fragments can block signaling effects. An example of this type of nanoparticle, that has been tested in a Phase I clinical trial, is a liposome encapsulating doxorubicin and containing the Fab' fragment of the antibody cetuximab (binds to EGFR)[28]. This nanoparticle is appropriate for treating cancers with overexpressed EGFR. The inclusion of targeting agents adds complexity to the nanoparticles, and the costs versus benefits of these agents have been discussed[29]. However, this type of additional functionality in nanoparticles can clearly be used to create nanoparticles that are designed to treat specific cancer types, e.g., those with upregulated surface proteins like Her2, EGFR, etc. Historically, it has been difficult to achieve functions from the targeting agents. Although recently, investigators have learned how to construct nanoparticles that can have multiple functions, including those of a targeting agent, where the functions work at the appropriate time and place along the delivery process rather than annihilating each other like in the past[30].

Within the next 5 years it is most likely that a number of new nanomedicines will become FDA approved.

What does the future hold for cancer nanomedicine?

Within the next 5 years it is most likely that a number of new nanomedicines will become FDA approved. The cancer nanomedicines that are nearing final clinical testing and approval are those carrying small molecule drugs. Additionally, within this time, there should be the first of several approved siRNA-based nanomedicines. These nanomedicines will not be to treat cancer, but rather for the treatment of liver diseases. However, they will lead the way for siRNA-based nanomedicines to be approved for cancer at a latter time (say within 10 years).

Because of the safety of nanomedicines, once they are approved, it is expected that they will be combined with numerous other therapeutics (including new immunotherapeutics) to provide more individualized and potent therapies to cancer patients. Thus, nanomedicines will be utilized in combination therapies to treat a broad spectrum of cancer types AND to treat specific tumor types, where the mode of deployment of the nanomedicine will depend only upon their specific designs and chemical configuration.

Mission of the NCI Alliance for Nanotechnology in Cancer Program

Nanotechnology is the application of materials, functionalized structures, devices, or systems at the atomic, molecular, or macromolecular scales. At these length scales, approximately the *1-100 nanometer range* as defined by the U.S. National Nanotechnology Initiative (NNI), unique and specific physical properties of matter exist, which can be readily manipulated for a desired application or effect. Furthermore, nanoscale structures can be used as individual entities or integrated into larger material components, systems, and architectures. Nanotechnology-based structures and devices are already enabling a large number of novel applications in various fields – including medicine.

Currently, scientists are limited in their ability to turn promising molecular discoveries into cancer patient benefits. Nanotechnology can provide technical control and tools to enable the development of new diagnostics, therapeutics, and preventions that keep pace with today's explosion in knowledge.

The Office of Cancer Nanotechnology Research (OCNR) within the Center for Strategic Scientific Initiatives (CSSI) at the National Cancer Institute (NCI) of the National Institutes of Health (NIH), develops and implements programs with and for the extramural research community related to the use of nanotechnology in medicine and cancer. The overarching goal of these initiatives is to discover and develop innovative nanotechnologies for application(s), ranging from discovery through to clinical translation phases, for the delivery of innovative clinically relevant technologies aimed at cancer prevention, diagnosis, control, and treatment. These initiatives include a programmatic effort known, collectively, as the NCI Alliance for Nanotechnology in Cancer, which aligns to several key areas of the National Cancer Institute's existing priority areas as displayed in **Figure 1**.

The OCNR's *NCI Alliance for Nanotechnology in Cancer* was designed to develop research capabilities for multidisciplinary team research, with the goal of advancing basic science, prevention, diagnostic, and/or treatment efforts from the research discovery to preclinical and early clinical development stages. The Alliance's development model calls for the most promising strategies discovered

and developed by its grantees to be handed off to potential for-profit partners for effective clinical translation and commercial development. Furthermore, to expedite translation into the clinical setting, it calls for the technologies to be characterized by the Nanotechnology Characterization Laboratory (NCL) in Frederick, MD.

The *Alliance for Nanotechnology in Cancer* is engaged in efforts to harness the power of nanotechnology to radically change the way we diagnose, treat and prevent cancer. As such, the *NCI Alliance for Nanotechnology in Cancer* is a comprehensive, systematized and multidisciplinary initiative encompassing the public and private sectors, designed to accelerate the application of the best capabilities of nanotechnological developments into the realm of contemporary oncology[31].

Purpose of Cancer Nanotechnology Plan 2015

The primary purpose of the *Cancer Nanotechnology Plan 2015* is to serve as a strategic document to the *NCI Alliance for Nanotechnology in Cancer* as well as a guiding document to the cancer nanotechnology and oncology fields, as a whole. Now in its third incarnation, this ***CaNanoPlan 2015*** has increased in scope, mostly, due to the fact that the field has significantly matured and expanded over the last decade. It includes contributions from researchers, clinicians, policy makers, and industrial experts in order to give a broad perspective on where the field is now and where it is heading in the future.

Figure 1. Graphical depiction of NCI Alliance for Nanotechnology in Cancer research areas (colored only) relative to the overall NCI priority areas.

Current State of the Program

In its first round (*Phase I, 2005-2010*), the Alliance focused on translational research (e.g., clinically worthy technologies) and developmental efforts to set the framework for the future. During this period, the program focused on multifunctional therapeutics, *in vivo* molecular imaging (imaging systems and contrast agents), and reporters of efficacy as well as on the areas of early detection, prevention, and control. The research covered a broad spectrum of cancer-specific targets[32]. The awards made during this period included, ***eight*** U54 (formally called ***Centers of Cancer Nanotechnology Excellence*** or ***CCNE***) and ***twelve*** R01 (formally called ***Cancer Nanotechnology Platform Partnerships*** or ***CNPP***) grants. The Alliance was overseen by the Coordination and Governance Committee (CGC), which consisted of its principle investigators and the National Cancer Institute program staff. Near the conclusion of the first round, strategies were re-assessed from lessons learned by the NCI, CGC, and the extramural communities to determine the best path forward for the next round[33,34].

In its second round (*Phase II, 2010-2015*), the Alliance re-balanced itself while maintaining translational research for its CCNEs with more basic research for its CNPPs. Also, the training and developmental efforts to proliferate the preparation of the next generation of multidisciplinary researchers in the field of cancer nanotechnology were expanded. This training component was viewed as an increasingly critical element to developing the multi- and trans-disciplinary scientists necessary to the future implementation of nano-enabled interventions in the practice of clinical oncology. In an attempt to emphasize cancers with the poorest survival rates and explore successful use of nanotechnology in therapies and diagnostics for them, Phase II of the program focused on brain, lung, pancreatic, and ovarian cancers. The awards made during this period included, ***nine*** U54 (*CCNEs*), ***twelve***

U01 (*CNPPs*), ***six*** R25 (formally called ***Cancer Nanotechnology Training Center*** or ***CNTC***), and ***seven*** K99/R00 *Pathway to Independence Award* grants. Nearing the expiration of this second phase in 2013, again a reevaluation was performed in order to formulate a path forward for the program, guided by similar principles as before[35,36].

To date, the communal output from the Alliance members has been substantial. Beginning with the output of robust science, the Alliance has published over *2,750* peer-reviewed journal articles that have been collectively cited over *83,500* times across the scientific literature spectrum generating an average impact factor of *7.7*. From the perspective of clinical translation, the Alliance researchers have filed over *220* patents/disclosures, filed many applications to the FDA with over *18* clinical trials approved, and formed over *85* companies that have collectively commercialized multiple products. This collective

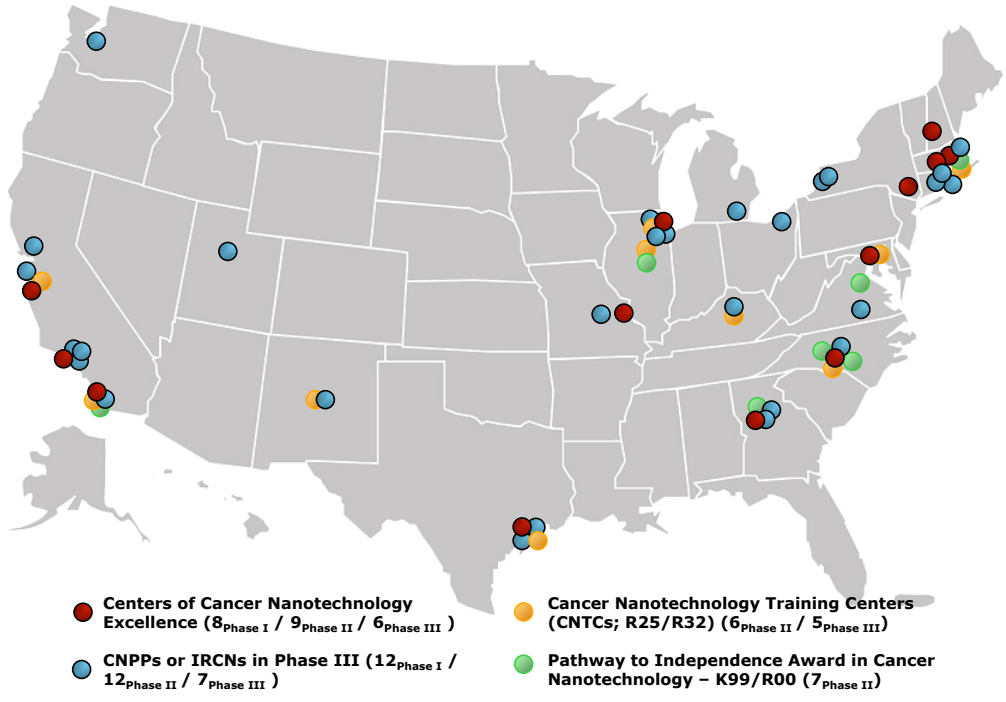

Figure 2. Map of United States as a geographical depiction of the locations of the NCI funded institutions (past and present, all represented) within the Alliance as of Fall 2015. CCNEs (red dots), CNPPs/IRCNs (blue dots), CNTCs (orange dots) and Pathway to Independence (green dots) all displayed circa their actual location in U.S.

output has come by way of NCI funding of over 1250 individual researchers and trainees. All of these statistics are direct results from work completed on Alliance-specific funded projects during only the 10-year period of the first two phases and are compiled in the **Infographic**.

Presently, the *NCI Alliance for Nanotechnology in Cancer* program is beginning its third round (i.e., Phase III), which began Fall 2015. The academic institutions that have been awarded grants during all three rounds to date are displayed, geographically, on the map in **Figure 2**. Although, this third round is similar overall to the previous, there are still several key differences. In this third phase, ***six*** U54 (CCNEs) have been awarded and the U01 granting mechanism has been altered from an RFA to a PAR for recurrent acceptance of applications including two application receipt dates per year through 2017. U01 grants are now formally termed Innovative Research in Cancer Nanotechnology (IRCNs) under this FOA, which reflects a shift in program focus towards addressing major barriers in cancer biology and/or oncology using nanotechnology and with an emphasis on fundamental understanding of nanomaterial interactions with biological systems and/or mechanisms of their *in vivo* delivery. CNTCs have also been transitioned to continual submission and are now funded via a T32 granting mechanism albeit through recurrent receipt dates. Although, the focus on training the next generation cancer nanotechnology experts has remained effectively unchanged. As of Fall 2015, ***seven*** U01 (IRCN) and ***five*** (CNTC) awards have been funded, although it is anticipated that more could be made over the course of next several years as more applications come in for the upcoming submission dates.

Nanotechnology Characterization Laboratory

In an effort to help advance the clinical translation of novel nanomedicines designed to improve therapeutic outcomes and enhance diagnostic capabilities, the National Cancer Institute, in concert with the Food and Drug Administration (FDA) and the National Institute of Standards and Technology (NIST), created the Nanotechnology Characterization Laboratory (NCL). The NCL has been pursuing preclinical characterization and development of these oncology-directed therapies and diagnostics for more than ten years now. In this time, NCL's multi-disciplinary team has worked with more than 100 of the world's foremost nanotechnology research organizations and evaluated

more than 300 different nanomaterials. Nearly a dozen NCL collaborators are now in human clinical trials with novel treatment strategies afforded through nanotechnology. NCL's unique setup has afforded an extraordinary opportunity to explore the biocompatibility trends and advantages and disadvantages of a vast array of nanoplatforms, cytotoxics, and targeting strategies in a relatively limited time span. Through sustained research and extensive educational outreach, the NCL strives to continually improve the pursuit of these much needed therapies, speeding their progression to clinical trials.

caNanoLab

The cancer Nanotechnology Laboratory (caNanoLab) is a web-based portal and data repository that allows researchers to submit and retrieve information on well-characterized nanomaterials including their composition, function, physical properties, and *in vitro / in vivo* experimental characterizations. Furthermore, information on the protocols used for these characterizations and links to any related publications may be similarly accessed. Initiated in 2006 by the National Cancer Institute as a collaborative effort between the NCI Center for Biomedical Informatics and Information Technology (CBIIT) and the NCI OCNR, caNanoLab serves as an established resource with an infrastructure supporting the structured collection of nanotechnology data to address the needs of the cancer biomedical and nanotechnology communities. While the majority of caNanoLab data has been entered through an in-house curator, individual users can submit data via web-based forms and an established, simple workflow. Submitters can customize the visibility of their data which ranges from private, sharable within a collaboration group, to open for public consumption. caNanoLab can also be used for discovery purposes by searching the results of all the publicly available data, protocols, and information about publications using webform-based queries. These results can be downloaded in spreadsheet-based reports for re-use and additional analyses. caNanoLab software is open source and available for download for local installation. Currently, the NCI instance of caNanoLab has information on 1,090 curated nanomaterial samples, 46 protocols , and 1,901 publications. Users are primarily from the U.S., but have grown to include users from several other countries such as Great Britain, Germany, China, the Netherlands, Spain, and Japan. In 2014, the number of unique portal visitors numbered over 3,000.

TONIC Consortium

The Alliance for Nanotechnology in Cancer established the Translation Of Nanotechnology In Cancer (TONIC) consortium in October 2011 to bring together public, private, and academic sectors interested in nanomedicine drug development, with the mission of accelerating the translation and development of nanotechnology solutions for the early detection, diagnosis, and treatment of cancer. TONIC members organized to combine their expertise to identify and evaluate the most promising technology candidates to develop a robust translational roadmap for the development of nanotechnology-based cancer products. The main goals of this partnership model include providing Alliance researchers insight into industry needs in technology platforms and drug targets, promoting collaborations between Alliance investigators and industry partners on promising pre-competitive and late-stage programs, and serving as a sustained forum for nanotechnology idea exchange. The partnership further provides TONIC members the opportunity to interact with regulatory authorities and the Nanotechnology Characterization Laboratory to promote the qualification, development, and regulatory acceptance of nanotechnologies in cancer. TONIC also encourages the sharing of consortium project results with the scientific community and independent verification opportunities to ensure data reproducibility and robustness.

Membership to the TONIC consortium remains free of charge, and for companies is limited to those that (1) have a successful track record of translating diagnostics and drug formulations and reaching their regulatory approval and, (2) are engaged in the development of nanotechnology-based formulations with application to imaging, diagnostics and therapy. In addition, these companies are expected to have a corporate structure with centralized operations and the capability and resources to effectively move along translational efforts. Currently, membership includes 14 corporate partners, and three patient advocacy groups, with participation by NCL and the FDA.

TONIC has organized several meetings and presentations at various venues over the past three years to educate Pharma and enhance awareness of nanotechnology platform opportunities in developing cancer solutions. It continues to participate in the annual Alliance principal investigators' meetings to promote networking and collaborations between industry and academic groups, and encourages the evaluation of external opportunities and platforms. The consortium has been credited with facilitating interactions

with NCL for TEVA and Astra Zeneca, two TONIC members. TEVA and NCL signed an agreement to initiate a collaborative study. Cytimmune credits TONIC for facilitating a research agreement with AstraZeneca to create a new nanomedicine using an AstraZeneca proprietary drug mounted on Cytimmune's PEGylated TNF gold nanoparticle platform. Moving forward, TONIC continues to take advantage of new opportunities to accelerate the consortium's mission of translating nanotechnologies to the clinic, and enhance academic-industrial partnerships.

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Section I: Emerging Strategies in Cancer Nanotechnology

Early-to-Late Stage Diagnosis: Nanotechnology-Based Interventions

Demir Akin, DVM, PhD and Sanjiv Sam Gambhir, MD, PhD
Department of Radiology, School of Medicine
Stanford University, Palo Alto, CA 94305

Introduction

The best chance of winning the war against cancer is to detect the disease at its earliest possible stages prior to there being increased cellular heterogeneity and physical spread of cancer cells from the primary site of origin. Finding cancer early is particularly challenging, as there are fewer numbers of cancer cells, and therefore lower concentrations of biomarkers at the cancer site and in bodily fluids, at an early stage along the natural progression path of the cancer. Furthermore, since most cancers are detected relatively late we often lack the ability to ideally characterize the true properties of early cancers, which are likely quite different than late cancers. Simply put, as there are more cancer cells present in advanced stage disease, in a similar fashion there are likely to be more changes in the genome, epigenome, proteome, and transcriptome when characterized *ex vivo*, as well as more protein targets for molecular imaging probes *in vivo*. All of these challenges can ideally be addressed by nanotechnology-based medical diagnostics as part of the *Nanomedicine* field. For its part, *Nanomedicine* promises unprecedented innovations for early diagnosis, staging, and therapy. It offers capabilities to perform simultaneous cancer detection and treatment in ways unachievable with other strategies. For example, nanotechnology has the potential to greatly impact *in vivo* diagnostics through molecular imaging for early cancer detection, even if, this approach must first be validated through the more tractable problem of impacting the management of later stage cancers. With its capacity to provide enormous sensitivity, multiplexing, throughput, and flexibility, nanotechnology has the potential to profoundly impact cancer patient management in the upcoming years.

Surgery is still the mainstay in medical management for both early and late stage cancers. Preoperative molecular diagnostic screening using both *in vitro* nano-enabled diagnostics tools and nanoimaging can detect and localize the tumor, exclude the patients who have metastasized beyond eligibility for a resection, identify the molecular signatures which can

be used to guide surgical procedure, screen the suitable cases whose biology is surgically most relevant, and orientate the surgeons to enable surgery planning.

Nanotechnology offers many other benefits for cancer early to late stage detection such as detailed single molecule and single cell analysis possibilities instead of 'bulk' measurements (**Figure 1**). Nanotechnology offers: (1) analytical sensitivity, (2) massive biomarker/analyte multiplexing ability, (3) low clinical sample volume operability, (4) capability to continuously monitor health and detect any deviation from it via implantable sensors, (5) capability for simultaneous cancer detection and therapy (theranostics), (6) solutions to visualize oncologic pathogenesis and its response to medical intervention in animal models via intravital fluorescence imaging, bioluminescence, and magnetic resonance imaging (MRI) and finally (7) cost benefits to the patients and the healthcare system at large.

Current Trends in Nanotechnology-Based Intervention for Early to Late Stage Diagnosis

A myriad of preclinical research grade nanobiosensors have already been developed, however, the ultimate goal of multiplexed, low-cost, high-throughput, reliable diagnostic devices for the clinic has yet to be fully realized. Having this capability in the clinic would undoubtedly allow for the improved detection of cancer with potential significant benefits to patients and the health-care system at large.

Often the vast majority of long-term cancer survivors have resectable tumors seemingly confined to the primary site at the onset of diagnosis and hence, they can benefit significantly from curative surgery, supporting that early cancer detection and intervention will increase the overall survival of patients. From a technological perspective, we have great nano-centric tools within our arsenal; disappointingly there are currently no reliable serum biomarkers with the sensitivity and specificity to accurately detect early pre-cancerous lesions. In many ways our technologies are ahead of our understanding of the underlying cancer biology. Furthermore, the heterogeneous nature of cancer and the inherently

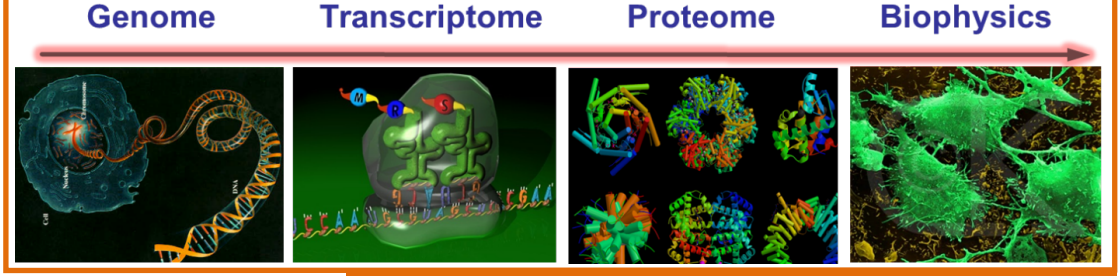

Figure 1. Nanotechnologies for comprehensive cancer cell analysis, ideally at single cell and single molecule sensitivity levels.

complex stromal microenvironment also present a challenge for identification of potential biomarkers. Hence, early diagnosis of tumors requires the simultaneous use of a panel of biomarkers for greater accuracy. In a recent mathematical modeling study[1] it was found that a tumor could grow unnoticed for more than 10 years and reach a spherical diameter of about 25 mm, before becoming detectable by current clinical blood assays. Further complicating it, the shedding rates of most current clinical blood biomarkers are found to be 10^4-fold too low to enable detection of a developing tumor within the first decade of tumor growth. These predictions well-align with clinical observations. Thus, currently there are no biomarkers suitable for screening of healthy general populations for possible occurrence of precancerous events. Routine surveillance of cancer is currently performed through classical cancer detection technologies, such as x-ray imaging based mammography for breast cancer, visible light colonoscopy for colorectal cancer, histo-pathological evaluation of Pap smears for uterine and genital cancers, and skin lesions by microscopic pathology, etc., none of which are presently enabled via nanotechnology. Currently, several preclinical diagnostic imaging tools are going through evaluation for their suitability as adjunctive technologies to the existing contemporary cancer diagnostic approaches. Some of these technologies are magnetic nanoparticle or gadolinium chelate-functionalized nanoparticle-enabled for high resolution MRI[2–4], nanoparticle and intrinsic contrast-based photoacoustic imaging[5,6], surface enhanced Raman spectroscopy-based endoscopy[7], cancer triggered self-assembling smart optical and MRI nanoimaging agents[8–10], micro-Nuclear Magnetic Resonance imaging[11], dual (*e.g.*, PET-Near Infrared fluorescence and PET-MRI)[12,13] and nano-enabled triple modality imaging (*e.g.*, MRI-Photoacosutics and Raman)[14]. A recent review summarizes the status of nanoimaging agents and the clinical trials associated with these approaches[15].

Currently, in the field of cancer nanotechnology-focused diagnostics, two very broad groups of devices and tools are emerging and there is strong and ongoing research in both. These groups are (1) benchtop or larger scale medical diagnostic devices and (2) miniaturized nano-based or nano-enabled diagnostic assays/devices that are designed and suitable for point-of-care or for patient's use at home directly or suitable for implantable, wearable, ingestible, inhalable uses. The medical expectations from the first group of devices is that they will be extremely robust, sensitive and specific as such they are suitable for confirmatory decision making that can both inform and guide clinical management of cancer. Nanoparticle-based imaging agents (*e.g.*, paramagnetic iron oxide or gold or silica-based nanoparticles, carbon nanotubes, surface enhanced Raman nanoparticles, etc.) and their associated detection/analysis instrumentation and nanoimaging devices (*e.g.*, nanoparticle assisted MRI, photoacoustic imaging, Raman spectroscopy) are examples of this category. On the other hand, the second group of cancer nanodiagnostic tools includes: nanocantilever, nanopore, nanowire, quantum dot, plasmonic nanoparticle-enabled micro/nanofluidic

devices, among many others. The medical expectations from these second group of point-of-care devices is that they will be cheap, produce rapid and reliable results, often during the same office visit and yield actionable results for seeking further medical evaluation. The first category of nanodiagnostic tools that are typically more suitable for later stage cancer and the second category of diagnostic tools are more applicable to early stage detection of cancer, recurrence, therapeutic efficacy monitoring, as well as general surveillance. There is a continued cancer nanotechnology research need for the improvement of and innovation in both of these categories of the medical diagnostic tools, which are inherently synergistic in principle from a medical benefits perspective.

Even with the progress resulting from early detection, the long-term prognosis of cancer patients is still limited by the occurrence of distant secondary metastases via circulating tumor cells (CTCs). Clinically occult micrometastases caused by these cells cannot currently be detected at primary diagnosis even by high-resolution diagnostic imaging approaches. The presence of CTCs in blood and bone marrow has shown to have therapeutic and prognostic impact for cancer[16–20]. It is postulated that CTCs could escape from chemotherapy by maintaining a dormant non-proliferating cell state (senescence) until the conditions are optimal to start expansion to manifest metastases[21]. Thus, the detection, enumeration and characterization of CTCs and their clusters (*i.e.*, 'liquid biopsy') remains as a viable candidate to investigate its potential to increase survival benefit for cancer patients, in particular, due to its ease of access and amenability for repeat sampling. A multitude of micro- to nano-scale technologies are now available to isolate an enrich CTCs[22,23], as well as highly sensitive and specific immunological and molecular assays[24,25] to characterize these cells at the single cell level in bone marrow and peripheral blood. These studies are providing insights into the critical steps of the initiation of the metastatic cascade.

...CDs offer significant potential as replacements for toxic metal-based quantum dots that have had difficulty with clinical translation.

Similar to CTC capture and characterization, extracellular vesicles released/secreted by cancer cells and loaded with cellular signals such as microRNAs and proteins, are emerging as important oncologic clues that can be obtained from clinical cancer samples (reviewed in Zocco *et al* 2014 and Webber *et al* 2015)[26,27]. The nondestructive isolation, enrichment, enumeration and intra-vesicular content analyses of these particles via the use of nanotechnology, such as nano-mechanical filters[28,29], nanoflare-based diagnostics (reviewed in Heuer *et al*, 2013, Prigodich *et al* 2012)[30,31], nanoproteomics analysis[32], bio-barcode-based analysis (reviewed in Pritchard, *et al* 2012)[33] are emerging as important

tool for cancer diagnosis, response to therapy and for prognostic surveillance. This field is currently expanding and it is expected to play a major role in cancer medical management in the near future.

Luminescent carbon dots (CDs) are emerging as new medical diagnostic tools as alternatives to quantum dots and other carbon-based nanomaterials such carbon nano tubes and graphene. These nanoparticles have well-defined, tunable surface functionalities, and their manufacture involves simple, fast, and cheap synthetic routes. Because of good biocompatibility, hydrophilicity, non-toxicity, resistance to photobleaching and -blinking, CDs offer significant potential as replacements for toxic metal-based quantum dots that have had difficulty with clinical translation.

Another novel development in the cancer nanotechnology field is the use of mass-encoded synthetic biomarker libraries for multiplexed monitoring of cancer in bodily fluids[34]. These exogenously administered 'synthetic biomarkers' are composed of mass-encoded tandem peptides conjugated onto nanoworm nanoparticles that leverage the intrinsic features of human disease and physiology for noninvasive urinary monitoring. These protease-cleavable peptide-based cancer sensors can target sites of disease, sample dysregulated protease activity and emit mass-encoded reporters into patient urine for multiplexed detection by mass spectrometry. It was shown that these agents can noninvasively monitor disease without the need for invasive core biopsies and the respective blood biomarkers.

The Future of Nanotechnology-Based Intervention for Early-to-Late Stage Diagnosis

Nanoscience applied to cancer research is proving to be a critical and encouraging approach for the eventual elimination or at least chronic control of cancer. Nanotechnology has been making a significant impact on cancer diagnosis and therapeutic management in revolutionary ways as exemplified in the NCI's 2010 Cancer Nanotechnology Plan (http://nano.cancer.gov/about/plan). Nanotechnology will continue to advance both *in vitro* diagnostics through genomic, cellomic, transcriptomic, proteomic and circulating tumor cell enumeration as well as exosome and microRNA analysis based nanosensors and for *in vivo* diagnostics via nanoparticles for molecular imaging. Moreover, *in vitro* diagnostics used in conjunction with *in vivo* molecular imaging is expected to markedly impact future cancer patient management by providing a synergy that neither strategy alone can offer. Indeed, the areas of earlier cancer detection and the prediction and monitoring of patient response to anti-cancer therapies could be impacted by this synergetic approach. Both represent very important applications for nano-enabled diagnostics with near-term clinical translational potential.

Specifically, the earlier detection of relevant cancers that are aggressive is still a major challenge for the cancer community. Earlier intervention of potentially aggressive cancers can greatly improve patient survival, quality of life and financial outcomes. These could be achieved via the synergistic use of highly sensitive and specific *in vitro* diagnostic devices to interrogate easily accessible clinical sample sources such as blood, urine, feces, sweat, tears, and saliva for multiple biomarkers (both protein and nucleic acid-based) and verify the presence and location of the tumor with nano-/molecular imaging *in vivo* using novel nanoparticles that allow signal amplification and multiplexing. As example, a cancer patient has cancer detected at much earlier stage through use of biomarkers derived from blood or other non-invasive samples and results from these *in vitro* tests are then verified by molecular imaging that simultaneously localizes tumor(s) prior to treatment. Additionally, post-treatment and potentially during treatment, the patients' response to therapy is measured to ensure the accurate differentiation of responders from non-responders can, which could be continually evaluated by blood analysis, without necessitating another tumor biopsy and/or molecular imaging.

The application of the above two approaches (combination of *in vitro* diagnostics with nanoimaging and the combination of *in vitro* diagnostics with benchtop ultrasensitive, specific nanodiagnostic technologies) in particular to the current unsolved oncologic challenges of detection of distant micrometastases, prognostic evaluation of tumor aggressiveness and its predicted response to a given therapy, differentiation of indolent tumors from the ones that have metastatic potential, tumor border demarcation during surgery are areas where there are significant gaps in our diagnostic abilities, hence, further and significant cancer nanotechnology efforts need to be spent on these critical areas to improve cancer patient outcomes within the next 5-15 years. Ideally, nanotechnology could make a huge impact in cancer by virtue of pre-emptive interventions to detect cancer early through continuous health monitoring via wearable, ingestible and implantable nanodiagnostics to detect deviation from health to pre-neoplastic conversion as early as possible. However, being able to get there will involve not only further nanotechnological advancements, but also, further improvements in the toxicological, biocompatibility and immunological concerns related to nanoparticles' use as cancer *in vivo* diagnostics. With appropriate level and timely financial commitments for nanoscience and nanotechnology research, the future of the *Cancer Nanotechnology* field is bright and full of opportunities as well as tremendous near-term rewards for patients.

Early-to-Late Stage Diagnosis: Detecting and Analyzing Circulating Tumor Cells

Jie-Fu Chen[1], Edwin M. Posadas[1], MD, Hsian-Rong Tseng[2], PhD
[1]Urologic Oncology Program, Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, California 90048
[2]Department of Molecular and Medical Pharmacology, California NanoSystems Institute, University of California at Los Angeles, Los Angeles, CA 90095

Circulating Tumor Cells (CTC)

The tissue-based evaluation of biopsy samples remains the gold standard for diagnosis and prognosis in clinical care and research. The bulk of published research focuses on tissue samples obtained by surgical excision or radiographically directed needle extractions. While these approaches have driven a tremendous amount of research, they are complicated by several issues. First, these extractions are both invasive to the patient and costly overall. Typically, serial biopsies are avoided for fear of complications from the procedure, but are essential in obtaining dynamic insight. Second, in cancers where metastatic tissue biopsies are problematic, research has relied upon historic primary tissues. Third, there is growing focus and concern for the impact of the tumor tissue's temporospatial heterogeneity.

As a measure to address these problems, circulating tumor cells (CTCs) have been proposed as they provide a means to sampling tumors across all present disease sites (they are perfused systemically in blood), including the primary tumor and metastases[35]. In addition to conventional diagnostic imaging and serum marker detection in cancer, the detection and characterization of CTCs in patients over the course of therapy creates new possibilities for personalizing cancer care by: (i) monitoring cancer progression, (ii) understanding the pathogenic mechanisms driving lethal disease and the dynamics of this evolving biology, and iii) guiding the implementation of the most effective treatment interventions and re-strategizing upon the emergence of resistance. Over the last decade, significant progress has been made in the areas of CTC detection, isolation, and characterization that has largely been driven by collaborative and interdisciplinary research efforts spanning across chemistry, materials science, bioengineering, and oncology. Recent technological advances in the field of nanotechnology offer powerful microfluidic systems and unique nanomaterials, which will enable a diversity of in-depth characterizations of CTCs with drastically reduced costs and ultimately bring the field of oncology closer to the goal of personalized care.

Conventional CTC Assays

The most widely used CTC detection assays include: (i) Immunomagnetic separation: these methods utilize capture agent-labeled magnetic beads to either positively select CTCs using a cell surface marker (*e.g.*, anti-EpCAM) or negatively deplete white blood cells (WBCs) using anti-CD45. The CellSearch™ Assay is the only FDA-cleared CTC diagnostic technology for metastatic breast, prostate, and colorectal cancers[36]. CellSearch™ Assay harvests CTCs with anti-EpCAM-coated magnetic beads, and the subsequent immunocytochemistry (ICC) process helps to identify CTCs (DAPI+/cytokeratin, CK+/CD45-) from nonspecifically captured WBCs (DAPI+/CK-/CD45+). Recently, several new systems (*e.g.*, MagSweeper, IsoFlux, Cynvenio, magnetic sifters, VerIFAST and AdnaGen/Qiagen) have been developed to further improve detection speed and efficiency. (ii) Flow cytometry: In conjunction with the use of fluorescent markers, flow cytometry is one of the most mature technologies for analyzing and sorting subpopulations of cells. However, this flow-based methodology is unable to provide the CTCs' morphological information to meet the gold standard set by pathologists. An improved method, known as ensemble-decision aliquot ranking, was developed to address this weakness[37]. (iii) Microscopy imaging. Microscopy imaging of ICC-treated blood samples allows for highly sensitive detection of CTCs, accompanied with their morphometric characteristics and protein expression. Currently, Epic Sciences is one of the leaders in the commercial sector, now providing CLIA-certified laboratory tests for both CTC enumeration and characterization. In contrast to the previous three approaches, which require the use of CTC markers, the following two approaches are recognized as label-free methods. (iv) CTC filters: Filter-based approaches have been established to trap CTCs according to their sizes. A wide collection of commercial kits/systems from Rarecells, ScreenCell, Clearbridge, and Creatv MicroTech etc. are now available to support research utility. Nevertheless, concerns regarding overlooking small-sized CTCs have been raised. (v) Dielectrophoresis: CTCs can be sorted from WBCs in the presence of a dielectrophoretic field, since the CTC's dielectric properties (depending on their diameter, membrane area, density, conductivity and volume) are different from those of WBCs. ApoCell's technology leverages these differences in a microfluidic flow channel to isolate CTCs. Silicon Biosystems' DEPArrayTM combines the use of microscopy imaging and dielectrophoresis sorting to identify and isolate pre-sorted CTCs, paving the way for downstream single-CTC molecular characterizations. (vi) Other methods: There are several outstanding review articles where side-by-side comparisons of a wide collection of CTC detection technologies are presented[38,39].

Microfluidics-enabled CTC Assays

The microfluidic affinity-capture devices demonstrated by the Massachusetts General Hospital team kicked off the research efforts devoted to the development of

nanotechnology-enabled CTC assays[40]. Their 1st-generation (gen) device (*i.e.*, CTC-Chip) featured chemically etched microposts on a silicon substrate, on which anti-EpCAM antibodies were covalently functionalized. These embedded microposts maximize the contact between the device surfaces and the flow through cells. Following CTC capture, ICC was conducted to identify CTCs from background WBCs. The CTC-Chips demonstrated significantly more gains in CTC enumeration performance than most of the conventional CTC assays. Thereafter, similar device configurations were adapted to create new microfluidic chips (*e.g.*, geometrically enhanced differential immunocapture, GEDI approach and Biocept's CTC assay), where different antibody capture agents were employed. Recently, a unique "Ephesia" approach based on microposts of capture agent- coated magnetic beads self-assembled in a microchip demonstrated combined advantages of both microfluidic and immunomagnetic cell sorting[41]. The MGH's 2nd-gen device (*i.e.*, herringbone-chip, HB-Chip) was made from an imprinted PDMS component on a glass slide[42]. Microscale herringbone patterns were engineered into the PDMS component to introduce microvortices, leading to enhanced contact between the CTCs and the antibody- coated chip surfaces. In addition to the commonly used ICC technique, the transparent nature of the HB-Chip allowed for imaging of the captured CTCs by standard clinical histopathological stains (*i.e.*, H&E stain),. Although the microfluidic setting improves CTC- capture performance, the majority of the microfluidic CTC assays suffer from depth of field issues when performing microscopy imaging due to the vertical depths of 3-dimensional device features. Time-consuming multiple cross-sectional imaging scans that generate large image files are required in order to avoid out-of-focus or superimposed micrographs. By coupling a pair of microelectrodes at the terminal of a plastic microfluidic chip, enzymatic release of the captured CTCs can be electrically counted without the issue of microscopy imaging[43]. In contrast to MGH's 1st and 2nd-gen devices, their 3rd-gen iChip represents a groundbreaking label-free approach, which combines negative immunomagnetic depletion processes with an inertial focusing setting in an integrated microchip[44]. Most importantly, this approach allowed for the recovery of unmanipulated CTCs with desired molecular integrity and viability, paving the way for downstream expressional profiling[45], as well as *ex vivo* culture and drug susceptibility tests[46]. Other microfluidic CTC assays based on unique principles, including micro-nuclear magnetic resonance (μNMR) platform[47], cell rolling[48], and Vortex technology[49] have also been developed and demonstrated. In addition to the microfluidic assays developed for the enumeration, molecular characterization, and *ex vivo* expansion of CTCs, a microfluidic device with designated sections for selectively capturing CTCs according to the amount of magnetic beads grafted on their surfaces has been created[50]. The device was employed to dissect CTCs into subpopulations according to EpCAM expression levels of individual CTCs.

Nanomaterials-enabled CTC Assays

It has long been documented that nanoscale components present in the tissue microenvironment, including extracellular matrix and cell-surface structures provide structural and biochemical support that regulates cellular behaviors and fates. Inspired by the nanoscale interactions observed in the tissue microenvironment, the UCLA team pioneered a unique concept of "NanoVelcro" cell-affinity substrates in which CTC capture agent-coated nanostructured substrates were utilized to immobilize CTCs with high efficiency[52]. The working mechanism of NanoVelcro cell-affinity substrates mimics that of Velcro™ – when the two fabric strips of a Velcro fastener are pressed together, tangling between the hairy surfaces on two strips leads to strong affinity between cell and nanosubstrates. Through continuous evolution, 3 generations of NanoVelcro CTC Chips (**Figure 2**) have been established to achieve different clinical utilities. The 1st-gen NanoVelcro Chip, composed of a silicon nanowire substrate (SiNS) and an overlaid microfluidic chaotic mixer, was created for CTC enumeration. Side-by-side analytical validation studies using clinical blood samples suggested that the sensitivity of the 1st-gen NanoVelcro Chip outperforms that of FDA-approved CellSearch™. In addition to SiNS, the general applicability of the NanoVelcro cell-affinity assay is supported by extensive research endeavors devoted to exploiting different nanomaterials, *e.g.*, polymer dots/nanotubes, TiO2 nanowires/nanoparticles, layer-by-layer-assembled nanostructures, gold clusters on silicon nanowires, Fe3O4 nanoparticles, and graphene oxide nanosheets to achieve high affinity capture of CTCs and other types of rare cells[53]. It is worth noting that NanoVelcro-like approaches allow immobilization of CTCs onto a relatively flat and small surface area, thus allowing subsequent microscopic imaging/identification of CTCs to be conducted quickly. Moving beyond CTC enumeration, UCLA's 2nd-gen NanoVelcro Chip (*i.e.*, NanoVelcro-LMD) was developed by replacing SiNS with a transparent substrate covered with polymer nanofibers[54]. The transparent NanoVelcro substrate retains the desired CTC capture performance, and allows for seamless integration with a laser microdissection (LMD) technique to isolate immobilized CTCs with single-cell resolution. The individually isolated CTCs can be subjected to single-CTC genotyping (*e.g.*, Sanger sequencing and next- generation sequencing, NGS) to verify CTC's role as a tumor liquid biopsy. Most CTC enrichment and isolation methods yield purified CTCs that are either fixed before isolation, damaged during the cell purification process, or irreversibly immobilized on an adherent matrix. Similar to MGH team's iChip, UCLA's 3rd-gen Thermoresponsive NanoVelcro Chip has demonstrated the feasibility to capture and release CTCs at 37 and 4°C, respectively[55]. By grafting thermoresponsive polymer brushes onto SiNS, the temperature-dependent conformational changes of polymer brushes can effectively alter the accessibility of the capture agent on SiNS, allowing for rapid CTC purification with desired viability and molecular integrity. The team has been exploring

the use of Thermoresponsive NanoVelcro Chips to purify viable CTCs for downstream molecular and functional analyses.

Future Scientific and Clinical Developments

Moving forward, future research endeavors in developing the Nanotechnology-enabled CTC assays will be driven by the needs of: i) acquiring a fundamental understanding of the nanointerfaces between CTCs (*e.g.*, how the underlying physical/chemical

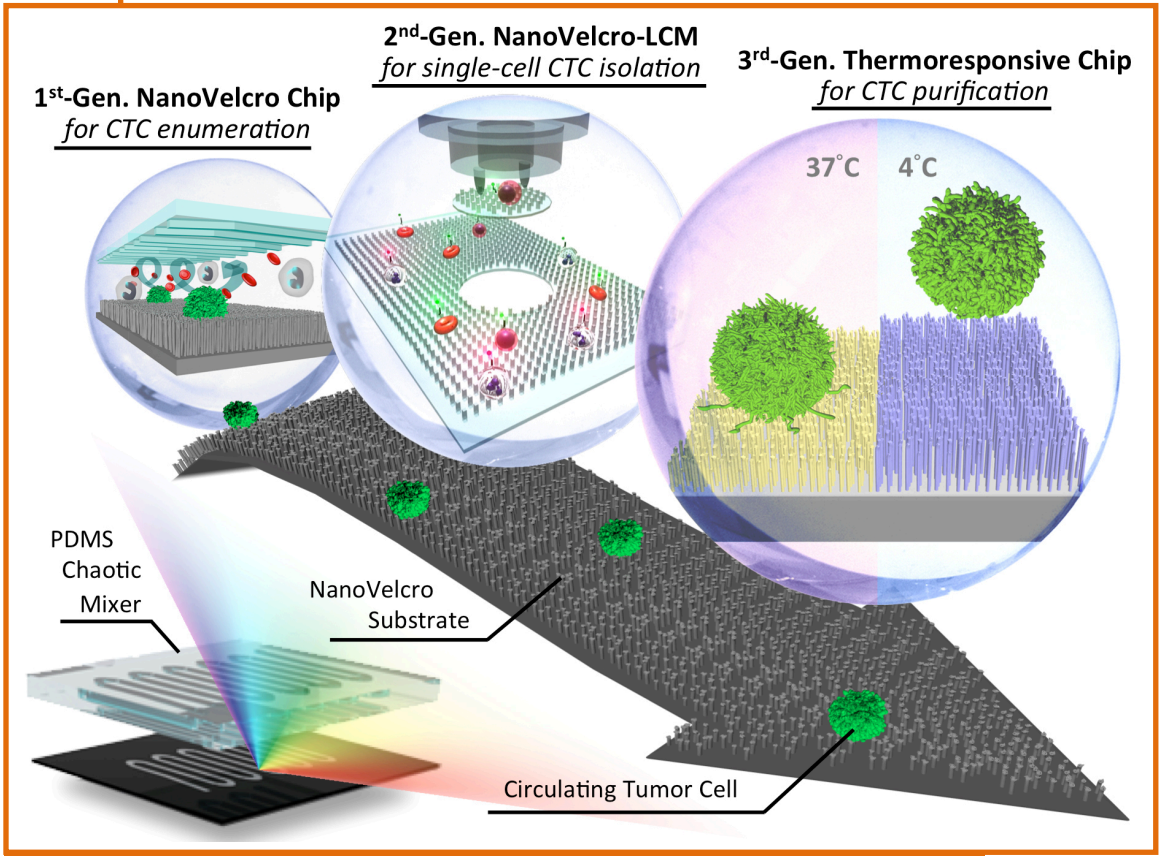

Figure 2. Conceptual illustration of the three generations of NanoVelcro CTC Assays developed by the UCLA team to achieve different clinical utilities. 1st-gen NanoVelcro Chip, composed of a silicon nanowire substrate (SiNS) and an overlaid microfluidic chaotic mixer, was created for CTC enumeration. In conjunction with the use of the laser microdissection (LMD) technique, 2nd-gen NanoVelcro-LMD technology, was developed for single-CTC isolation. The individually isolated CTCs can be subjected to single-CTC genotyping. By grafting thermoresponsive polymer brushes onto SiNS, 3rd-gen Thermoresponsive NanoVelcro CTC Chips were developed for purification of CTCs via capture and release of CTCs at 37 and 4°C, respectively. The surface-grafted polymer brushes were responsible for altering the accessibility of the capture agent on NanoVelcro substrates, allowing for rapid CTC purification with desired viability and molecular integrity. (*Reprinted with permission from Tseng et al, 2014*)[51]

properties of any given nanosubstrate affect their CTC-capture performance, as well as the viability and molecular integrity of captured CTCs); ii) developing new CTC-capture/release mechanisms governed by physiologically compatible stimulations for instant isolation/purification of CTCs with desired viability and molecular integrity in order to set the stage for conducting downstream *ex vivo* characterization, as well as molecular analysis; iii) exploiting a broad diversity of multi-omic analytical technologies (that could be from other research initiatives within NCI Nanotechnology Alliance Program) with single-cell resolution to characterize the heterogeneous CTC pool; iv) exploring the use of rare-cell culture techniques that will enable *ex vivo* expansion of purified CTCs for in-depth studies (*e.g.*, xerograph models and drug susceptibility tests); v) studying other types of circulating rare cells (*e.g.*, tumor associated macrophage and stromal cells) and non- cellular particles (*e.g.*, exosomes), which also carry information about the tumor microenvironment.

Following development of these technologic advances, challenges remain in utilizing these new assays to address unmet needs in the areas of cancer biology and, most importantly, clinical oncology. Research endeavors should be devoted to: i) performing multi-omic molecular characterizations on CTCs together with concurrent tumor tissues (including primary and metastatic sites if available) to establish CTC-tumor relationship that will become the foundation for using CTCs as liquid biopsy[35]. Consequently, CTCs can then be used as surrogate tumor tissue for providing relevant information to guide implementation of cancer treatment; ii) dissecting CTC subpopulations according to their distinct phenotypes (*e.g.*, molecular fingerprints, morphological characteristics, and behaviors) in order to address the issue of heterogeneity in tumor/CTC pool. For instance, a subpopulation of CTCs with defined small nuclei (*i.e.*, vsnCTCs) was discovered to strongly correlate with the presence of visceral metastasis in prostate cancer, offering a new way to detect the onset of the most lethal disease progression[56]; iii) conducting analyses on serial CTC samples through monitoring the dynamic change of CTC subpopulations and their multi-omic molecular signatures to better understand the evolution of cancer, which is currently limited by the difficulty of obtaining tumor tissues; iv) effectively generating and applying CTC-derived cell lines as well as xerograph models to better understand the oncogenic/resistant mechanism, and evaluate a wide range of treatment options that can poetically benefit individual patients. Validation in appropriately powered studies will be needed as these ideas translate directly into the clinical setting. Ultimately, the regulatory and commercial efforts will be required to bring these tools to the population at large.

Conclusion and Outlook

Early successes in the field of nanotechnology have shown great promise for addressing the existing unmet needs in clinical oncology. As the scientific understanding of the dynamic and complex biology of cancer evolves, it has become clear to clinical scientists and cancer biologists that characterizing this dynamic biology may add an important dimension to clinical data. Oncologists practicing cancer care in this evolving biologic environment are already accustomed to handling temporal variation of data. Monitoring the dynamic alterations of biological variables, which themselves follow a distinct and biologically relevant rhythm, is a fundamental part of clinical medicine. Given the limitations of performing serial biopsies or the limited data obtainable in single biomarker panels, to date, this type of dynamic characterization has been possible only in animal models or in limited biomarker panels. The promise that the analysis of CTCs and other circulating entities holds is in the ability to study the dynamic biology that bares the greatest relevance: that of the individual patient. In this era of molecular medicine that has brought us beyond the cell to the level of DNA, RNA, and proteins, it has become exceedingly clear that no two patients are identical and no two cancers are identical. Having a non-invasive means of dissecting these differences bridges the gap between the laboratory and the clinic. While these ideas are young, the successes seen in this field provide ample cause for continued work and fuel the enthusiasm for launching integrated transdisciplinary research in this transformative field.

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Early-to-Late Stage Diagnosis: Nanoflares for Intracellular mRNA Detection

Chad Mirkin, PhD
Department of Chemistry
Northwestern University, Evanston, IL 60208

Spherical nucleic acids (SNAs)[57] have recently emerged as a powerful tool in biomedicine with far-reaching implications in the fields of cancer research and oncology. SNAs are typically composed of nanoparticle cores (*e.g.*, gold[58], silver[59], iron oxide[60], infinite coordination polymers[61], silica[62]), densely functionalized with highly oriented oligonucleotide shells (*e.g.*, single- or double-stranded DNA[58], siRNA[63], mRNA[64], PNA[65], LNA[66], RNA/DNA hybrids[67]) (**Figure 3**). Core-less or hollow versions of these structures have also been synthesized (*e.g.*, crosslinked alkyne polymers[68], liposomes[69]), some of which are composed purely of biologically compatible components. Many of the novel chemical and physical properties that make these materials useful in cancer research and oncology stem from the unique architecture of the oligonucleotide shell and are core-independent. Indeed, SNAs are recognized by Class A scavenger receptors and enter cells (over 60 tested to date) as a single-entity without the use of ancillary transfection agents[70–72]. They also are resistant to enzymatic degradation and show no apparent toxicity or immunogenicity[73–75]. SNAs also exhibit a high affinity for complementary DNA strands (100 times higher than that of free DNA of the same sequence in solution)[76]. SNAs are highly modular and the composition of their cores as well as the sequence, length, and density of their oligonucleotide shells can be tailored; in the context of cancer research and oncology, this means that SNAs can be designed to target almost any gene, including those associated with a wide variety of cancer types, in extracellular and intracellular biodetection and therapeutic schemes. SNAs were first synthesized in the Chad Mirkin laboratory at Northwestern University in 1996, and they were first formulated as nanoflare constructs in 2007 by the same lab.

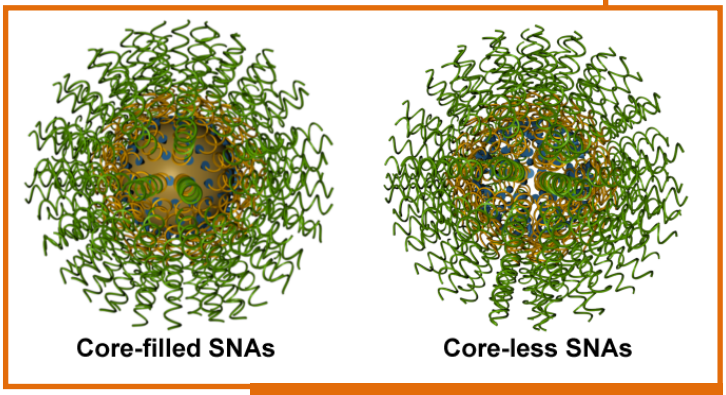

Figure 3. Gold nanoparticle-filled (left) and core-less (right) spherical nucleic acid (SNA) structures.

Based upon SNAs, these new constructs, termed NanoFlare, possess many of the

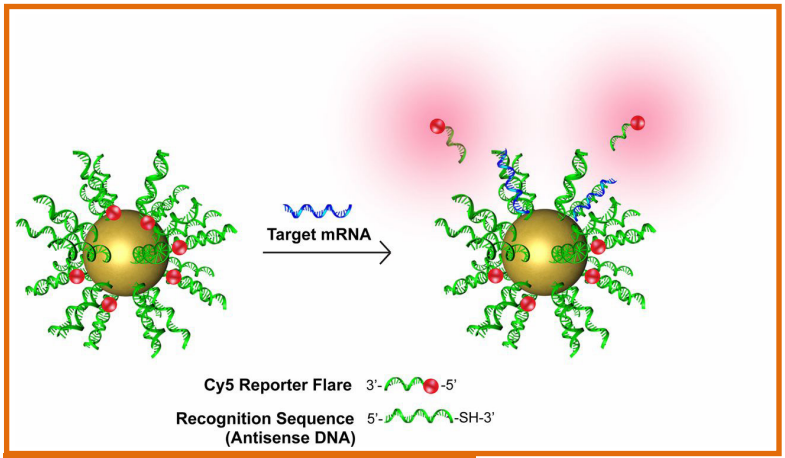

Figure 4. Schematic of Nanoflare structure and function. (*Reprinted with permission from Halo et al, 2014*)[79]

aforementioned useful chemical and physical properties[77]. Specifically, NanoFlares are gold nanoparticle-based SNAs that are hybridized with short, fluorophore-labeled complementary DNA strands (**Figure 4**). Their usefulness as a diagnostic is simple, when hybridized the fluorophores are held in close proximity to the gold nanoparticle and their respective fluorescence output is quenched. However, when a nanoflare encounters a longer, complementary target (*e.g.*, mRNA strand) in a cellular environment, it displaces one of the shorter "flare" strands and the fluorescence signal is observed. As such, these novel nanomaterials have proven to be highly useful probes for intracellular mRNA detection with exceptionally low limits of detection (*e.g.*, sub-pM). When coupled with flow cytometry, NanoFlares currently constitute the only means of interrogating the genetic content of live cells and sorting them based on such content. NanoFlares are also capable of engaging in gene regulation as potent antisense, siRNA, and microRNA delivery vehicles; indeed, these structures have been proven to have theranostic potential as they could be used to both detect and treat cancer, simultaneously[78].

In initial proof-of-concept studies, it was demonstrated that NanoFlares could be used to detect oncogenes – specifically survivin, an anti-apoptotic gene that is up-regulated in a range of cancer types – for example, in a breast cancer cell line (SKBR3) in a highly sensitive and sequence-specific manner[77]. Indeed, increased fluorescence was observed when NanoFlares targeting survivin were added to SKBR3 cells expressing survivin compared to when either NanoFlares bearing a non-complementary sequence were added or cells that did not express survivin (C166 cells) were used (**Figure 5**). These results demonstrate how researchers can use NanoFlares to distinguish cancerous cell populations based on the expression of an mRNA target of interest. Further, in the context of cancer research and oncology, it would be useful to track the up- or down-regulation of multiple genes at once. Thus, more advanced nanoflare systems have been developed that allow a single nanoflare to target multiple genes (*e.g.*, two[31], three[80], or four[81]) in cervical and breast cancer cell lines. These multiplexed NanoFlares also allow quantitative information to be obtained, the signal-to-noise level to be reduced, and to mitigate the effects of cell-to- cell variability.

More recently, NanoFlares were designed to target markers (*i.e.*, vimentin and fibronectin) of the epithelial-to-mesenchymal transition (EMT), an integral part of cancer metastasis. Coupled with flow cytometry, they also were used to capture live breast cancer circulating tumor cells (MDA-MB-231) from human whole blood samples and from an orthotopic murine model of metastatic triple negative breast cancer[79]. Furthermore, these NanoFlares were used to retrieve GFP-positive cells in a HER2+ mouse model of breast cancer and subsequently cultured into mammospheres (**Figure 6**), which are spherical clusters formed only from cancer stem cells. These results suggest that it may be possible to isolate and further culture live CTCs from human patients *ex vivo*, providing the opportunity to study cancer cell heterogeneity and its relation to patient outcomes. Simultaneously, these results demonstrate the ability of NanoFlares to survey the metastatic potential of cells in the blood stream. This approach provides an unprecedented opportunity to isolate cancer stem cells based on the presence of genetic markers and may improve cancer diagnosis and prognosis.

In 2012, nanoflares were commercialized by AuraSense, LLC, a company founded by Chad Mirkin. Two years ago, AuraSense entered into a multi-million dollar partnership with EMD Millipore to commercialize them under the trade name SmartFlares™ for use in *in vitro* cell assays. SmartFlares™ are now available as research tools to investigators with over 1,700 different versions sold in over 230 countries. Over the next 5-15 years, the number of flares available through EMD Millipore is expected to increase, and subsequently nanoflares will move beyond the research setting to the clinic to be used for medical diagnostic purposes. Concurrently, there is an initiative to quantify and track the spatial location of mRNA in cells, as this is highly related to cellular function. As such, it is anticipated that drugs coupled to nanoflare systems

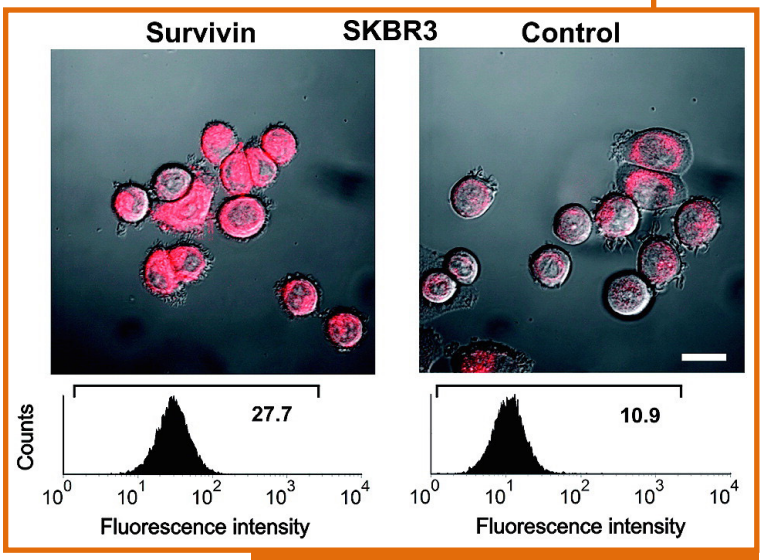

Figure 5. Intracellular testing of nano-flares. Differential contrast and fluorescence image of survivin-expressing SKBR3 cells treated with survivin-specific nano-flares (top left panel) and noncomplementary nano-flares (top right panel). Scale bar is 20 µm. Flow cytometry data are shown below each image. The bold numbers to the right of the histogram are the total mean fluorescence of the cell populations. (*Reprinted with permission from Seferos et al, 2007*)[77]

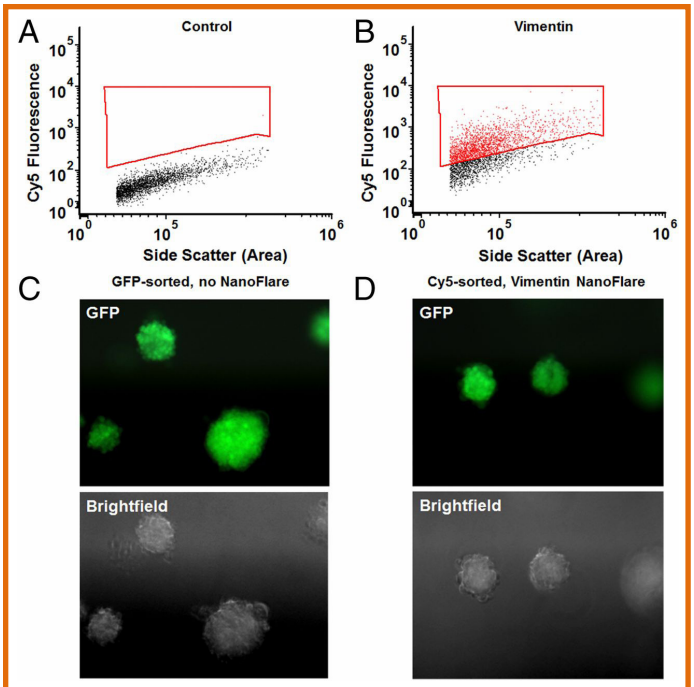

Figure 6. Cell isolation and mammosphere formation post NanoFlare treatment and flow cytometry analysis. Representative scatter plots show Cy5 fluorescence (NanoFlare) of GFP recurrent cells spiked into (A) untreated human whole blood or (B) Vimentin NanoFlare-treated blood. Upon treatment with NanoFlares, Cy5 fluorescence of GFP-positive cells increases 5.4-fold. Cells in the red gate in the Vimentin sample were sorted for mammosphere culture. Cells retrieved from blood form mammospheres (C) untreated or (D) Vimentin NanoFlare-treated. (*Reprinted with permission from Halo et al, 2014*)[79]

will allow therapy to be administered based on the genetic content of the cell, in a highly targeted manner. These research directions are already underway and will have significant implications for the field of cancer research and oncology.

Intraoperative Imaging

Michelle Bradbury, MD, PhD
Department of Radiology and Neuroradiology
Memorial Sloan-Kettering Cancer Center, New York, NY 10065

Introduction

In the operating theatre, there is an urgent need for implementing new image-directed visualization tools that will enhance surgical vision, facilitate minimally invasive surgical procedures, and dramatically alter surgical outcomes of oncological patients. Early detection, staging, and treatment of cancer are essential to minimizing morbidity and mortality. Each year, nearly 13 million new cancer cases and 7.6 million cancer deaths occur worldwide[82]. The cornerstone of clinical cancer care rests on surgical management. However, intervention is often limited to tumors diagnosed in an early stage as outcomes are notably poorer when surgery is no longer a treatment option[83]. Adjuvant radiation and/or chemotherapy are typically added for specific indications including locally invasive tumors and/or spread to regional lymph nodes. The challenge has been in the lack of clear 'surgical vision,' which impacts the ability of the operating surgeon to accurately and specifically identify the extent of malignancy[83,84], macroscopic/microscopic tumor burden[85–88], or remnant disease, notably at the site of surgical removal (*i.e.* surgical margin). Complete assessment of surgical margins will be based upon the quality and extent of tissue sampling[89]. Collectively, these factors will affect therapeutic outcome, prognosis, and treatment management. Moreover, despite technical advances that have enabled large-scale imaging instruments, such as PET-CT and MRI, to meaningfully impact preoperative cancer diagnostics and staging, they are either not practical for intraoperative settings or offer limited utility in terms of achievable spatial resolution and/or sensitivity. Alternatively, newer molecular imaging probe designs (*i.e.*, engineered optically- active nanomaterials), coupled with state-of-the-art device technologies, may enhance cancer care, provide real-time imaging guidance, and lead to new, more efficient approaches for early-stage detection and treatment.

A key goal of cancer surgery is to reliably distinguish cancer from normal tissues at an early stage to pursue a surgical cure while maximizing safety, limiting damage to vital structures, preserving cosmesis, and increasing throughput. The current standard of care relies upon palpation and visual inspection[90]. Although anatomic structures can be efficiently identified, such evaluations depend on successful discrimination of a narrow range of spectral features (*i.e.*, contrast) or subtle textural differences, rather than elucidating molecular processes

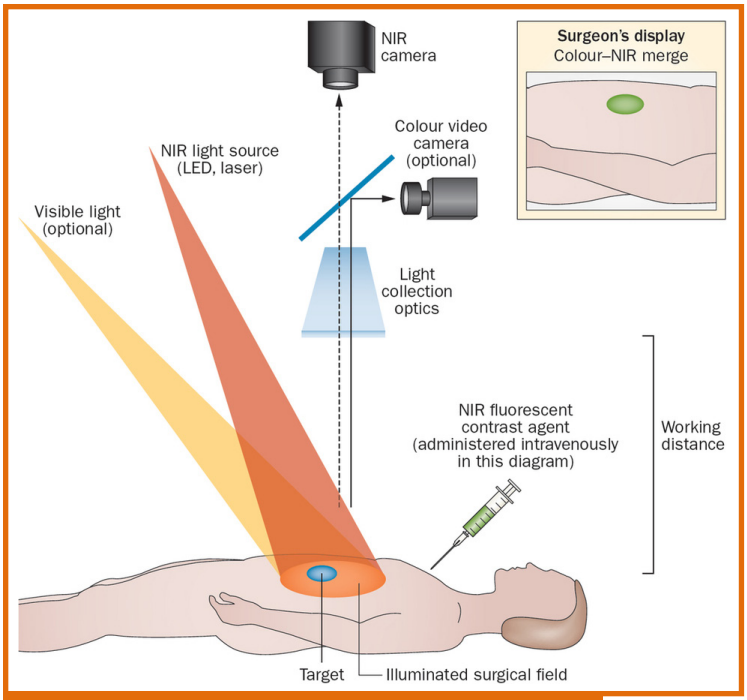

Figure 7. Mechanics of NIR fluorescence imaging. During surgery, an NIR optically-active agent is visualized using a fluorescence camera system. All systems must have adequate NIR excitation light, collection optics, filtration and a camera sensitive to NIR optical emissions. Optimal imaging systems include simultaneous visible (white) light illumination of the surgical field, which can be merged with NIR optical images. The display can be a standard computer monitor, goggles, or a projector. Current imaging systems operate at working distances that enable illumination of a sizable surgical field. LED, light-emitting diode (*Reprinted with permission from Vahrmeijer et al, 2013*).

defining a given disease stage[91]. This leads to a higher risk of incomplete surgical resection and/or soft tissue injury.

These limitations may be overcome by the application of improved intraoperative optical imaging approaches, which have traditionally been hampered by (1) the small number of imaging agents available in the near-infrared (NIR) spectrum, (2) high background autofluorescence that restricts depth and detection sensitivity, (3) large spectral overlap between optical agents preventing concurrent detection of multiple targets (*i.e.*, multiplexing), and (4) rapid photobleaching that reduces the imaging duration[15]. However, significant progress is being made on a number of fronts. Fueled by the emergence of an increasing number of new, diverse, and clinically promising NIR fluorescence probes, including particle-based agents, that can enhance soft tissue contrast, detection sensitivity, and depth penetration, some of these key drawbacks are being addressed, noting that these probes require an intraoperative optical imaging system with clinical grade accuracy (**Figure 7**). In addition to offering exquisitely sensitive real-time detection sensitivities, the higher resolution offered by these systems has enabled lesions to be detected down to sizes smaller than 10 µm, which truly revolutionizes imaging capabilities by dramatically increasing the sensitivity and specificity of detection over human vision[92]. Such tools can be seamlessly integrated with minimally invasive, robotic-assisted surgical equipment to enable navigation to target sites deep within the body. Unlike other imaging modalities, the combination of optically-active, disease-targeting probes and state-of-the-art multichannel camera systems offers

the possibility of interrogating real-time biological processes and identifying one or more novel biomarkers for (1) imaging (*i.e.*, cancerous nodes, surgical margins, remnant tumor); (2) staging; and (3) treatment response (**Figure 8**). Such markers can be further validated in the clinical trials setting. Collectively, the potential of these technologies to improve patient outcomes, minimize surgical risk, promote clinical throughput, and lower health care costs represents a significant clinical advance, and promises to transform the current practice of surgical oncology.

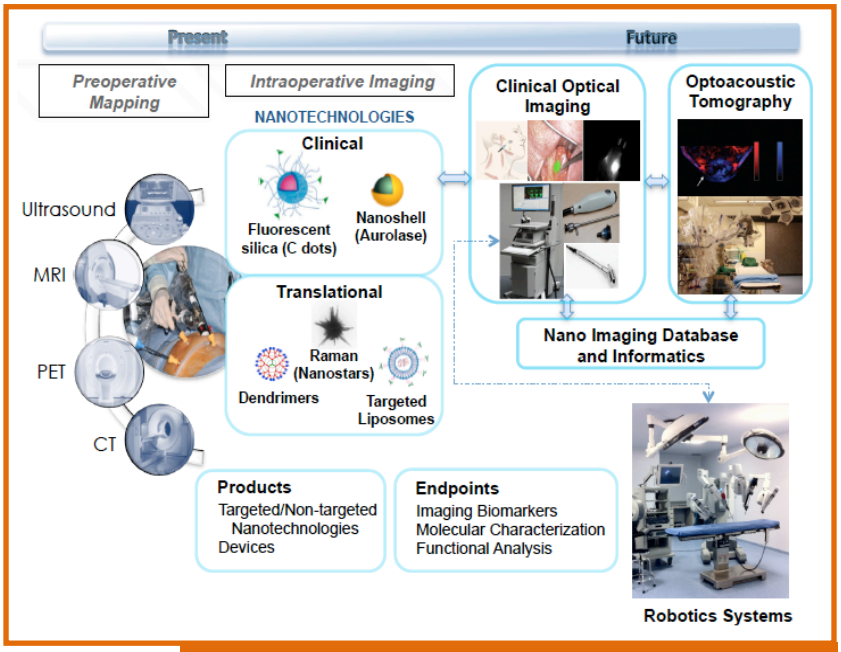

Figure 8. Present and future of NanoOncology Image-guided Surgical Suite. Preoperative conventional imaging tools are used to screen for disease and inform optically-driven minimally-invasive and open surgical procedures. Clinically available particle platforms can be monitored in real-time using portable multichannel camera systems. Representative translational probes and devices for future clinical use are also shown. In the future, the operating surgeon will select suitable probe-device combinations for specific indications, and be provided with structural, functional, and/or molecular-level data regarding tissue status for further treatment management.

Intraoperative Imaging Via Nanotechnology

A significant volume of work, however, has been performed utilizing endogenous tissue contrast, which is restricted to examination of only very small fields-of-view, or by administering non-specific optical agents[93,94]. The latter class of agents have included particle-based probes (*i.e.*, quantum dots)[95] and fluorescent dyes, such as indocyanine green (ICG)[96,97], an FDA-approved NIR dye for selected clinical indications. However, the lack of selective targeting found with these agents limits their utility for many applications aimed at detection of strictly cancer-bearing tissues. Thus, to enhance surgical vision during image-guided procedures, as well as impart labeling specificity, NIR optical probes targeting tumor-selective biomolecules are desired. Towards this end, a number of targeted molecular products, including dye-bound antibodies and peptides, can be applied as visualization tools for improving examination of tumor borders or localization of tumor deposits by attaching to upregulated cancer receptors[98–100]. Although not yet reaching full potential in surgical

practice, early potential benefits of optical imaging have been shown in clinical studies utilizing targeted molecular probes, albeit conjugated to visible dyes. However, such dyes reduce contrast resolution and depth penetration due to higher absorption and scatter in this part of the light spectrum[101,102].

More recently, the emergence of diverse classes of NIR fluorescent nanoparticle platforms, designed to improve the sensitivity, accuracy, and reliability of lesion detection over that of organic dyes, has revealed exciting new possibilities for probing and characterizing new molecular targets and novel biomarkers within human subjects[15]. The ability to tailor and refine the physicochemical and photophysical properties of these materials in a well-controlled and iterative fashion can favorably modulate their biological activities, resulting in one or more characteristics that improve upon those exhibited by simple molecular agents. These characteristics include multivalency enhancement (potency) as a consequence of simultaneous interactions of multiple targeting ligands with cell surface receptors, improved target retention, extended plasma residence time, bulk renal clearance, and improved pharmacokinetic profiles. Moreover, in some cases, the encapsulation of dyes within the particle structure has led to significantly enhanced brightness and photostability relative to the native dye, in addition to increasing tissue penetration depths (up to several centimeters)[103]. Collectively, these adaptations can improve target-to-background ratios and *in vivo* detection sensitivities following particle administration, the ultimate goal being to identify and remove all cancer cells. Finally, the ability to create multimodality platforms by incorporating more than one contrast-producing moiety into the particle design can yield multiparametric imaging data that validates potential biomarkers, potentially altering current standard of care.

Given these diverse, highly versatile, and integrated particle surface designs, coupled with improved state-of-the-art optical clinical camera systems, key surgical indications can be performed more reliably and accurately. Current applications have mainly focused on (1) selective mapping of cancerous lymph nodes, (2) precise identification of surgical borders (crucial landmarks), (3) accurate detection and treatment of remnant disease, and (4) reliable assessment of tissue function (*i.e.*, perfusion). For SLN mapping, the principal aim is to map the lymphatic drainage of exogenous agents and highlight only cancer- bearing nodes for selective resection. The primary factor controlling lymphatic transport is the agent size. An optimal size is one that is small enough to exhibit rapid lymphatic transport to the SLNs and other downstream nodes, yet large enough to be retained, typically around 5–10 nm[87,104]. One such sub-10 nm hybrid (PET-optical) cancer-targeting imaging platform is shown in **Figure 9**. A second surgical indication, the mapping of surgical margins, involves precise delineation of the tumor extent. The presence or absence of tumor cells at the site of resection is a key determinant of treatment success or failure, and is often used

to determine the need for adjuvant therapy. Positive margins are a negative prognostic indicator for many solid cancers[83]. Furthermore, surgical margins are often evaluated by immediate intraoperative analysis of the specimen, which can lengthen operating time and/or lead to incomplete readouts due to suboptimal specimen quality or inadequate sampling, the result being a positive surgical margin and poor outcome[89]. One such triple-modality (*i.e.*, MR-photoacoustic-Raman imaging, MPR) particle has sought to address this issue by efficiently and accurately delineating brain tumor margins (**Figure 10**)[14].

In addition, newer higher resolution whole-body optical imaging strategies, such as multispectral optoacoustic tomography (MSOT) (**Figure 8**), which detects optical absorption by means of ultrasound, have grown in popularity due to the concurrent development of clinical imaging systems[91,95]. These methods utilize multiple optical wavelengths and spectral demixing algorithms to permit imaging at depths greater than those typically achievable with fluorescence imaging. In addition, these methods can detect a broad range of novel light-absorbing nanoparticles (gold nanorods)[105], among other entities (*i.e.*, endogenous chromophores, organic dyes)[91], to yield high resolution optical assessments of targets deep to the tissue surface, as well as provide functional measures of viability and/or perfusion.

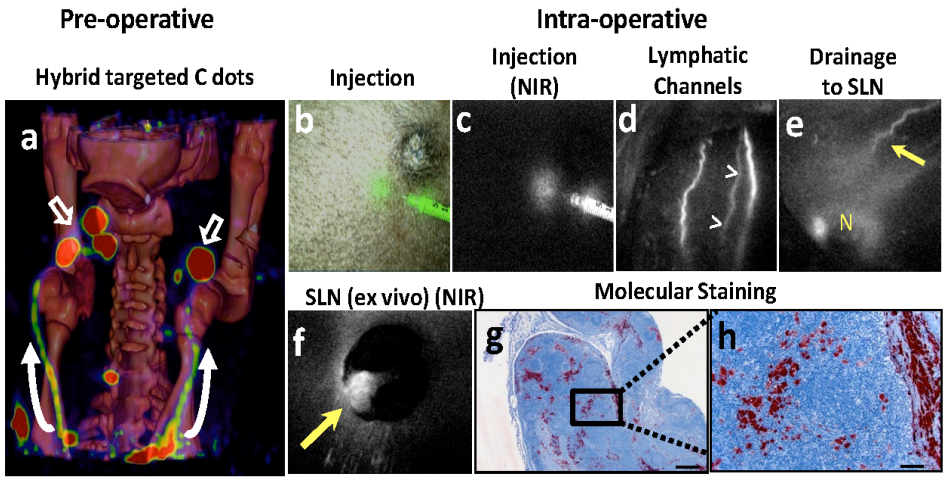

Figure 9. Mapping of Metastatic Lymph Nodes Using a Clinically Translated Hybrid PET-Optical Silica Nanoparticle (C dots). (a) Volume-rendered pre-operative PET-CT fusion images of the neck shows metastatic lymph nodes (red) bilaterally and lymphatic channels after injection of ultrasmall (6 nm diameter) integrin-targeting C dots into melanoma miniswine. (b,c) Intraoperative SLN mapping with two-channel NIR optical imaging of the exposed nodal basin. Local injection of fluorescent C dots displayed in dual-channel model (b) RGB color (green) and (c) NIR fluorescent channels (white). (d,e) Draining lymphatics (arrowheads) distal to the injection site extending toward the node (N). (f) Image of excised SLN in the NIR channel. (g) Low-power view of HMB45-stained (red) SLN confirms the presence of metastases (black box, bar = 500 µm). (h) Higher magnification reveals HMB-45+ expressing melanoma cells (bar = 100 µm) (*Reprinted with permission Bradbury et al, 2013*).

Future of Intraoperative Imaging Via Nanotechnology

It is anticipated that fluorescence-enhanced surgical vision, despite its limitations, will significantly impact and likely transform conventional surgical practice in oncology over the next 5 to 15 years by increasing the sensitivity and accuracy of surgical procedures, such as evaluation of surgical margins, mapping of local and distant cancerous lymph nodes, and detection of microscopic disease. Rather than relying on visual and tactile cues for guiding disease assessment and therapeutic management, the surgeon will utilize a growing array of dedicated intraoperative treatment tools in the form of targeted optically-active particle probes and portable multichannel optical devices. Nanoparticle surface versatility and their unique physicochemical and biological properties will play a key role in this field, providing new opportunities to probe critical cancer targets and identify potential biomarkers that can be validated in clinical trials. Although in its infancy, a variety of particle therapeutic strategies are currently being developed for effectively treating disease in the intraoperative setting. The future implementation of such tools in clinical practice should lead to improved patient outcomes and reduced surgical risks. The foregoing developments are also expected to promote acceptance of optical technologies and, as a consequence, accelerate the growth of minimally invasive surgical procedures, with the intent of maximizing functional outcomes and limiting treatment-related morbidity. Identification of normal tissue markers may also enable particles to be engineered with specific ligands and fluorescent labels for highlighting poorly visualized vital structures (*i.e.*, nerves). In addition to their expected utility for real-time intraoperative procedures, the application of these optical technologies

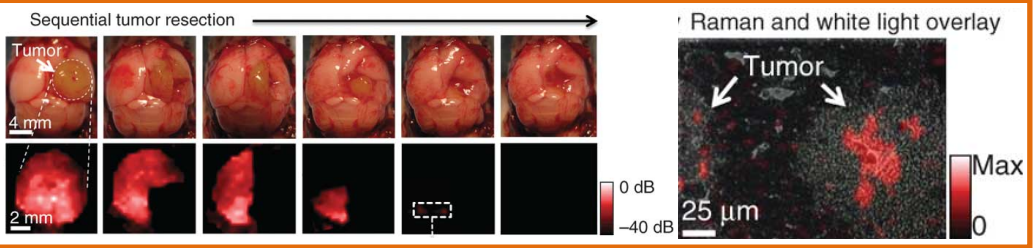

Figure 10. Raman-guided intraoperative surgery using Raman imaging nanoparticles (MPR). (a,b) Living tumor-bearing mice underwent craniotomy. Quarters of the tumor were sequentially removed (photographs, a), and intraoperative Raman imaging was performed after each resection step (b) until the entire tumor had been removed, as assessed by visual inspection. After gross tumor removal, small foci of Raman signal were found in the resection bed (dashed white square). Raman microscopy image (right) of dashed white square depicts Raman signal within an infiltrative tumor, indicating the selective presence of MPRs. Raman color scale (red): –40 dB to 0 dB (*Reprinted with permission from Kircher et al, 2012*).

may additionally aid inspection of resected tissue specimens, leading to less time-intensive evaluations and improved clinical throughput.

Despite the significant data generated to support the translational developments of new, optically-active particle probes for intraoperative cancer treatment, advancing such agents into the clinic has been challenging, particularly those exhibiting molecular specificity[106-108]. Importantly, FDA-IND approvals have been issued for both targeted particle drug[106] and device[109] technologies, and such developments are paving the way for translating additional targeted optically-active technologies to the clinic for use in image-guided surgeries. Furthermore, as tumor heterogeneity is an important consideration for selecting a targeting ligand, 'cocktails' of multiple cancer-targeting particle probes will be increasingly utilized, each probe incorporating a different ligand and optical dye for improving detection and staging accuracy. Enabling simultaneous visualization of these cocktails will require implementation of state-of-the-art multichannel fluorescence camera systems that can detect fluorescence from multiple wavelengths. Several of these camera systems are already in clinical use. As additional novel particle probes are developed and camera systems continually evolved to permit both structural and functional assessments, the true clinical value of these combined technologies will ultimately be realized. Promising higher resolution techniques, such as optoacoustic imaging, may be increasingly implemented to overcome instances where degradation of the emitted fluorescence signal is observed, notably when interrogating complex tissue compositions.

Nanoparticle surface versatility and their unique physicochemical and biological properties will play a key role...

Finally, the need to establish standardized quantitative metrics for intraoperative decision-making is paramount, and is at a very early stage of development. Often these assessments are of a qualitative nature, and the chosen endpoints may depend on many factors, including the nanomaterials probe selected and the device providing the measurements. It is expected that the optical imaging community will address these issues in the near future, as they will significantly hamper efforts to make effective comparisons among different probe-device combinations for a specific indication. Implementation of well-designed outcomes studies will also be critically important for widespread dissemination and acceptance of image-guided optical technologies in standard surgical practice.

Targeting the Tumor Microenvironment

Kinam Park[1], PhD, Bumsoo Han[2], PhD, and Murray Korc[3], MD
[1]Weldon School of Biomedical Engineering and [2]School of Mechanical Engineering
Purdue University, West Lafayette, IN 47907
[3]Division of Endocrinology, Department of Medicine
Indiana University, Indianapolis, IN 46202

The Big Picture

Personalized medicine, or precision medicine, relies on the selection of the correct drugs, or drug combinations, based on the disease-specific genetic traits. Selecting the proper drugs is the first step toward precision medicine, but its completion needs effective delivery of the selected drugs to the target (*e.g.*, tumor). Recent progress in nanotechnology has made drug delivery more efficient compared with the control solution formulation, but subsequent effectiveness of the drugs delivered is still in question. Nanoparticulate drug delivery systems are designed and tested for the ultimate goal of developing clinically useful formulations to treat various cancers. Thus, the usefulness of nanoparticle formulations needs to be considered in the context of treating cancers (*i.e.*, improving efficacy and safety) in human patients.

Benefits of Nanoparticle Formulations

Over the last few decades, various nanoparticles have been prepared for treating cancers. One large benefit to using nanoparticle formulations is in the ability to avoid non-aqueous solvents when administering hydrophobic drugs to patients, resulting in fewer side effects, even if the efficacy remains the same. This has been exemplified by the success of Abraxane® (based on nanoalbumin particles) and Doxil® (PEGylated liposome formulation), which in large part, rely on delivering anticancer drugs without using organic solvents. Although, nanoparticle formulations, or for that matter any formulation, can deliver drugs to the area near target tumors, but the subsequent delivery to the tumor cells is hindered by the complex microenvironment of tumors. Drug efficacy occurs only after the drug is absorbed into target tumor cells. Thus, it is important to understand the tumor microenvironment (TME) to achieve or improve upon the desired drug efficacy.

Understanding the Tumor Microenvironment (TME)

The tumor microenvironment comprises a highly heterogeneous mixture of tumor and stromal cells embedded in an extracellular matrix with many cytokines, growth factors, inflammatory cells and macrophages[109]. The current difficulty of developing new anticancer

drugs and drug delivery systems partly stems from the lack of a clear understanding of the delicate interplay between tumor and stromal cells in the complex TME[111]. Here, pancreatic ductal adenocarcinoma (PDAC) is used as the fundamental, albeit extreme, example of this in order to portray the importance of improved targeting to TME.

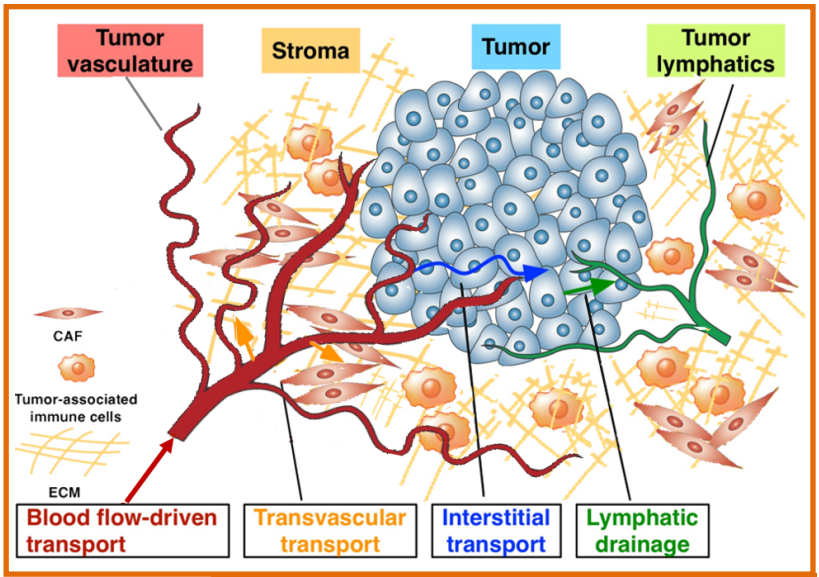

Figure 11. Transport of drug molecules and nanoparticles in the TME of PDAC. Drugs and nanoparticles can only reach the target tumors via multiple transport processes in the TME. PDAC has a very complex TME with dense stroma composed of cancer-associated fibroblasts (CAFs), tumor-associated immune cells, and dense ECM structure.

PDAC consists of two components, the malignant epithelial cell population and a complex, large stromal compartment. **Figure 11** describes a highly desmoplastic PDAC tumor which is infiltrated with activated cancer-associated fibroblasts (CAFs) and inflammatory cells. CAFs release collagens, laminin, and fibronectin. The complex extracellular matrix (ECM) includes dense collagen types I and III bundles, hyaluronic acid (HA), fibronectin, desmin, cytokines, growth factors, and the matrix metalloproteinase family of proteases. The exact roles of the stromal compartment are still not clearly established, but it certainly provides an immense physical barrier to the multiple transport steps for effective drug delivery. Overcoming the transport barriers presented by both stroma and tumor for effective delivery requires ingenious design of nanoparticles, at least beyond the nanoparticle design paradigms currently in clinical use due to their size and surface functionalities. Moreover, interactions between tumor cells and various cell types in the stroma may affect the drug response of tumor cells. The outcome of these interactions is highly context-dependent, and further understanding of dynamic cancer biology and oncology is critical. The current idea of targeted drug delivery using nanoparticles addresses only a very small portion of this complexity. As such, any new paradigm should comprise tools for overcoming the enormous complexities of the TME.

Future Needs to Efficient Delivery of Anticancer Drugs Through Priming of the TME

The TME has enhanced stiffness, increased HA content, and elevated hydrostatic pressure, all of which are known to reduce effective intratumoral drug delivery. For drugs to be effective, they must reach the target tumor cells through the TME or the stromal surrounding. Thus, solid tumor priming, *i.e.*, modulating the abnormal TME, is promising idea for enhancing the antitumor efficacy. The strategies of solid tumor priming includes vascular normalization using anti-angiogenic treatment, solid stress alleviation by induced apoptosis and stromal normalization, and using tumor-penetrating peptides[112]. Of these stromal normalization is attractive because it can be achieved by using relatively benign components.

Stromal HA is known to be a key factor making the too TME dense for proper diffusion of drug molecules, not to mention nanoparticles. This provides a means to enhance the permeation of nanoparticles through TME by treating PDAC first with hyaluronidase[113]. Calcipotriol, a synthetic, highly potent derivative of vitamin D that does not cause hypercalcemia, was recently reported to reduce the activation of pancreatic stellate cells and their conversion to CAFs by activating the vitamin D receptors that are expressed in these cells, thereby decreasing desmoplasia[114]. When used in combination with gemcitabine, calcipotriol prolonged survival in a genetically engineered mouse model (GEMM) of PDAC by decreasing fibrosis, increasing intra-tumoral vasculature, and enhancing gemcitabine delivery into the tumor. Importantly, Calcipotriol has been shown to exert anti-proliferative and pro-differentiation effects, as well as immune-modulating effects[114]. Interpretation of these results is complicated by a very recent finding that vitamin D may also promote tumor chemoresistance to gemcitabine, *underscoring the need to improve our knowledge on how to target the stroma*[115].

While the stroma-targeting approach has been successful in GEMMs of PDAC, it did not work in clinical trials. The successful treatments observed in mouse models seldom translate into clinical success. There may be several reasons for this discordance between findings in humans and in GEMMs of PDAC. The TME in mouse is likely to be very different from that in human. In addition, the amount of a drug delivered after HA priming was simply not adequate in clinical trials. Disrupting stromal layer alone may not be sufficient to kill tumor cells without delivering sufficient drugs. Since tumors are highly heterogeneous, delivering a single drug might have not been effective. Indeed, the heterogeneity of gene alterations in the cancer cells and the complexity of the stromal components mandate the design of novel multi-targeted and multi-drug dosing approaches.

Future Needs for New In Vitro Test Methods

Effective tumor treatment requires testing various priming agents in combination with delivery of multiple drugs, either simultaneously or sequentially. This involves a very large number of studies, and it makes animal testing expensive and time consuming. Moreover, small animal data may not be good predictors of clinical outcome. Thus, it is essential to develop *in vitro* test methods that can represent the microenvironment of human tumors.

Recent advances in tissue engineering and microfluidic technologies present an opportunity to realize *in vitro* platforms alternative to animal testing.

Recent advances in tissue engineering and microfluidic technologies present an opportunity to realize *in vitro* platforms alternative to animal testing. These platforms enable mimicking complex and multiple transport processes of drug delivery systems including circulation in the blood, extravasation from blood vessels to the tumor region, and diffusion of drug to the target tumor[116]. Tumor cells can be grown in 3D matrices with other relevant stromal cells to more closely recapitulate the complexity of solid tumors in patients. The current ability of forming 3D perfused tumor tissue needs to be advanced further to create an accurate TME, which accurately represents that of human tumors. This requires the design of 3D co-culture systems in which cancer cells, CAFs, and other stromal cells are grown within the necessary ECM components, yielding a delicate balance of biological, chemical and physical parameters relevant to human tumors.

Exact duplication of the human TME in microfluidic systems may not be feasible in the near future, but the TME-on-Chip can be used to systematically study the significance of given biological, chemical and physical parameters on the efficacy of nanotechnology-based drug delivery system and priming agents. Eventually, it should serve as a useful screening system for testing a large number of priming agents and drug combinations for personalized medicine.

Overcoming Specific Biological Barriers: Stromal

Huan Meng[1,3], PhD, Jeffrey I. Zink[2,3], PhD, Timothy Donahue[4-6], MD, Caius Radu[5,6], MD, Zev Wainberg[7], MD, and Andre Nel[1,3], MD, PhD
[1]Division of NanoMedicine, [2]Department of Chemistry and Biochemistry, [3]California Nanosystems Institute, [4]Departments of Surgery, [5]Molecular and Medical Pharmacology, [6]Ahmanson Translational Imaging Division, and [7]Hematology and Oncology
University of California at Los Angeles, Los Angeles, CA 90095

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer-related deaths in the United States and its 5-year survival rate has remained unchanged (6%) over the past decades (*Cancer Facts & Figures 2014*, www.cancer.org). Due to the inevitable late diagnosis and early metastasis, chemotherapy is the only approved option for the majority of PDAC patients, with the standard of care involving the use of nucleoside analog gemcitabine or a more potent (but more toxic) four-drug regimen, oxaliplatin, irinotecan, 5-fluorouracil, and leucovorin (a.k.a FOLFIRINOX). Chemotherapy failure can be partly explained by the presence of an abundant dysplastic stroma, serving as a physical and biological barrier for drug access and unfavorable pharmacokinetics. It is appropriate, therefore, to consider the important stromal contribution to drug delivery and chemoresistance and sidestepping this barrier to improve survival outcomes[117]. This short overview will address the inhibitory role of the stroma in the treatment of PDAC, including the consideration for the use of nanocarriers to potentially engineer past this obstacle. We provide a perspective and guidance towards the implementation of nanotherapeutic approaches that could prove useful to improve therapeutic delivery and efficacy of gemcitabine and FOLFIRINOX.

Overcoming Tumor Stroma is Important to Cancer Nanotherapeutics

Because the stromal volume in PDAC is the highest among solid tumors (~70% of the total tumor volume), this requires special consideration in the treatment of this deadly disease[117]. Not only is the stroma poorly vascularized, but the existing vessels exhibit low permeability due to a high pericyte coverage, which blocks the extravasation of drugs, molecular therapeutics, and even nanocarriers to the tumor site (**Figure 12A**)[118]. The stroma also contributes to chemo-resistance and an unfavorable pharmacokinetic/pharmacodynamic (PK/PD) profile[117], including the expression of a high content of cytidine deaminase (CDA), which leads to gemcitabine inactivation, limiting its half-life to as little as 0.28 hours

(**Figure 12A**)[119]. Moreover, the intracellular activation of gemcitabine is dependent on phosphorylation by the rate-limiting kinase, deoxycytidine kinase (dCK) to generate the active metabolites, dFdCDP and dFdCTP (**Figure 12A**)[120]. It is believed that chemo-resistance to gemcitabine in PDAC is due in part to decreased expression of dCK. Another important stromal contribution is its pro-tumorigenic effect through supportive cell types that promote cancer cells proliferation and metastasis via complicated cross-talk mechanisms. Given this background, it is important to consider overcoming the challenges of the stromal barrier to address drug delivery and unfavorable PK/PD to the cancer site, including the improvement of intratumoral distribution, bioavailability, and overcoming drug resistance.

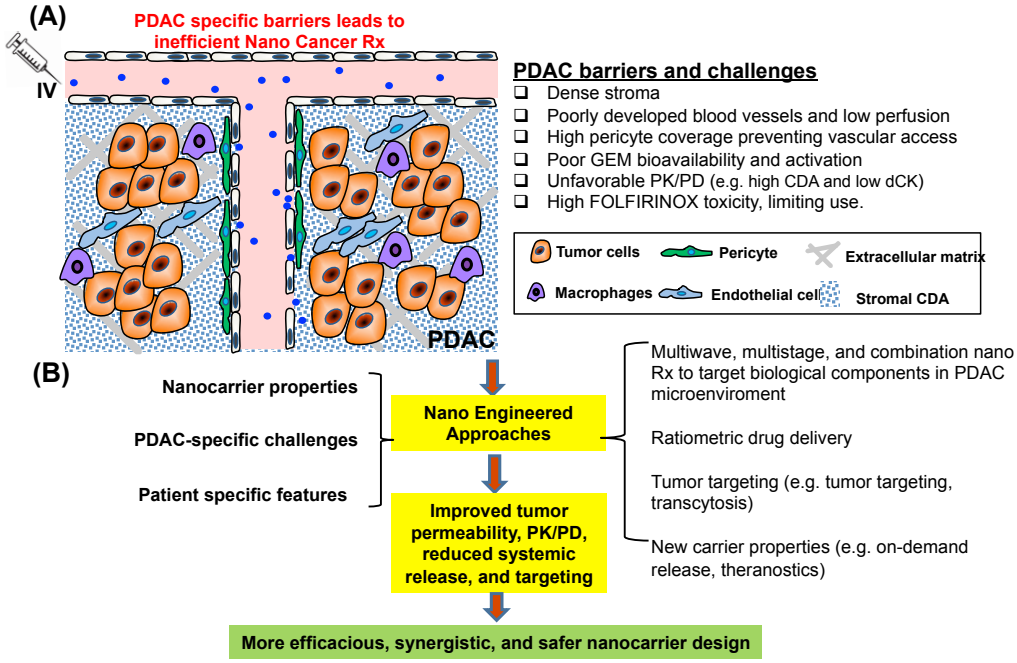

Figure 12. (A) Schematic to show the barriers and challenges that are responsible for failed chemotherapy in PDAC, including as a result of an abundant dysplastic stroma, which serves as a physical and biological barrier. This includes interference in vascular access and the presence of a high local concentration of deaminase activity, which leads to in activation of GEM. **(B).** We propose an engineered approach using nanocarriers, which can overcome stromal vascular gate or suppress the stromal abundance by the delivery of drugs that suppress pericyte coverage or decreases the stromal volume and abundance of deaminase activity. Moreover, a combination of these features could be used in synergistic designed nanocarriers. It is also a possible to include tumor targeting or the use of peptides that induce transcytosis across the stromal barrier.

The Current State of Overcoming Stromal Barriers in Cancer Nanotherapy

A number of stromal treatment strategies are currently being considered to improve PDAC treatment. These efforts have involved the use of enzymatic degradation, pharmacological suppression, tumor vasculature modification/intervention, and stromal targeting peptides. The first approach is the introduction of stromal-directed agents that obliterate the dense stromal microenvironment to improve drug delivery[113]. An ongoing clinical trial has demonstrated that the combination of gemcitabine with PEGylated hyaluronidase (PEGPH20) can ablate hyaluronan and overcome the stromal barrier, allowing chemotherapeutic drug access to the cancer site[121]. While PEGPH20 showed promising results pre-clinically and in some clinical studies, success is dependent on the dosing schedule as well as the specificity of this treatment[122]. In April 2014, FDA announced a clinical hold due to dosing and safety (*e.g.*, induction of thromboembolic event) concerns about the use of PEGPH20 in a Phase II clinical trial (www.halozyme.com). Although the clinical study resumed in September 2014, no update is available at this time. The second approach is to consider the use of pharmacokinetic suppression, as illustrated by the FDA granting approval for the use of the albumin-bound paclitaxel nano-complex, Abraxane®, in PDAC; co-administration of this therapy promotes gemcitabine survival outcome by 1.8 months. The proposed mechanism of Abraxane® action is the suppression of stromal density and reduced expression of CDA at the tumor site[123,124]. While the efficacy of this treatment is premised on using conventional therapeutic doses of each drug, it is not designed to deliver a ratio-dependent drug combination, which is an important consideration due to differences in the PK, distribution and elimination of the synergistic drug combination. This provides the opportunity to consider the ratiometric design of a single gemcitabine/Abraxane carrier to achieve *in vivo* synergy. The third approach is to use vasculature modification to improve drug delivery. In this category, there are a number of options, including targeting of the transforming growth factor beta (TGF-β) pathway, which promotes pericyte coverage of vascular fenestrations, among its pluripotent biological effects[125]. Intervention in the TGF-β signaling pathway using receptor kinase inhibitors or monoclonal antibodies have shown promising results to enhance vascular access and delivery of cancer drugs and nanocarriers to the tumor site[126,127]. However, the use of free inhibitor or antibody may require relatively high-dose/frequency and/or "off-target" effects due to the limited tumor targeting of these agents. Vasculature access can also be improved by stromal depletion through the use of antifibrogenic drugs, such as losartan (a clinically approved angiotensin II receptor antagonist)[128] and Hedgehog inhibitors[129], leading to decreased contractile elements, lowering of the interstitial fluid pressure[130] or a transient increase in intratumoral vascular density. While it has been shown that small 30 nm drug-loaded polymeric micelles can

permeate the stromal barrier to deliver antitumor drugs in PDAC without the need for targeting, the use of small particles may come at the expense of a reduced drug loading capacity[131]. The last approach is to develop stromal targeting therapy. This includes the recent discovery that iRGD peptides can increase PDAC vasculature access[132]. The exposed "CendR" motif, upon cleavage from the iRGD peptide, interacts with NRP-1 kinase receptor, which is capable of triggering transcytosis of macromolecules and liposomes, without the need of covalent conjugation of the peptide to the nanocarrier. This pathway is likely analogous to the vesiculo-vacuolar organelle, which has been observed in tumor vasculature during performance of electron microcopy[133].

Future Perspective in Overcoming Stromal Barriers

Because of the challenges of conventional chemotherapy for PDAC and the realistic expectation that there are no imminent changes in the treatments for metastatic disease, there is a unique opportunity for the use of nanotechnology in the treatment of this disease over the next 5-15 years. This is evidenced by the introduction of classic (*e.g.*, liposome and polymer) as well as novel (*e.g.*, inorganic-based) nanocarriers for this purpose. Although the use of small particles that rely on size-exclusion principles has shown promising results, nanotherapeutics are poised to make an even bigger impact because nanocarriers can be designed to deliver single or synergistic drug combinations, target, image and deliver, as well as allowing for engineered approaches to treatment. We define an "engineered approach" as the dynamic integration of the drug delivery properties with additional nanocarrier properties that address tumor-specific challenges, such as the stromal barrier (**Figure 12B**). Such an engineered approach could be particularly relevant to stroma-rich cancers in which the tumor stroma and other inferring biological components result in heterogeneous treatment effects in the tumor microenvironment. It is possible to design stromal targeting nanocarriers to enhance the efficacy of existing cancer drugs such as small molecules, peptides and proteins. One example is the introduction of a proof-of-principle "two-wave" platform in which a small molecule inhibitor of the TGF-β receptor kinase was used to decrease pericyte coverage at PDAC vascular fenestrations, allowing 2nd wave access of gemcitabine-laden liposomes, which could enter the tumor site to enhance gemcitabine tumor killing[134]. We postulate that the use of multiwave, multistage, and combination nanotherapeutics could have a translational impact on PDAC therapeutics in the clinic[135–137]. Another approach would be to design nanocarriers that can deliver synergistic drug combinations in a ratiometric fashion. In this sense 'ratiometric delivery' is defined as the *in vivo* release of a drug combination from a nanocarrier, with the purpose of providing a fixed drug ratio at the target site[138]. One example is the combination of a drug that exerts therapeutic effects on the suppression of the stroma (*e.g.*, paclitaxel) and a drug that kills PDAC cancer cells (*e.g.*, gemcitabine). In this regard, we have recently demonstrated

the design of a lipid bilayer supported mesoporous silica nanoparticle that can achieve ratiometric delivery of gemcitabine (trapped in the porous interior) with a sub-cytotoxic dose of paclitaxel incorporated into the lipid bilayer[139]. This synergistic combination resulted in the suppression of the tumor stroma and CDA expression in subcutaneous and orthotopic PDAC models in mice, providing more effective tumor shrinkage than free gemcitabine plus Abraxane. This type of nanocarrier could also be useful for treatment of other cancers with the same drug combination. Moreover, we envisage that this carrier can be further improved through the addition of incremental design features, such as on-demand release, theranostics, and promotion of transcytosis with iRGD peptides[132]. It is important, however, to consider the design complexity against the cost of each component and the ability to achieve GMP level manufacturing production volumes.

...nanocarriers could prove useful for addressing the toxicity of FOLFIRINOX.

It is possible to develop nanocarriers for precision medicine and addressing patient-specific response differences for treatment with gemcitabine and FOLFIRINOX. This could include the use of drug profiling, PK, drug uptake and metabolic effects in treatment design (*e.g.*, consideration of the delivery of a diphosphorylated version of gemcitabine to patients that have a relative low expression of dCK enzyme) leading to intracellular gemcitabine activation. To achieve this integration of nanotherapeutics with clinical-based approaches for PDAC, we have assembled a multidisciplinary team to advance the clinical tools, infrastructure and imaging approaches for delineating gemcitabine-responsiveness in PDAC patients (*e.g.*, PET scanning and intratumoral drug profiling)[120]. This could constitute the basis of future translational studies that build on the development of nanocarriers that can address patient-specific disease characteristics in orthotopic implant models in animals.

In addition to influencing the stromal barrier, nanocarriers could prove useful for addressing the toxicity of FOLFIRINOX. While this regimen has an increased response rate compared to gemcitabine (31.6% *versus* 9.4%), FOLFIRINOX is far more toxic and therefore restricted to patients with good performance status[140]. Encouraged by the promising results of MM-398 (an irinotecan liposomal formulation in Phase III trials)[141], single and multi-drug nano formulations are being developed to provide toxicity reduction, while maintaining efficacy. This could lead to FOLFIRINOX usage in more patients, with the ability to enhance the efficacy by combining this treatment with the "engineered approaches" described in the foregoing section. It is possible to envisage the use of engineered and targeted approaches (**Figure 12B**) to stromal therapy in preclinical studies over the next 5 years, assisted by the use of the transgenic KPC model and patient-derived orthotopic tumors. GMP-level

manufacturing, quality control and initiation of Phase I into clinical studies are achievable within 10 years. FDA approval and the introduction of at least one nanocarrier platform are envisaged after 15 years.

Overcoming Specific Biological Barriers: The Blood-Brain Barrier to Target Primary and Metastatic Brain Tumors

Julia Ljubimova, MD, PhD, Eggehard Holler, PhD, and Keith Black, MD
Department of Neurosurgery and NanoMedicine Research Center
Cedars-Sinai Medical Center, Los Angeles, California 90048

Clinical Problems in Glioma Treatment

Gliomas are the most common primary brain tumors; grades III (*anaplastic astrocytoma*) and IV (*glioblastoma multiforme, GBM*) are characterized by increased cell and vessel density, cellular atypia and high mitotic activity. Malignancy grade is directly related to endothelial proliferation[142]. Despite considerable clinical and scientific efforts, patient survival still remains at 15.8 months on average. Little progress in pharmacological brain cancer treatment is due to the inability of many drugs to cross the blood-brain barrier (BBB) mostly formed by brain vascular endothelium. The BBB was discovered by Edwin E. Goldman more than 100 years ago. It protects the brain from environmental "noise", but, when the pharmacological treatment is needed, the same barrier prevents the brain influx of most drugs useful for the brain cancer treatment. Over a century-long scientific effort to circumvent the BBB has failed to answer many questions about drug delivery through the most powerful biological barrier in the body.

Nanomedicine Advances in Overcoming the Blood Brain Barrier

Glioma-derived signals triggering an intense angiogenesis in the tumor are not completely understood. Importantly, GBM and BBB interactions occur via extracellular proteins. For instance, the imbalance of tenascin and fibronectin in the tumor contributes to vessel formation[143]. We have described a switch of vascular basement membrane protein laminin isoforms in GBM from laminin-421 detected in normal brain to laminin-411, which may lead to higher rate of recurrences and shorter patient survival (Ljubimova *et al.* 2004, Cedars-Sinai Medical Center, clinical trial). The overexpression of laminin-411 in gliomas may contribute to increased glioma invasion (**Figure 13**). One clinical complication is the development of vasogenic brain edema, which dramatically increases the intracranial pressure (ICP) due to the BBB leakage[144]. Brain tumor-related edema can be a life-threatening complication of glioma growth, and so far, its treatment has relied on the use of corticosteroids.

Using systemically administered novel nanobiopolymer, Polycefin, anti-laminin drugs were delivered through the BBB, which dramatically reduced GBM size and normalized

brain cancer vasculature[145]. After the BBB crossing, polymeric nanobioconjugate release molecular inhibitors into the cytoplasm of glioma cells *in vivo* preventing the syntheses of laminin-411. Inhibition of this ECM protein decreased the tumor size by 90%. It has further been shown that the molecular mechanism of action of the endosomal drug releasing unit trileucine peptide (Leu-Leu-Leu) is based on pH sensitivity[146]; nano drug toxicity was found to be negligible and scale-up production has already begun. These nano drug treatments may significantly protect the brain from edema developing (**Figure 13**).

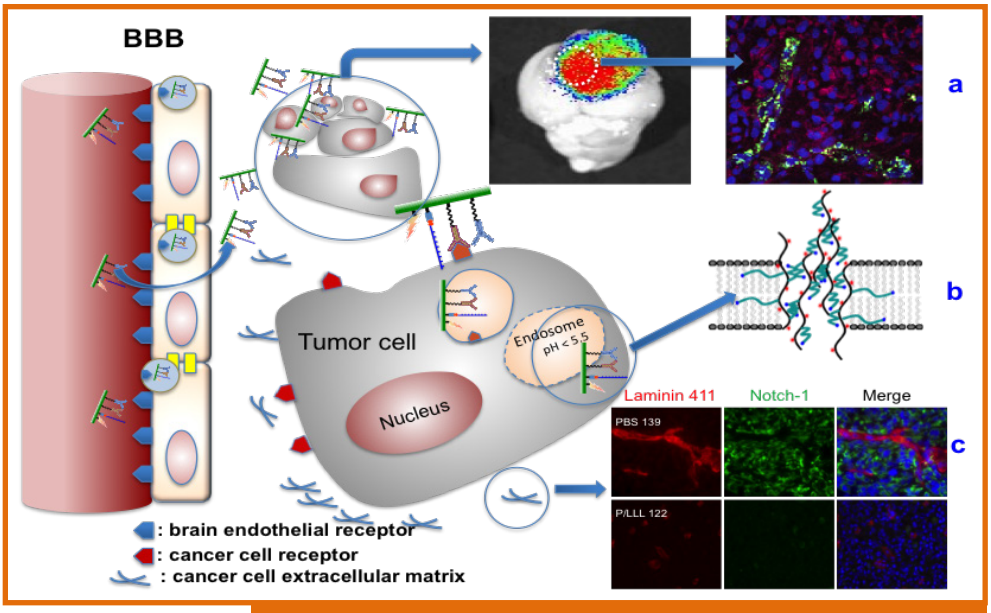

Figure 13. Multifunctional nanoconjugates for drug delivery into brain tumors. a, The nanoconjugates specifically target and accumulate in brain tumor (left), and cross BBB through receptor mediated transcytosis confirmed by confocal microscopy (right); b, Nanoconjugates are delivered into the cytoplasm by pH-dependent endosome membrane disruption and antisense oligonucleotide drugs are released; c, Successful inhibition of brain cancer stem cell marker Notch-1 as a result of inhibition of glioma-overexpressed vascular laminin-411.

Recently, the combination treatment of glioma-bearing animals with polymeric nano drugs showed significant life prolongation[147]. The polymeric nanoparticles were used for convection-enhanced intratumoral delivery of herpes simplex virus type I thymidine kinase DNA combined with the prodrug ganciclovir. An obstacle in brain tumor treatment is the limited ability for the delivery of a number of therapeutic and immunoregulatory molecules. For instance, therapeutic monoclonal antibodies, such as trastuzumab for breast and ovarian cancer, cetuximab for lung and breast cancer, and rituximab for lymphoma are effective for primary tumor treatment however cannot penetrate the BBB to reach the brain, and thus fail to treat their respective metastases in the brain. However, these antibodies can be used for brain drug delivery when they are part of 'nano-vehicles' capable of crossing

the BBB. Nanotechnology can master these problems with nanomedicines designed to cross the BBB and deliver drugs and/or immunostimulatory agents directly to a brain tumor and the respective immune cells in its microenvironment. Taking these possibilities into consideration Polycefin nano drug variants were engineered to treat human EGFR-positive triple negative breast cancer[148] and HER-2/neu positive breast tumors[149] in nude mice. The same nano drugs were similarly used to treat brain metastases from triple negative and HER2/neu positive breast cancer metastases to the brain). Furthermore, primary HER2/neu positive breast cancer has been successfully treated with a combination nanodrug that blocked HER2/neu synthesis and provided an immune system boost by directly targeted IL-2 at the same time. In this case, IL-2 was delivered as part of fusion monoclonal antibody against HER2/neu positive breast cancer[150].

Overall, the development of versatile biodegradable and non-toxic nanobioconjugate based on naturally derived polymalic acid[151] with its ability of targeting brain and breast human tumors in preclinical cancer models, inhibiting the expression of tumor-specific markers, normalizing vasculature, reducing invasion, and blocking their growth, resulted in significantly increased tumor-bearing animal survival. Additional recent nanodelivery systems/methods studied to deliver drugs across the BBB, include: focused ultrasound (FUS) disruption, SR-mediated endocytosis, and targeted adsorptive-mediated transcytosis among several others[152–158].

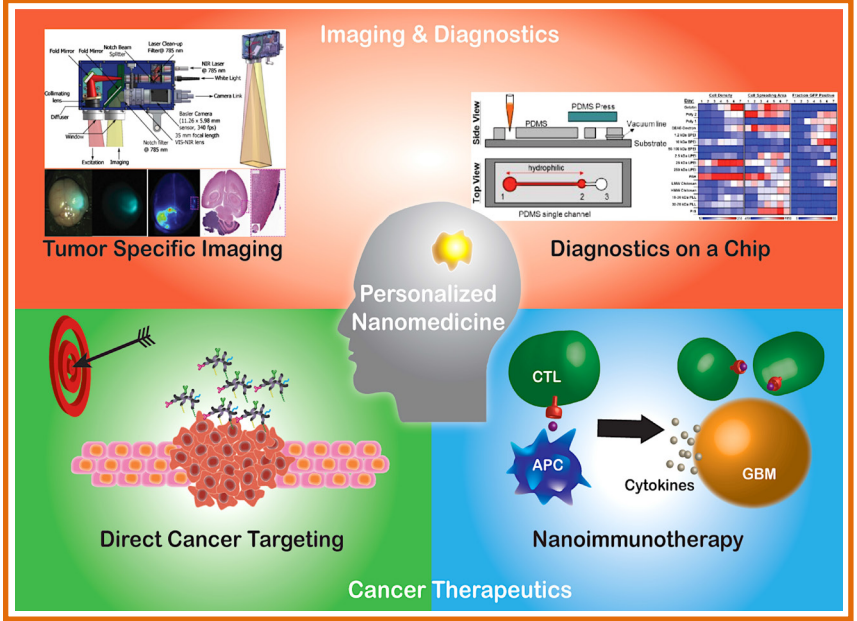

Figure 14. Brain tumor diagnostics and treatment.

Future Scientific and Clinical Developments

Treatment of brain metastases

Progress in treatment of primary cancers has led to improved patients' survival but has also increased the chance of residual tumor cells to metastasize, in particular to the brain. Melanoma, breast and lung cancer form brain metastases in up to 50% of cancer cases, with 3 to 6

months median survival. Therefore, brain metastasis treatment becomes a major issue for brain cancer management.

Personalized nanomedicine

During the last two decades, the dominant model of cancer based on genetic changes has been the chief conceptual foundation for developing targeted therapies. However, cancer immunology is currently coming back and may soon provide new mainstream cancer therapies[159]. We believe that tumor-targeted nano drugs can combine cancer genetics providing tumor cell markers, and immunotherapy providing anti-cancer immune response to treat each cancer patient individually (**Figure 14**).

Diagnostic and targeting

Current targeting strategies of nano drugs and imaging agents are based on monoclonal antibodies that will be substituted by peptides in the future to reduce immunogenicity and production costs. Significant advances of nanotechnology in cancer treatment give hope for the use of its achievements to treat a variety of other human diseases. Notable examples include neurodegenerative disorders, such as Alzheimer's and Parkinson's disease, which are on the rise due to the aging of the world population.

Significant advances of nanotechnology in cancer treatment give hope for the use of its achievements to treat a variety of other human diseases.

Non-Intravenous Routes of Delivery: Aerosol Therapy for Cancer Management

Gregory R. Robbins[1], PhD, Catherine A. Fromen[7], PhD, Tojan B. Rahhal[1,3], J. Christopher Luft[1,4], PhD, Andrew Z. Wang[1,5], MD, Chad V. Pecot[1,6], MD, and Joseph DeSimone[1,2,3,4], PhD
[1]Lineberger Comprehensive Cancer Center, [2]Department of Chemistry, [3]Department of Chemical and Biomolecular Engineering, [4]Eshelman School of Pharmacy, [5]Department of Radiation Oncology, and [6]Department of Medicine
University of North Carolina, Chapel Hill, NC 27599
[7]Department of Chemical Engineering
University of Michigan, Ann Arbor, MI 48109

Nanoparticle-based inhalation drug delivery holds several advantages over intravenous drug delivery. First, inhalation is less invasive and drug administration is more rapid than intravenous. Second, inhaled therapeutics enter circulation directly and avoid the first pass through hepatic clearance. Lastly, nanoparticles allow for tunable drug release in the lung that can provide long-term treatment with fewer administrations[160]. Additionally, nanoparticles can be used to program the local mucosal immune response and re-purpose resident immune cells for tumor immunotherapy[161,162]. Historically, aerosol delivery of nanoparticles has been considered inefficient due to the low particle mass impacting aerodynamic properties and airway deposition. However, recent advances in particle fabrication and inhaler designs are changing this outlook[163]. This document will discuss the existing science and future directions for aerosol cancer treatment using nanoparticle chemotherapy, chemopreventatives, and cancer vaccines (**Figure 15**).

Aerosol Chemotherapy

Inhalation chemotherapy offers the potential for higher drug concentrations in the lung[163–166]. Additionally, aerosol delivery allows for enhanced access to the intra-thoracic lymphatic system either through direct drainage or intra-cellular transport. Preclinical studies have suggested that there may be benefits to aerosol chemotherapy. Inhaled liposomal formulations of chemotherapies have demonstrated superior efficacy over traditional routes for the treatment of lung metastases in preclinical models[167]. Other formulations such as aerosol particles of 5-fluorourcil (5-FU), paclitaxel, carboplatin, and gemcitabine have also been studied preclinically[164,168–174]. Clinically, chemotherapeutic drugs have been delivered to the lungs through the use of nebulizers for both free drug and liposome formulations. The liposome formulations have encapsulated 9-nitrocamptothecin, doxorubicin, and cisplatin[175–177]; however, clinical trial results to date are inconclusive and suggest utilizing caution with this approach.

Delivery of Chemopreventatives to the Lung

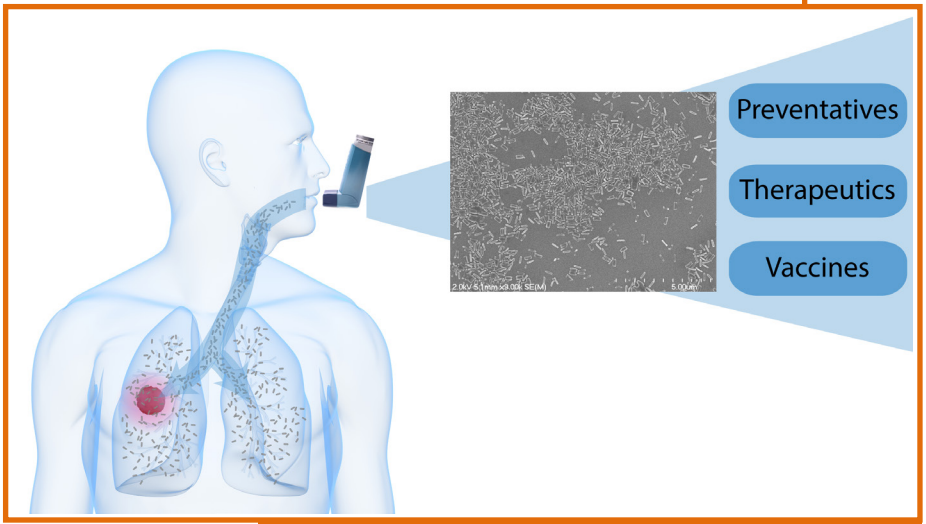

Figure 15. Depiction of aerosol based delivery of chemopreventitives, chemotherapeutics, or cancer vaccines via nanotechnology delivery with SEM image (inset) of nanoparticles designed for aerosol delivery route.

While chemotherapeutics are intended to alter disease progression following tumor establishment, chemopreventative agents are pharmaceutical interventions aimed at halting, or reversing disease progression[178–181]. Chemopreventatives can be given at a tumors' primary stage to high-risk patients, a secondary stage to patients with an identified pre-malignancy state, or a tertiary stage to prevent a secondary occurrence of the tumor[178]. To date, there have been numerous clinical trials targeting lung cancer, with minimal, or even negative, impact on disease progression. These trials have included mainly dietary supplements including various anti-oxidants, vitamins, and retinoids. Pre-clinical studies administering inhaled corticosteroids as a chemopreventative reduced cancer formation in mouse models; however, these findings did not translate to humans[182–185]. Despite these negative data, there is cause for optimism in this approach. There have been considerable successes in preclinical models involving aerosol delivery of selenium and cyclooxygenase inhibitors delivered at the primary stage[178–181]. Aerosol liposomal formulations of interleukin-2 (IL-2) have resulted in disease remission or maintenance in canine cancer models, and a number of clinical trials using nebulized IL-2 show slightly decreased tumor occurrence in humans[166,182]. Inhaled delivery of interferon, granulocyte-macrophage colony-stimulating factor (GM-CSF), and cyclosporine have also demonstrated efficacy in pre-clinical studies and, to some extent, in humans with no adverse systemic effects. Furthermore, use of oral iloprost in a randomized Phase II, placebo controlled trial for heavy smokers, has demonstrated the ability to decrease endobronchial dysplasia[186].

Lung Targeted Nano-Based Cancer Vaccines

Modulating the local immune environment of the tumor and surrounding tissue to enhance tumor eradication may be further achieved through a cancer vaccine. An ideal cancer vaccine would direct the power and precision of the patient's own immune system toward tumor elimination while providing immunological memory for rapid elimination of subsequent malignancies. The biggest challenge for cancer vaccine development is convincing the immune system that the tumor is harmful and needs to be eliminated while minimizing collateral damage in healthy tissues[183]. Achieving tumor specific immune responses requires immune targets that are exclusively (or at least preferentially) expressed by tumors, termed tumor associated antigens (TAA). The hope is that vaccines combining TAAs and immune modulating adjuvants will instruct the immune system to eliminate tumor cells.

Recent clinical trials for lung cancer vaccines incorporating non-small cell lung cancer (NSCLC) TAAs and strong immune modulators have shown measurable increases in patient survival (~3 month increase OS versus placebo control); however, none were curative[183]. Potential explanations for modest efficacy include patient selection and vaccination timing; however, another major consideration is the route of vaccine delivery. Some vaccines required multiple injections via parenteral routes[184]; however, recent pre-clinical studies using lung targeted nano-based vaccines suggest that pulmonary vaccine delivery may provide more robust immune responses with implications for targeting cancer[162,185].

Pre-clinical infectious disease models using a variety of nano-based vaccines provide protection from subsequent pathogen challenge[162,185–189]. Two of these studies directly compared pulmonary and parenteral vaccine administration and found that direct immunization of the lung provided better protection than injection at distal sites[162,188]. Part of the protective immune mechanism works through activation of cytotoxic T cells (CTLs) that seek out and eliminate cancerous cells. In addition to CTL activation, several of these vaccines also promoted TNFα and IFN-γ cytokine production, which are known to promote an anti-tumor environment by inhibiting suppressive tumor associated macrophages[162,185,190]. The added benefit of an efficacious cancer vaccine is that these immune cells roam the body and have the capacity to target sites away from the primary tumor, which has major implications for metastatic control. Support for this hypothesis includes a study in which a nano-vaccine delivered to the lung was able to eliminate melanoma in the flank and establish long-term tumor rejection and survival[162].

Future Directions for Aerosol Delivery of Nanoparticles in Cancer Management

Nanoparticle therapeutics in the lung represent an area of great potential, especially for treating cancer. To date, most aerosol therapies have involved delivery of 1-5 µm sized particles, due to their aerodynamic properties and their assumed deposition in the lung[191]. Indeed, even the chemotherapy liposome formulations evaluated in clinical trials were on the order of ~1 µm[164,167,192]. More recent nanoparticle formulations (<200 nm) could offer tremendous benefits to the three aspects of cancer management mentioned here: drug delivery (including enhanced tumor uptake), mucosal diffusion, and lymph trafficking[160]. However, delivery concerns will need to be addressed in order for nanoparticles to deliver and deposit at high efficiencies in the airways. Controlled aggregation or a "Trojan horse" approach may be required for effective delivery, with independently tunable aerodynamic properties for controlled deposition in the region of interest within the lung[173]. Additionally, advancement of particle-based lung therapies will require continued optimization of inhaled delivery devices[165,193].

Of the potential applications for aerosol cancer management, nanoparticle delivery of cancer vaccines may be best situated to make the greatest impact within the next decade. The extensive research and success in particle formulations for intravenous nanoparticle therapies can be readily translated to lung administration with minimal reformulation, while current clinical evaluations of aerosol liposome formulations establish precedence for use of a particle approach for direct vaccine delivery. The biggest challenges moving forward will be choosing the most specific TAA's, overcoming immune tolerance mechanisms and avoiding immune pathology in an already vulnerable patient population. Overcoming immune tolerance may require co-administration of therapeutic antibodies to disrupt normal lymphatic checkpoint mechanisms (anti-CTLA4, anti-PD1, anti-PDL1) and allow the vaccine to establish an immune response[194]. Another challenge will be establishing the safety of the nanoparticle platforms, especially in combination with immune adjuvants with a goal of inducing strong immune responses without damaging lung tissue. Ultimately, studies assessing patient tolerance to pulmonary-targeted nano-vaccines will be critical to the use of safe adjuvant combinations.

Aerosol chemotherapy faces a steep uphill battle to fruition. There are two deeply rooted schools of thought regarding inhaled chemotherapeutics and it is likely to remain a controversial issue. Most clinicians believe the direct delivery of highly toxic chemotherapeutics to the lungs exposes the patient to unacceptable risk, and could inflict further damage to an already susceptible tissue. The opposing argument points to the urgent need for alternative approaches for lung cancer treatment. Thus moving forward,

nanoparticle aerosol delivery of chemotherapeutics will require substantial and strategic preclinical and clinical research to discern the practical application of these therapies.

Chemopreventative agents have demonstrated success in preclinical models...

Chemopreventative agents have demonstrated success in preclinical models, but the difficulties in identifying target patient populations makes widespread chemoprevention in a primary stage cancer challenging. Evaluation of lung specific biomarkers and further characterization of the lung cancer progression will help identify patient populations likely to benefit from chemoprevention; however, dosing at a secondary or tertiary stage following the identification of pre-malignant lesions or prevention of a secondary occurrence may be more tractable. Winterhalder *et al.* suggest that cell surface receptors, such as EGFR and HER2, may be important targets to halt progression of epithelial lung cancer; given the history of systemic nanoparticle formulations targeting these pathways, this may be a tractable first nanoparticle approach[181]. Finally, there are many genetic factors in lung cancer that could be potential targets for gene therapy that are considered "undruggable" using conventional approaches, which are also ideally suited for nanoparticle formulations[195,196].

The nanoparticle approaches discussed here represent novel lung cancer management strategies that may also apply to other cancers. Additionally, topics discussed here may be better suited as combination therapies with more traditional approaches including surgical resection, chemotherapy, and radiation. We anticipate that many of these approaches will be first investigated in recurrent or late-stage disease following alternative interventions. Success in these situations may ultimately lead to a paradigm shift that utilizes aerosol-only based approaches.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will conduct further preclinical studies on direct lung chemotherapeutics use and efficacy; develop chemopreventatives to better establish effects on lung cancer progression; and identify and validate drug targets for local lung cancer vaccine therapy. Looking further ahead over the next 5 years, researchers will identify tumor associated antigens and adjuvant combinations that target lung related tumors for nano-based cancer vaccines; and carry out perspective studies on effects of direct lung therapy, positive or negative. In the next 10 years, researchers will establish a clinical development program for aerosol treatment of lung cancer, utilizing chemotherapy, chemopreventatives, and nano-based cancer vaccines.

Non-Intravenous Routes of Delivery: Oral

Eric Pridgen, PhD
School of Medicine
Stanford University, Palo Alto, CA 94305

Introduction

Nanoparticles (NPs) have the potential to make a tremendous impact on the treatment of cancer. Combining biological understanding with engineering and materials science principles has led to the development of nanomedicines for the treatment of cancer that are now entering clinical trials[197–199]. However, NPs are currently limited to parenteral methods of administration. In addition, many chemotherapeutic agents and biological therapeutics are limited to parenteral administration because of low bioavailability. Injection-based therapies can suffer from poor patient compliance and reduced efficacy due to the pain and inconvenience associated with the treatment regimens. Therefore, alternate routes of administration, such as transdermal, nasal, buccal, pulmonary, and oral, are under investigation as a means to improve these therapies. Of these alternate routes, oral is considered the most desirable, especially for long-term treatment of diseases, because of the convenience and improved compliance[200].

In clinical studies with cancer patients, most favored oral over intravenous chemotherapy because of the increased convenience as long as efficacy was not compromised[201–203]. The convenience of taking medications at home was especially convenient for patients that lived far from hospitals and clinics[204]. Several trials have demonstrated that oral-based therapies can be as efficacious as parenteral administration, but offered additional advantages. In one trial, oral administration of Tegafur-uracil (UFT) was compared with intravenous administration of 5-fluorouracil (5-FU) for the treatment of metastatic colorectal cancer[205]. The oral administration was associated with decreased incidence of drug-related adverse effects without compromising efficacy. Other studies have shown that intravenous methods required more frequent hospitalizations that were expensive, time intensive, and required intravenous access[206]. Oral formulations have advantages for physicians as well, providing flexibility and adaptability to tune dosing schedules to individual patients based on efficacy and toxicity[204]. Without the intensive demands on staff required by intravenous administration, studies in the United Kingdom showed that switching from intravenous to oral chemotherapy allowed a 7-fold increase in patients treated[207]. Finally, reducing hospital or clinic visits as well as costs associated by using oral formulations could reduce overall costs for cancer treatments[208–210]. Indeed, cost-benefit studies conducted in Europe and Canada examining oral versus standard intravenous regimens for colorectal cancer suggested

significant savings with the oral route despite the higher cost of the orally formulated therapies[211].

While oral delivery is highly desirable, it presents many challenges due to the number of barriers presented by the gastrointestinal tract before therapeutics are absorbed and enter the bloodstream. These barriers include extreme pH environments ranging from 1 to 8[212] and enzymatic degradation, which limit the absorption of biologic therapeutics such as proteins and nucleic acids. In addition, there is a transport barrier presented by the intestinal epithelium, which is a polarized cell monolayer that tightly regulates the transport of material from the external environment (intestinal lumen) to the *lamina propria*[213]. This intestinal epithelium is covered by a mucus layer, which protects the epithelial surface by trapping pathogens and foreign particulates and rapidly clearing them[214]. Therapeutics that reach the intestinal cell surface and enter the cells must than bypass the cells metabolic systems and P-glycoprotein (P-gp) drug efflux pumps, which can cause low bioavailability for many small molecule drugs such as chemotherapeutic agents[215]. Finally, if the therapeutics cross the intestinal transport barrier, they must avoid immune cells that patrol the *lamina propria* in order to reach the bloodstream and the mononuclear phagocyte system of the liver in order to reach other organs in the body.

Polymeric NPs are a well-studied option for oral delivery that can aid in overcoming many of the intestinal barriers

Polymeric NPs are a well-studied option for oral delivery that can aid in overcoming many of the intestinal barriers. The NPs are stable in the GI environment and can protect encapsulated therapeutics from the pH environment, enzyme degradation, and drug efflux pumps[200,216]. However, intestinal absorption of NPs is highly inefficient because the physicochemical parameters, particularly size, of NPs prevent their transport across cellular barriers such as the intestinal epithelium. To improve the absorption efficiency of NPs and make oral administration practical in the clinic, additional strategies are necessary to overcome the intestinal epithelial barrier.

Oral Delivery Strategies

There are several pathways across the intestinal epithelial barrier that could be used for oral delivery[217]. One option is the paracellular pathway, which is a major passive permeation pathway across the intestines and allows diffusion of small molecules in the space between epithelial cells. The tight junctions between epithelial cells regulate the permeability of this pathway based on the size and charge of the molecules[218,219]. Another option is the

transcytosis pathway, which is an active transport pathway that relies on receptors specific for a molecule to guide the molecule through the cell in endosomes without entering a degradation pathway. Because of their large size, NPs are restricted to this pathway.

One approach for oral delivery that has been extensively evaluated is the use mucoadhesive materials (**Figure 16A**). These are polymers such as chitosan[220], polyacrylic acid (PAA)[221], and poly(fumaric-co-sebacic) anhydride[222] that interact with the mucus layer covering the epithelial cells. Adherence to the mucus layer increases the residence time and contact of released drug with the underlying epithelium, resulting in increased drug concentrations at the site of absorption[223]. In addition to increasing the concentration of therapeutics near the epithelium, many mucoadhesive polymers increase intestinal absorption by acting as permeation enhancers, reversibly opening tight junctions between epithelial cells to allow enhanced paracellular transport[224]. Since the tight junctions are less than 20 nm in diameter, NPs are unable to pass through this pathway, but small molecule therapeutics can cross the epithelium[225]. One disadvantage of this approach is that the permeation enhancer activity

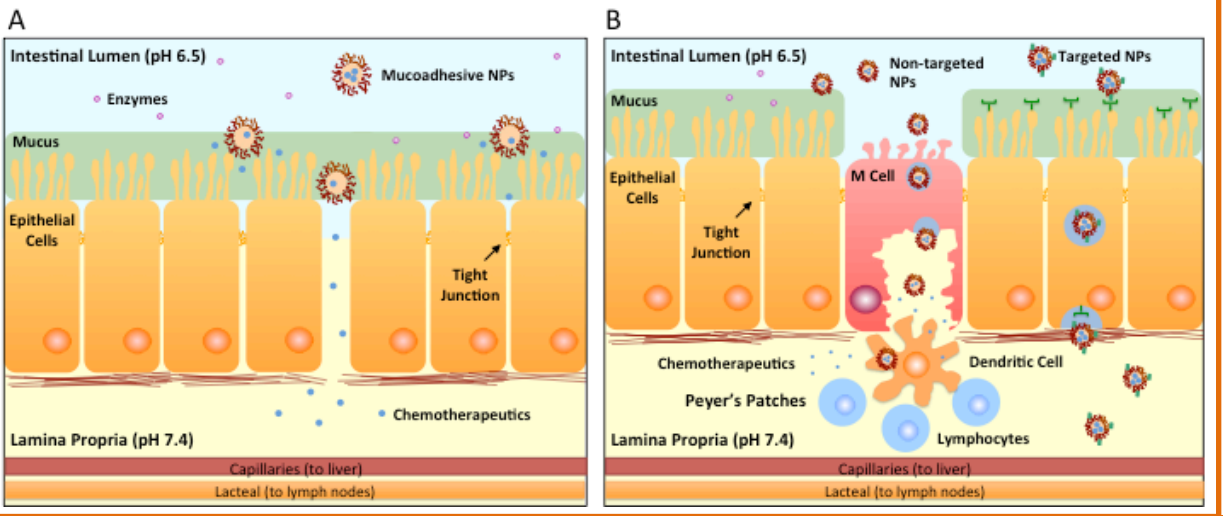

Figure 16. Schematic illustration of strategies for oral delivery. (A) Mucoadhesive materials used to form NPs adhere to the mucus layer above the epithelial cells and release therapeutics at high concentrations near the surface of the epithelial cells. In addition, they are able to reversibly open tight junctions to allow paracellular transport of therapeutics between the cells and across the epithelial barrier into the *lamina propria*. (B) The transcytosis pathway is an active transport pathway that transports material across cells in endosomes while evading degradation pathways in the cell. Examples of transcytosis pathways include M cells, which are responsible for transporting antigens across the intestines for immune surveillance and are associated with Peyer's Patches. Other examples include the vitamin B12 receptor pathway and the FcRn pathway, where NPs targeted to the specific receptors are trafficked across the epithelial cells and released in the *lamina propria*.

is non-specific, potentially allowing toxins and other pathogens present in the intestines to cross the intestinal barrier once the tight junctions are open[226,227]. Another limitation is that the surface area for absorption through the paracellular pathway is less than 0.1% of the total intestinal epithelium surface area, which could limit the capacity for absorption of therapeutics[228].

Targeting NPs to natural transcytosis pathways is another approach used for oral delivery (**Figure 16B**). It offers a way to cross the intestinal barrier without affecting the intestinal epithelium barrier integrity. There are several mechanisms that have been studied for transcytosis of NPs. The most extensively studied is the M cell transcytosis pathway. M cells are associated with Peyer's Patches, which are organized components of the gut-associated lymphoid tissue (GALT). The role of M cells is to transport antigens across the intestines through a non-degradative pathway for immune surveillance[229,230]. This pathway is attractive because M cells have reduced protease activity, lack mucus secretion, and have a sparse glycocalyx[231]. One potential problem with this approach is that since M cells are closely associated with immune cells in the *lamina propria*, NPs crossing the intestines through this pathway may be engulfed by immune cells before reaching the bloodstream and releasing their cargo[232]. Absorption by M cells may also be limited because M cells only make up a small percentage (5-10%) of the non-absorptive epithelium in humans[233,234].

Other strategies have focused on targeting NPs to receptor-mediated transcytosis pathways that are not associated with the GALT, which may help NPs evade immune cells after crossing the epithelium. One example is the vitamin B12 receptor, which traffics vitamin B12 across the intestinal epithelium[235]. NPs targeted to this pathway have been shown to successfully deliver biologic payloads to the bloodstream, although transport of NPs has not been demonstrated yet[236,237]. One potential drawback of this approach is that vitamin B12 absorption does not occur until the distal section of the ileum, requiring NPs to maintain stability and not release their cargo while traveling through most of the small intestine. Another example is the neonatal Fc receptor (FcRn), which transports IgG antibodies across the intestinal epithelium[238,239]. This receptor is expressed throughout the intestines. NPs targeted to the FcRn were able to cross the epithelium and circulate in the bloodstream to several different organs, including the liver, spleen, lungs, and kidneys, along with releasing a therapeutic payload[240].

Clinical Impact

While oral delivery has been extensively studied and many strategies have had success in animal models, there has not been much success translating the research into practical clinical solutions. Most of the effort has focused on developing technologies for oral delivery

of insulin. However, NPs are flexible in terms of the molecules that can be encapsulated and changes to formulations could easily result in NPs capable of delivering chemotherapeutic molecules. In addition, NPs can encapsulate protein therapeutics and small interfering RNA (siRNA), which are emerging treatment modalities for cancer. The major limitation to translation is that the technologies developed are not efficient enough to make them practical for the clinic. More recent technologies such as NPs targeting the B12 receptor and FcRn have demonstrated higher efficiencies, but only in animal models at this point.

There are currently several technologies that are entering early-stage clinical trials for oral delivery of therapeutics. These include Oramed's oral formulation consisting of permeation enhancers that is now entering Phase II clinical trials. Novo Nordisk is developing an absorption enhancer technology that is entering Phase I trials. Entrega is developing a mucoadhesive technology that is still in early stage development. Each of these technologies is focused on enhancing transport through paracellular pathways, which would enable drugs, but not NPs, to cross the intestinal epithelium.

As nanomedicines are shown to be effective for cancer therapy in clinical trials, future efforts should focus on translating technologies to the clinic that utilize the transcytosis pathway. These technologies could enable the NPs carrying chemotherapeutics to cross the intestinal epithelium and reach circulation. In this case, the advantages of NPs in the bloodstream could be utilized for the treatment of cancer, such as passive or active targeting of tumor cells, delivery of multiple therapeutics in a controlled or triggered release manner, and selective biodistribution of the therapeutics to the tumor to reduce side effects. Future research should also focus on discovering other natural transcytosis pathways that could be used to transport NPs across the intestines. This could include studying how some bacteria are able to cross the intestines and the subsequent rational design of NPs that could mimic those processes. In addition, new technologies such as microneedle-based pills have shown promise in improving bioavailability of biologics in initial animal studies, but need further study to determine clinical feasibility[241].

...researchers will develop NP delivery vehicles targeted to transcytosis pathways that specifically encapsulate and deliver chemotherapeutic agents...

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers

will optimize the physicochemical parameters of NPs targeted to transcytosis pathways to maximize bioavailability after oral administration; and conduct research into alternate transcytosis pathway receptors and alternative technologies such as microneedle-based pills. Looking further ahead over the next 5 years, researchers will develop NP delivery vehicles targeted to transcytosis pathways that specifically encapsulate and deliver chemotherapeutic agents; and evaluate the performance of permeation enhancer and mucoadhesive technologies currently entering clinical trials. In the next 10 years, researchers will gain FDA approval for permeation enhancer and mucoadhesive technologies that are successful in clinical trials; conduct clinical trials on NP delivery vehicles targeted to transcytosis pathways for cancer treatments; and study how patient-to-patient variability, diet, fasting states, and disease states affect the performance of these technologies in humans in order to determine the robustness of these technologies.

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Section II: Unique Modalities for Nanotherapeutics

Optimizing Nanoparticle Delivery of Chemotherapeutics

Alberto Gabizon[1], PhD and Irene Ninh La-Beck[2], MD
[1]Oncology Institute, Shaare Zedek Medical Center
Hebrew University-School of Medicine, Jerusalem, Israel
[2]Department of Immunotherapeutics and Biotechnology, School of Pharmacy
Texas Tech University Health Sciences Center, Abilene, TX 79601

Chemotherapeutics in Cancer Therapy

Chemotherapy can be defined as the use of cytotoxic drugs that attack or interfere non-specifically with critical components of the cell. Chemotherapeutic drugs include at least 3 well-known categories: agents that damage the DNA template directly or indirectly; agents that damage microtubules; and, agents that inhibit DNA, RNA, or protein synthesis (antimetabolites). In addition to their lack of specificity, various pharmacologic factors seriously limit drug distribution and penetration to tumors and neutralize the activity of chemotherapy. This group of agents could tremendously benefit from a delivery system to improve its tumor specificity and reduce its toxicity to normal tissues. However, it is now often questioned whether chemotherapy will be abandoned and replaced entirely with biological and immunological therapies in the near future. While important advances have been made in the areas of biological therapy and immunotherapy of cancer, chemotherapy remains a critical tool of cancer treatment with a large contribution to cancer cures in the adjuvant setting and an important contribution to life extension in the metastatic setting. Improvements in safety and efficacy of chemotherapy are definitely a worthy endeavor since they will have a dramatic effect on the well-being of our patients, their quality of life during treatment, and their ability to face the hardship of therapy and complete successfully the protocol regimes. Moreover, chemotherapy is also likely to remain an important component of a multimodality therapeutic approach, together with biological therapy and immunotherapy, to improve the antitumor response rates in a broad array of cancer types. There are many examples of the continuing role of chemotherapy and its critical added value to biological therapy. One of them is exemplified by the combination of chemotherapy with anti-HER2 antibodies (Trastuzumab) in HER2-positive breast cancer, which is required for optimal antitumor response. From a tumor response rate of only 12% for single agent Trastuzumab, the response rate climbs to 56% when doxorubicin and cyclophosphamide are combined with Trastuzumab[1]. While this combination of doxorubicin with Trastuzumab was problematic because of a major rise in cardiac complications, a number of subsequent studies have shown that replacing doxorubicin with liposomal

doxorubicin can avoid or minimize cardiac toxicity[2]. This example emphasizes the valuable contribution of chemotherapy to targeted therapies and the need to refine the formulations of chemotherapy for optimal results.

Towards "Smart" Chemotherapy with Nanoparticle Delivery

Nanomedicine is a platform to allow sophisticated and smart drug delivery within the size window of a submicroscopic system that enables delicate and complex interactions with cancer cells and their biological milieu. Nanoparticles and some macromolecules are the main tools of nanomedicine[3]. Pegylated liposomal doxorubicin (PLD) was the first nanoparticle-based cancer chemotherapeutic approved by the FDA. PLD together with nanoparticle albumin-bound paclitaxel (NAB-paclitaxel) are probably the cancer nanomedicines that have made, so far, the most important clinical impact[4,5], excluding antibody-drug conjugates, generally considered to be a separate group of complex drugs.

Transforming the administration of a drug in free form, several angstroms across, into a 100-nm diameter nanoparticle loaded with thousands of drug molecules and with ~1 million-fold greater volume is a formidable pharmaceutical challenge that will have major pharmacological implications. However, from the clinical point of view, the only questions that have any significance when using nanopharmaceuticals are: Is the safety profile of the drug improved? Is the efficacy of the nano-engineered drug superior to the standard treatment or best performing comparator? To achieve these objectives, the nanoparticle-based approach should ideally fulfill two critical parameters:

a. Stable association of drug and carrier in circulation, and release of active drug in tissues, at a satisfactory rate, for anti-tumor activity. This parameter appears to have been satisfactorily met by pegylated liposomal doxorubicin (PLD)[6].

b. Enhanced drug delivery to tumors via the nanoparticle formulation. For this to occur, first, the nanodrug or nanopharmaceutical must have a long circulation time to increase the number of potential passages through the tumor microvasculature. Second, the nanoparticle physical size has to be in the optimal size regime to allow extravasation across tumor blood vessels, which usually display higher permeability than normal blood vessels. The size window that will exploit the difference in permeability between normal and tumor blood vessels appears to be between 20 to 200 nm.

Successful control of these two parameters in the drug nano-formulation allows sparing normal tissues from toxicity and in boosting the antitumor effect with an overall increase of the therapeutic index. Some nanomedicines have failed to meet these requirements because

of either short circulation time, poor drug retention, or insufficient drug release[7–9]. Yet, other nanomedicines have been able to make a positive clinical contribution despite only minor changes in drug pharmacokinetics. This is the case of NAB-paclitaxel which avoids the acute toxicities associated with Cremophor EL® vehicle used in solvent-based paclitaxel, and has been found useful in various indications.

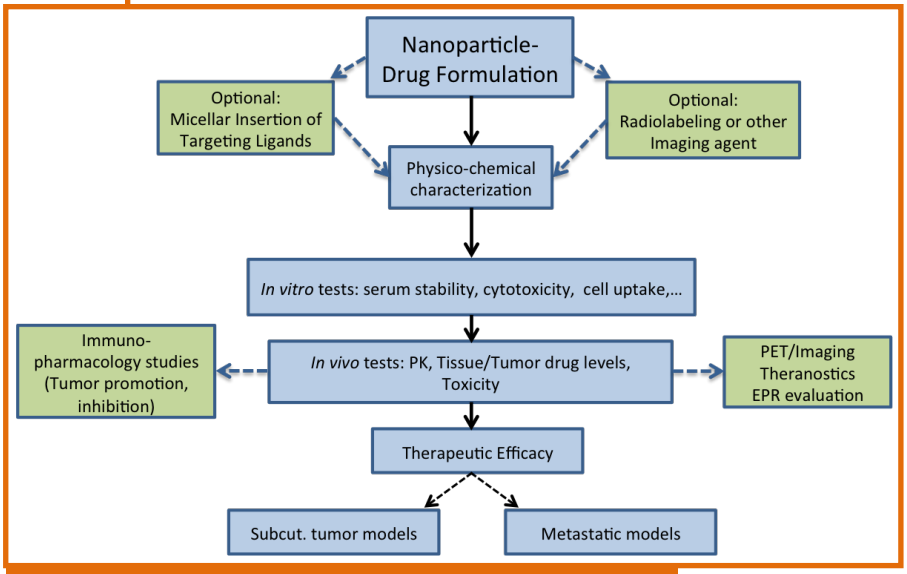

Figure 1. Schematic model of a work plan for rational development of nanoparticle-based chemotherapeutics.

High microvascular permeability is an important and frequent feature of tumors usually referred to as Enhanced Permeability and Retention (EPR) effect, and is a key component for nanoparticle transport into tumors[10]. EPR appears to be a particular feature of tumor-driven neoangiogenesis. While EPR is observed in most models of implanted experimental tumors, large variations have been observed in human cancer depending on tumor type, tumor size, tumor site, and other factors, such as previous chemotherapy, antiangiogenic therapy, and radiotherapy. EPR may also be modulated by pharmacologic mediators. In some instances, tumors or their metastases derive their blood supply by a process known as co-option of normal blood vessels which results in blood vessels less permeable and less responsive to anti-angiogenic treatments and, consequently, less likely to display the EPR effect[11]. The high response rate of Kaposi Sarcoma, a tumor with high vascular permeability, to relatively low doses of PLD suggests that EPR is critical for the antitumor activity of nanodrugs. While this hypothesis has a strong pharmacologic rationale, it has not been tested rigorously, and we cannot discard that tumors with low EPR will still respond to nanodrugs better than to free drugs.

Smart delivery of chemotherapeutics may be simply achieved by controlling release rate of the active agent and by changes in tissue distribution, without necessarily including a targeting component specific for cancer cells. In fact, all the nanopharmaceuticals approved for clinical use belong to the non-targeted category. A scheme for development of nanoparticle-based chemotherapeutics is shown in **Figure 1**.

Targeted Nanomedicines

Our understanding of the molecular processes underlying the pathologic behavior of cancer cells has progressed enormously in the last decade. Overexpressed receptors in the membrane of tumor cells, may offer a potential Trojan horse for targeting specific ligands or antibodies and delivering a cytotoxic drug cargo. Probably, the best example of a successful clinical translation of this approach is the antibody-drug conjugate known as T-DM1 which combines Trastuzumab, an anti-HER2 antibody, with emtansine, a potent and highly toxic chemotherapeutic, and has conferred a significant disease-free survival advantage to patients with HER2-positive breast cancer[12].

Targeted delivery of a large payload of drug via ligand-directed nanoparticles to cancer cell-specific receptors is probably the most valuable objective of nanomedicine. A comprehensive and in-depth review of this subject has been recently published[13]. Indeed, the most logical improvement of nano-based drugs is the coupling of a ligand to the surface of the nanoparticle to target to a specific cell-surface receptor. This would be followed by internalization and intracellular delivery of the small-molecule drug cargo. Examples in this direction are the targeting of PLD to HER2-expressing or folate-receptor expressing cancer cells using respectively a specific anti-HER2 scFv or a folate conjugate anchored to the liposome surface, or the targeting of polymeric nanoparticle of docetaxel to PSMA, a marker of prostate cancer[14–16]. Yet, another example is the tumor vascular targeting of liposomes with endothelium-specific peptides associated to liposomes[17]. A major advantage of targeted nanocarriers over ligand-drug bioconjugates is the delivery-amplifying effect of the former, which can deliver to the target cell at a ratio of ~1000 drug molecules per single ligand-

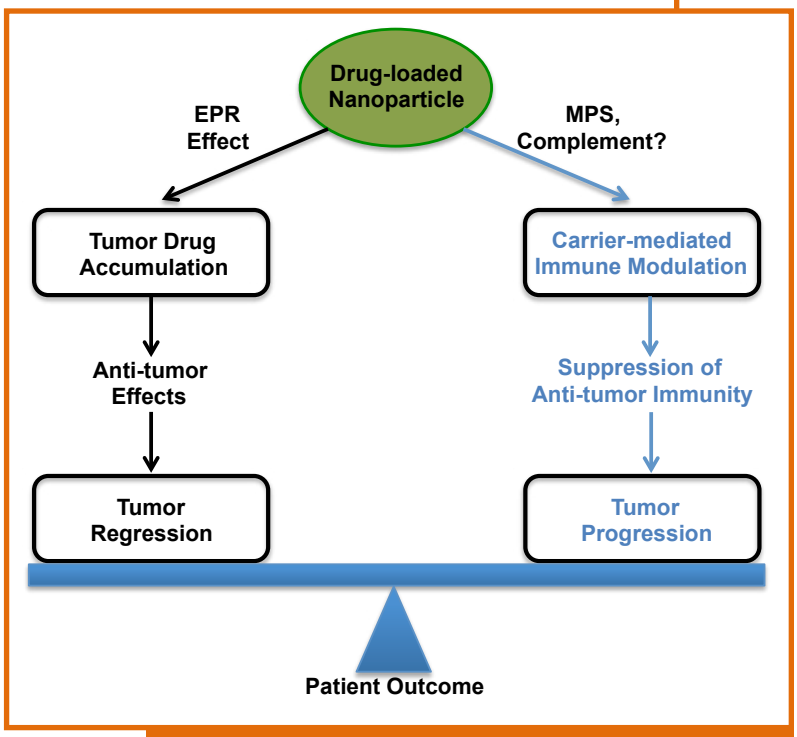

Figure 2. Nanoparticle carrier interactions with the immune system may suppress antitumor immunity, thereby attenuating the antitumor effects of the drug cargo. A mechanistic understanding of the mechanisms of carrier-induced immune modulation will enable the development of systematic tools that may help to realize the full clinical potential of nanoparticle-based therapies.

receptor interaction. In addition, the multivalent conjugation of targeting ligands on the surface of nanoparticles is presumed to enhance binding to the desired target. Targeting ligands, particularly small molecule ligands, can significantly enhance target-specific avidity of nanoparticles by several orders of magnitude through multivalent interactions[13].

Interaction of Nanoparticles with the Host

Nanoparticles, including liposomes, are known to interact with the immune system to varying extents[18]. These interactions can affect drug pharmacokinetic parameters and may have significant clinical consequences. The majority of intravenously administered nanoparticles are rapidly cleared by the mononuclear phagocyte system (MPS) through internalization by phagocytic cells such as hepatic Kupffer cells and splenic macrophages. Notably, peripheral blood monocyte count and phagocytic function have been shown to correlate with PLD clearance rates in patients[19], and similar correlations have been observed with other pegylated liposomal formulations (S-CKD-602, and SPI-077) in preclinical rodent and canine models[20]. Thus uptake and sequestration of nanoparticles in cells and organs of the MPS is a major barrier limiting the circulation half-life and, hence, tumor accumulation of carrier-mediated drugs.

In addition to interactions with the MPS, it is well established that nano-carriers interact with serum proteins such as IgG, IgM and the blood complement proteins, which contribute to opsonization of the carrier and enhance clearance by the MPS. Importantly, activation of complement proteins also generates anaphylatoxins (C3a, C4a, C5a) which can stimulate release of inflammatory mediators by immune cells leading to complement activation-related pseudoallergic reactions (CARPA) in swine and canine models, and several formulations of nanoparticles in clinical use (Doxil, DaunoXome, AmBisome, Abelcet, Amphocil) have been shown to cause hypersensitivity reactions consistent with CARPA. Clinically, it was shown that PLD activates complement in the peripheral blood of cancer patients and that the extent of complement activation correlated with the development of acute infusion reactions[21]. Therefore, undesired interactions with circulating serum proteins can also affect the pharmacokinetics and tolerability of carrier-mediated drugs.

Coating of nanoparticles with poly-ethylene glycol (PEG) (“pegylation”) has become widely used to reduce opsonization, improve stability in plasma, and prolong circulation time which are important requirements for effective tumor targeting. However, these approaches may not abolish immune reactions to nanoparticles. In addition, recent evidence suggests that PEG is not immunologically inert. Several groups have demonstrated that the initial systemic administration of pegylated nanoparticles induces production of anti-PEG IgM antibodies that enhance immune recognition and clearance of the second dose of nanoparticles in

preclinical models. Interestingly this "accelerated blood clearance" (ABC) phenomenon has not been reported in patients and its clinical relevance is currently unclear. In fact, the opposite has been observed in patients treated with PLD, where clearance rates decrease with repeat administration, up to 30% by the third cycle[22].

Recently, it was shown that nanoparticle-induced complement activation could promote C5a-dependent tumor growth in tumor bearing mice, presumably through the recruitment and activation of immunosuppressive leukocytes. Yet, the nanoparticles used in these studies were intentionally designed to activate specific complement pathways[23]. It is not known whether clinically relevant nanoparticulate carriers, which activate complement in the peripheral blood, also induce complement activation in the tumor tissue, or how this impacts tumor growth. However, new evidence with a pegylated liposomal carrier similar to the PLD carrier, showed that these liposomes significantly enhanced tumor growth in an immune competent murine tumor model[24]. This was associated with suppression of antitumor immunity as indicated by blunting of cytokine production in tumor-associated macrophages and cytotoxic T cells, and diminished tumor antigen specific immune responses. Moreover, tumor microvessel density was significantly increased, consistent with enhanced angiogenesis. Collectively, these findings suggest that carrier-induced immune modulation could attenuate therapeutic efficacy of the nano-encapsulated drug (**Figure 2**), which may partially explain why there has been an insufficient improvement in anticancer efficacy in many of the clinical studies with nano-drugs despite their major pharmacologic advantages over free drugs[25].

It is possible that during preclinical development, the prevalent use of rodent models with immune defects and the dearth of *in vivo* immune functional studies may have downplayed the consequences of the interactions between drug carriers and the immune system. It is also possible that manufacturing of the nanomedicines themselves were not as pure as initially thought with various solvents left behind in the formulations. Either way, incorporation of fully immune competent tumor models along with systematic immune functional studies may yield more accurate insight and analytical tools, that may help to realize the full clinical potential of nanoparticle-based therapies[26].

Cancer Nanodrugs in Clinical Use or Clinical Testing

Table 1 shows a list of nanoparticle-based drugs approved for cancer treatment by the FDA and/or the EMA. As seen in Table 1, the number of nanopharmaceuticals in clinical use has been slowly albeit steadily rising and includes chemotherapeutics of various classes, such as anthracyclines, taxanes, vinca alkaloids, and DNA topoisomerase-1 inhibitors. Most of these formulations are liposome based. Two of them, Depocyt and Mepact, are large

liposomes above the ultrafilterable range and probably should not be considered *bona fide* nanomedicines. Also included in Table 1 is NaL-Iri, which has not yet been approved although it has completed phase 3 trials for the 2nd line therapy of pancreatic cancer and met its primary objective of improved survival rates.

The early and positive preclinical and clinical experience with liposomal delivery of anthracyclines is probably one of the reasons for the dominance of liposomes in the field. Liposomes still remain as one of the most attractive particulate systems for cancer nanomedicine applications. A liposome formulation of doxorubicin, PLD (known as Doxil/Caelyx or Lipodox in generic version), is currently approved for various indications and in wide clinical use[4]. PLD has significantly reduced acute toxicity, as well as cardiac toxicity as compared to free doxorubicin precisely because of its unique pharmacokinetic characteristics. Probably the most significant clinical value added of PLD is the evidence of a major (~3-fold) risk reduction of cardiotoxicity as compared to free doxorubicin enabling risk-free, extended treatment[2].

In addition, many other promising nanochemotherapeutic products are under clinical testing or about to be clinically tested. These include: polymeric nanoparticles of docetaxel in targeted and non-targeted form which have a significantly different pharmacological profile from the solvent-based docetaxel formulation; pegylated liposomal formulations of various cytotoxic drugs including eribulin and a prodrug of mitomycin C; a HER2-targeted version of PLD (MM-302); a low-temperature, release-sensitive, liposomal doxorubicin formulation; and a liposome formulation of co-encapsulated cytarabine and daunorubicin at fixed molar ratio[16,27–32].

Table 1: Nanoparticle-based products for cancer approved by FDA and/or EMA

Product	***Indication in cancer***
Pegylated Liposomal Doxorubicin	Kaposi Sa., Ovary, Breast, Myeloma
Liposomal Daunorubicin	Kaposi Sa.
NAB-Paclitaxel (Abraxane)	Breast, Lung, Pancreas
Liposomal Doxorubicin	Breast
Liposomal Vincristine (Marqibo)	Adult A.L.L.
Low-pegylated Liposomal Irinotecan (NAL-IRI)	Pancreas (Phase 3 completed, awaiting NDA)
Liposomal Cytarabine (DepoCyt)	Lymphomatous meningitis
Liposomal Mifamurtide (Mepact)	Osteosarcoma

The Future of Nanoparticle-Based Chemotherapeutics - Quo Vadis?

Two fundamental aspects of nanomedicines remain to be clarified in upcoming years: we need an improved understand of the interaction of nanoparticles with the immune system and to learn how to manipulate it for the benefit of the patient; and, we need to understnad how relevant is the EPR effect in human cancer, particularly in metastases, and what role does it play in the performance of nanopharmaceuticals.

It is likely that we will witness a more extensive use of the currently approved nanotherapeutics at the expense of conventional use of chemotherapeutics. In addition, other nanodrugs in clinical development may be approved in the coming years, expanding the classes of drug available in nanopharmaceutical form. Nanodrugs designed to exploit the EPR effect best, with optimal stability and drug release profiles, are likely to perform better although safety improvements will remain a key aspect dictating clinician preference. The use of targeted nanomedicines is probably going to be on the rise, particularly when there is a need to improve the cell uptake of a specific pharmaceutical agent.

Two fundamental aspects of nanomedicines remain to be clarified in upcoming years:...

The use of nanoparticles to deliver therapies, other than chemotherapeutic drugs, is also foreseeable, especially for agents with problematic *in vivo* delivery. In the case of siRNA, the nanoparticle protection is crucial. Recently published studies suggest that for some biologic agents such as tyrosine kinase inhibitors[33], or, immunomodulators such as aminobisphosphonates[34], nanoparticle-based delivery may also improve their *in vivo* performance in combination with chemotherapy or adoptive lymphoid cell therapy respectively.

Another area where nanoparticles could have a future impact is co-encapsulation of drugs[35]. Synchronized co-delivery of drugs co-encapsulated in the same particle or encapsulated separately in particles with identical physico-chemical and pharmacokinetic characteristics. Ideally, the drugs chosen should have synergistic or complementary anti-tumor effects with minimal overlap of toxicity profiles.

The co-administration, on the same nano delivery platform, of a therapeutic and a diagnostic or tracking agent, such as a PET-emitting radionuclide, is referred to as a Theranostic. This approach could enable real-time monitoring of the fate of a nanoparticle and its drug

payload. In essence, providing an insight as to the degree of cancer targeting achieved in each specific cancer individual. By imaging the nanoparticle, the EPR effect can then be predicted in each specific case and correlated with clinical response. This would provide direct clinical data to determine whether selecting patients based on their EPR tumor activity could lead to improved therapeutic benefit of nanoparticle based therapy[36].

Finally, the use of nanomedicines in conjunction with loco-regional approaches to therapy (e.g., hyperthermia, radiofrequency ablation, radiotherapy) is a small niche, but has potential opportunities in specific applications that will increasingly attract clinical testing and adoption[37].

RNAi Therapeutics

Alexander H. Stegh, PhD
The Brain Tumor Institute, Robert H. Lurie Comprehensive Cancer Center
Northwestern University, Chicago, IL 60611

RNAi as a Tool for Precision Cancer Medicine

Precision cancer medicine, i.e., the design of therapeutic regimens informed by tumor genotyping, continues to be a central paradigm in modern cancer research. The most recent FDA approval of crizotinib and vemurafenib for the treatment of ALK-translocated lung cancer and BRAF-mutated melanoma, represents the latest proof-of-concept that oncogenomics-driven drug design can improve cancer prognosis[38,39]. High-throughput interrogations of cancer genomes have evolved with unprecedented pace. Bioinformatics, functional cancer biology and genetics continue to identify oncogenes and tumor suppressors that drive or contribute to the pathogenesis of cancer. The design and clinical testing of small molecules inhibiting 'druggable' targets, such as BRAF or ALK, embodied the initial promise of precision medicine, but the vast majority of the dauntingly complex oncogenome has yet to be translated into meaningful therapeutic strategies. How can the activity of multiple unprecedented, non-enzymatic targets with unknown *modi operandi* be modulated?

RNA interference (RNAi) comes to mind, as a potent mechanism to silence aberrant oncogene expression by blocking the translation of their encoding mRNAs. Without prior knowledge of oncogene function, sequence-specific microRNAs (miRNAs) or small interfering (si) RNAs can be designed to selectively target oncogenic pathways, which drive unabated growth, apoptosis resistance, neo-angiogenesis and enhanced migration/invasion of tumor cells. siRNAs are generated by cleavage of long double-stranded (ds) RNAs into ~20 nucleotide-containing siRNAs by the enzyme Dicer. Unwinding of siRNAs into two single-stranded (ss) RNAs, incorporation of the guide strand into the RNA-induced silencing complex (RISC), and binding of siRNAs to complementary mRNAs triggers the degradation of endogenous mRNA by Argonaute, the catalytic component of the RISC complex (reviewed by Hannon and Rossi 2004)[40]. Structurally similar to siRNAs, mature miRNAs are non-coding RNAs, which typically exhibit incomplete base pairing to the target mRNA, and inhibit translation of multiple mRNAs via binding to their untranslated regions (reviewed by Di Leva et al. 2014)[41]. Thus, the level of expression of single miRNAs can influence multiple biologic processes. In contrast, siRNAs bind the coding portion of the mRNA with complete base-pair match and induce mRNA cleavage only in a single, specific target. Due to the negative charge of the RNA backbone, siRNA or miRNA oligonucleotides require delivery systems to

overcome negatively charged membranes, and to prevent rapid renal and hepatic clearance, the degradation of si/miRNAs by nucleases, and toxicity and immunogenicity of the RNA payload.

Preclinical Evaluation of RNAi-Based Therapeutics – Recent Developments Utilizing Nano-Enabled Approaches

The first clinical proof-of-concept that systemically delivered siRNA reduce oncogene expression via an RNAi mechanism in humans[42] motivated the development of several RNAi delivery platforms, which target a wide array of oncogenes in many different cancers.

Spherical nucleic acids (SNAs) (i.e., 13 nm polyvalent gold nanoparticles functionalized with siRNAs or miRNAs) were preclinically evaluated to deliver Bcl2-Like12 (Bcl2L12)-targeting siRNAs (**Figure 3**) and mature miR-182 sequences to intracranial glioblastoma[43,44]. Bcl2L12 is potent caspase and p53 inhibitor with near ubiquitous expression in primary GBM specimens[45–49]. miR-182 is a tumor suppressive miRNA, which regulates apoptosis, growth and differentiation programs via transcriptional repression of Bcl2L12, c-Met, and Hypoxia Inducible Factor 2 alpha (HIF2α) to enhance therapeutic susceptibility, and to decrease expansion and multipotency of glioma-initiating cells[44]. siBcl2L12 and miR-182-based SNAs robustly penetrated glioma-initiating cells via scavenger receptor-mediated endocytosis. In an *in vitro* blood-brain barrier (BBB) model involving the co-culture of human primary brain microvascular endothelial cells separated from astrocytes by a semi-permeable filter insert, Cy5.5-labeled SNAs passed through the endothelial cell layer and filter, and rapidly entered the astrocytes. Systemic administration into Sprague-Dawley rats and non-human primates have not resulted in SNA-related differences in body or organ weight, nor in an inflammatory response in the brain

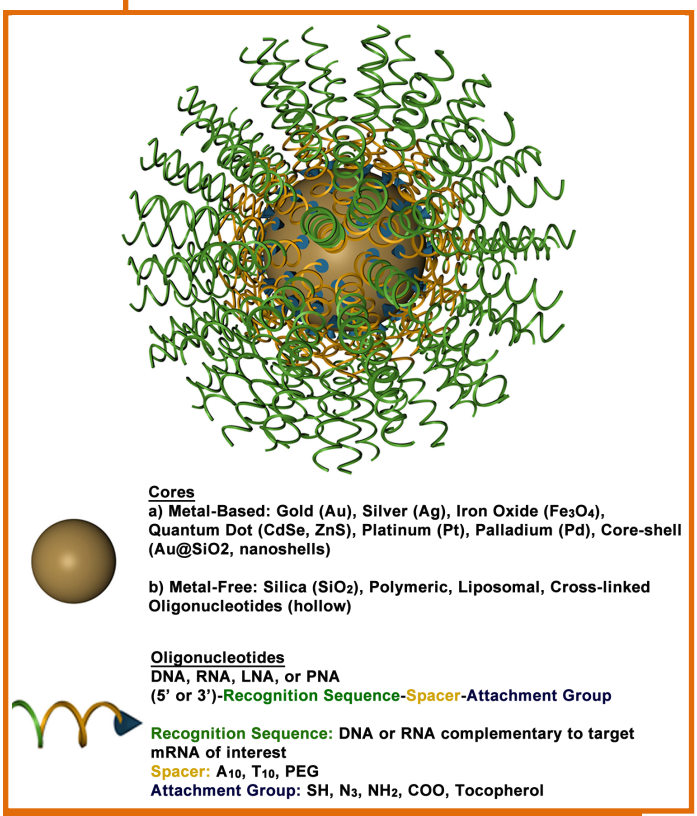

Figure 3. Schematic representation of a Spherical Nucleic Acid (SNA) nanoconjugate. The surface of a variety of different core materials including metal nanoparticles (e.g., Au, Pt), liposomes and polymers, can be functionalized with highly oriented nucleic acids (*Reprinted with permission from Barnaby et al., 2015*)[54].

or in reticuloendothelial system (RES) organs, as shown in published[43], and unpublished data. Importantly, si/miRNA-based SNAs crossed the blood-tumor barrier and accumulated in glioma elements relative to normal brain tissue likely via enhanced permeability and retention of the tumor-associated vasculature. Accumulation and pervasive dissemination into extravascular tumor parenchyma translated into robust intratumoral protein knockdown, increased intratumoral apoptosis, impaired tumorigenicity, and prolonged survival of GIC-derived xenogeneic mice[43,44].

Jacks and colleagues developed a combinatorial RNAi regimen using lung-targeting polymeric nanoparticles made of low-molecular-weight polyamines and lipids to deliver siRNA and miRNA mimetics to lung adenocarcinoma cells *in vitro* and to tumors in a genetically engineered mouse model (GEMM) driven by KRas activation and p53 deletion[50]. The lead compound is a nanoparticle with multilamellar structure, which was synthesized by reacting with a 15-carbon lipid tail in ethanol[51], mixed with $C_{14}PEG_{2000}$. Delivery of miR-34a and siRNAs targeting KRas reduced lung cancer progression more effectively than either small RNA alone, and synergized with cisplatin-based chemotherapy to prolong survival of animal subjects[50].

Accumulation and pervasive dissemination into extravascular tumor parenchyma translated into robust intratumoral protein knockdown...

Bhatia and colleagues developed a tumor-penetrating nanocomplex (TPN) with siRNAs specific for the ovarian cancer oncogene inhibitor of DNA binding 4 (ID4)[52]. For tumor delivery, the nanoconjugate was co-functionalized with a tandem tumor-penetrating and membrane-translocating peptide, which enabled robust and pervasive delivery of siRNA to the tumor parenchyma. Subsequently, treatment of ovarian tumor-bearing mice with ID4-specific TPN suppressed growth of the established tumors and significantly improved survival. Similar to TPN-mediated ID4 knockdown, inhibition of the DNA repair enzyme poly(ADP-ribose) polymerase 1 (PARP1) with siRNA-based lipoids is an effective treatment for ovarian cancer. Intraperitoneal (i.p.) administration of siPARP1 lipoids promoted apoptosis, and increased animal subject survival in BRAC1-deficient, but not the wildtype allografts *in vivo*[53].

Using a genetically engineered breast cancer model, driven by SV40-large T antigen under the control of the C3(1) component of the rat prostate steroid binding protein (PSBP) to direct SV40 expression to the mammary gland, computational gene network modeling identified HoxA1 as a putative driver of early breast cancer progression. RNAi-mediated

suppression of HoxA1 in mammary tumor spheroids increased acinar lumen formation, reduced tumor cell proliferation, and restored normal epithelial polarization. *In vivo*, intraductal delivery of siRNA-based lipoid nanoconjugates targeted to HoxA1 into FVB C3(1)-SV40TAg mice triggered robust reduction of breast cancer progression associated with reduced cell proliferation rates, and sustained expression of estrogen and progesterone receptors[55].

Future Challenges and Directions

The confluence of progress in many different areas of cancer research, i.e., high-throughput oncogenomics, the development of physiologically relevant cell and animal models as testing platforms for gene function and gene-specific therapeutics, and the emergence of RNAi-based nanotechnological strategies, have positioned the field well to implement precision cancer nanomedicine into clinical practice. With currently 24 different RNAi-based therapeutics in 43 different clinical trials, critical questions and challenges for the next 5 to 10 years have become very apparent, i.e., to identify the most critical target genes that drive or contribute to cancer initiation, progression, metastasization and therapy refractoriness, as well as to further improve and comprehensively evaluate efficacy, specificity, and biocompatibility of RNAi nanotherapeutics in the most relevant cell and animal models. Specifically, several important areas for development include the following.

RNAi Nanoconjugates as Tools for Discovery Sciences

With the number of gene aberrations ranging from thousands to hundreds of thousands, the genomic and genetic landscape of cancer is complex. Only a subset of genes drive the initiation and maintenance of cancer. In addition, tumors show specific, spatially and temporally controlled genetic changes, which are influenced by cooperative oncogenic and tumor suppressive signatures, and further modulated by heterotypic tumor-stroma interactions, and patient-specific germline mutations. Genome-wide RNAi and cDNA complementation screens are constantly evolving to determine cancer gene function and their genetic context, and will continue to provide lists of candidate genes that require further in-depth testing in cell and animal models. For preclinical evaluation, established or patient-derived cancer cells, together with murine cancer cell lineages are engineered to over- or underexpress the gene of interest, and these cell systems are then channeled into a variety of functional assays determining the impact of gene dosage on cellular transformation, growth, apoptosis sensitivity and migration/invasion. By orthotopically injecting these cell systems into immunocompromised or syngeneic hosts, subsequent *in vivo* experiments then evaluate the impact of cancer gene overexpression and knockdown on tumor progression. Nano-RNAi should be developed as a tool for discovery science to

evaluate gene function and its impact on cancer progression in cells *in vitro* and in animal models *in vivo*. Instead of generating cell transfectants stably or transiently expressing small hairpin (sh) RNAs and siRNAs, or engineering cells with a gene-specific knockout harnessing the CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology, RNAi-based nanoconjugates can be administered to cells, graft and genetically engineered cancer models, to determine cancer gene function *in vivo*.

Further Developing RNAi-Based Nanotherapeutics

While a plethora of RNAi-based nanoconjugates have emerged in the past 10 years as fundamentally novel classes of therapeutics that can robustly and safely delivery RNAi to tumor sites, structure-activity relationships that dictate nanomaterial activity (RNAi delivery to cells, target gene knockdown) are only beginning to emerge. This incomplete understanding is based in part on the difficulty in generating structurally defined materials, and in rapidly evaluating the cellular impact of these nanomaterials in a massively parallel fashion. Design rules have to be determined that optimize the development of RNAi nanoconjugates for therapeutic applications. Unlike small molecule-based therapeutics, where millions of compounds are surveyed in an initial high-throughput screen, and thousands are tested under optimized conditions in various cell culture models, nanomedicinal evaluations typically focus on a defined subset of candidates only. Furthermore, deep mechanistic and biological studies are required to fully understand some of the fundamental properties underlying gene knockdown (is gene knockdown truly mediated by an RNAi mechanism, or is it due to rather unspecific toxic effect of the conjugate?) cellular entry, endosomal escape, tissue dissemination, and low-level cellular and organismal impact. With more comprehensive screenings of cancer cell-specific surface markers, the modification of RNAi nanoconjugates with ligands or antibodies to facilitate tumor-specific uptake, beyond the EPR effect, has to be optimized to further increase conjugate efficacy while reducing the potential for adverse side effects associated with systemic administration. Due to the dependence of the cancer phenotype on multiple deregulated pathways, co-extinction strategies have to be developed that concomitantly silence multiple oncogenes and oncogenic pathways. In particular, the concept of therapeutic synergy between siRNAs and miRNAs has to be exploited further, as recent study in ovarian and lung cancer showed significant cooperativity in reducing tumor progression when compared with either monotherapy alone[50,56]. The design of such combination therapies, and the development of multimodal si/miRNA nanoconjugates have to be optimized, and evaluated *in vivo* for efficacy, pharmacokinetics, pharmacodynamics,

...nanomedicinal evaluations typically focus on a defined subset of candidates only.

and toxicology in the relevant grafts and GEMMs. Finally, we have to understand and harness synthetic lethal interaction of si/miRNAs with conventional chemotherapy (e.g., DNA-damage-inducing agents), targeted pharmaceuticals that inhibit critical driving oncogenes, such as (receptor) tyrosine kinases, and possibly immunotherapies. It will be critical to determine the molecular mechanisms that act as roadblocks preventing chemo- and RTK-targeted therapies from inducing tumor-specific apoptosis and regression, and enabling cancers to escape immune surveillance. We then can target these roadblocks using RNAi-based nanomaterials, and can envision using hybrid conjugates co-functionalized with chemotherapeutics, small molecules, biotherapeutic antibodies and si/miRNA sequences to concurrently target driving oncogenes and their downstream signaling.

Milestones to address these critical areas that researchers should be able to be achieve over the next 5-10 year time frame include many aspects. In the next 5 years, researchers will comprehensively determine structure-function relationships of RNAi nanoconjugates with high-throughput methods; determine the potential synthetic lethal interaction between cancer genes and extant chemo-/targeted therapies to identify those genes required for therapy resistance; develop and preclinically evaluate multimodal nanoconjugates for the concurrent delivery of small RNAs and chemo-/targeted therapies; preclinically develop combination regimens of immunotherapies and RNAi-based nanomaterials; and develop RNAi nano-conjugates as tools for discovery sciences to characterize oncogene function in cells and animal models. Looking further ahead over the next 10 years, researchers will perform clinical testing of multiple RNAi-based nanoconjugate combinations, in conjunction with established therapies; and potentially there should be FDA approval of several RNAi conjugates and RNAi-based combinatorial regimens.

X-ray Induced Photodynamic Therapy

Hongmin Chen, PhD and Jin Xie, PhD
Department of Chemistry
University of Georgia, Athens, GA 30602

Introduction to X-PDT and its Importance to Oncology

Photodynamic therapy (PDT), as a relatively new cancer treatment methodology, has attracted wide attention. PDT uses a photosensitizing drug that is activated by exposure to light of a specific wavelength. While they display minimal toxicitiy in the dark, photosensitizers, upon light activation, produce cytotoxic reactive oxygen species such as singlet oxygen ($^1O_2$) and hydroxyl radicals, leading to cancer cell death. PDT is minimally invasive and highly selective. Unlike ionizing radiation, PDT can be applied repeatedly to the same diseased sites without causing incurred resistance. PDT can also be applied in conjugation with other treatment modalities to facilitate tumor management. For instance, PDT is being evaluated in the clinic to treat prostate cancer patients who have failed radiotherapy.

One major limitation to PDT, however, is the shallow penetration depth. Even with new generations of photosensitizers, it is challenging for PDT to treat tumors of large volumes (> $1cm^3$) or ones located deep under the skin. This restraint is a major cause behind the limited impact and current role of PDT in the clinic. To address the issue, there have been many efforts on developing two-photon PDT and upconversion nanoparticle-mediated PDT. However, because the excitation source is near-infrared light, their potential therapeutic outcomes are still heavily surface-weighted.

Very recently, our group and others have exploited the possibility of using X-ray as an energy source to activate PDT. We termed this methodology X-ray inducible PDT, or X-PDT. Unlike visible or near-infrared light, X-ray affords excellent tissue penetration ability and is widely used in clinical diagnosis and therapy. X-PDT can thus, to a large degree, transcend the depth limitation of conventional PDT (~ 1 cm), permitting deep-tissue therapy[57]. For X-PDT to work, there are several requirements. First, a scintillating transducer, which converts X-ray photons to visible photons. Second, a photosensitizer, whose excitation wavelength is well matched to the emission of the scintillator. Third, a carrier, which can co-deliver the scintillator and photosensitizer, and ensure that the two components are spatially close enough for efficient energy transfer. As simple as it sounds, it is difficult to meet all three requirements using conventional methods.

This puzzle is solved by advances in nanotechnology, which allow for preparation of nanoscale scintillators and carriers. **Figure 4** shows an example of such an integrated nanosystem, consisting of a nanoscintillator core made of $SrAl_2O_4$:Eu (SAO), a photosensitizer merocyanine 540 (MC540), and a silica capsule that encapsulates the two. Upon X-ray irradiation, the SAO core converts X-ray photons to visible photons via a physical phenomenon known as X-ray excited optical luminescence (XEOL). Due to excellent spectral overlap between the emission and the excitation of MC540, the photons emitted by SAO are absorbed by MC540 deposited in the silica matrix. This produces reactive oxygen species, including hydroxyl radicals and singlet oxygen ($^1O_2$), causing death of cancer cells.

Current State of the Art in X-ray Inducible PDT

The number of studies on X-PDT is relatively small but is increasing. In addition to this group's work, other groups have exploited different scintillator materials using similar or different designs. For instance, the Chen group has investigated X-PDT with Cu-cysteine[58], LaF_3:Ce[59], and ZnS:Cu,Co[60]. The Shi group reported that Ce(III)-doped $LiYF_4$@SiO_2@ZnO nanoparticles upon ionizing irradiation can generate hydroxyl radicals to kill cancer cells[61]. Recently, Kotagiri et al. observed that Cerenkov radiation from radionuclides can be harnessed to activate TiO_2 nanoparticles, an oxygen-independent nanophotosensitizer, to produce radicals and kill cancer cells[62].

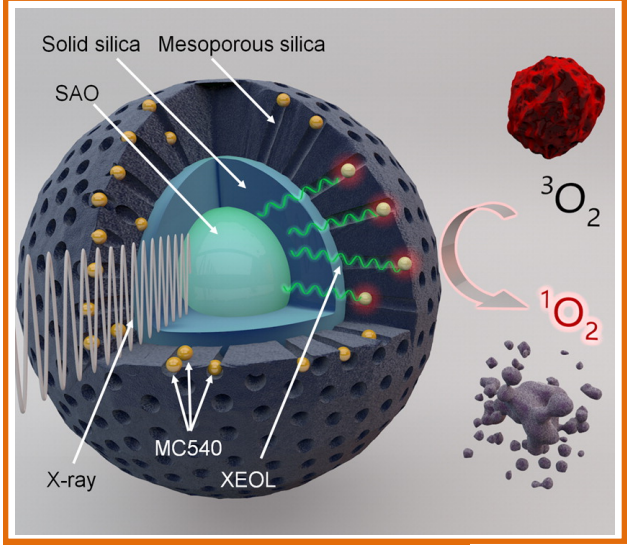

Figure 4. X-PDT, mediated by MC540 loaded and silica coated SAO nanoparticles (or M-SAO@SiO2 nanoparticles). Upon X-ray irradiation, SAO works as a transducer, relaying energy in the form of X-ray excited optical luminescence (XEOL) to MC540 to activate it and produce cytotoxic 1O2. M-SAO@SiO2 nanoparticles can be conjugated with a tumor targeting motif to further enhance the selectivity against cancer cells (*Reprinted with permission from Chen et al, 2015*).

X-PDT treated cells often display blebbing, swelling, and morphology changes, suggesting PDT-induced necrosis as the dominant cell killing mechanism. This is different from ionizing irradiation, in which cell death is often caused by apoptosis. However, it does not mean that there is no contribution of ionizing irradiation in X-PDT. While $^1O_2$ is produced in nanoparticle-rich compartments such as the cell membrane and endosomes/lysosomes, other organelles are under the impact of ionizing irradiation. Hence, X-PDT is essentially a combination therapy of PDT and ionizing irradiation. Previously, several groups

have studied PDT and radiation combination therapy and observed a synergistic effect between the two[63–66]. This is because the two modalities act on different targets: PDT often damages cell membranes whereas ionizing irradiation targets DNA. Due to distinctive cell killing routes, each modality suppresses the cell repair mechanism of the other, leading to enhanced treatment outcomes. The same synergy is believed to play a role in X-PDT.

From this perspective, X-PDT is not only a PDT derivative, but also a type of radiation therapy derivative. It however, affords several benefits over conventional ionizing irradiation. First, X-PDT can kill cells that are resistant to radiotherapy (e.g., glioma cells[57]). This is because the main cell killing mechanism of X-PDT is PDT-induced cell damage rather than radiation caused DNA damage. Second, low irradiation doses. Like PDT, X-PDT achieves good tumor control within in a few or even single treatment sessions[57]. The total irradiation dose is often less than 10 Gy. The dose is much lower than traditional radiotherapy, in which case a total dose of 60-80 Gy is often needed[67,68]. Third, low irradiation dose rates. It is known that irradiation induced toxicities are positively correlated to dose rates[69]. In X-PDT, irradiation doses per fraction are often comparable to conventional radiotherapy (e.g., 2-5 Gy); however, the irradiation is given out over a span of 15-30 min (typical for PDT), as opposed to minutes or even less in radiotherapy. This leads to dramatically lowered dose rates and potentially reduced toxicities. Fourth, high selectivity. In X-PDT, the treatment is mediated by not only irradiation but also the respective nanotransducers. With proper surface coating and by conjugating with a tumor targeting ligand, nanotransducers may accumulate in tumors with high efficiency. This dual selectivity, in conjugation with low irradiation doses and dose rates, are expected to minimize normal tissue toxicities, a major concern in radiotherapy.

Future Scientific and Clinical Developments

While X-PDT has demonstrated good efficacy and benefits, there is a lot that we don't know about this new therapeutic modality. As discussed above, X-PDT is essentially a combination therapy of PDT and ionizing irradiation. However, exactly how the two modalities interplay and whether we can improve the synergy by tuning irradiation parameters and/or changing nanotransducer targets is largely unknown. These need be elucidated in future studies.

The nanoscintillator is the key to X-PDT. It will be important to exploit ways to improve their energy conversion and safety profiles. These include: (1) change scintillator materials to ones that have a larger X-ray absorption cross-section and higher X-ray-to-visible-photon conversion efficiency as well as optimized spatial positioning of the molecular entities involved; (2) reduce the overall size of the nanotransducers; this however, should be balanced against the loss in energy conversion efficiency. It is noted that many of the

One solution to the problem is to use coatings to coat hydrolytic scintillator cores so as to slow down, but not prohibit hydrolysis.

reported nanotransducers in X-PDT have a relatively large size, which is suboptimal to tumor targeting; and (3) strike a balance between short-term stability and fast biodegradation of nanoparticles. Many scintillator materials are hydrolytic, quickly reducing to constituent ions when exposed to water. Water resistant scintillators do exist, but then the issue becomes the too slow degradation *in vivo*. One solution to the problem is to use coatings to coat hydrolytic scintillator cores so as to slow down, but not prohibit hydrolysis. Taking $SrAl_2O_4$:Eu nanoparticles for instance, it was found that after silica coating, the particles can maintain stability in physiological environments for 3-7 days and are then gradually degraded. Other materials/coating strategies should be exploited to modulate the stability and degradation of scintillators *in vivo*.

So far, X-PDT has been demonstrated mostly *in vitro* or with subcutaneous models. In future studies, it is important to evaluate the methodology in more clinically relevant tumor models. X-PDT holds the potential of clinical translation as an alternative to irradiation therapy in the next 10-15 years. It is important to compare the two modalities in the clinic to assess benefits and drawbacks of X-PDT with regard to treatment efficacy and side effects. It is also interesting to evaluate the capacity of X-PDT to treat tumors refractory to or ones that have failed radiotherapy. In radiotherapy, pre-treatment functional imaging (e.g., PET) is often performed to stage tumors and guide irradiation planning. However, functional imaging is not permitted in an irradiation room, and a change in patient position from prescans may occur, leading to setup errors. Many scintillator materials contain high-Z-value elements, making them visible under on-board CT. It is thus possible to use these nanoscintillators to not only regulate PDT but also guide the irradiation so as to minimize normal tissue damage. These possibilities should also be investigated to facilitate clinical translation of X-PDT.

Targeting Undruggable Targets

Anil K. Sood[1], MD and Gabriel Lopez-Bernstein[2], MD
[1]Department of Gynecologic Oncology and [2]Department of Experimental Therapeutics
University of Texas MD Anderson Cancer Center, Houston, TX 77030

The Importance of Targeting Undruggable Targets to Cancer Research/Oncology

Over the last few decades, advances in surgery, chemotherapy, and targeted drugs have led to improvements in progression-free and overall survival increases for many cancer types[70]. However, cure rates have remained largely unchanged. To accelerate the gains in clinical outcomes, large-scale efforts such as the Cancer Genome Atlas (TCGA), Clinical Proteomic Tumor Analysis Consortium (CPTAC), Cancer Target Discovery & Development (CTD[2]), and others were launched. These efforts have produced very high quality data due to the stringent requirements for sample quality and have clearly increased the pace of discovery for novel targets. However, to date, most of the knowledge is correlational in nature and large-functional data are needed. Challenges to rapid translation include the need for rapid, reliable, and effective functional data. While genetically engineered mouse models (GEMMs) remain a key tool in our armamentarium to determine the effects of various molecular pathways on biological processes, such models can have limitations (e.g., lengthy time, expense) and do not always reflect the biology of advanced stage human tumors. Therefore, other approaches such as 3-D, patient-derived xenografts, and orthotopic model systems remain an important component of biological validation and drug development.

The growing knowledge from the large-scale "omics" efforts has produced highly complex maps of genetic dysregulation in cancers. Moreover, these functional and biological systems have produced a plethora of targets that appear attractive for therapeutic development. However, many of the targets are not druggable by conventional strategies. Many important targets are difficult to inhibit with small molecules and furthermore require lengthy development phases that often fail. In addition, many small molecule inhibitors lack specificity and can be associated with intolerable side effects. While monoclonal antibodies have shown substantial promise against specific targets (e.g., VEGF, EGFR), their use is limited to either ligands or surface receptors. Some oncogenic proteins (e.g., Ras) activate pathways leading to altered transcription while others (e.g., Myc) are themselves transcription factors that directly control the expression of genes essential for proliferation, survival, and metastasis. Attempts have been made to develop pharmaceutical inhibitors against some of these factors, but many are still widely considered "undruggable".

Collectively, these and other observations have led many investigators to consider alternative strategies, such as RNA interference (RNAi), for inhibiting these targets.

Current Status in the Targeting of Undruggable Targets

Since the first report of RNAi in the late 1990s, there has been a massive expansion in efforts to apply it for therapeutic applications. Among these, short interfering RNA (siRNA) allows for highly selective silencing of target(s) of interest. Non-coding RNAs such as microRNAs (miRNA) can be used to target a larger array of targets. Moreover, combinations of siRNA and miRNA offer opportunities for "co-extinction" to maximize therapeutic efficacy while avoiding activation of redundant/compensatory pathways. While the promise of RNAi-based therapeutics is enormous, challenges (e.g., potential off-target effects and toxicity, requirement for delivery, endosomal uptake, activation of adaptive pathways) also exist[71]. Among these, perhaps the biggest challenge is achieving efficient systemic delivery. Naked siRNA becomes degraded rapidly and cannot be delivered into the tumor efficiently. However, these are precisely the kinds of concerns that can be overcome with biocompatible nanotechnology platforms. Already, several such platforms have yielded promising results in both pre-clinical and clinical settings for oncological and other clinical needs. For example, Davis and colleagues demonstrated in a landmark paper the ability of a cyclodextrin-based nanoparticle (CALAA-01) to deliver RRM2-targeted siRNA in patients with melanoma[42]. Other studies with delivery of miR-122 for HCV infection[72] and lipid nanoparticles for delivery of siRNAs targeting VEGF and KSP in cancer patients have also demonstrated promising clinical results[73]. The DOPC nanoliposomal platform has already shown promise for delivery of Grb2-targeted anti-sense nucleotides[74] and has also been introduced into phase 1 testing for EphA2-targeted siRNA. Additional platforms are likely to build on these initial experiences and allow for robust delivery of RNAi-therapeutics.

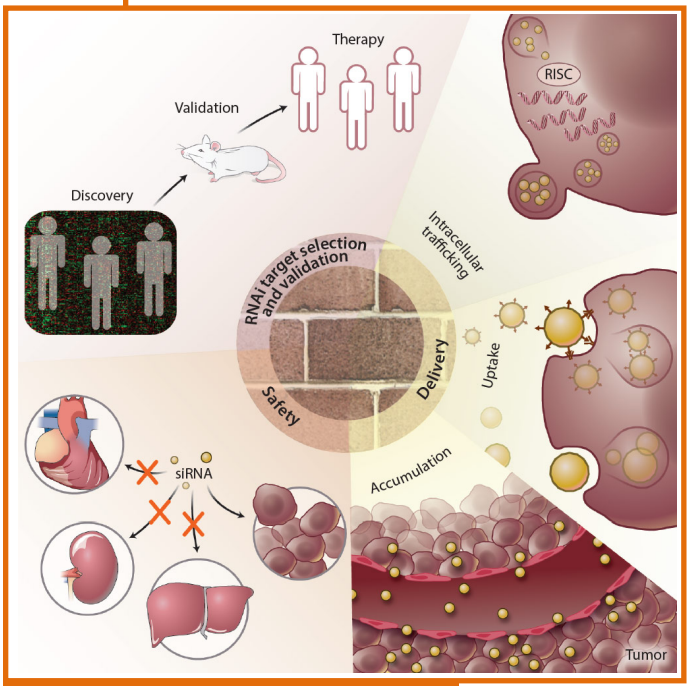

Figure 5. Strategies for targeting undruggable targets that rely on careful target discovery followed by developing nanoparticle systems that allow for highly efficient systemic delivery into the tumor microenvironment while sparing delivery into normal organs such liver, kidneys and heart (*Reprinted with permission from Wu et al., 2014*).

The success of RNAi-therapy depends, in part, on careful selection of targets for such approaches and delivery to the appropriate sites. Several key targets (e.g., KRAS, MYC) are already widely considered to be important. Additional efforts in the selection of targets, have incorporated systems biology approaches where genomic and proteomics screens can be merged with functional and clinical data to identify the highest priority targets[75,76]. In such an approach, following a systematic effort aimed at target selection, validation studies are carefully carried out (**Figure 5**). The biological validation studies are ideally carried out in a portfolio of model systems that can recapitulate human disease and hopefully inform success and potential for toxicity in subsequent clinical studies. The nanoparticle systems should be selected based on several criterial including biocompatibility, efficiency of delivery, safety profile and pharmaceutical feasibility (e.g., ability to scale-up, nucleotide incorporation and cost efficiency).

Future Scientific and Clinical Developments

We are clearly at a crossroads of a massive amount of information and a need to converge disciplines to understand the biological and clinical significance of such data. The ability to convert such data into personalized medicine regimes is still in its infancy. Success will require multi-disciplinary teams that include biomedical engineers, cancer biologists, pharmacologists, and translational as well as clinical scientists.

One major future opportunity will be in improving frequency of dosing and careful planning of clinical trials.

The achievements so far have demonstrated important proof-of-concept studies for RNAi-based therapeutics and have identified opportunities for future work. One major future opportunity will be in improving frequency of dosing and careful planning of clinical trials. Most of the current delivery platforms require frequent dosing to maintain sustained gene silencing. While such therapies are feasible to deliver in clinical trials, sustained delivery methods could ideally reduce the number of clinic visits required for treatment. Some of these delivery methods (e.g., multistage vectors, dual-assembly nanoparticles) have shown preclinical evidence of sustained delivery. But, additional work will be required to refine these approaches for clinical testing.

Given the genomic chaos and instability present in many solid tumors, it is not surprising that bypass or redundant molecular pathways are activated following many of the current therapeutics. Such adaptive mechanisms require an iterative process whereby careful preclinical testing and information-rich early-stage clinical trial designs utilize systems

biology approaches. Either Phase 0 or Phase 1 trials with pre- and post-treatment biopsies are an important avenue to learn about adaptive changes. Moreover, Phase 0 studies offer another unique opportunity for assessing the delivery of nanoparticles directly to the tumor site. Then, using sophisticated model systems, rational combinations could be rapidly developed. Adaptive trial designs can further help to limit the number of patients in the inactive-dose cohorts with the test article and allow faster transition to phase 2 clinical trials. Nanotechnology-enabled RNAi therapies are ideally suited for carrying out "co-extinction" of adaptive pathways. Questions related to packaging multiple RNAi molecules in same nanoparticles vs. loading them separately, but co-administering them is similarly worthy of additional future investigation.

It is unlikely that biologically-targeted drugs will replace the existing therapies such as chemotherapy and radiation. Opportunities exist, however, to identify and block targets that can amplify the anti-tumor response to these traditional therapies. These combinatorial approaches will likely offer new avenues for not only improving response rates, but perhaps even cure rates. Another opportunity resides in enhancing immune therapies. Check-point inhibitors (e.g., anti-CTLA-4, anti-PD-1) have resulted in remarkable efficacy in a fraction of patients with various tumor types, in particular melanoma[77]. There are many reasons why others do not respond to such therapies at present, but silencing "undruggable targets" among others related to immune-tolerance represents an opportunity for expanding the reach of immunotherapies.

Many of the existing delivery methods result in a fraction of the payload being deposited into the tumor with a large fraction going to other organs, especially liver. Understanding the physico-chemical properties that allow for enhanced delivery into the tumor represents an important area of investigation. Moreover, exploiting targeted delivery of nanoparticles decorated with peptides, aptamers or other approaches might enhance therapeutic ratios. Clinical regulatory pathways are needed to allow these targeted delivery methods to move into clinical testing.

Drug Reformulation

Stephan Stern, PhD
Nanotechnology Characterization Laboratory
Cancer Research Technology Program, Leidos Biomedical Research, Inc.
Frederick National Laboratory for Cancer Research, Frederick, MD 21702

Reformulation via Nanotechnology

Reformulation of legacy drugs offers an efficient pathway for commercialization of nanotechnology platforms. Nanotechnology-based medicine, as a relatively new area of science, does not have the well-defined regulatory path of traditional drugs. Since the development of a new chemical entity utilizing nanotechnology further compounds regulatory scrutiny, the reformulation of existing drugs represents a logical first step toward market. An alternate formulation of an existing drug that is no longer under patent can be developed under the FDA 505(b)(2) regulatory path that utilizes existing safety data, and has less associated development cost and time than that of a new chemical entity under the traditional 505(b)(1) application process. The 505(b)(2) regulatory path was codified in the "Drug Price Competition and Patent Term Restoration Act" (1984) statues with the specific goal of offering cheaper alternatives to the branded products, but has had the, perhaps, unintended consequence of expediting commercialization of new drug formulation technologies that offer therapeutic improvement of existing drugs.

Nanotechnology reformulation can overcome many of the liabilities of current oncology drugs, including insolubility, rapid metabolism, poor bioavailability and off target toxicity. The earliest successful commercialization of nanotechnology was encapsulation of doxorubicin in a nanoscale liposome, approved by the FDA in 1995 (**Figure 6**). Liposomal doxorubicin, Doxil® (Janssen Biotech, Inc.), decreases systemic free doxorubicin concentrations, reducing cardiac exposure and associated cardiotoxicity[78]. The success of this formulation is highlighted by the recent approval of the first Doxil generic, Lipodox® (Sun Pharmaceutical, FDA approval 2013). Liposome reformulation strategies are also being used to deliver synergistic combinations of oncology drugs, an example being Celator's combination cytarabine-duanorubicin liposome (CYT 351) that is currently in phase III clinical trials for treatment of acute myeloid leukemia.

Current Enabling Technologies

Liposomal doxorubicin commercialization was followed by cremophor-free formulations of the highly insoluble drug paclitaxel, initially as an albumin nanoparticle, Abraxane® (Abraxis BioScience), approved in the US 2005, and later a polymeric nanomicelle,

Genexol-PM® (Samyang Genex Company), approved in Korea 2007[79]. Abraxane is a 130 nm nanoparticle composed of human donor-derived albumin, while Genexol-PM is a 25 nm micellar particle composed of monomethoxy poly(ethylene glycol)-block-poly(D,L-lactide) (PEG-PDLLA) copolymer. By removing cremophor from the legacy paclitaxel formulation, Taxol® (Bristol-Myers Squibb), these nanotechnology reformulations demonstrated dramatic improvements in dose tolerability, as cremophor-dependent dose-limiting hypersensitivity reactions were no longer observed. This allows maximum tolerated doses of >300 and 260 mg/m^2 for Cynviloq and Abraxane, respectively, in comparison to 175 mg/m^2 for the legacy Taxol formulation. In addition to eliminating unwanted hypersensitivity side effects, these new cremophor-free formulations are effective against malignancies that the legacy Taxol formulation was not. Abraxane received orphan drug status for treatment of late-stage pancreatic cancer in the US in 2013 and has projected sales of $1.5-2 billion (Celgene Presentation at UBS Global Healthcare Conference, May 19, 2014 pp.9)[80]. Genexol-PM is currently in development in the US under the brand name of Cynviloq™ (Sorrento Therapeutics, Inc.) as an alternate formulation of Abraxane under the 505(b)(2) regulatory pathway for the treatment of advanced pancreatic cancer[81]. This use of the 505(b)(2) pathway for development of an alternate formulation of a marketed nanotechnology formulation is an example of how approval of nanotechnology formulations can further expedite approval of other nanotechnology formulations.

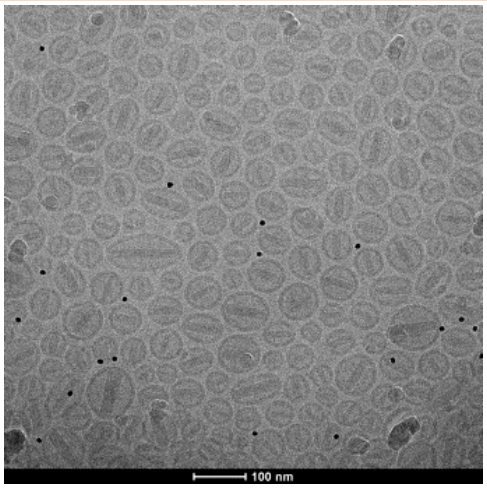

Figure 6. Cryo-transmission electron microscopy image of Doxil liposomal doxorubicin (*courtesy of Dr. Ulrich Baxa, Electron Microscopy Laboratory, Frederick National Laboratory for Cancer Research, 2015*).

The success of these reformulation efforts have solidified the advantages that nanotechnology offers the pharmaceutical industry, driving the implementation of nanotechnology earlier in the discovery phase of drug development. Many pharmaceutical companies now have in house nanotechnology formulation efforts underway, or are partnering with nanotechnology companies to optimize leads and even resurrect failed molecules. For example, a nanotechnology reformulation technique that has become so commercially acceptable that it is now used routinely in development of oral drugs is the Nanocrystal™ technology first developed by the Elan Corporation. The first commercial nanocrystal formulation was a reformulation of sirolimus, Rapamune® (Wyeth Pharmaceuticals, Madison, NJ), approved in 2000[82]. Nanocrystal formulation can increase bioavailability of oral formulations by reducing drug particle size, resulting in a dramatic increase in

surface area, and therefore drug dissolution rate (**Figure 7**)[83]. Other advantages can include enhanced dose linearity and consistency. The Elan nanocrystal technology is also being used for parenteral drug delivery, and an intramuscular nanocrystal reformulation of the schizophrenia drug paliperidone palmitate was approved in 2009.

Future Developments

As described above, the earliest use of nanotechnology to improve oral bioavailability was for incremental increases in the bioavailability of drugs already approved for oral administration through the use of nanocrystal technology. Recent formulation efforts are now focusing on the more difficult challenge of overcoming biological barriers, formulating molecules with little or no inherent bioavailability, such as protein therapeutics. One such example is the work of Robert Langer's lab on oral insulin, utilizing receptor mediated transport to overcome the gastrointestinal mucosal barrier[84]. These researchers utilized a polymeric nanoparticle construct targeting gastrointestinal FcRN receptors to stabilize and deliver insulin to the systemic circulation (**Figure 8**). Optimization of this uptake pathway could revolutionize both protein and small molecule therapeutics, no longer requiring costly and invasive intravenous administrations. Another example of utilization of receptor-mediated transport to cross biological barriers is glutathione-targeted doxorubicin liposome designed to increase uptake across the blood-brain barrier. These glutathione-targeted doxorubicin liposomes developed by BBB Therapeutics are currently in phase II clinical trials for treatment of brain metastasis and glioma[85].

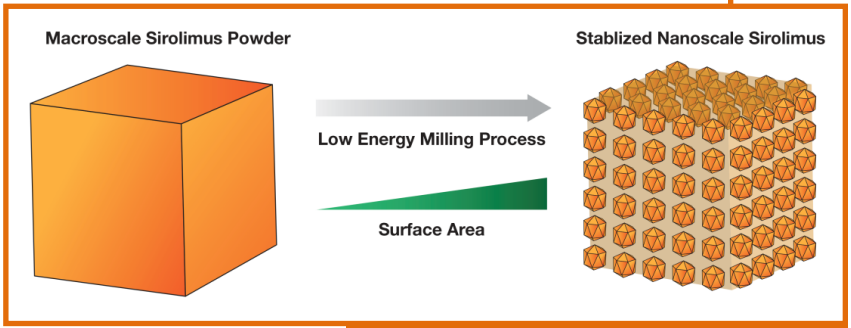

Figure 7. The Elan Nanocrystal™ technology.

Clearly, the future of nanomedicine resides in targeted therapies that allow for exquisite selection of diseased over healthy tissues. This was and continues to be the unrealized potential of this technology. The most notable advance in this area has come from Bind Therapeutics' progression of PMSA-targeted polymeric nanoparticles containing paclitaxel, Bind-014, to the clinic[16]. Bind's Accurin™ platform consists of a PMSA targeting *S,S*-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid small molecule, attached to a mixed pegylated poly(d,l-lactide) (PLA) and poly(d,l-lactide-*co*-glycolide) (PLGA) nanoparticle. In addition to paclitaxel, Bind also has a vincristine formulation under late stage development,

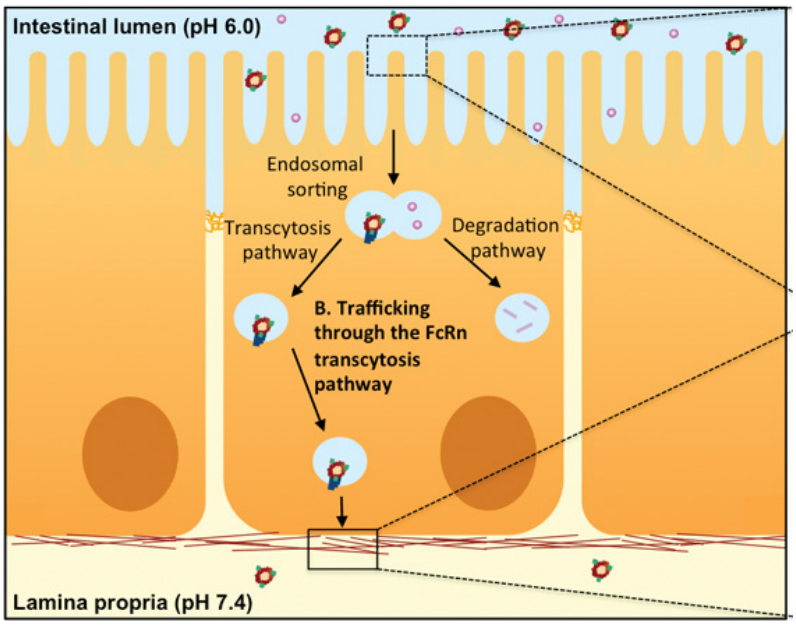

Figure 8. FcRN receptor-mediated nanoparticle uptake. (*Reprinted with permission from Pridgen et al., 2013*).

and is partnering with several pharmaceutical companies, including Pfizer, AstraZeneca, Roche, Merck, and Amgen, for development of their proprietary small molecules. Success of the Accurin platform will undoubtedly lead to further development of targeted therapies and new avenues for targeted reformulation. As has been the case in the past, reformulation will continue to lead commercialization of novel nanotechnology platforms.

With the joint efforts of investigators at academic institutes and within industry, several advances should come to fruition over the upcoming 5-10 year time frame. In the next 5 years, researchers will have begun streamlining of drug reformulation by identification of optimal drug physicochemical properties that result in successful reformulation for each nanomedicine class; and begin commercialization of actively targeted-nanoparticle reformulations. Looking further ahead over the next 10 years, researchers will generate reformulation of intravenously administered small molecule and protein-based therapies for oral and inhalation administration.

Nanotherapeutic Solutions for Metastatic and Disseminated Cancers

Nalinikanth Kotagiri, PhD and Samuel Achilefu, PhD
Mallinckrodt Institute of Radiology
Washington University School of Medicine, St. Louis, MO 63110

Metastasis Remains the Bane of Successful Cancer Therapy

Cancer metastasis accounts for over 90% of all cancer associated death and suffering, representing the single biggest challenge to the management of cancer[86]. Although the advent of novel therapies and effective combination regimens has increased overall patient survival, many of these interventions are only palliative and an overwhelming number of cancer patients succumb to the disease[87]. Several factors can be attributed to this undesirable outcome, including the inefficiency of using conventional chemotherapeutics to treat small clusters of disseminated malignant cells or therapy-resistant metastases[88]. The three major sites of most cancer metastasis are the lungs, liver, and bone marrow (**Figure 9**).

Although small drugs and nanotherapeutics are readily delivered to the liver and lungs, the protective bone marrow niche provides a conducive environment for metastatic cells to undergo intrinsic genetic and epigenetic cellular changes that eventually lead to drug resistance[88]. When present in small clusters, the small tumor surface area relative to surrounding uninvolved tissue reduces the efficacy of treatment at the typically low concentrations of drugs that reach the metastatic tumor cells. Further complicating the treatment response is the high expression of cell membrane-based efflux transporters, such as P-glycoprotein 1 and multidrug resistance-associated protein 1, which effectively expel the drugs before they can exert therapeutic effects on the cellular machinery[89]. Moreover, the serious side effects caused by conventional chemotherapeutics, particularly to the bone marrow stem cells, are limiting factors. As efforts to uncover the biological mechanisms of cancer metastasis and resistance to therapies continue to provide new insight into the metastatic niche, it is obvious that new therapeutic approaches are needed to increase treatment efficacy, prevent relapse, and provide a cure with minimal off-target toxicity. These goals can be accomplished by harnessing the multivalent and multifunctional attributes of nanoparticles to design novel nanotherapeutics with the capacity to irreversibly trigger cancer cell death.

Cancer Nanotherapeutic Strategies for Metastatic and Disseminated Tumors

Nanotherapeutics have considerable advantages over conventional chemotherapeutics, including the ease of controlling their circulation times in blood, as well as their *in vivo* stability, bioavailability, and bioactivity. These properties can be employed to address some fundamental limitations of small molecule chemotherapeutics in treating metastatic tumors. For example, nanotherapeutics are frequently used to improve the bioavailability and local concentration of existing drugs that are highly effective against metastatic cancer cells via passive targeting. This approach is most effective in large metastases of the liver and lungs, where an enhanced permeability and retention (EPR) effect is achievable. However, EPR uptake is ineffective for small and poorly vascularized micrometastases (tumors <2 mm in size), which are frequently found in the bone marrow and at early stages of metastasis elsewhere. Efforts to address this challenge have focused on nanoparticle formulations designed to target cancer biomarkers selectively. Although the mechanism of tumor uptake is not fully understood at this point, albumin-bound paclitaxel (Abraxane), represents an interesting coupling of EPR and cancer-targeted approaches to deliver drugs to tumor cells. Clinical studies demonstrate that this nanoparticle-bound drug exhibited a blood circulation half-life more than 100 times longer than that of the small molecule paclitaxel alone. Response rate (74% vs 39%) and progression-free survival (14.6 vs 7.8 months) using the nanotherapeutics were higher than for the unbound drug in patients with metastatic breast cancer[90].

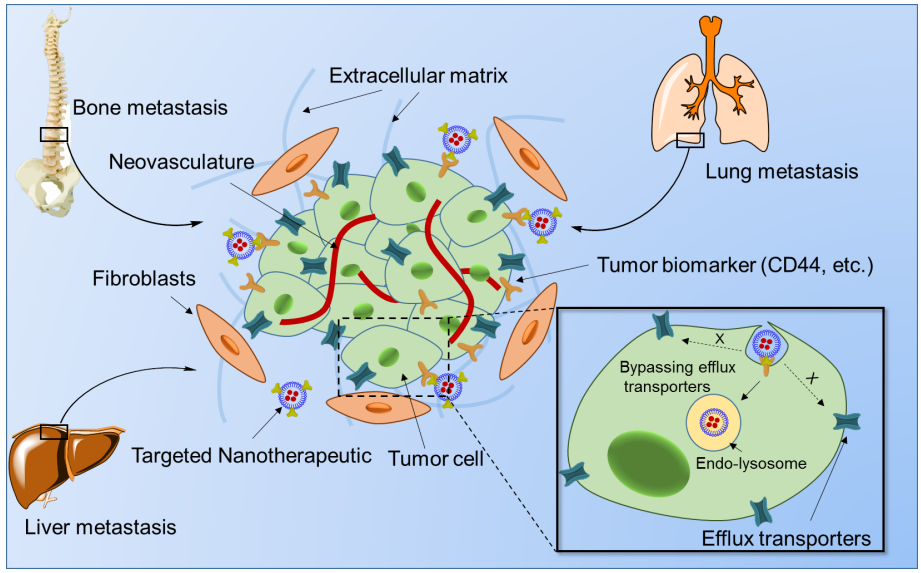

Figure 9. Major sites of cancer metastasis and the respective nanotherapeutic targeting strategies.

Some disseminated tumors, such as multiple myeloma, which can serve as a model of bone marrow metastasis, and particularly drug resistant phenotypes, commonly found in niches such as the bone marrow microenvironment, are not responsive to Abraxane nanotherapy. For example, adhesion of multiple myeloma

cells to the bone marrow stroma results in cell-adhesion-mediated drug resistance (CAM-DR). Thus, a dual-function ligand that simultaneously targets the tumor cells and inhibits adhesion to surrounding stroma would improve treatment outcome. This goal was achieved in a recent study by loading self-assembling micellar nanoparticles with doxorubicin and functionalizing the micelle surface with very late antigen-4 (VLA-4) peptide, which served as an anti-adhesion molecule. This formulation not only selectively delivered doxorubicin to the tumor cells, but also overcame CAM-DR. The micellar nanoparticles preferentially homed to tumors in the bone marrow with ~10-fold higher drug accumulation and tumor growth inhibition with a reduced overall systemic toxicity compared to the small molecule drug alone[91]. An alternative approach incorporates antisense drugs into polymeric nanoparticles for targeting the genes of osteopontin and bone sialoprotein, which are overexpressed in bone metastases of mammary carcinomas. These nanoparticles protect the drugs against nuclease degradation, thereby enabling sustained release of antisense therapeutics and a significant decrease in the incidence of bone metastasis[92].

The effectiveness of some drugs is hampered by the high efflux rate in drug resistant phenotypes of metastatic cells expressing P-glycoprotein 1 and multidrug resistant transporters. Despite several studies demonstrating the efficacy of Vincristine sulfate (VS) in cancer therapy, the high efflux rate by these transporters decreases the intracellular resident time for effective therapy. To overcome this impediment, VS was encapsulated in polymeric nanoparticles, causing it to be taken up through clathrin and caveolae mediated endocytotic pathways and allowing it to bypass the efflux transporters. The ensuing accumulation and retention of VS nanotherapeutics in metastatic cancer cells resulted in a ~21-fold increase in cytotoxicity compared to VS alone[93].

Cancer is a highly heterogeneous disease with distinct cell subpopulations that are phenotypically and biochemically diverse.

Future Challenges

Cancer is a highly heterogeneous disease with distinct cell subpopulations that are phenotypically and biochemically diverse. Given their different capacities to grow, differentiate, develop drug resistance, and form metastases, understanding tumor biology is critical for the development of successful therapies. Biomarker discovery and identification is an important aspect of this progress and an indispensable step in the development of targeted nanotherapeutics. However, significant variations between primary and metastatic cancer from the same patient further complicate the development of a consensus strategy to

treat the disease. The ability to target multiple cancer biomarkers and deliver combinatorial therapy favors the use of nanotherapeutics to maximize treatment outcome. An emerging frontier in cancer therapy is in understanding the contribution of tumor environment to its survival and metastasis. Some studies suggest that several factors alter a secondary site before the homing of migrating tumor cells. Sometimes the metastatic tumor cells remain dormant and undetectable after the primary cancer is removed, leading to relapse. With current knowledge of cancer-type specific metastatic patterns, it will be possible to develop nanotherapeutics that can reside in the secondary tissue for prolonged periods to achieve preventive or augmented nanotherapy. In addition, this treatment paradigm could be enhanced by other forms of therapy, such as gene silencing and immunomodulatory techniques to provide a multipronged strategy to combat cancer, with minimal morbidity effects to the patient. Phototherapy appears to be effective in treating metastasis, but the limited penetration of light has hampered the use of this technique in clinics. A recent study postulates that Cerenkov radiation from radionuclides used in positron emission tomography could serve as a depth-independent light source for cancer therapy in the presence of photo-sensitive nanomaterials that generate cytotoxic radicals upon exposure to light[62]. Application of this concept to the treatment of circulating tumor cells and metastases could improve treatment outcome, especially for chemotherapy resistant metastasis.

Nanotechnology Solutions to Overcome Plasticity and Resistance Using Epigenetic and MicroRNA-Based Reprogramming

Lara Milane, PhD and Mansoor Amiji, PhD
Department of Pharmaceutical Sciences
Northeastern University, Boston, MA 02115

Tumor Plasticity and Therapeutic Resistance

Plasticity is an inherent characteristic of cancer and a plays a vital role in cancer initiation and sustenance. The cellular changes that transition a normal cell into a cancer cell can be defined as cellular plasticity; likewise the perpetual adaptions that cancer cells undergo to survive can be classified as cellular plasticity. In this sense, tumor plasticity enables therapeutic resistance and could be considered a survival response. As cells that continually transform to maintain their immortalization, cancer cells are the ultimate biological representation of "survival of the fittest," through their inherent plasticity they are able to adapt and survive in inhospitable conditions (low oxygen, nutrient deprived) and even evade the effects of cytotoxic drugs and biologics. In 2000 and in a 2011 follow-up review, Hanahan and Weinberg took a comprehensive approach to characterizing cancer and defined the six hallmarks of cancer as; the ability to sustain proliferative signaling, the ability to evade growth suppressors, activation of invasion and metastasis, replicative immortality, induction of angiogenesis, and resistance to cell death[94]. An important feature of solid tumor masses is their cellular heterogeneity, this is caused by survival adaptations of cells (plasticity) and the inherent genome and proteome dysregulation characteristic of cancer cells; tumor heterogeneity undoubtedly contributes to drug resistance. Multi-drug resistance (MDR) can be innate (biologically inherent to the cancer cell) or acquired (after drug exposure); as discussed below, epigenetic factors and microRNA contribute to both innate and acquired MDR as well as to tumor plasticity. Cancer cells employ a variety of mechanisms of MDR including decreasing drug influx into the cell, increasing drug efflux, increasing DNA repair, increasing drug metabolism, and decreasing apoptosis[95]. Tumor heterogeneity is a challenge to the clinical treatment of solid tumors as tumor sub-populations of cells respond differently to treatment, which can increase the development of acquired MDR and metastasis. Tumor plasticity enables drug resistance and cell survival despite aggressive therapeutic treatment.

Epigenetic and Phenotypic Reprogramming

In recent years, the role of epigenetics in genotype expression has been elucidated and we are beginning to understand the significance of epigenetics in cancer development and regulation. Epigenetics refers to a heritable (mitotic and meiotic), stable change in gene expression without a modification of the DNA sequence[96]. The most common epigenetic changes include direct chemical modifications of DNA (methylation), histone modifications, and chromatin remodeling. Epigenetic modifications regulate cell differentiation, maternal and paternal inheritance patterns, gene expression responses to environmental factors and stress, seasonal gene expression, and cancer development[97]. When the human genome project completed in 2003, there were still many questions that the vast "decoding" could not seem to answer; how do our experiences, the food we eat, the environment we are exposed to, and daily stress exert a genetic effect? How can these variables lead to cancer? How does parental imprinting occur? The epigenome has evolved as an answer to these questions. If DNA is thought of as the same set of ingredients that every cell has, the epigenome can be thought of as the recipe – what each cell makes with those ingredients; an old, memorized family recipe that is passed down from generation to generation. Given the governing role of the epigenome in gene expression, the contribution of epigenetic changes to cancer initiation, progression, plasticity, and resistance is not surprising[97]. Although tissue-specific and patient specific epigenetic variations have been noted in tumors, in general, the cancer epigenome displays hypomethylation and hypermethylation at site-specific CpG islands (cytosine clusters) within gene promoters[97].

...the role of epigenetics in genotype expression has been elucidated and we are beginning to understand the significance of epigenetics in cancer development and regulation.

Also in recent years, the powerful contribution of microRNAs (miRNAs) to cancer has been discovered. MicroRNAs are 18-25 nucleotide, noncoding RNAs that negatively regulate gene expression at the post-transcriptional level. RNA polymerase II or III transcribes a primary microRNA (pri-miRNA) in the nucleus, the pri-miRNA is cleaved by a Drosha/DGCR8 complex to form precursor miRNA (pre-miRNA) which is transported into the cytoplasm, then Dicer processes the pre-miRNA into mature miRNA for incorporation with RISC (the Argonaute containing RNA-induced silencing complex)[98]. It is this miRNA-RISC complex that blocks gene expression by either degrading target mRNA or by hybridization to the 3' untranslated region of the target mRNA[98]. Over 2,500 miRNAs have been identified and many have multiple targets; although

many miRNAs are down regulated in different cancers (such as the miR-34 family), miRNAs that are overexpressed in many cancers have been coined "onco-miR's;" these oncogenic microRNAs include miR-155 and miR-21[99]. Validated oncogenic miRNAs such as miR-21 have been demonstrated to contribute to drug resistance, as has miR-19 and the miR-221/222 family[100].

There is a dynamic feedback circuit between epigenetics and miRNAs where the epigenome regulates the expression of miRNAs and certain miRNA's control mediators of the epigenome such as histone deacetylases, DNA methyltransferases, and polycomb group proteins (regulate linage delineation)[101].

Nanotechnology-Based Delivery Strategies for Reprogramming

A recent study validated epigenetic targeting with nanoparticle based therapies as an approach to reverse MDR. The study combined decitabine (a DNA hypermethylation inhibitor) loaded nanoparticles with doxorubicin loaded nanoparticles and demonstrated that combination therapy improved the efficacy of treatment and decreased the expression of DNA methyltransferase isoforms in the tumor bulk and in cancer stem cell populations in an MB-MDA-231 xenograft model in mice[102]. Using nano-based delivery systems to co-administer epigenome modifiers with standard chemotherapeutics has clinical potential as a strategy for reducing tumor plasticity and stem-like properties while reversing drug resistance. Likewise, combination therapy with chemotherapeutics and microRNA mimetics delivered in nanoparticle based formulations have demonstrated reversal of MDR through down regulation of ABC transporters (drug efflux pumps)[103]. MicroRNAs demonstrated to down regulate ABC transporters include miR-451, miR-27a, miR-223, miR-331, miR-326, miR-297, miR-487a, and miR-181a[103]. A variety of nanoparticle platforms have been explored for miRNA mimetic delivery, nanoparticles are ideal for nucleic acid delivery as they offer levels of protection as well as the ability to surface functionalize the vector for active targeting to tumor tissue. In April of 2013, the first clinical trial (phase 1) of a microRNA mimetic began in patients with liver cancer and hematological malignancies[104]. MRX34 consists of a miR-34 mimetic administered in "Smarticles"; pH responsive liposomes that exploit the lower pH of tumors to facilitate uptake[104]. As endogenous miR-34 regulates over 20 oncogenes, pre-clinical studies have demonstrated MRX34's ability to restore tumor suppression[104]. Cationic liposomes have been used to deliver miR-29b in pre-clinical lung cancer models, as miR-29b targets the cyclin dependent protein kinase 6 oncogene in lung cancer, treatment with the liposomes resulted in sixty percent tumor growth inhibition in a mouse model[105]. A variety of lipid and cationic polymer based nanoparticle systems have been developed for miRNA delivery in pre-clinical pancreatic cancer models[106]. More elaborate systems such as a liposome-polycation-hyaluronic acid nanoparticle system surface modified with

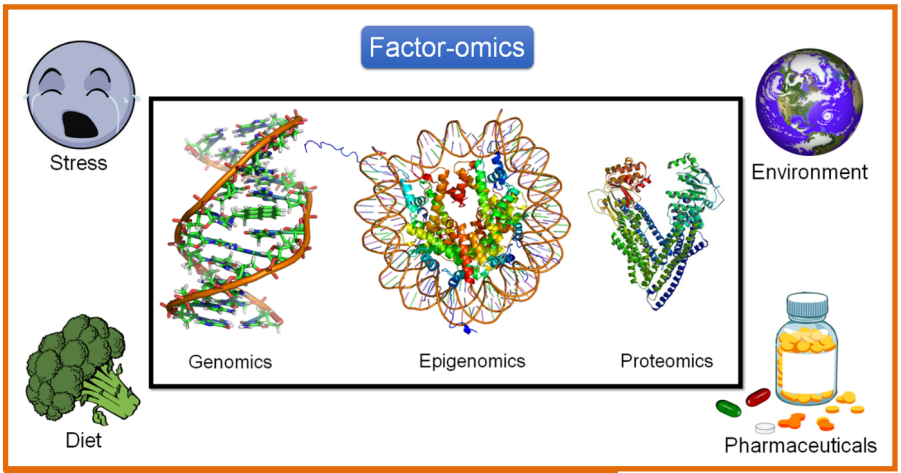

Figure 10. Emergence of "factor-omics" as a field, classifying and studying the environmental, dietary, physiological, and pharmacological factors that influence the epigenome, post-transcriptional gene expression, and the proteome. Genomics is the foundational field, proteomics is the translational product of the genome, the epigenome regulates gene expression (and hence, proteomics), and factor-omics will detail the environmental, nutritional, physiological (such as stress), and pharmacological factors that influence the genome, epigenome, and proteome.

a single chain antibody fragment to actively target GC4 (a metastatic melanoma epitope) for combination delivery of siRNA and miRNA have been developed and have demonstrated efficacy in reducing tumor growth and inhibiting metastasis[107]. Nucleic acids require delivery vectors such as nanoparticles to avoid immune system clearance and degradation and achieve therapeutic concentrations at the target site; the clinical application of microRNA relies on nanotechnology to enable therapeutic delivery. In addition to therapeutic applications, nano-based sensors are also being explored for cancer biomarker detection of circulating microRNAs and circulating tumor DNA[108,109]. In a 2011 article in *Nature Nanotechnology*, Li-Qun Gu and fellow researchers reported the development of a nanopore sensor capable of sub-picomolar detection of target microRNA in the plasma of lung cancer patients[109]. The nanopore used in this study was the α-haemolysin protein pore; synthetic nanoprobes are sure to follow in coming years[109]. More recently, researchers have developed a gold nanoparticle based sensor with peptide nucleic acid probes that exploit localized surface plasmon resonance to detect tumor-specific epigenetic variations in human serum samples[108]. Profiling a patient's disease from their plasma sample is a remarkable advancement in clinical oncology and could provide a powerful means of assessing and tailoring treatment.

Future of the Field

In this era of "omics" we anticipate the development of the next "omics" field; a field we will dub "*factor-omics*" for now (**Figure 10**), a field studying and classifying the factors that affect the epigenome, post-transcriptional gene expression, and the proteome. This field has already begun although has yet to be unified in a cohesive way, as with genomics, proteomics and epigenetics, this will occur naturally as the science progresses. Studies detailing the genetic, epigenetic, and post-translational effects of environmental, nutritional, physiological, and pharmacological factors have been well under way for some time, yet the key to evolving this field will be reviewing the results of the studies and making collective observations that can form the foundational science of the field. A second significant anticipated advancement in this arena will be the clinical application of nanotechnology-based sensors for microRNA and epigenetic cancer biomarkers.

A second significant anticipated advancement in this arena will be the clinical application of nanotechnology-based sensors for microRNA and epigenetic cancer biomarkers.

With the joint efforts of investigators across the spectrum, several advances should come to fruition over the upcoming 5-10 year time frame. In the next 5 years, researchers will have performed scientific studies/reviews to classify and interpret the environmental, physiological, and pharmacological factors that influence the epigenome and proteome; perform clinical evaluations of microRNA nano-sensors for cancer biomarker screening; and research investigational nano-therapeutics that reverse MDR using microRNA and epigenetic approaches. Looking further ahead over the next 10 years, the establishment of "factor-omics"; a field classifying and studying the environmental, physiological, and pharmacological factors that influence the epigenome, post-transcriptional gene expression, and the proteome will occurred. As genomics is the foundational field, proteomics is the translational product of the genome, and the epigenome regulates gene expression (and hence, proteomics), factor-omics will detail the environmental, physiological, and pharmacological factors that influence the epigenome and proteome; clinical application of microRNA nano-sensors for cancer biomarker screening; and clinical testing of nano-therapeutics that reverse MDR using microRNA and epigenetic approaches.

Exosome-Mediated Communication in the Tumor Microenvironment and Metastasis

Lara Milane, PhD and Mansoor Amiji, PhD
Department of Pharmaceutical Sciences
Northeastern University, Boston, MA 02115

Tumor Exosomes and Content

Although exosomes were first discovered in 1987[110], it wasn't until recent years that the importance of exosomes in cellular communication has been elucidated. Exosomes are 30-100 nm vesicles shed by cells as a process of cell signaling and communication. In recent years it has been discovered that cancer cells produce and shed more exosomes than normal cells[111]. Exosomal release is one of three possible fates for multivesicular bodies (MVB). Multivesicular bodies are formed when plasma membrane receptors are marked for recycling or degradation through ubiquitination; early endosomes are formed through plasma membrane internalization and as internal vesicles form within the endosome, the endosome transitions to multivesicular bodies[111]. The three fates for multivesicular bodies are; recycling through the trans-Golgi network, lysosomal degradation, or secreted through exocytosis or through fusion with the plasma membrane (exosome release). Exosome secretion through exocytosis is mediated through intracellular Ca^{2+} levels while factors such as extracellular/intracellular pH gradients can effect release and uptake[112,113]. Much investigation has focused on exosome content and determining if exosome content is a deliberate process in cell signaling; exosome content is rich in enzymes, microRNA, transcription factors, heat shock proteins, MHCs, cytoskeleton components, signal transducers, and tetraspanins (transmembrane proteins). It is most commonly accepted that exosome content is determined non-specifically under multivesicular formation and not through a deliberate sorting and packaging process[111]. But is this really the case? Are most biological processes not deliberate? From a metabolic perspective, it would be a vast waste of cellular energy for exosome content *NOT* to be deliberate. Perhaps there is a missing piece we have not had insight to yet, indeed, the function of the endosomal sorting complex required for transport (ESCRT) in sorting ubiquitinated proteins provides insight to a possible sorting process[114]. Perhaps in healthy cells exosome release is one of three cellular fates for MVB, but in cancer cells, exosome release is exploited as a deliberate means of cell communication and to specifically achieve metastasis. The existence of this missing piece – the confirmation that cancer cells use exosomes as a deliberate mechanism of communication is likely to be proved or disproved within the next five years.

Exosome-Mediated Cell-Cell Communication

Exosomes are taken up by recipient cells through receptor-mediated endocytosis, pinocytosis, phagocytosis, or through fusion with the cell membrane resulting in direct release of contents into the cytoplasm. If cancer cell exosomal content is not selected randomly, but is a deliberate process, then exosomes can be thought of as the cancer cells elevator pitch to the outside world – *this is what I want you to know and why*. On the other hand, if the current paradigm is correct where exosomal content is not selective, and is just a random sample of the cellular content then exosomes can be thought of as an informational press release to the public – *this is the news, this is what I am doing right now*. Either way, it is a powerful means of communication that is utilized by cancer cells more than normal cells. Despite the intent of the message, what is the result of these messages?

Among other effects, such as transferring drug resistance, a demonstrated result of exosomal communication is metastasis. The metastatic process consists of a series of events that include the epithethial-mesenchymal transition (EMT; mobilizing cells) and the mesenchymal-to-epithelial transition (MET; establishing a secondary tumor site). Cancer exosomes have been demonstrated to deliver functional proteins, complexes, and RNA that promote both EMT (such as HIF-1α) and MET (such as miR-200).

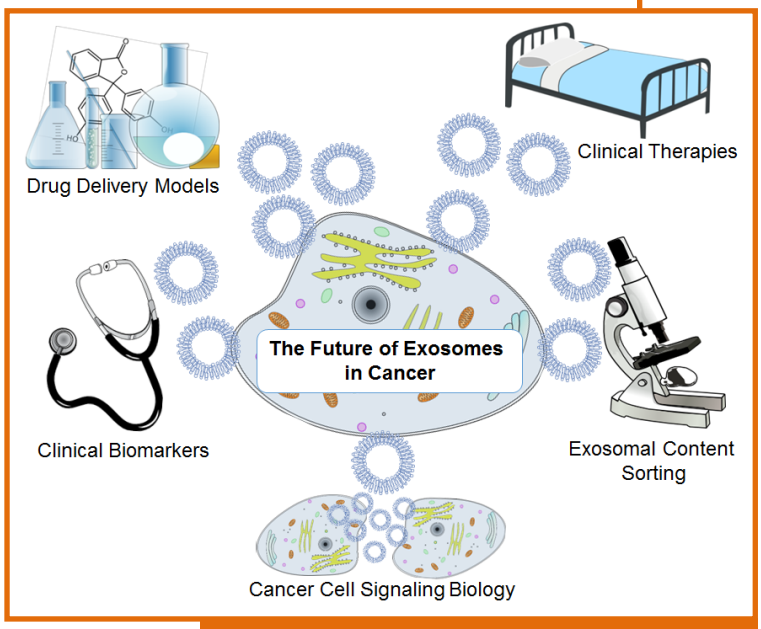

Figure 11. The future of exosomal research in cancer will entail fast-tracked clinical therapies and diagnostics for clinical biomarkers, deeper insight into cancer cell signaling particularly from highly heterogeneous tumors, studying exosomes as a model for drug delivery, and answering the highly debated question of exosomal content sorting and selection as a deliberate or non-selective process.

Metastasis: Epithethial-Mesenchymal Transition (EMT)

Hypoxia Inducible Factor-1α (HIF-1α) has gained attention over the past ten years as a powerful transcription factor contributing to oncogenic, aggressive, and drug resistant phenotypes in cancer. Under hypoxic conditions and under conditions of cell stress HIF-1α translocates from the cytoplasm to the nucleus where it forms an active transcription complex with HIF-1β binding to hypoxia responsive

elements on over fifty target genes including growth factors, drug efflux pumps, glucose transporters, cadherins, and factors that promote invasion and metastasis[115]. Our own studies have demonstrated a correlation between HIF-1α expression, multidrug resistance, and aggressive tumor phenotypes[115]. HIF-1α also contributes to epithethial-mesenchymal transition (EMT)[116]. A recent study by Pagano and Shackelford demonstrated that HIF-1α is excreted in a functional form from nasopharyngeal carcinoma cells infected with Epstein-Barr virus[116]. The study illustrated that transfection of nasopharyngeal carcinoma cells with latent membrane protein 1, the primary oncogene of Epstein-Barr virus, increased HIF-1α in secreted exosomes[116]. Using HA-tagged HIF-1α expression vectors in a series of *in vitro* studies the researchers demonstrated that exosomal HIF-1α was transcriptionally active in recipient cells. This, and similar studies, have demonstrated that exosome content can be altered through genetic and phenotypic modifications in the donor cell and these alterations can have profound effects on cell signaling through exosomal release and uptake.

Metastasis: Mesenchymal-to-Epithelial Transition (MET)

One of the most groundbreaking exosomal studies in recent years was the eloquent investigation conducted by Judy Lieberman at Boston Children's Hospital. Lieberman et al demonstrated that exosomes and ectosomes (larger vesicles formed by cell membrane budding) released from metastatic cancer cells can transfer metastatic capability to non-metastatic cells and this capability appears to be mediated through the microRNA-200 family, known regulators of mesenchymal-to-epithelial transition (MET)[117]. The study used extensive *in vitro* and *in vivo* techniques and through the meticulous selection of experimental conditions, resulted in a foundational exosomal and microRNA study. For example, the study selected cells with distinct metastatic capabilities (metastatic 4T1E mouse cells and metastatic human cells CA1a and BPLER cells and poorly metastatic 4T07 mouse cells and poorly metastatic human mesenchymal MB-231 cells) to study *in vivo* metastatic induction in mouse and human xenograft models. The study optimized the use of fluorescent cell labeling in many experiments; for example, to distinguish between metastatic lesions formed from circulating tail-vein injected cells from primary tumor cells, GFP-expressing primary orthotopic breast cancer tumors were developed in mice and firefly luciferase and mCherry expressing tumor cells were injected via tail-vein-injection[117]. Collectively, the *in vitro* and *in vivo* analysis demonstrated that exosomes and ectosomes from highly metastatic cells can increase the metastatic capabilities of local and distal poorly metastatic cells through the uptake of MET regulating miR-200[117].

Exosome Content Modulation and Application

An interesting phenomena that was noted in the Lieberman study was that micro-RNA's delivered in exosomes are sometimes associated with Ago2, indicating these miRNA's may be contained in RNA-induced silencing complexes (RISC) which results in their immediate activity in recipient cells[117]. In the Pagano and Shackelford's studies of HIF-1α exosomal delivery, HIF-1α was delivered both as an inactive (uncomplexed) and active (complexed) form[116]. Our current understanding of exosomal content is that it is non-specific and dependent on the cellular content. It may be, just as years ago introns were considered to be "junk DNA", that we just do not have a complete understanding of this process yet. It may be that as we learn more about exosome formation and communication that the process is revealed as a deliberate and selective mechanism of cellular communication.

From a drug delivery perspective, exosomes are nature's own nanoparticles delivering an array of functional proteins and nucleic acids. Exosomes are innate "stealth" carriers that can have profound effects on recipient cells. Exosomes can benefit the field of medicine and therapeutics in two ways; studying exosomes as a biological model for "drug" delivery and manipulating exosomes for therapeutic outcomes and as diagnostic tools (**Figure 11**).

...exosomes are nature's own nanoparticles delivering an array of functional proteins and nucleic acids.

The methods for altering exosome content are electroporation, direct chemical transfection of exosomes, transfection of exosome donor cells, activation of exosome donor cells, and direct incubation of exosomes with loading cargo[118]. Elaborate investigational studies, such as Lieberman's miR-200 exosomal study are being conducted, and this exosomal research has been so exciting and promising, exosomes seem to have fast-tracked their way into clinical trials. Several clinical trials have already completed globally to explore the medical promise of exosomes as cancer therapeutics. The most recently completed exosome clinical trial in the United States was a pilot study of an immunotherapy vaccine for malignant gliomas[119]. The Phase I trial was conducted by David Andrews at Jefferson University Hospital and consisted of extracting the patient's own tumor cells, treating them with an antisense oligodeoxynucleotide against insulin-like growth factor type 1 receptor (IGF-1R/AS-ODN), placing the treated cells in a biodiffusion chamber, implanting the device in patients abdomens and relying on exosomes released from the chamber to communicate and initiate an immune response (T-cell activation) against the tumor[119]. A second Phase 1 trial of this therapy is underway as the majority of patients (8/12) in the first trial elicited a positive clinical response[119]. Other clinical trials recruiting

patients in the US include a study investigating the use of plant derived exosomes to deliver curcumin to colon tumors and normal colon tissue and a study evaluating circulating exosomes as prognostic and predictive biomarkers for gastric cancer patients. Exosomes are indeed proving to be effective, innate, *cellular nanoparticles* that can be manipulated for therapeutic applications, used as cancer biomarkers, and studied as ideal models for drug delivery.

Several milestones should come to realization over the upcoming 3-10 year time frame. In the next 3-5 years, researchers will have standardized methods for isolation and study of Exosome communication in the immune/tumor interface, intra-tumoral communication, extracellular matrix composition, and metastasis; should have a definitive answer, is exosomal content deliberately selected in cancer cells as a mechanism of cell communication, invasion, and metastasis?; be studying exosomes as "native" nanoparticles as a model for drug delivery; and clinical trials for therapeutic and biomarker applications of exosomes. Looking further ahead over the next 10 years, the establishment of tools and methods for biomarker screening; began therapeutic intervention at the immune/tumor interface, intra-tumoral communication, extracellular matrix composition, and metastasis; studied exosome signaling from distinct cancer cell populations, MDR cells, cancer stem cells; and clinical approval and marketing of exosomal therapeutics and diagnostic tools.

Measuring Therapeutic Response to Cancer Immunotherapy via Nanotechnology

James Heath, PhD
Department of Chemistry and Nanosystems Biology Cancer Center
California Institute of Technology, Pasadena, CA 91125

Cancer Immunotherapy was the Science Breakthrough for the Year 2013[77], with tremendous promise and excitement surrounding two immunotherapy classes. Class 1 is comprised of immune checkpoint inhibitors[120,121], such as for the programmed death (PD)-1/L1 blockade, or anti-CTLA-4. These drugs can increase the susceptibility of cancer cells to immune system attack. Class 2 is adoptive cell transfer (ACT)[122,123], which seeks to strengthen the anti-tumor immune system function. ACT of chimeric-antigen-receptor (CAR) engineered T cells is now being pursued within a number of major pharmaceutical companies as an effective treatment for leukemias and lymphomas. The clinical testing of PD-1/L1 blockade has been carried out in multiple cancers, but has been led by work in melanoma[124], and has demonstrated a new era in cancer treatment[125,126]. It is fair to say that cancer immunotherapy has, in just the past two years, altered the conversation around cancer therapies from that of 'treatments' to that of 'cures.' However, it is still in its very early days yet, and immunotherapies have only been shown to provide powerful treatments for a subset of cancers, and even within those subsets, only for specific patient populations. Even for those patients who exhibit strong anti-tumor responses to immunotherapies, only a fraction (albeit a large one) exhibit durable responses. Thus, in order for the profound benefits of cancer immunotherapy to be extended to increasingly larger patient populations, there are a number of technological challenges to be addressed, and there are important roles for cancer nanotechnology to play. Here we outline two of many such challenges.

In Vivo Biomarkers

As with any therapy, it is challenging to identify potential immunotherapy responders from non-responders. The most promising prognostic biomarker is that of a pre-therapy anti-tumor immune response, in the form of CD8+ T-cells infiltrating into the growing margins of the tumor. Patients that exhibit such a baseline immune response are significantly more likely to respond to PD-1/L1 blockade therapies[127], and it is an absolute requirement for patients seeking ACT therapies that utilize *in vitro* expanded populations of tumor-infiltrating lymphocytes[122]. For melanoma patients, obtaining tissue biopsies for the analysis of CD8+ T cell infiltrates is straightforward, but for many tumors, such biopsies are not readily obtained. Thus, an *in vivo* imaging probe of CD8+ T cells would provide a powerful diagnostic tool for stratifying patients. If it is a positron emission tomography (PET) probe, then

antibodies are unlikely to serve this purpose, as their retention time in the body provides unwanted competition for the half-life of the ^{18}F-radiolabels commonly used. In addition, commercially available anti-CD8+ monoclonals do not exhibit particularly high affinities for the target. A high affinity, and a low off rate, are both important metrics, because many patients who exhibit a baseline anti-tumor immune response only have a low number of CD8+ T cell infiltrates. Other *in vivo* biomarkers include the emerging list of immune checkpoint molecules that are being explored for expanding immunotherapy to cancers such as prostate or breast. Thus, there is a unique opportunity here for nanotech solutions that can provide for rapid clearance, high target avidity, and tumor penetration.

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Neoantigens and the Design of ACT Therapies

In any cancer immunotherapy, the major tumor cell killers are CD8+ T cells. The killing function of those T cells is activated following a highly specific interaction between the T cell receptor (TCR) and a tumor antigen presented by tumor cells (**Figure 12**). Very recent findings are pointing to the importance of neoantigens in illiciting strong and highly specific anti-tumor T cell responses[128–131]. Neoantigens are fragments of proteins from the cancer cells that contain genetic mutations, and so differ from self-antigens. The very strong implication is that if one knows the tumor antigens present within a patient's tumor, and one knows sequence of the TCR α/β chain gene that encodes a TCR that recognizes those antigens with high avidity, then one can design a personalized, and potentially highly effective ACT therapy for that patient. In terms of guiding this technology discussion, we'll assume that one has access to tumor tissue from the patient. The key information for designing a personalized ACT therapy regimen for the patient is the following:

- *Which T cell populations, as defined by specific TCR receptors, have clonally expanded within the tumor?* That information identifies the cells that have 'seen' tumor antigen.

- *What are the tumor antigens that are promoting this clonal expansion?* If the tumor antigens are neoantigens, then they are likely safe immunotherapy targets. If they are not, then they must be evaluated with great caution.

- *What are the TCR α/β gene sequences that encode recognition for the specific neoantigens?* This is the information that is required for genetically engineering the T cells for the actual ACT.

There has been a recent flurry of activity in this area, but no approach has come close to yielding all three pieces of information, and most only yield one of the three pieces[132,133]. As such, here are the major challenges.

First, the tumor exome may be mined to identify potential neoantigens using existing software, and the number of neoantigens for a given tumor is likely on the order of 20-200. One can build a tetramer library based upon these 20-200 neoantigens[134], but the best cytometry approaches for tetramer-based T cell sorting based are 20-plex, and so barely touch the required range of multiplexing[133]. Even those methods require that the T cells infiltrates from the tumor be expanded *in vitro*. Next, identification of those T cell populations that have clonally expanded within the tumor requires analysis of infiltrating lymphocytes directly from the tumor – i.e., without expansion *in vitro*. One may obtain only 10^4-10^5 T cells from a tumor biopsy. This is not enough for standard cell analysis tools, but may be enough for nanotech tools. Finally, once the T cells that recognize a specific neoantigen are identified, the TCR α/β genes must be sequenced at the single cell level. The TCR gene is very challenging to sequence, but methods for TCR gene sequencing with reasonable (~50%) yield have been reported[135–137]. No existing technology can *simultaneously* solve these three challenges. This should motivate a challenge to the cancer nanotechnology community, specifically, for an analytical/diagnostic modality that can help provide such a solution, in the next 5-10 years.

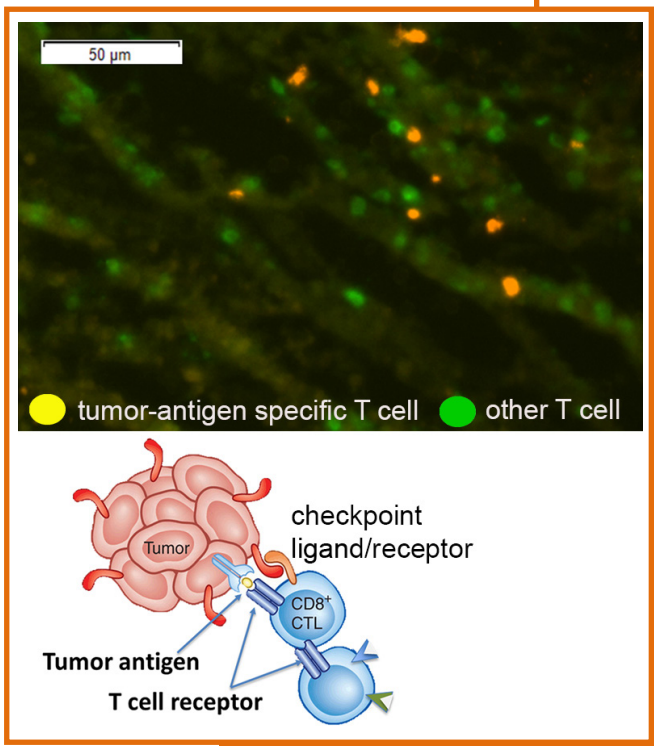

Figure 12. Tumor antigen-specific T cells are imaged in this fluorescence micrograph of a tumor from an in vivo immunotherapy model. Details of tumor/T cell interactions are shown in the drawing below.

Enhancing Cancer Immunotherapy with Nanotechnology

Andrew Z. Wang[1-4], MD and Leaf Huang[2-4], PhD
[1]Department of Radiation Oncology, [2]Lineberger Comprehensive Cancer Center, [3]Eshelman School of Pharmacy, and [4]Carolina Center for Cancer Nanotechnology Excellence
University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Cancer Immunotherapy

Cancer immunotherapy utilizes the patient's own immune system to treat cancer, now a powerful novel strategy in cancer treatment. Antibodies blocking negative immune regulatory pathways, such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death 1 (PD-1), have substantially improved clinical outcomes in patients with metastatic melanoma[125,138,139]. Moreover, these agents have been shown to be effective in many other cancers, including head and neck, lung, kidney, bladder, and liver cancer[140]. In addition to checkpoint blockade agents, dendritic cell therapy and chimeric antigen receptor (CAR) T-cell therapies have also achieved clinical success[141,142]. Lastly, recent clinical data suggest that some cancer vaccines may also provide survival benefit. Such successes have generated high interest in developing strategies to further improve cancer immunotherapy.

While highly effective, the major limitation of checkpoint inhibitor therapeutics is the low rate of long-term, durable responses. Most patients eventually develop resistance and progressive disease. CAR-T cells are difficult to engineer and have high toxicity (frequently fatal) if the targeted antigens are also present on normal cells. Lastly, current dendritic cell therapy has low potency and the therapeutic benefit is only realized several years after treatment. Thus, there is ample opportunity for the development of novel therapeutics and strategies to improve cancer immunotherapy.

Nanoparticles and Cancer Immunotherapy

Nanoparticles, because of their virus-like size, readily elicit an immune response upon local or systemic administration. Without pegylation or other anti-fouling surface modification, nanoparticles are rapidly taken up by macrophages and other antigen presenting cells (APCs) and lead to immune activation. While this innate nanoparticle property has been detrimental to drug delivery applications, it is highly favorable for cancer immunotherapy. Taking advantage of this property, nanoparticles can be utilized to deliver tumor antigens to APCs. Moreover, immune responses to NPs can be modulated by adjusting the size and shape of nanoparticles[143,144]. Nanoparticle-bound antigens have been shown to elicit greater

immune responses than free antigens. In addition, nanoparticles can also act as immune adjuvants, enhancing response when given together with cancer vaccines.

Cancer immunotherapy can also capitalize upon the drug delivery property of nanoparticles. Nanoparticles can be formulated to deliver pro-inflammatory/pro-immune molecules with tumor antigens to enhance immune reactions. Such co-delivery is more likely to activate APCs and thus result in robust immune responses.

Current Approaches using Nanotechnology to Enhance Cancer Immunotherapy

...nanotechnology holds great potential in improving cancer immunotherapy.

Despite being a new area of investigation, nanotechnology has been explored by a number of research groups to improve cancer immunotherapy. A common approach has been the use of nanoparticles to improve tumor antigen presentation by APCs *in vivo*[145]. Using mouse tumor cells (such as B16 melanoma cells) overexpressing ovalbumin (OVA) protein, several groups have shown that nanoparticle-delivered OVA is more effective than OVA itself in eliciting immune responses. Such data suggest that nanoparticle-antigen combinations can be effective cancer vaccines. To further enhance immune responses, immune-activating molecules such as CpG have been co-delivered with tumor antigens[146]. The investigators showed that co-delivery of antigen and adjuvant are several-fold more effective than each agent given separately.

Another strategy to improve cancer immunotherapy has been the use of nanoparticles to activate immune cells. Fadel et al. recently reported the use of carbon nanotubes containing immune activating molecules (e.g., IL-2) to activate T-cells[147]. Such activated T-cells were then able to delay tumor growth. In a separate study, Perica et al. engineered nanoparticles that mimic APCs and utilized these nano-APCs to activate T-cells[148]. Nanoparticles have also been used to directly activate dendritic cells (APC)[149]. These studies suggest a role for nanoparticles in cell-based cancer immunotherapy.

In addition to improving antigen presentation, nanoparticles have also been used for their drug delivery properties. Tumor microenvironments are frequently immune suppressive, and nanoparticles can deliver therapeutics to overcome immune suppression. Park et al. demonstrated the proof-of-principle of this approach by delivering a TGF-β inhibitor and IL-2 and showing that these drugs delayed tumor growth and improved survival using a mouse model of melanoma[150]. Xu et al. further demonstrated this approach using nanoparticles

to deliver a TGF-β inhibitor to the tumor microenvironment to enhance tumor vaccine effects[151]. These studies suggest that drug delivery approaches can be combined with vaccine and immune activation approaches described above.

Future Directions

Nanoparticle-based cancer immunotherapy is a new and exciting field. It holds high potential in making direct impact on cancer care. To fully realize the potential of this approach, studies are needed to systematically characterize nanoparticles properties (e.g., size, shape and surface properties) that are optimal for immune activation and cancer immunotherapy.

Immune activation against tumor cells is a highly complex process (**Figure 13**). Because of unique properties of nanoparticles, they can be applied to improve each of these steps. Nanoparticle therapeutics can induce tumor cell death and in turn increase antigen release. They can be utilized to improve antigen presentation and activation by the APCs. Nanoparticles can also deliver pro-immune/pro-inflammatory agents to tumors and tumor microenvironments to enhance the cancer immunotherapy response. Lastly, nanoparticles can be utilized to "train" dendritic and cytotoxic T-cells *ex vivo* for cancer immunotherapy.

Given the exciting clinical data with checkpoint blockade inhibitors, approaches that combine nanomedicine and checkpoint blockade inhibitors are most likely to make immediate clinical impact. Future studies should focus on which checkpoint blockade agents and regimens are synergistic with nanoparticles and how nanoparticle-based agents can be integrated into checkpoint blockade treatments (e.g., timing of nanoparticle administration).

Cancer vaccine is another application where nanomedicine can make immediate impact. Nanoparticles can be formulated using biodegradable and biocompatible GRAS (generally regarded as safe) materials, which enables rapid clinical translation. However, existing clinical literature suggest that cancer vaccines targeting a single tumor antigen have limited benefits. Therefore, future work should focus on the development of multi-antigen cancer vaccines.

Cancer vaccine is another application where nanomedicine can make immediate impact.

Other applications for nanoparticles in immunotherapy include the development of tumor-targeting T cells as well as CAR-T cell treatments. In addition, they can also improve dendritic cell treatments. These applications require better understanding of nanoparticle properties as well as tumor immunotherapy (e.g., which tumor antigens more likely to elicit antitumor responses). As the field of cancer

immunology evolves, nanomedicine approaches will likely become more effective and more clinically relevant.

In summary, nanotechnology holds great potential in improving cancer immunotherapy. There are many known and potential applications of nanoparticles in immunotherapy. We also expect many novel applications for nanoparticles in cancer immunotherapy that have not been discussed given the rapidly evolving field of immunology. Future success in this field will depend on the full integration of cancer biology, cancer immunology and nanomedicine in this research space.

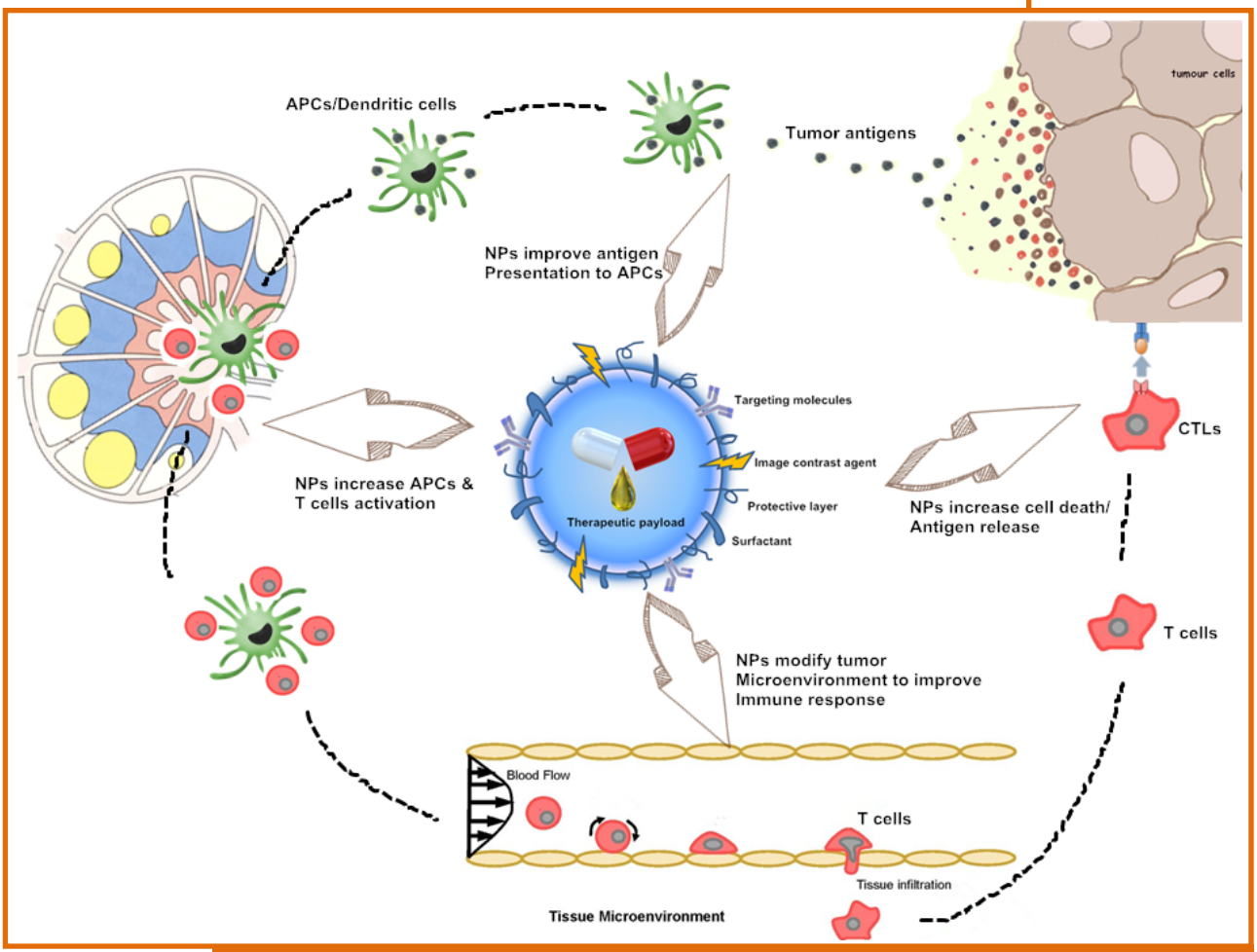

Figure 13. Depiction of the complex pathway involved in cancer immunotherapy. Nanoparticle delivery vehicles can play a role at multiple points along this pathway.

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Section III: Novel Nanomaterials for Diagnosis and Therapy

Mesoporous Silica Constructs

Kimberly Butler, PhD[2] and C. Jeffrey Brinker, PhD[1,2]
[1]Advanced Materials Laboratory
Sandia National Laboratory, Albuquerque, NM 87185
[2]Department of Chemical and Biological Engineering
University of New Mexico, Albuquerque, NM 87106

Introduction

Specific drug delivery is one of the greatest challenges in cancer medicine. Targeted delivery of drugs encapsulated within nanocarriers can potentially ameliorate a number of problems exhibited by conventional 'free' drugs, including poor solubility, limited stability, rapid clearing, and, in particular, lack of selectivity, which results in non-specific toxicity to healthy cells and prevents the dose escalation necessary to eradicate diseased cells and overcome drug resistance. However, the physical and chemical properties of the nanocarrier, including size, shape, internal structure, and surface properties, play major roles in determining biodistribution of the carrier *in vivo*, biological interactions, cargo loading and release, biodegradation, and toxicity[1]. The optimal biodistribution and biological interactions of the nanocarrier can vary between different cancers (and individuals) making the ideal nanocarrier one in which the physical and chemical properties can be controlled and essentially tuned for the specific application[2]. An additional very necessary feature of an effective nanocarrier is the efficient loading and controlled release of the therapeutic cargos, which can range from small molecules to plasmids that have highly variable charge, polarity, and hydrophobic/hydrophilic character. Finally, a nanocarrier's potential to include imaging agents as well as drugs grants the possibility of creating 'theranostics', which allows both drug delivery and the monitoring of the course of therapy to be achieved with a single nanocarrier. In the context of creating a tunable nanocarrier, mesoporous silica nanoparticle constructs, developed over the past decade, have a distinctive *combination of features* that could enable their development as 'universal' nanocarrier platforms, of which, are simultaneously drug and disease agnostic.

Creation of Mesoporous Silica Nanoparticle Constructs

Mesoporous silica nanoparticles (MSNP) are composed of periodic arrangements or uniformly sized mesopores (ranging in diameter from 2 to >20-nm) embedded within an

amorphous silica framework and characterized by exceptionally high internal surface areas ranging from 500 to over 1200 m^2/g^3. MSNP are synthesized by two major routes: solution based synthesis or evaporation-induced self-assembly. Using solution based colloidal self-assembly it is possible to synthesize uniformly sized populations of MSNP with spherical, prismatic, torroidal, rod-like, or hollow shapes[4–8] with dimensions spanning 25-nm to over 250-nm, while in many cases maintaining low polydispersity indices <0.1[9]. Using evaporation induced self-assembly[10], it is possible to generate in a single step spherical MSNP with a predictable power law particle size distribution spanning 25-nm to over 250-nm. The highly tunable synthesis of MSNP allows for the selection of the size, size distribution, and shape most applicable based on the proposed delivery route and target biodistribution (**Figure 1A-D**).

During synthesis, the MSNPs can be modified to increase their functionality, for example their interiors can be constructed in a core/shell manner to introduce metal or metal oxide nanoparticles as imaging agents (**Figure 1E**). Core-shell MSNPs have seen many recent

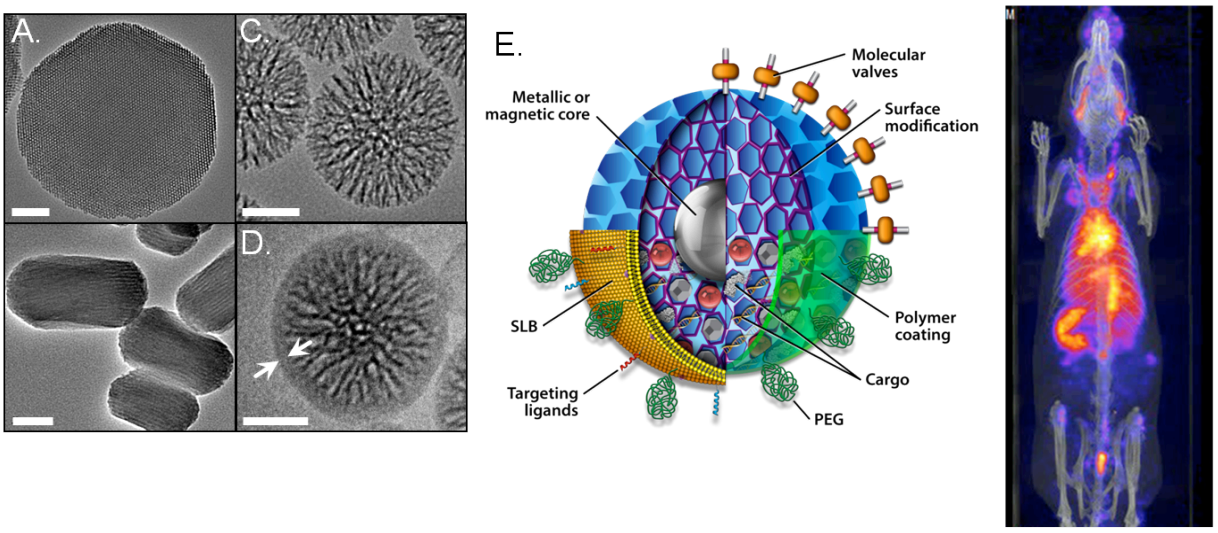

Figure 1. Mesoporous silica nanoparticles shape, pore size, lipid coating, functionalization and use. TEM images of spherical mesoporous silica nanoparticles with 2 nm pores (A), rod shaped mesoporous silica nanoparticles with 2 nm pores (B) and ~150 nm spherical mesoporous silica nanoparticles with 8 nm pores (C). CryoTEM of spherical mesoporous silica nanoparticles with 8 nm pores and a lipid bilayer coating highlighted by the white arrows (D). Scale Bars = 50nm. Schematic of a multifunctional mesoporous silica nanoparticle showing possible core/shell design, surface modifications and multiple types of cargo (E). SPECT image of radiolabeled 50nm mesoporous silica nanoparticles 5 hours post IV injection (F) (*Schematic (E) reprinted with permission from Tarn et al., 2013, TEM and SPECT images courtesy of Paul Durfee, University of New Mexico, Natalie Adolphi, University of New Mexico, and Yu-Shen Lin, Oncothyreon*).

applications in theranostics and allow for combined therapy and imaging simultaneously[11,12]. During or post-synthesis, the MSNP cores can also be loaded with fluorescent dyes with emissions spanning the visual range including; fluorescein isothiocyanate (FITC), rhodamine B isothiocyanate (RITC) and Cy3 as well as near-IR dyes such as AlexaFluor 700 and DayLight 680. The resulting MSNPs are extremely bright and optically stable enabling high-resolution multichannel optical imaging and quantitative multispectral flow cytometry. These labeled MSNPs provide a **unique** opportunity to examine the interaction between cells and nanocarriers along with MSNP biodistribution and delivery to tumors offering a direct measurement of these two important criteria during any regulatory approval[13,14].

Mesoporous Silica Nanoparticle Modification

MSNP functionality can be introduced by modifying silanol groups (ΞSi-OH) present both within the pore interiors and on the outer surface. Silanol groups are chemically accessible and can be easily reacted with alkoxy or chlorosilane derivatives to introduce organic functionality. Modification performed in single step or multi-step procedures provides an almost unlimited ability to 'tune' the charge, polarity, and hydrophobic/hydrophilic character of the pore and exterior particle surfaces, provide sites for further chemical conjugation or chelation with targeting and control ligands, and to couple imaging agents including radio labels for SPECT imaging (**Figure 1F**). Chemical moieties can also be adsorbed onto MSNP, especially facilitated by negatively charged SiO- groups, resulting from deprotonation of surface silanol groups at neutral pH, which result in attractive electrostatic interactions with positively charged moieties.

Introducing functional groups on the MSNP exterior surface gives rise to additional surface properties. They can be further reacted as linkers to attach larger molecules or used to adsorb coatings through noncovalent interactions. For the latter case, polymers are commonly employed on MSNPs[13,15,16]. Due to the intrinsic negative charge of the silica surface resulting from deprotonation of surface silanols, bare nanoparticles can be electrostatically functionalized with a positively charged polymer. Polymers or other surface bound functional groups can also be used to retain cargo within the MSNP and aid in colloidal stability that is required keep MSNPs highly dispersed for biomedical applications. An alternative means of surface coating MSNPs is by fusion with phospholipid bilayers to form a construct referred to as a *protocell*[14,17]. The cryo-TEM image (**Figure 1D**) shows a mesoporous silica particle core prepared by EISA enveloped by a conformal, 4-nm thick supported lipid bilayer (SLB). The properties of the SLB can be varied widely using lipids with differing fluidities or melting transition temperatures and headgroup chemistries that dictate charge and chemical reactivity. Membrane-bound components like cholesterol along with PEG can be introduced to control the fluidity and stability of the SLB, and it can be chemically

conjugated with ligands to effect targeting and internalization (*vide infra*) (**Figure 2**). As with polymer coatings, the SLB can serve to retain cargo introduced into the MSNP interior and aid in colloidal stability for biomedical applications. *Protocells* however have the advantage that acidification, as occurs in a tumor microenvironment or endosome, serves to permeabilize/destabilize the supported lipid bilayers triggering release of cargo[14,18].

Cargo Loading, Targeting and Cargo Delivery

Three major features of mesoporous silica constructs; high surface area, controllable pore size, and the ability to tune the charge of the particle, make them ideal for loading of varied cargo. Small molecule drugs and biological entities such plasmids or mRNA cargo present a large size range, which requires variable pore sizes for cargo loading. Using surfactants or block copolymers as structure directing agents in conjunction with swelling agents, it is possible to control pore size[19] from ~2-nm to over 20-nm, while hollow or toroidal particles provide even larger pore sizes (**Figure 1A-D**).

The tunable surface characteristics in combination with the high surface area allows for the simple loading of high concentrations of diverse classes and combinations of cargos that can be delivered by endocytosis or macropinocytosis[20]. The uniform arrangement, size, and connectivity of the porosity established by self-assembly confer to a MSNP very high BET (i.e., Brunauer–Emmett–Teller theory) surface areas ranging from 500 to over 1200 m^2/g. Surface area is important because it is the drug accessible surface area that dictates the drug loading capacity of an MSNP.

MSNPs can accumulate in tumor targets through both passive and active targeting. Passive targeting schemes rely on the enhanced permeability of tumor vasculature (the so-called enhanced permeability and retention (EPR) effect) to direct accumulation of nanocarriers at tumor sites, but the lack of cell-specific interactions needed to induce nanocarrier internalization decreases therapeutic efficacy and can result in drug expulsion and induction of multiple drug resistance (MDR). In terms of passive targeting, coating of MSNPs with a cationic polymer (e.g., PEI) significantly facilitates their uptake into tumor xenografts[16]. More recently, combining size control of MSNPs and PEI/PEG copolymer coating resulted in enhanced EPR effect in a xenograft tumor model[15].

To limit the degree of nonspecific binding while enhancing specific internalization by the target cell or tissue, MSNPs can be actively targeted toward an intended region (**Figure 2A**). Active targeting employs ligands that bind specifically to receptors overexpressed on the cancer cell surface. Bioactive ligands, such as folate, RGD peptide, and transferrin have been employed due to their respective receptors being overexpressed on many

different cancer cell types[21]. In general, high specificity and binding affinity require a high concentration of surface-conjugated ligands to promote multivalent binding effects, which results in more efficient drug delivery through receptor-mediated internalization pathways. However, high ligand densities can promote nonspecific interactions with endothelial and other noncancerous cells and increase immunogenicity, resulting in opsonization-mediated clearance of nanocarriers via the mononuclear phagocyte system (MPS). In this regard, the MSNP supported lipid bilayer construct (i.e., *protocell*) provides some potential advantages because its fluid SLB enables targeting ligand recruitment to target cell surface receptors, promoting high avidity with a low overall peptide concentration (**Figure 2B**).

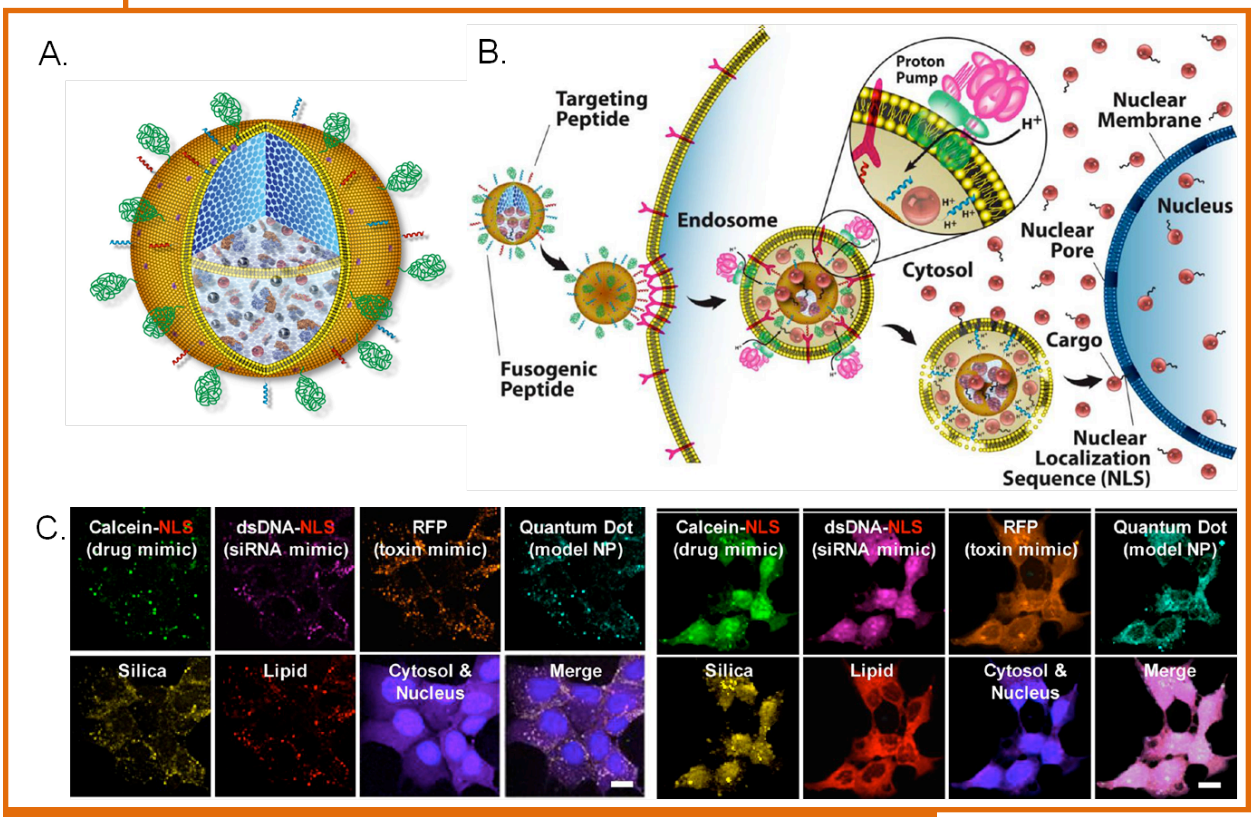

Figure 2. (A) Schematic of the protocell showing the MSNP core containing various cargo; such as drugs, nucleic acids and fluorophores, and coated with a lipid bilayer which has been functionalized by targeting ligands and PEG. (B) Schematic diagram depicting the successive steps of the multivalent binding and interanalization of targeted MSN –supported lipid bilayers, followed by endosomal escape and nuclear localization of MSNP-encapsulated cargo. (C) Hyperspectral confocal imaging of targeted delivery of multicomponent cargos in protocells to Hep3B cells for 15 minutes (left panel) or 12 hours (right panel) at 37°C. Alexa Fluor 532-labeled nanoporous silica cores (yellow) were loaded with calcein (green), an Alexa Fluor 647-labeled dsDNA oligonucleotide (magenta), RFP (orange), and CdSe/ZnS quantum dots (teal). Cargos were sealed in the cores by fusion of Texas Red-labeled DOPC liposomes (red) (*Reprinted with permission from Tarn et al., 2013*).

Thus, simultaneously with porosity, tunable surface and internal chemistry of the MSNP allowing for the inclusion of multiple cargos, MSNPs with lipid or polymer coating and cell type-specific targeting create a very robust single multifunctional nanocarrier platform (**Figure 2C**).

The highly tunable nature of MSNPs has also provided an ideal platform for the development of even more advanced nanocarriers with specific and controlled release of their cargo. The uniform pore size coupled with facile surface chemical conjugation has enabled modification of the pore entrances or interiors with responsive (light, pH, redox, etc.) molecular machines that can serve as gates[22] or 'stir bars' or molecular logic[23] to effect environmentally triggered release and control of the release rate profile.

The highly tunable nature of MSNPs has also provided an ideal platform for the development of even more advanced nanocarriers with specific and controlled release of their cargo.

Biocompatibility and Toxicity

A critical issue for any potential nanocarrier for medical applications is toxicity. The toxicity of silicon dioxide, both crystalline and amorphous, has been studied for more than a century, especially as it relates to *silicosis*, and recently, the toxicity of silica nanoparticles has been extensively investigated, due in part to the high surface-to-volume ratio of nanoparticles that could potentially lead to enhanced cellular interactions and different pathways of toxicity compared with coarse grained silica[15]. There is a general consensus that toxicity of MSNPs and amorphous silica in general is associated in part with the surface silanol groups, which can hydrogen bond to cellular membrane components or, when dissociated to form SiO^- (above the isoelectric point of silica ~pH 2-3), interact electrostatically with the positively charged tetraalkylammonium-containing phospholipids, both processes leading to strong interactions and possibly membranolysis[24].

Based on the high surface-to-volume ratio of silica NPs, it might be anticipated that they would show in general higher toxicity compared with their bulk counterparts (e.g., crystalline or amorphous). However in the case of MSNPs, the intrinsic porosity of the MSNP surface reduces the extent of hydrogen bonding or electrostatic interactions with cell membranes[24]. Considering both former and latter facts about silica in a nanoparticulate form, it would seem unclear as to the potential toxicity that MSNPs would display. With this in mind, many studies have been performed recently to address this.

Although the porosity of MSNPs should decrease their toxicity due to the decreased surface interaction, studies of the toxicity of MSNPs have shown widely variable ranges of toxicity. One potential reason for the variability in toxicity studies is the surfactant used to template the pores is toxic and variable amounts of this surfactant can remain within the pores of the MSNP depending on the processing[25]. A recent study which used FTIR to confirm that the template surfactant had been removed prior to testing MSNPs for toxicity found survival of all mice treated with up to 1000mg/kg by IV injection and followed for 14 days[26]. The survival of all the animals treated with a very high dose of MSNPs that did not retain surfactant shows the lack of toxicity of the silica framework of the MSNP itself. Potential toxicity is further mitigated by the high drug loading capacity of MSNPs, which greatly reduces needed dosages compared with other nanocarriers. Studies of drug loaded MSNPs in mice have shown that they are well tolerated and demonstrated no histological changes in organs at therapeutic doses such as 1mg/kg IV injection[26]. Mice treated with MSNPs with or without a PEG coating at higher doses, such as 20mg/kg IV injection, also demonstrated no signs of toxicity and no organ damage visible by histology[27]. Additionally, the ability to modify the surface of MSNPs with polymers or lipids will alter and potentially reduce toxicity of MSNPs. Finally, the ability to add targeting will further modify and reduce toxicity as the MSNPs are directed specifically to the target cells or tissues of interest and will have reduced nonspecific interactions within the body as a whole. Regardless, it is important to test all proposed nanocarriers in their final form for toxicity as well as to take into account the highly tunable and variable options presented by the MSNP platform. In addition to toxicity, the biocompatibility of the nanocarrier must also be taken into account. In this area, the porous structure of the MSNPs further enhances their biocompatibility as the high surface area and low extent of condensation of the MSNP siloxane framework promote a high rate of dissolution into soluble silicic acid species, which are found to be nontoxic[25]. The breakdown of the MSNPs overtime into nontoxic species supports the potential of repeat and long term use of the MSNPs to deliver drugs as the MSNP can be cleared from a biological system, overtime, in a nontoxic way. Examination of animals treated with both PEG coated and unmodified MSNPs showed excretion of the silica in both feces and urine[27]. The safety of MSNPs is also supported by the fact that amorphous silica is Generally Recognized as Safe (GRAS) by the FDA. Recently amorphous silica nanoparticle 'C-dots' (*Cornell Dots*) were FDA approved for diagnostic applications in a stage I human clinical trial[28]. The FDA clearance for a clinical trial of silica nanoparticles should accelerate the acceptance of amorphous colloidally derived silica's for applications in medicine.

In Vivo Application of Mesoporous Silica Nanoparticles to Cancer Models

The study of MSNP as nanocarriers has advanced in recent years to studying the capacity of MSNPs to successfully deliver cargos to *in vivo* animal models of human cancers. Some of current studies have focused on the use of the enhanced permeability and retention (EPR) effect found in tumors. Meng *et al.* showed that the addition of PEG to the surface of MSNPs loaded with doxorubicin allowed 12% of the particles to accumulate within a tumor xenograft. In this study, the treatment response, of mice bearing squamous cell carcinoma xenografts, to the PEG coated doxorubicin MSNPs were compared to free doxorubicin, which showed an increased efficacy of the MSNPs versus the free drug. The mice in the study also showed reduced side effects, including reduction in weight loss as well as reduced liver and renal injury from the drug loaded MSNPs versus the free doxorubicin treatment[15]. More recent studies have begun to take advantage of the ability to add targeting moieties to the surface of the MSNPs. He *et al.* targeted polymer coated MSNPs to cervical cancer cells by conjugating transferrin to the MSNPs and increased the uptake of the MSNPs by also conjugating TAT cell penetrating peptide to the surface of the MSNPs. These targeted MSNPs were able to successfully deliver selenocysteine as a synergistic chemo- and radiotherapy agent to cervical cancer xenografts. Selenocysteine is a potential anticancer agent whose clinical development has been hindered by low selectivity, solubility and stability issues, which potentially could be overcome by loading the selenocystine into MSNPs. Mice treated with the targeted selenocystine MSNPs had dose dependant decreases in tumor volume at lower doses than mice treated with free selenocystine, showing the increased efficacy of the targeted MSNPs versus free drug[26]. The use of MSNPs has even been explored for increasing vascular access in difficult cancer types such as pancreatic ductal adenocarcinoma (PDAC). PDAC elicits a dense stromal response that limits the vascular access to the tumor and contributes to chemotherapy resistance. Polyethyleneimine (PEI)/polyethylene glycol (PEG) coated MSNPs containing the TGF-β inhibitor, LY364947, were delivered first to decrease pericyte coverage of the vasculature. The MSNPs were then followed by treatment with liposomes containing gemcitabine, a first line chemotherapy agent. The high loading capacity and pH-dependent LY364947 release from the MSNPs facilitated rapid entry of IV-injected gemcitabine containing liposomes and MSNPs at the PDAC tumor site. This two-wave approach provided effective shrinkage of the tumor xenografts compared to the treatment with free drug or gemcitabine-loaded liposomes only[29]. As shown by these studies, the utility and the variety of MSNPs for increasing drug delivery and specificity is increasing rapidly. As such, MSNPs have promise for decreasing toxicity for many chemotherapy agents and potential for increased efficacy in difficult to treat cancers.

Future Developments

The modular design of mesoporous silica constructs promises a new drug and disease agnostic platform technology for customized delivery and controlled release of multiple types of cargos and cargo combinations. Packaging within MSNP will enable the re-purposing of drugs that have to date failed clinical trials due to poor solubility, high toxicity, and/or susceptibility to degradation. MSNP supported lipid bilayers (so-called *protocells*) have the further advantage that the bilayer can retain and protect fragile and/or highly soluble cargos and enable triggered release of the cargo upon acidification within the tumor or tumor microenvironment. The modularity of the MSNP size, shape, pore size and surface chemistry further suggest applications in personalized medicine requiring individualized cargo combinations, targeting, and release profiles. However the modularity and versatility of MSNP may pose difficulties in pursuing FDA approval as new standardized protocols will be needed to establish structure, cargo content, PK/PD, and degradation profiles.

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Milestones to address these critical areas that researchers should be able to be achieve over the next 5-15 year time frame include many aspects. In the next 5 years, researchers will establish standardized procedures to characterize the physicochemical properties of MSNPs including purity, cargo loading and release, and biodegradation; Determine the size, shape, and surface chemistry dependence of the biodistribution, biodegradation and toxicity (e.g. maximum tolerated dose) of non-targeted MSNP depending on the route of administration and cancer model in small animals and dogs; Demonstrate the *in vivo* performance of targeted MSNP for delivery of multiple types of cargo to tumors and circulating and metastatic cancers in small animals; Perform PK/PD studies of select MSNP and targeted MSNP in small animals to correlate therapeutic efficacy with MSNP nanostructure and cargo loading and release characteristics; and conduct Phase 0 clinical trials of select non-targeted MSNP for delivery of small molecule cargos such as doxorubicin, paclitaxel, or cisplatin and cargo combinations. Looking further ahead over the next 10 years, researchers will conduct phase 0, I, and II clinical trials for select MSNP/cargo combinations and optimize MSNP performance (BD and PK/PD) via re-engineering of physicochemical properties; gain FDA approval of at least one MSNP-based therapeutic; and conduct phase 0, I, and II clinical trials for targeted MSNPs and MSNP theranostics and optimize *in vivo* performance. Looking further ahead over the next 15 years, researchers could gain FDA approval of at least twenty MSNP-based therapeutic systems including targeted MSNP, combination cargos, and theranostics; and conduct phase 0, I, and II clinical trials for personalized MSNPs with individualized cargos and targeting.

In Vivo Self-Assembly/Disassembly of Nanoparticles for Cancer Imaging and Drug Delivery

Jianghong Rao, PhD
Department of Radiology
Stanford University, Palo Alto, CA 94305

Introduction

Nanoparticles have been shown to offer great detection sensitivity because of their unique physical, optical, electrical, and magnetic properties. Enormous efforts have been made in designing and synthesizing a variety of nanoparticles and applying them to cancer imaging. However, translation of nanoparticles-based contrast agents to clinical cancer imaging has been challenging, as summarized in a recent opinion paper authored by the NCI Alliance for Nanotechnology in Cancer Imaging working group[30]. Intravenous infusion is the most common delivery strategy for anticancer therapy or imaging applications. Injected nanoparticles have often met hurdles, such as non-specific uptake by the reticuloendothelial system (RES) and long-term retention in the body leading to chronic toxicity. The tools available to mitigate these effects are limited. A commonly used approach to reducing RES uptake and increasing circulation times is steric stabilization of particle dispersions by polyethylene glycol (PEG) coating. However, long circulation times achieved by PEG-coated "stealth" particles do not necessarily lead to enhanced accumulation deep into tumors because the relatively large size of nanoparticles attenuates transvascular transport and interstitial penetration (**Figure 3** left). To overcome these challenges, nanoparticle design and delivery have to be optimized, which is the main focus of the nanoimaging field. We have been exploring a unique approach to developing novel nanotechnology that will have high translational potential to clinical cancer imaging.

Our new, unique approach explores the concept of directly building nanoparticles inside living cells from small molecular weight building blocks taken up by target cells, as outlined in **Figure 3** (right). Small molecules typically have good transvascular transport and interstitial penetration into tumor (**Figure 3** middle), but unfortunately they are poorly retained at the target site and easily washed out. This new strategy seeks to combine the advantages of nanoparticles and small molecules for cancer imaging and drug delivery. More specifically, small molecules are injected through intravenous infusion, so they will diffuse into the interstitial space after crossing through the vascular vessels in the tumor. To enhance their retention in the tumor, they are activated by tumor-specific biomarkers already present and self-assemble into nanoparticles. At other tissue locations, where the cancer-specific biomarkers are absent, activation and the subsequent self-assembly does

not occur. Thus, the injected small molecules are poorly retained relative to the assembled nanoparticles at the tumor site. This new nanotechnology will help provide solutions to many challenges encountered in nanotechnology based drug delivery and cancer imaging.

Current State in the In Vivo Self-Assembly of Nanoparticles

This concept was first demonstrated in fluorescence imaging of the activity of a furin-like convertase in cell culture[31]. The success was enabled by a novel bioorthogonal reaction between an aromatic cyano group and a 1,2-aminothiol group[32]. The amino and thiol groups are conjugated with a masking group, and only after activation by the target enzyme to generate the free cysteine, will condensation take place to form macrocycles. These macrocycles have very affinity for each other and not the surrounding medium, thus readily self-assemble into nanoparticles. The end result being extended signal enhancement and retention in the local region where they assembled. Two modes have been established in the molecular cascade which enable this nanoparticle self-assembly: intermolecular condensation[31,33,34] and intramolecular cyclization[35–39]. Both initial condensations are specific, and with the subsequent intramolecular cyclization, it is free from any potential competition by endogenous free cysteine[35].

Since then, it has been shown that this approach can be applied to image many molecular targets and is compatible with a range of imaging modalities such as fluorescence[37], photoacoustic[34], magnetic resonance imaging (MRI)[33,38,39], and positron emission tomography (PET)[36]. For example, we have successfully synthesized a [^{18}F]-labeled caspase-sensitive nanoaggregation PET tracer ([^{18}F]-C-SNAT), and have validated it for PET imaging of caspase-3 activity with a doxorubicin-induced tumor apoptosis model in nude mice bearing HeLa tumor xenografts[36]. Using a super-resolution fluorophore, we have directly visualized the assembled fluorescent nanoparticles in apoptotic tumors, and thus fully validated the working mechanism *in vivo*[37]. We have shown that different biomolecules such as caspase-3/7[36–38], furin[32,34,35], beta-galactosidase [unpublished], and redox changes[33,39] can specifically remove the masking groups to trigger the condensation reaction and self-assembly.

These studies have clearly demonstrated that this *in vivo* target biomolecule-triggered self-assembly platform could be transformative for clinical cancer imaging. Because the nanoparticles are generated *in situ* at the cancer target site, the small molecule precursors will not encounter the same challenges faced with current injected nanoparticle-based *in vivo* diagnostic contrast agents. Rather, these nanoparticles are selectively synthesized at the tumor site to enhance imaging contrast.

Notably, a group at Brandeis University has developed a different chemical system, albeit based on the same concept, to generate pericellular and intracellular nanofibers for antitumor activity. The monomers used in this system are small peptides that are highly water-soluble. These small peptides are the substrate of a target enzyme such as alkaline phosphatase found in the cell. Upon the enzymatic processing of the small peptides, they will self-assemble into nanofibers through hydrophobic interactions at a site that is near the enzyme. With respect to their potential efficacy, it has been reported that the formation of nanofibers can lead to death of cancer cells *in vitro* through disruption of the dynamics of microtubules[40].

Another group at the University of Toronto has explored this *in vivo* nanoparticle assembly concept through a biotin-streptavidin interaction[41]. In their studies, poly(ethylene glycol) (PEG)-grafted small nanoparticles bearing biotin and streptavidin-conjugated fluorescent probes are injected sequentially. Both are diffusive and permeable to the tumor vasculature, and upon co-localization, they assemble into nanoaggregates, which is mediated via the strong biotin-streptavidin interaction, and enhance retention at the tumor site.

Future Scientific and Clinical Developments

Our current research has established an *in vivo* self-assembly nanoplatform for cancer diagnostics. To further advance this novel platform, one very critical component would be to introduce a novel design element that would allow for a gradual *disassembly* of the assembled nanoparticles into small molecules again, at the end of imaging. The purpose of this would be to allow

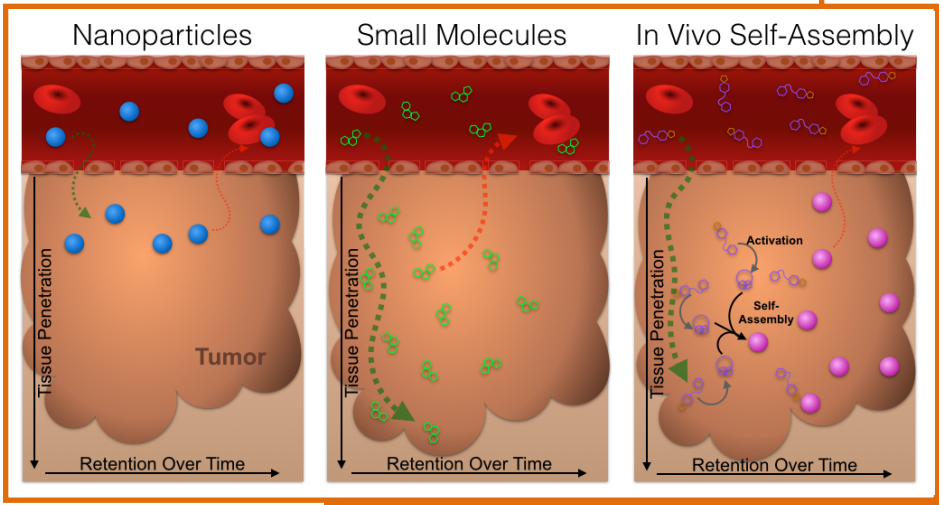

Figure 3. Schematic of transvascular transport and interstitial penetration of three types of intravenously injected materials. Left: nanoparticles cross the leaky tumor vasculature and are trapped well, but poorly penetrate due to its large size. Middle: small molecules (e.g., drugs) diffuse and penetrate deeply, but are poorly retained. Right: a new type of small molecules can be activated to self-assemble into nanoparticles after diffusion and penetration into tumor.

the nanoparticles to be eliminated from the body post-imaging. As such, over the next 5 years, this will be a primary focal point in this field, i.e., to establish *in vivo* disassembling technology and integrate it into the current self-assembling platform for cancer imaging in pre-clinical animal models. This self-assembly/disassembly nanoplatform will be applied to a range of cancer-specific targets and produce a number of imaging probes successfully evaluated in small animals.

In the next 10 years, those most promising Phase 0 candidates should be able to be further translated into human applications in the clinic as they will reach IND stage for clinical testing. It is expected that the unique feature—*in vivo* self-*assembly*/*disassembly* of nanoparticle—of these nanoplatforms should overcome the challenges commonly associated with injected nanoparticles, such as the transendothelial barrier to delivery, and minimize the acute and chronic toxicity, which is the primary reason for an optimistic view of their facile translation to the clinic.

...the small-molecule nature of these agents should present an important advantage for commercialization and large-scale production.

In the next 15 years, some of these agents will gain FDA approval for clinical applications such as cancer diagnosis, patient stratification, treatment monitoring and imaging-guided surgery. Moreover, the small-molecule nature of these agents should present an important advantage for commercialization and large-scale production.

DNA/RNA-Based Nanostructures for Cancer Nanomedicine

Hao Yan, PhD and Yung Chang, PhD
Biodesign Institute
Arizona State University, Tempe, AZ 85287

Nucleic Acid Nanotechnology

Over the past several decades, nucleic acid molecules (DNA, RNA and their chemical cousins and derivatives) have emerged as highly programmable building blocks for nano-construction due to the increasing knowledge of their three-dimensional (3D) conformations and intra- and inter-molecular base pairing interactions[42]. A variety of design rules and assembly methods have been developed to engineer self-assembling nucleic acid nanostructures of increasing complexity[43,44]. DNA nanostructures ranging from periodical lattices to discrete objects of various sizes have been constructed using a rich library of DNA nanostructure motifs and different assembly strategies[43]. DNA origami, a method that uses a number of short, single-stranded DNA (ssDNA) oligonucleotides to direct the folding path of a long ssDNA 'scaffold' strand, has enabled the construction of spatially addressable and geometrically sophisticated 2D and 3D DNA nanostructures with near-quantitative yield[45–47]. As the sister molecule to DNA, RNA has also shown great promise in engineering rationally designed nanostructures. The canonical and non-canonical base pairing interactions, as well as the greater diversity of tertiary structures resulting from a rich library of naturally existing RNA structural motifs, have led to an emerging field of RNA nanotechnology[44,48,49]. Nucleic acid analogs such as PNA (peptide nucleic acid), LNA (locked nucleic acid), GNA (glycol nucleic acid) and TNA (threose nucleic acid), and chemical modifications of nucleic acids have all brought useful properties, including improved chemical, biological and thermo-stability to nucleic acid nanostructures. The structural properties of nucleic acid, which allow it to serve as a versatile construction material, have also been exploited to create dynamic nanodevices ranging from small switchable structures to structures that display complex motions[50]. In addition, logic gates and molecular computing based on nucleic acid building blocks have opened up great opportunities to implement sense-compute-actuate mechanisms into nucleic acid based nanosystems[51]. This is highly desirable for developing intelligent molecular devices for biological and medical research.

Nucleic Acid Nanostructures for Cancer Nanomedicine

The ability to engineer designer DNA nanostructures with high programmability and accurate spatial and dynamic control has allowed researchers to explore novel applications

in cancer nanomedicine. Nucleic acid nanostructures are attractive materials for this purpose, not only because of their inherent design modularity, structural programmability and biocompatibility, but also because nucleic acid molecules of a particular sequence can be modified to selectively bind, distinguish and communicate with target cells to trigger controlled delivery of therapeutic agents. With the development of various chemical conjugation methods, it is now technically feasible and convenient to present functional molecules, such as proteins or peptides, nucleic acids (aptamers, anti-sense RNA, siRNA etc.), inorganic nanoparticles (metallic, semiconducting and magnetic nanoparticles) and organic fluorophores at selected sites on nucleic acid nanostructures for making programmed theranostic devices. For example, researchers recently developed a DNA nano-barrel with single stranded aptamer locks that were opened to expose the loaded antibody cargo only in the presence of target cells[52]. Performing molecular computation directly on the surface of cells, or in cellular environments, will facilitate *in vivo* targeting and drug release. Recently, Rudchenko, Stojanovic and colleagues engineered DNA strand displacement cascades that detected the presence of certain biomarkers on the surface of cells[53]. In another report, Hemphill and Deiters successfully engineered oligonucleotide logic gates to detect specific microRNA inputs in live, mammalian cells[54]. As more complex and robust nucleic acid based computing systems are developed, it may be possible to integrate them into cellular systems to control and trigger cellular functions, such as gene expression, or to interfere with the metabolic pathways. By combining nucleic acid computation-based target cell detection with reconfigurable nucleic acid nanostructure-based drug containers, it may be possible to create a nucleic acid-based nanorobot that can interface and communicate with living cells to develop smart cancer therapy.

A critical step in administering effective drug therapy is the initial delivery of the therapeutic agents into cells. It was found that some nucleic acid nanostructures can be directly and efficiently internalized into live cells without transfection agents[55]. Although the underlying mechanisms still remain to be explored, such cell-penetrating nucleic acid nanostructures, in combination with targeted ligand-receptor recognitions, may lead to the development of universal cellular delivery systems. Pure DNA nanostructures have already displayed higher structural stability and resistance to nuclease digestion[56,57], compared to double helical DNA molecules. Recent studies further demonstrated that enclosing DNA nanostructures with PEGylated lipid bilayers leads to enhanced protection against nuclease digestion with decreased immune activation and significantly improved pharmacokinetic bioavailability[58].

There are several studies that have utilized the unique structural and geometric features of DNA nanostructures to deliver DNA or RNA molecules into cells (**Figure 4**). Examples include the delivery of DNA nanostructure-scaffolded CpG oligonucleotides *in vivo* to trigger immune responses[59] and delivery of siRNA both *in cellulo* and *in vivo* for regulation

of protein expressions[60]. DNA nanostructures carrying chemical drugs such as Doxorubicin have demonstrated great value in not only efficient drug delivery, but also simultaneously circumventing the drug resistance problem in chemical therapy[61].

Several unique properties, such as higher thermostability and synthesis scalability through *in vitro* and *in vivo* transcription, have made RNA-based nanostructures appealing molecular scaffolds for cancer therapy applications. In addition, the chemical stability of RNA nanostructures has been greatly enhanced by introducing chemical modifications such as the 2'-Fluro substitution to the 2'-OH group. It has been shown that a RNA-based nano-scaffold displays favorable pharmacokinetic profiles *in vivo* and shows no toxicity in mice[62]. Exemplified by the utility of the phi29 pRNA nanostructure system, RNA nanoparticles carrying various ligands such as siRNA, micro-RNA, and aptamers have shown great promise in targeted delivery of cancer therapeutics[63]. More recently, a multi-module

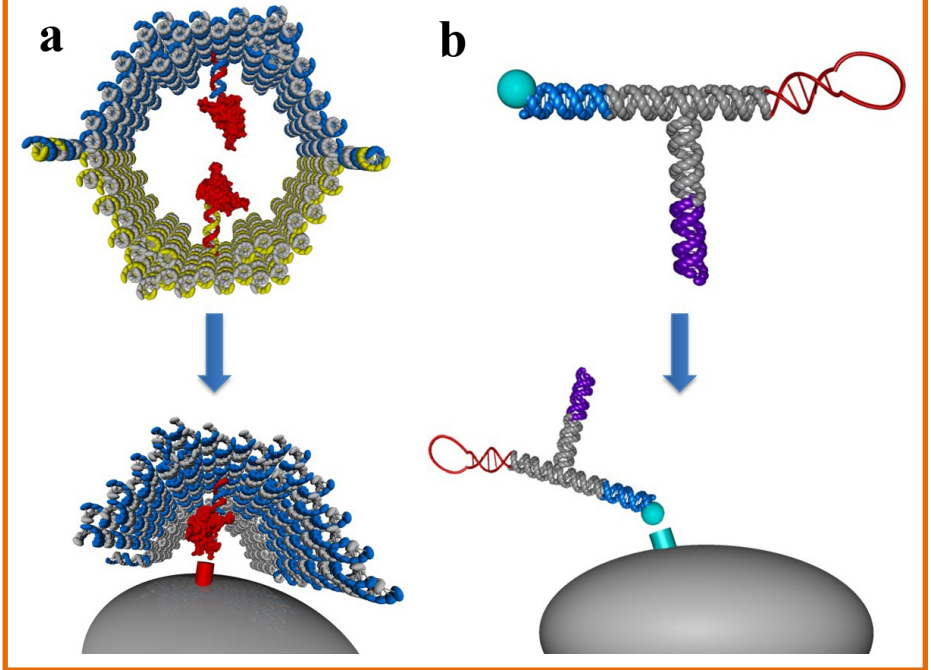

Figure 4. Programmable multi-functional nucleic acid nanostructures for cancer therapeutics. (a) Schematics illustrating the use of a DNA nanocage for targeted recognition of cancer cells. Top: Closed DNA nanocage loaded with an antibody payload. The cage is set to the closed state using structural switching DNA aptamer locks. The aptamers recognize the receptor molecules on the cancer cell surface to trigger the unlocking of the cage to expose the antibody to the target cell. Other payloads, such as chemical drugs, siRNA, and micro-RNA may also be loaded to create multi-functional targeted cancer therapeutics. (b) Illustration of a multi-functional three-way RNA junction motif carrying folate for cancer cell recognition, malachite green dye binding aptamer for cell imaging and siRNA for cancer cell gene expression regulation.

...nucleic acid based nanostructures can also be explored for cancer immunotherapy, ranging from immune activators, tumor-specific vaccines to immunosuppression blockers.

pRNA nanoparticle functionalized with folate acid was constructed to actively target metastatic cancer cells, demonstrating its benefits in treating cancer metastasis[64].

Given the intrinsic adjuvant activity of DNA and RNA molecules, nucleic acid based nanostructures can also be explored for cancer immunotherapy, ranging from immune activators, tumor-specific vaccines to immunosuppression blockers. Initial research in this direction includes the assembly of model vaccines using nucleic acid nanoscaffolds that display multiple immunogenic molecules and deliver immune-stimulating molecules to cells[59]. Yan, Yung and co-workers have demonstrated good immunogenicity of DNA-scaffolded vaccines. With a growing number of immune activators and check-point blockers being identified, one can use nucleic acid based-nanostructures to rationally assemble these molecules for elicitation of stronger and more effective anti-tumor immunity. Thus, the application of nucleic acid based nanostructure platforms for directed assembly of synthetic vaccines and immune modulators has great potential to revolutionize cancer immunotherapy. Furthermore, many chemotherapeutic drugs have been shown to enhance anti-tumor immunity, *via* an induction of immunogenicity of cell death and selective killing of immunosuppressive cells. Thus, programmable nucleic acid based nanostructures are best suited for the development of combined chemo- and immunotherapeutics in our fight against cancer.

Future Developments

To realize the full capability of using nucleic acid nanostructures for cancer research and treatment, several critical issues need to be addressed and carefully investigated. First, although initial studies have shown that some nucleic acid nanostructures (modified or unmodified) do not trigger strong immune responses, the safety of a larger spectrum of nucleic acid nanostructures must be established before practical use in clinical trials, given the adjuvant nature of DNA and RNA. Second, the use of nucleic acid based nanostructures for diagnostic and therapeutic applications rely on the complete clearance or degradation of the nucleic acid nanostructures within a reasonable amount of time. Depending on the type of application, it is important to investigate the bio-distribution, pharmaco-kinetic and dynamic (PK/PD) profiles of the nucleic acid nanostructures so that the nanostructures can be improved to achieve an optimal balance between efficient delivery and sufficient

retention time *in vivo*. Third, a set of design rules and parameters needs to be generalized for the nucleic acid nanostructure geometry, dimension, dynamics of reconfigurability, functionalization and chemical modification to develop the most effective nanodevices for different purposes of cancer therapy (e.g. structures need to be tuned to achieve balanced drug loading capacity and efficient targeted delivery; positions of recognition ligands on the nanoscaffolds need to be optimized to achieve improved affinity with minimized non-specific binding etc.). Fourth, a central obstacle to transforming nucleic acid nanostructures into clinical solutions is the cost of synthetic oligonucleotides. Researchers have made significant progress in producing RNA nanostructures through *in vitro* and *in vivo* transcription[65,66], and replicating small DNA nanostructures *in vivo*[67]. Further efforts are required to develop robust protocols to scale up the production of nucleic acid nanostructures of various designs through transcription, replication or through reducing the cost of nucleic acid oligo synthesis.

Indeed, a great advantage of using nucleic acid nanostructures for cancer nanomedicine is the ability to create multi-functional dynamic nanodevices with high programmability and intrinsic sequence/spatial addressability. There is plenty of room to take full utility of such a unique advantage for cancer nanomedicine. For example, nucleic acid nanostructures hold great potential to design and construct a set of novel, multifunctional, programmable anti-cancer vaccines that are specifically targeted to the tumor and programmed to release anti-cancer therapeutics and immune modulating factors at the tumor site to induce a robust, systemic immune response that will cause a sustained tumor regression. When such designs are integrated with molecular computing and programming, smart molecular doctors and personalized cancer therapeutics are within reach in the foreseeable future. Upcoming breakthroughs would require a multi-disciplinary effort from chemistry, biology, materials sciences, computer science, physics and clinical studies to push the boundaries of this exciting research area.

There is plenty of room to take full utility of such a unique advantage for cancer nanomedicine.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will evaluate the *in vivo* stability, bio-distribution and pharmaco-kinetics for a wide spectrum of nucleic acid nanostructures; identify optimal nucleic acid nanostructures with predictable behaviors *in vivo*; and develop robust and standard protocols to functionalize nucleic acid nanostructures to display therapeutic functions and targeted *in vivo* delivery properties. Looking further ahead over the next 5 years, researchers will evaluate the safety issue of the nucleic acid nanostructures which have demonstrated optimal *in vivo* behaviors;

develop multifunctional nucleic nanostructures and validate their initial uses in targeted cancer therapy and cancer vaccine development; and develop methods to scale down the cost of nucleic acid nanostructures and standardize protocols to make high yield synthesis of homogenous nucleic acid nanoparticles with designed functionality. Over the course of the next 10 years, researchers will conduct clinical trials of a variety of nucleic acid nanostructure-based cancer therapeutics; and integrate nucleic acid nanostructure-based therapeutics with molecular computing and programming to develop smart therapeutics in response to the cellular and tissue environments of various cancer and cancer matastasis.

Cooperative Nanosystems

Sabine Hauert, PhD[1-4] and Sangeeta N. Bhatia, MD, PhD[1-3]
[1]Harvard–MIT Division of Health Sciences and Technology, [2]David H. Koch Institute for Integrative Cancer Research, and [3]Institute for Medical Engineering and Science
Massachusetts Institute of Technology, Cambridge, MA 02139
[4]Engineering Mathematics Department
University of Bristol, Bristol BS8 1TR, UK

More Than the Sum of Its Parts

Bioengineers are currently designing increasingly sophisticated nanoparticles that can deliver treatments and diagnostics selectively to tumors[68,69]. Much of the field's focus has been on engineering the functionalities of individual nanoparticles to improve their transport[70], to target them to the tumor vasculature[71,72] or extracellular matrix[73], to deliver therapeutics[74,75], diagnostics[76], or heat[77,78] to the tumor environment, and to reprogram cancer cells[79] or the immune system[80]. However, the behavior of each nanoparticle depends not only on its design (size, shape, charge, material, cargo, and coating), but also on the interactions that occur in the body as a result of these design components. Thus, it is the collective, or 'systems' behavior of trillions of such nanoparticles interacting in a complex tumor environment that will define their success as diagnostic or treatment agents[81].

Predicting and engineering these collective nanoparticle behaviors is empirical and not always intuitive. For example, nanoparticles that are optimized to strongly bind and accumulate in cancer cells may mostly build up in the most proximal cells they encounter after leaking into the tumor environment. The resulting collective behavior is poor tissue penetration, leaving deep seeded tumor cells untreated[82–84]. Weaker nanoparticle binding, although detrimental to the function of the individual nanoparticle, could still lead to a better outcome by the system as a whole. Further engineering these behaviors on the level of single nanoparticles could result in emergent cooperative behaviors typically seen in self-organized systems[85].

Self-organized systems in nature, including those formed by social insects, animals, and cells, are able to perform complex behaviors through the local interactions of many simple agents and their environment[86–89]. The field of swarm robotics[90,91] has long taken inspiration from nature to engineer minimal robots that use simple rules to interact with their neighbors and local environment to solve complex real world problems[92–95]. Cooperative behaviors relevant to nanomedicine applications include amplification, optimization, mapping,

structure assembly, collective motion, synchronization and decision-making. By tapping into the field of swarm engineering, we may be able to produce behaviors that go beyond the functionalities of the individual nanoparticles and towards efficient, modular, and predictable system-based outcomes.

State-of-the-Art in Cooperative Nanosystems

Nanoparticles can cooperate implicitly, directly through self-assembly and disassembly, or through stigmergy (**Figure 5**). These behaviors have been useful to improve nanoparticle transport, accumulation, and distribution in tumor tissues towards development of treatment and diagnostic applications.

Most nanoparticle systems implicitly cooperate, in which each nanoparticle is designed to optimize its individual functionality[96]. The collective impact of the nanoparticles as treatment or imaging agents is assumed to be the sum of the independent nanoparticle effects. Understanding the system level behavior of implicit cooperators may add insight that can improve outcome predictions. Emphasis could be placed on studying whether the nanoparticles can collectively distribute throughout a tumor environment or accumulate at effective levels in, or around, targeted cells[70]. Similarly, combination therapies aimed at preventing resistance can be composed of different types of nanoparticles that independently target varied signal pathways, or even subpopulations within the tumor[97–99].

In addition to implicit cooperation, nanoparticles that physically interact harbor a more direct means of cooperation. Nanoparticles in this class of particles typically self-assemble or disassemble to modify their kinetics, or to collectively transport combined treatment and imaging agents to tumors. For example, rapidly diffusing imaging agents are able to anchor in tumors by binding to previously injected gold nanoparticles that have been given time to accumulate outside the vasculature via the EPR effect[40]. Similarly, small (10 nm) gold nanoparticles engineered to release conjugated doxorubicin in acidic tumor environments can subsequently self-assemble to form larger gold aggregates that are then available for use in photothermal therapy[100,101]. *In vitro* experiments reveal that nanoparticles capable of self-assembly in response to enzymatic activity may be able to perform logic computations towards the diagnosis of tumor state[102]. In another example, larger nanoparticles (100 nm) are able to disassemble into smaller nanoparticles once inside the tumor environment in response to enzymatic activity, thereby improving their circulation time, accumulation in the tumor, and ability to penetrate deep in the tissue[103]. Other multi-stage nanoparticles such as nested nanoparticles, mother ships, and nanocells are all able to overcome transport barriers through the release of nano-based components in tumor environments[104–106].

In contrast to collective behaviors mediated by direct interactions between nanoparticles, many swarm systems found in nature communicate by modifying the environment. This concept is called stigmergy[86]. Ants deposit and sense chemical signals to form trails that lead to sources of food[87]. Termites are able to build complex structures by modifying and locally sensing their physical environment[94]. In a similar way, nanoparticles have been designed to modify their physical environment or deposit signals. Gold nanorods that accumulate in a tumor, upon heating to sub-lethal temperatures with NIR light, can improve perfusion of angiogenic vessels and in some cases upregulate receptors used in targeting, which in turn improves the delivery of a second wave of nanoparticles, such as liposomes and magnetic nanoworms, to tumors for treatment and imaging purposes[107,108]. Gold nanorods heated through NIR light can also cause a clotting cascade in tumors[109]. This biological cascade serves as a signal to communicate the location of the tumor to circulating nanoparticles, thereby leading to a 40-fold increase in the amount of chemotherapeutic delivered to the tumor when compared to a non-communicating system[109]. Nanoparticles that aim to normalize the vascular bed, or degrade the extracellular matrix can improve the transport of secondary nanoparticles[110,111]. Nanoparticles can also be designed to release either a cargo or energy, which can directly interact with neighboring nanoparticles. As an example, gold nanorods activated through NIR light emit heat in tumors to trigger the release of chemotherapeutics contained in thermally sensitive drug carriers[112].

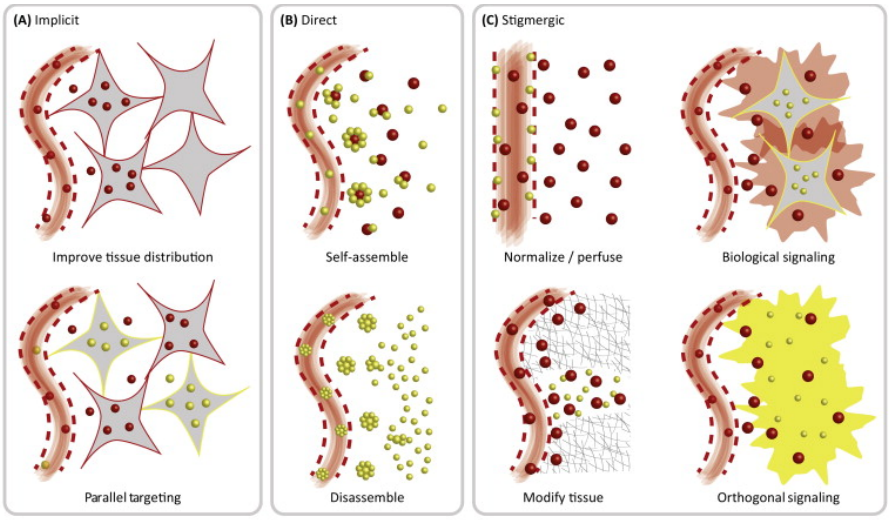

Figure 5. Mechanisms of cooperation in cancer nanomedicine. Nanoparticles can cooperate implicitly to improve their tissue distribution, directly through self-assembly and disassembly to change their distribution, or by communicating through the environment (stigmergy). Using stigmergic interactions, nanoparticles can impact perfusion or tissue density to improve the delivery of secondary nanoparticles. They may also communicate by initiating a biological cascade that can be sensed by other nanoparticles, or send an orthoganal signal (energy, chemicals) to activate secondary nanoparticles. (*Images and text reused with permission, Hauert and Bhatia, 2014*).

Systems Nanotechnology

The practice of engineering and predicting the collective behavior of large numbers of nanoparticles that interact in complex tumor environments is typically non-intuitive, even for simple nanoparticle designs. By harnessing a systems approach, bioengineers could start by automatically exploring potential nanoparticle designs using crowdsourcing (http://nanodoc.org) and machine learning[113], then modeling the resulting collective behavior in simulation[70,82,83,114], followed by testing the best candidates experimentally through fast prototyping of both the nanoparticles[115,116] and their environment[117], and finally validating the collective behaviors *in vivo* with feedback on their outcome provided by high resolution imaging[118]. Through this systems-based process (**Figure 6**), we expect nanoparticles to become more robust in their ability to react to environmental feedback by changing their motion and trajectory, thereby achieving increasingly swarm-like behaviors. Growing expertise in control of nanomaterials, achieving a deeper understanding of cancer biology, and ongoing advances in the modeling and automation of nanosystems are all contributing to the field's first steps in this direction.

More broadly, we anticipate that lessons learned from efforts made to design cooperative nanosystems will also prove useful in the engineering of naturally swarming biological components, such as cells of the immune system[119] or synthetic bacteria[120] in order to improve tumor treatment and diagnostics.

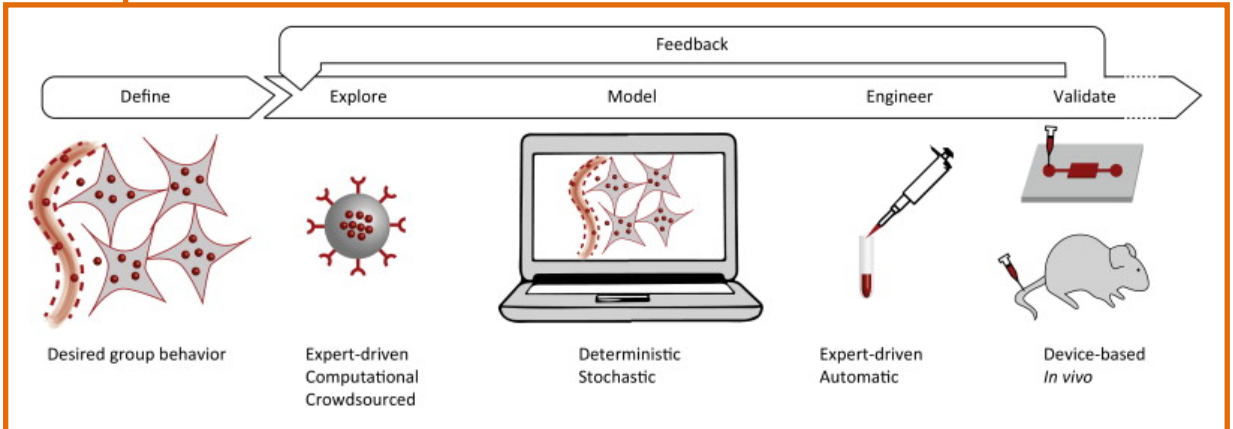

Figure 6. Systems approach to the design of cooperative nanomedicine. Starting from a desired group behavior, tools are needed to explore possible nanoparticle designs, model their resulting cooperative behaviors in simulation, engineer the nanoparticles, and validate them *in vitro*, and *in vivo*, before clinical translation. (*Images and text reused with permission, Hauert and Bhatia, 2014*).

Multimodal Imaging Constructs

Moritz F. Kircher, MD, PhD
Department of Radiology
Memorial Sloan-Kettering Cancer Center, New York, NY 10065

Introduction

With the aim in mind to create molecular imaging beacons that can be "seen" by multiple imaging methods, nanoparticles have several key advantages over small molecule contrast agents: (1) It is possible to integrate multiple contrast agents into the a single nanoparticle, and therefore combine their complementary strengths (e.g., whole body imaging and high resolution during intraoperative imaging). It is not possible, however, to simply mix the contrast agents together and expect reasonable signal to be generated for each modality. Most contrast agents require a particular environment to achieve optimal performance. Nanoparticles are small enough so they can be tuned to reach tissues of interest, but also large enough so that the particular needs of each contrast agent can be met within the same particle. (2) Their size range is ideal so that they can be coated with a variety of surface modifying moieties. These moieties can range from antibodies, affibodies, peptides or small molecules in order to induce binding of the particles to a specific target of interest. Here, the clustering of a large number of such targeting moieties on the relatively small surface of the nanoparticle can amplify their targeting abilities via multivalency effects. Nanoparticle surfaces can also be passivated with other moieties (e.g., polymers), through which one can influence and fine-tune the blood half-life and overall whole body biodistribution. (3) Nanoparticles can also be "armed" with many different therapeutic functions, be it that they deliver drugs at the target site or that they serve as photothermal agents that can destroy tumor cells via heat induction.

Current State for Multimodal Imaging Via Nanotechnology

There has been significant progress in the design and application of multimodal nanoparticles since 2010. One of the first nanoparticles that were in clinical trials for imaging purposes are superparamagnetic iron oxide nanoparticles (SPIONs)[121,122]. While several different versions with slightly different chemical compositions were in clinical trials for lymph node imaging with MRI these never received full FDA approval, and were subsequently taken off the market[121]. It is well known, however, that the iron contained in SPIONs is incorporated into the iron pool of the human body upon degradation of the particles, and the formulation as a nanoparticle can be more efficient than elemental iron in replacing iron in humans. This lead to the FDA approval in 2009 of a modified formulation

(Ferumoxytol) for the treatment of iron deficiency anemia in adult patients with chronic kidney disease. While not yet approved for imaging purposes, this has lead to a renaissance of clinical studies using SPIONs as an MRI contrast agent (e.g., NCT01336803). Given the many preclinical studies that used SPIONs as a platform for multimodal imaging, such as by adding a fluorochrome or radiotracer, this also rekindles the hope that such multimodal nanoparticles will eventually receive approval for diagnostic imaging purposes[123,124].

Several nanoparticle therapeutics made of other materials such as gold, silica or both, are currently in advanced stages of clinical trials[125]. These advances are not only representing milestones in the field of nanotherapeutics, but also increase the likelihood of nanoparticles of similar size and composition to be approved for imaging purposes. In fact, in 2010 the FDA approved an IND for the first in human testing of so-called 'Cornell dots' or C dots (NCT01266096). C dots are silica nanoparticles that are less than 8 nm in size, contain fluorochromes in their core, and can be functionalized with radiotracers for PET imaging for dual modality detection of melanoma metastases[28]. This was the first time that the FDA approved a clinical trial using an inorganic material in the same fashion as a drug in humans.

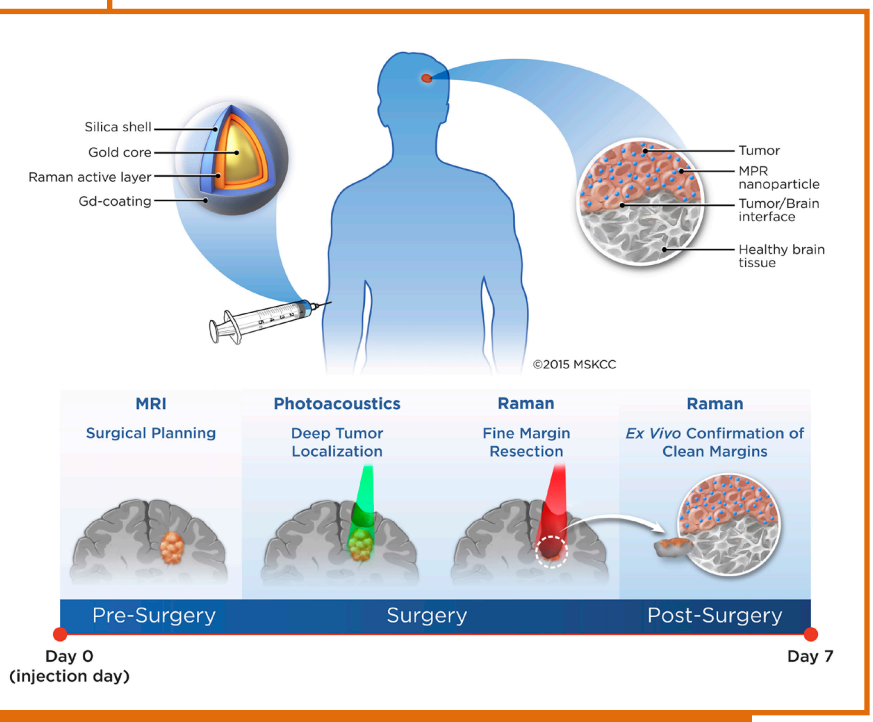

Figure 7. Principle of a triple-modality MRI-photoacoustic-Raman nanoparticle and its envisioned clinical use. The nanoparticle is injected intravenously. In contrast to small molecule contrast agents that wash out of the tumor quickly, the nanoparticles are stably internalized within the brain tumor cells, allowing the whole spectrum from preoperative MRI for surgical planning to intraoperative imaging to be performed with a single injection. T1-weighted MRI depicts the outline of the tumor due to the T1-shortening effect of the gadolinium. During the surgery, photoacoustic imaging with its greater depth penetration and 3D imaging capabilities can be used to guide the gross resection steps, while Raman imaging can guide the resection of the microscopic tumor at the resection margins. Raman could also be used for rapid confirmation of clean margins in the operating room instead of the time-consuming analysis of frozen sections.

Major advances have also been made in the

preclinical arena, of which only few can be mentioned in this short summary. These comprise improvements to existing modalities, integration of multiple modalities into the same nanoparticle, and the establishment of new imaging modalities. As an example of the latter, "surface-enhanced Raman scattering" (SERS) nanoparticles were shown for the first time to allow imaging of cancer and image-guided tumor resection[126]. It was also shown that such SERS nanoparticles could be transformed into multimodal molecular imaging agents, by adding detectability from both MRI and photoacoustic imaging. This triple-modality approach was developed, with the goal in mind, to perform more precise brain tumor imaging and image-guided resection (**Figure 7**). While the MRI capabilities allow for preoperative planning, intraoperative photoacoustic imaging can provide a surgeon with a roadmap for the gross resection steps, while SERS imaging indicates whether or not the tumor tissue has been completely resected at the microscopic level[126,127]. Because SERS provides such a specific signal (Raman "fingerprint"), it is ideally suited for high precision cancer imaging. This has more recently been demonstrated with a new generation of "surface-enhanced resonance Raman scattering" (SERRS) nanostars that are orders of magnitude brighter and allow imaging of microscopic disease in multiple different cancer types[128,129]. New synthetic protocols now allow the creation of multiple layers of silica, each fine-tuned in thickness and each containing a different contrast agent (patent pending). This principle allows incorporating a large number of contrast agents into the same nanoparticle, while also allowing optimal placement of each contrast agent within the particle architecture. For example, a SERS reporter has to be placed as close as possible to the noble metal core, while a fluorochrome has to be placed at a certain distance to avoid quenching of the fluorescence. An MRI contrast agent is ideally placed at the nanoparticle surface to allow interaction with water molecules. This principle is illustrated in **Figure 8**.

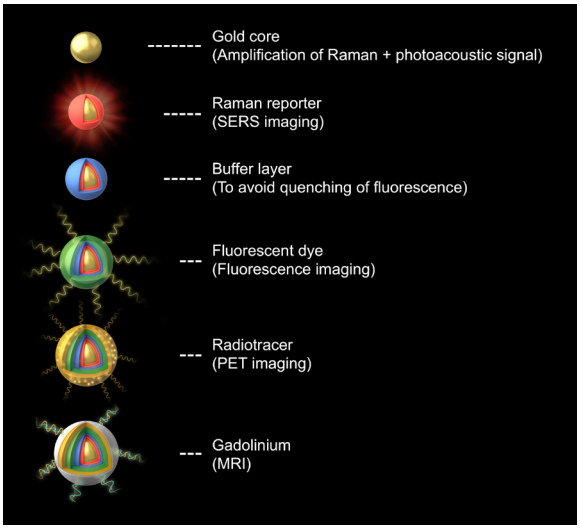

Figure 8. Synthesis of multimodal nanoparticles via a multilayer silication method. Addition of multiple layers of silica with finely tuned thickness as a strategy to incorporate many different imaging modalities into the same nanoparticle, while optimizing the signal intensity of each modality.

Future Challenges in Multimodal Imaging

The main challenge for nanoparticle imaging agents is and remains the regulatory approval by the FDA. Multimodal nanoparticles are facing significantly greater hurdles in the approval process than small molecule agents that would suffice for isolated PET, CT, MRI or fluorescence imaging. The most difficult hurdle for nanoparticles that are not small enough to be cleared via the kidneys is that sufficient proof has to be presented to the FDA that the retention of the nanoparticles in the body does not represent a health risk. Most intravenously injected nanoparticles are cleared from the blood by the organs of the reticuloendothelial system, such as the liver, spleen and lymph nodes, and are retained in these organs for extended amounts of time. In the case of SPIONs, Ferumoxytol has proven to be degraded over time, which facilitated regulatory approval. For those nanoparticle compositions that do not degrade or are eliminated from the body over time, it has to be shown that the retention does not cause any adverse effects. To this end, the recent development of novel artificial organoids that closely recapitulate human organs might offer a great avenue to accelerate such studies without having to risk the health of human patients.

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Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will conduct large animal studies of currently available multimodal imaging agents; initiate more clinical trials; and continue the development of next generation nanoparticle imaging agents. Looking further ahead over the next 5 years, researchers will test the newest generations of multimodal nanoparticles in artificial organs, which are expected to exist by then and should facilitate the translation into the clinics; and complete the currently ongoing clinical trials, analyze results and detail the lessons learned. In the next 10 years, multiple clinical trials should have been completed, including those that originated from initial testing in artificial organ systems. This should give a good indication about how well toxicity profiles can be predicted from studies in artificial organ systems, with the hope that parts of the current phases of the FDA required clinical trials can be replaced with testing in those novel model systems.

Theranostics: Smart, Multi-Functional Materials for Diagnosis and Therapy

Jinwoo Cheon, PhD
Department of Chemistry
Yonsei University, Seoul, Korea

Overview

Current orthodox in the treatment of cancer involves surgical resection of large tumor areas followed by non-selective radiation therapy or chemotherapy. Such procedures can cause severe side effects from their non-specificity for tumor cells and concurrent damage to the immune system, rendering patients susceptible to other diseases. Moreover, the cancer frequently returns in refractory forms, resistant to current therapeutic approaches. Owing to the lack of effective late-stage cancer therapies, early detection and appropriate treatment is critical.

For the past two decades, the interesting and unique nanoscale delivery model and its respective tools have proven to be effective in medicine, especially in the field of cancer research and oncology. There has been much work to harness the tunable physicochemical properties of nanomaterials for diagnosis and therapy, such as real time visualization of cells/tissues and the precise delivery of therapeutic molecules to the targeted area. The diagnostic properties of nanomaterials (e.g., high plasmonic effect, enhanced MRI contrast effect, strong fluorescence, etc.) can enable early detection of small-sized tumors with exceptionally high sensitivity[130,131]. Furthermore, the multivalent characteristics of various nanomaterials allow for accurate tumor-specific imaging with the aid of a targeting moiety and synergistically integrated multi-modalities[132,133]. The improved targeting ability has also been advantageous from a therapeutic perspective, by which nanomaterials can selectively deliver therapeutic molecules to the tumor site, thereby increasing the therapeutic efficacy and reducing required dosages to minimize unwanted side-effects[71].

The distinct advantage of nanomaterials over conventional small molecules is their tunable physicochemical properties. Their size, shape, composition, and surface control can be adjusted to optimize their application in diagnosis and therapy. For example, rationally designed nanomaterials with specific dimensions and appropriate surface characteristics (e.g., neutral PEG and zwitterion) can circulate in blood vessels for a long time without opsonization by evading detection from macrophages and preferentially accumulate in tumor tissues via extravasation[134–136]. When incorporated with targeting moieties, the nanomaterials can be even more accurately delivered to the tumor site.

These phenomena are used for tumor-specific imaging (e.g., iron oxide for MR imaging and gold for highlighting tumor borders during brain surgery). As a method for enhancing diagnostic accuracy, multi-modal imaging (e.g., PET-CT and PET/SPECT-MRI) using different complementary modalities has been widely studied[133,137]. For example, nanoparticles functionalized with radioisotopes, known as multi-modal nanoparticles, have the potential to enhance diagnostic accuracy by increasing sensitivity of detection and adding the precision of anatomical localization[138]. Recently, magnetic particle imaging (MPI)-MRI demonstrates the potential for real-time visualization of tumor and cancer-related events (e.g., angiogenesis) with nano-molar sensitivity and anatomical details[139,140].

For therapy, the most promising and common application of these phenomena is the transportation of drug molecules. One example is BIND®, a targeted therapeutic nanoparticle, which in clinical trials has effectively reduced tumor sizes at lower doses than traditional chemotherapy[141]. The nanoparticles hold the chemodrugs without leakage during circulation and release them only upon reaching the targeted tumor. Some types of nanomaterials have additional therapeutic capabilities, such as the transformation of external energy to heat (e.g., iron oxide for magnetic fields and gold for light). These heat-generating therapies are known as photothermal ablation and magnetic hyperthermia, and they have been effectively used in cancer treatments[137,142]. The hyperthermia-based therapy has regulatory approval in 27 European countries[143].

Following treatment, nanomaterials can also be utilized to assess treatment efficacy and aid in making a prognosis (e.g., complete removal, regrowth, or metastasis of tumor). Nanosystems that can provide real-time diagnosis, in tandem with therapy and/or prognosis using multi-functional nanomaterials, are called *theranostics*. Research to combine the diagnostic and therapeutic characteristics of nanomaterials within a single platform, is being actively pursued. Currently, a wealth of research is being conducted in this area to improve cancer diagnosis and therapy. However, it is still only at the initial stages of the developmental pipeline.

Clinical Significance

From a diagnostic point of view, real-time monitoring of cancer-indicative markers (e.g., from genes and/or proteins) would allow for the administration of preemptive medicines at the moment pre-cancerous symptoms are found. A nanoparticle pill that Google is currently developing is a representative example of real-time monitoring[144]. When patients swallow a pill containing magnetic nanoparticles decorated with biomolecules for the identification of cancer or heart disease, the nanoparticle can detect and report signs of targeted disease through a wearable device. This proactive monitoring concept can switch the treatment

paradigm from the curative to the preventive. Even in cases where prevention fails, there is still a large benefit to early cancer detection. It keeps more effective treatment options available, which offers the best opportunity to be cured.

From a therapeutic point of view, the targeted delivery of therapeutic molecules to a tumor using nanomaterials can potentially enhance the efficacy of therapy and significantly reduce systemic toxicity, such as that experienced with Abraxane®, the FDA-approved paclitaxel albumin-stabilized nano-formulation[145]. When combined with the imaging capabilities of nanomaterials, the therapy can be monitored for maximum accumulation time, effective release of the drug, and the patient's response to treatment. This in turn allows for more informed decision-making on timing, quantity, type of drugs, and choice of treatment procedure, as well as an evaluation of an individual's response to treatment. This could be the basis for the future of personalized cancer treatment.

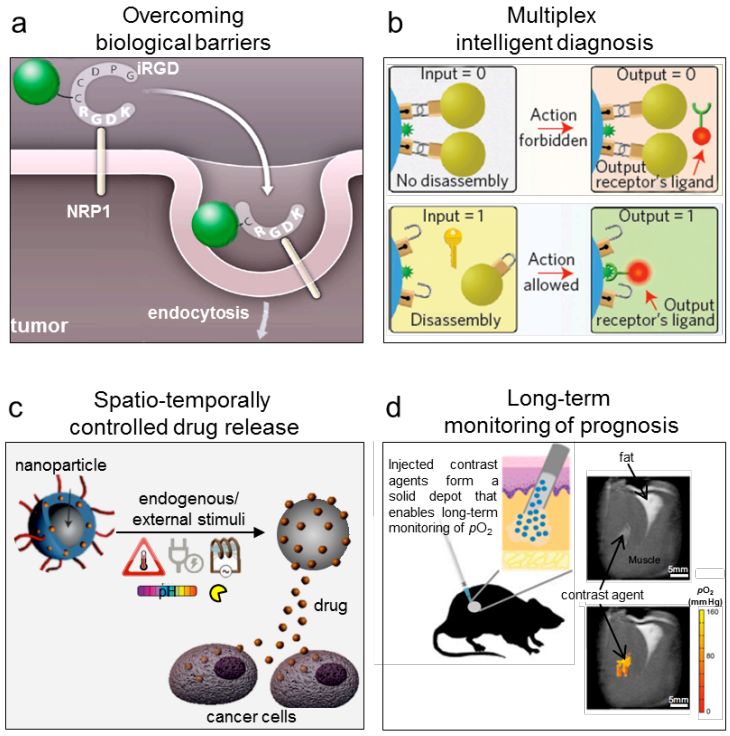

Figure 9. Challenges for future theranostic nanomaterials. (a) Nanomaterials should possess capabilities to overcome hurdles in tumor-specific delivery. One possible approach can be iRGD which allows nanomaterials to access a tumor by penetrating endothelial and tumor tissues. (b) Nanomaterials delivered to tumors should provide comprehensive information about tumor microenvironments. Logic-performing nanomaterials enable smart diagnostics by detecting and processing multiplexed molecular signatures. (c) Based on diagnostic information, nanomaterials should initiate spatio-temporally controlled therapy in response to external or endogenous stimuli. (d) After completing therapy, the non-toxic nanomaterials can be left inside the body and continuously give prognostic information (e.g., oxygen level). (*(a) Reprinted with permission from Feron, 2010; (b) from Nikitin et al., 2014; (c) from Mura et al., 2013; and (d) from Liu et al., 2014*).

Future Challenges

Although current theranostic nanomaterials have great potential, next-generation design concepts and their effective implementation strategies are required (**Figure 9**). Future nanosystems should be able to pass through biological barriers (e.g., BBB, hypoxic tumor regions, stroma, etc.) to reach any tumor sites of the body. One possible approach can be integrating nanomaterials with functional

peptides (i.e., tumor-penetrating peptides) which allow the nanomaterials to reach deep inside an extravascular tumor[146,147]. Magnetic targeting might be another potential solution if the magnetic force exerted on the nanomaterials can be made strong enough to overcome the drag force of blood flow[148,149]. This requires precise control of the direction and intensity of the applied external magnetic field.

When the theranostic nanomaterials arrive at the target site, they should provide quantitative and comprehensive information on the multiple molecular signatures of cancer cells. Current single target-specific imaging and qualitative sensing are not adequate for accurate diagnosis because tumorous environments are complex and heterogeneous[150]. Therefore, nanomaterials should be developed to have multiplexing and logic capability that detects numerous molecular signatures and intelligently reports them to us for accurate diagnostic results[151]. Considering the expression level of those signatures, such diagnostic nanomaterials should possess high sensitivity (e.g., at least pico-molar) for cancer-related biomolecule detection[126].

The nanomaterials have to be designed to sensitively and precisely respond to the corresponding stimuli.

After the diagnosis, spatio-temporally controlled therapeutic action should only start upon reaching the target region in order to lessen collateral damage. The remote trigger of the action can be either multiple and logical combinations of endogenous tumor microenvironments (e.g., pH and enzymes), or exogenously controlled physical stimuli (e.g., light and electromagnetic field)[152,153]. The nanomaterials have to be designed to sensitively and precisely respond to the corresponding stimuli. Simultaneous or sequential execution of therapeutic methods from one nanomaterial also needs to be pursued to overcome cancer resistance (e.g., multidrug resistance)[154]. Finally, when the therapy is complete, the remaining nanomaterials need to be able to assess the treatment's efficacy and aid in making a prognosis[155]. They should of course be fully biodegradable or clearable over time, and in order to meet regulatory requirements, their safety should be ensured for prolonged use through investigation of their clearance (e.g., renal and biliary routes, etc.).

Milestones to address these critical areas that researchers should be able to be achieve over the next 5-15 year time frame include many aspects. In the next 5 years, researchers will establish new sets of design principles to control physical, chemical, structural, and biological properties of nanomaterials for improved sensitivity and specificity in tumor microenvironment monitoring, cancer detection, and therapeutic effect; understand

sub-cellular level interactions between nanomaterials and cancer cells for effective tumor targeting; and evaluate the diagnostic and therapeutic effectiveness of developed nanomaterials by employing *in vitro/in vivo* models. Looking further ahead over the next 10 years, researchers will devise nanomaterials that overcome the biological barriers that limit accessibility to tumors; create nanomaterials with optimal circulation time for enhanced tumor accumulation with minimal off-target effects; endow a multiplexing capability to nanomaterials to identify multiple targets for diagnostic imaging/therapy in real-time; verify the ability to reproducibly initiate therapeutic activity only at tumor/cancer cell sites *in vivo*; and determine nanomaterial safety by characterizing biodistribution, PK/PD depending on size, shape, surface chemistry, etc. In 15 years, researchers will have optimized the theranostic properties of nanomaterials, specifically for prevention/early-detection of cancer, monitoring of cancer heterogeneity, and significant increment in therapeutic index; establish nano-regulatory with industries and the FDA; and make several highly effective nanotechnology based imaging and/or therapeutic agents in the late stage of clinical trials or in the market.

Theranostics: Targeted Theranostics in Cancer

Lily Yang, MD, PhD
Department of Surgery
Emory University School of Medicine, Atlanta, GA 30322

Introduction

The major challenges in the effective treatment of cancer patients are low efficiency in drug delivery and intrinsic drug resistance in highly heterogeneous human tumors[156,157]. Chemotherapy drugs have short blood half-lives and limited amounts of drugs can be delivered into tumors despite high doses of drugs being administrated to patients that cause severe systemic toxicity. Therefore, improvement of drug delivery into tumor cells should be one of the most important strategies for enhancing therapeutic responses in human cancer.

At present, nanoparticle formulated chemotherapy drugs, such as Doxil (liposome encapsulated doxorubicin) and Abraxane (paclitaxel-albumin protein complex), are FDA-approved nanotherapeutic agents for drug delivery into tumors, which utilize the enhanced permeability and retention (EPR) effect mediated by leaking tumor vessels[158–160]. Various non-targeted or targeted liposome and polymeric nanoparticle drug carriers are in preclinical developments and clinical trials[75,161]. Although those nanotherapeutics have shown promising anti-tumor effects and reduction in systemic toxicity in animal tumor models and in cancer patients, lack of novel approaches for timely assessment of efficiency of intratumoral drug delivery and response remains an issue. It is well known that human tumors are heterogeneous in vasculatures, tumor stromal components, and abnormalities of tumor cells, which contribute to significant differences in physical barriers for drug delivery and intrinsic barriers in drug sensitivity. Therefore, effective cancer therapy not only requires new drug delivery approaches, but also personalized evaluation of drug delivery and the subsequent early tumor response, in individual patients, using noninvasive tumor imaging. This 'precision' version of oncology would make it possible to maximize effectiveness of therapeutic agents by selecting the most efficient drug delivery approach while simultaneously minimizing systemic toxicity through timely replacement of ineffective therapeutic agents.

Current advances in the development of multifunctional nanoparticles with the abilities of targeted drug delivery and imaging intratumoral drug accumulation and distribution, i.e., *theranostics*, offer a unique opportunity for the integration of targeted and image-guided cancer therapy using a single nanoparticle platform[162,163]. First, imaging properties allow for

determining whether a cellular target is expressed by tumors and if this targeted approach is able to deliver sufficient nanoparticles into a specific tumor by non-invasive imaging (**Figure 10A**). In so doing, the cancer patients with the highest likelihood of a clinical response to the targeted theranostic nanoparticle can be selected. This is particularly important for patients with tumors, which are not easily accessible for biopsy. To overcome drug resistance, two or more therapeutic agents can be loaded to a single nanoparticle for targeted delivery into tumor cells, simultaneously, to enhance the synergistic effect of the drugs. This approach has clear advantage over conventional combination chemotherapy since drug molecules with different chemical properties vary in their pharmacokinetics, bioavailability, and stability. Encapsulation or conjugation of drugs to theranostic nanoparticles will significantly improve the blood half-lives of drugs, and protect drug molecules from binding to serum proteins and becoming inactivated by enzymes, leading to targeted delivery of large amounts of active drug molecules into tumor cells.

Following systemic delivery, non-invasive imaging modalities, such as MRI, PET, ultrasonic, photoacoustic, and optical imaging, can be used for determining nanoparticle-drug delivery efficiency (**Figure 10B**). Using an imaging modality with high resolution and anatomic information, it is feasible to monitor early tumor responses following targeted therapy to identify imaging signatures that predicate a good or poor response such that ineffective drugs will be replaced with more potent therapeutics in a timely manner (**Figure 10C and D**). Finally, targeted delivery of multimodal imaging theranostic nanoparticles enables intraoperative detection and removal of drug resistant tumors using image-guided surgery (**Figure 10E**).

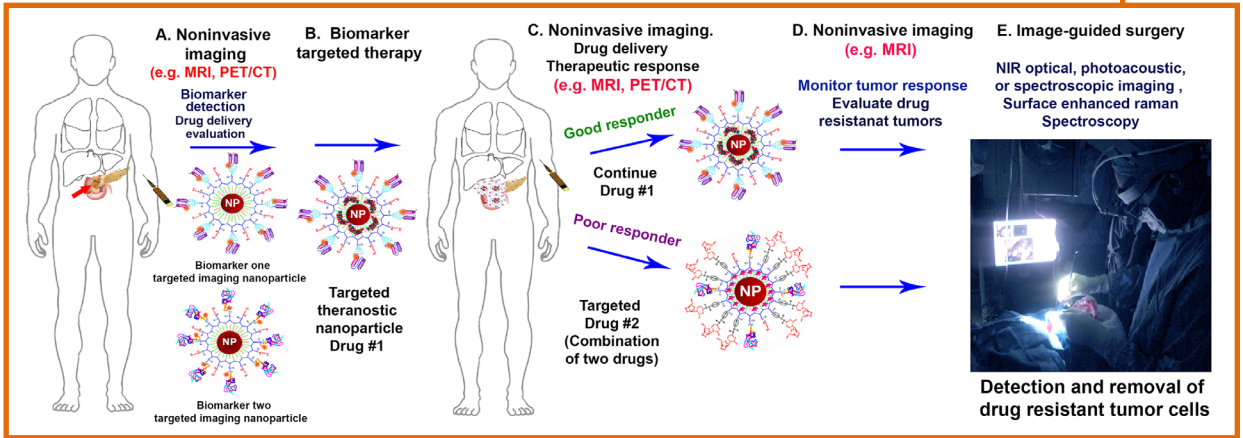

Figure 10. Clinical paradigm for theranostic nanoparticles. An outline of steps [A-E] along the clinical path of which theranostic nanosystems would display their inherent importance in oncology.

The development and translation of image-guided and targeted therapy using theranostic nanoparticles have clinical significance in the treatment of several aggressive cancer types, such as triple negative breast, pancreatic, ovarian, lung, colon, and liver cancers. For example, neoadjuvant chemotherapy has been given to triple negative breast cancer (TNBC) patients before surgery. About 22% of TNBC patients showed a good therapeutic response (pathologic complete response) and an excellent prognosis[164]. TNBC patients with drug resistant tumors following neoadjuvant therapy have a high incidence of tumor recurrence and a poorer survival. Image-guided neoadjuvant therapy using theranostic nanoparticles will allow for the selection of more potent therapeutics for individual patients while reducing systemic toxicity. Additionally, the integration of image-guided and targeted therapy using theranostic nanoparticles offers the possibility of reduction of tumor burdens of un-resectable pancreatic cancers, including over 50% of pancreatic cancer patients with locally advanced diseases[165], for potentially curative surgery. Optical image-guided surgery enables for complete removal of drug resistant tumors in those patients. Therefore, success in the development of targeted theranostic nanoparticles and innovative imaging approaches has the potential to change the paradigm of future clinical management of cancer patients.

The importance of theranostics in cancer therapy has promoted rapid advances in the development of various types of theranostic nanoparticles.

Current State of the Art

The importance of theranostics in cancer therapy has promoted rapid advances in the development of various types of theranostic nanoparticles. However, challenges in the development of such a class of multifunctional nanoparticles are well recognized. As a drug carrier, it is necessary to select nanomaterials that are biodegradable with low toxicity even after repeated administrations at high doses. It requires high drug loading and conditional drug release in tumor cells. Production of strong and lasting imaging signals is also required. Active targeting to cell surface receptors highly expressed in tumor cells is critical for increasing not only drug delivery into tumor tissues, but also into tumor cells by endocytosis. Theranostic nanoparticles targeting multiple cell types in the tumor, such as tumor endothelial cells, stromal fibroblasts and macrophages, and tumor cells have been shown to enhance intratumoral delivery of targeted nanoparticles[166]. Examples of the cellular receptors that are highly expressed in tumor stromal and tumor cells are uPAR, IGF-1R, folate receptor, and integrin αvβ3. Several examples of cellular receptors that are highly expressed in tumor cells include EGFR, HER2, MUC1, and CEA.

Theranostic nanoparticles have been produced by conjugation and encapsulation of radiotracers to nanoparticles for PET imaging or gadolinium for MRI[167]. Those approaches are used for converting liposomal, polymeric, silica, and dendrimer nanoparticles into theranostic agents. PET/CT detects targeted delivery of radioisotope labeled nanoparticles with high sensitivity. However, repeated administrations of large amounts of radioactive agents and exposure to high doses of ionizing radiation in combination with CT imaging are the major concerns. Relatively short half-lives of radioisotopes require the theranostic nanoparticles to be administrated into the patients in a short time after labeling with radiotracers. This also makes it difficult to monitor therapeutic responses, which often take days or weeks.

Near infrared (NIR) fluorescent dye conjugated or encapsulated nanoparticles are promising optical imaging probes for image-guided surgery, which represents another theranostic application. The effect of pH-sensitive or protease-activated polymeric nanoparticles carrying NIR dyes on identification of tumor margins for surgical resection has been demonstrated in animal tumor models[168,169]. Results from a recent clinical trial using RGD peptide conjugated ultra-small fluorescent silica nanoparticles labeled with a radiotracer (iodine) showed that it is safe for systemic administration in human melanoma patients and the nanoparticles were cleared through renal excretion[28].

Metallic magnetic iron oxide and gold nanoparticles are commonly used theranostic nanoparticle platforms in preclinical studies. Biodegradable magnetic iron oxide nanoparticle (IONP) with MRI contrast is one of the most promising theranostic nanoparticles for clinical translation. Therapeutic agents are conjugated to or encapsulated in the surface coating of the nanoparticles. Targeted theranostic IONPs have been developed and their effects on tumor growth and MRI of nanoparticle-drug delivery have been demonstrated in preclinical studies[170–172]. In comparison with other imaging modalities, MRI has imaging depth and high-resolution 3D-imaging capability for interrogation of heterogeneous intratumoral drug distribution. IONPs can serve as both T_1 and T_2 contrast agents depending on the core sizes and MRI scan methods[173–175]. IONPs are relatively stable in the tumor for an appropriate length of time for monitoring tumor responses to therapy by MRI. In combination with clinical contrast enhanced MRI imaging signatures of the early tumor response may be identified. A drawback of MRI is relatively high costs. Further

...MRI has imaging depth and high-resolution 3D-imaging capability for interrogation of heterogeneous intratumoral drug distribution.

improvements of T_1-contrast imaging approaches should increase sensitivity and specificity of detecting small tumor lesions in organs with a low MRI contrast, such as the liver and lung. Targeted IONPs conjugated with NIR dyes can be used for intraoperative detection of drug resistant tumors[166,176].

Theranostic applications of gold nanoparticles have been developed[77,177]. Targeted delivery of gold nanoparticles generates plasmonic photothermal bubbles that promote drug release from nanoparticle drug carriers in the endosome of cells[178]. Although gold-based theranostic nanoparticles have been produced and tested in animal tumor models, there is a concern about its low biodegradability and lack of a well-defined mechanism of clearance following systemic delivery in large therapeutic doses.

A multi-spectral imaging approach using a Raman endoscopic imaging device and tumor targeted surface-enhanced Raman scattering (SERS) gold nanoparticles has been developed for cancer detection and image-guided resection. Feasibility of multiplexed tumor imaging using SERS has been demonstrated in animal tumor models and in excised human colon tissues[179]. Image-guided hyperthermia treatment using NIR signals produced by photosensitizing agents conjugated to metallic nanoparticles has also been tested in animal tumor models[180]. Accumulation of the nanoparticles in tumors allows for image-guided therapy by precisely applying a laser to the tumor sites.

Future Science and Clinical Development

Clinical development of theranostic nanoparticles has to address challenges that are common for all cancer therapeutics and nanoparticle drug delivery systems as well as unique requirements for its dual therapeutic and imaging applications. Research areas that may have the most impact on clinical translations includes: (1) Development of ultra-small and biodegradable nanomaterials with high imaging signal strengths, high drug loading capacity, and conditional drug release ability; (2) Innovative targeting approaches and nanoparticle designs that significantly enhance passive and active targeting for intratumoral drug delivery, avoid non-specific uptake by macrophages, and have the ability of overcoming tumor stromal barrier for improving drug delivery into tumor cells; (3) Combined delivery of potent therapeutic agents for the treatment of drug resistant tumors; and (4) understanding mechanisms of nanoparticle-drug delivery and interactions of targeted theranostic nanoparticles with tumor cells and tumor microenvironment in animal tumor models that are highly relevant to human cancers, such as human patient tissue derived xenograft (PDX) tumor models and transgenic mouse tumor models. Finally, large-scale production of Good Manufacturing Practices grade theranostic nanoparticles for human use will be the major challenge. It requires the production of consistent nanoparticle core and coating, efficiency

in drug loading, and conjugation of large amounts of endotoxin-free and bioactive targeting ligands to the nanoparticles.

With the joint efforts of the NCI Alliance of Nanotechnology for Cancer and investigators at academic institutes and within industry, several advances should come to fruition over the upcoming 5-15 year time frame. In the next 5 years, researchers will complete preclinical studies for 5 to 6 targeted theranostic nanoparticle platforms; File IND applications for 3 to 4 of the above nanoparticles for Phase I clinical trials; and begin 2 phase I clinical trials for image-guided surgery using targeted imaging nanoprobes. Looking further ahead over the next 10 years, researchers will generate 3 to 4 new theranostic nanoparticles and image-guided cancer therapy protocols in Phase 1 clinical trials; 1 to 2 Phase II/III clinical trials using an integrated image-guided and targeted therapeutic clinical protocol for personalized cancer treatment; and receive FDA approval of 1 targeted imaging nanoparticle for image-guided surgery. Even further out over the next 15 years, researchers will complete 1 to 2 Phase 11/III trials; gain FDA approval of 1 theranostic nanoparticle and associated image-guided therapy protocol; and initiate 5 to 6 new clinical trials using theranostic nanoparticles and image-guided treatment protocols.

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Section IV: *In Vitro* Empirical Models to Understand *In Vivo* Response

Nanostructured Materials as Models for Cell Motility and Metastasis

Daniela Kalafatovic, PhD and Rein V Ulijn, PhD
Advanced Science Research Center (ASRC)
City University of New York, New York, NY 10031

Introduction

Metastasis, i.e. cancer cells migrating from the primary tumor to a distant site in the body, where secondary tumors develop, is a major contributor to mortality[1]. Despite progress, many questions remain unresolved regarding the mechanisms involved. It is now clear that it is not just the cells, but also their environment - and in particular the dynamic interplay between them - that dictates whether metastasis is likely to occur. Thus, there is a need for well-defined model systems that enable determinants of metastasis to be studied systematically. We summarize recent breakthroughs and future opportunities for nanostructured materials to contribute to this area.

Metastasis, adhesion and migration

Stages of the development of metastases (**Figure 1**) can be summarized as follows: (1) detachment of cancer cells form the primary tumor by reduced adhesion to neighboring cells; (2) invasion through surrounding tissues by clearing the path to allow cell migration; (3) intravasation of cells through the vasculature to enter the bloodstream and remaining in circulation under flow; (4) attachment to endothelial tissue and subsequent extravasation to the secondary site; (5) proliferation and establishment of secondary tumor[2]. Changes in interactions of cells with their environment, typically adhesion and migration, are critical at every step. Adhesion in this context can refer to cell-cell and/or cell-matrix (ECM) interactions. Migration for our purpose can be either adhesion-dependent or -independent, and may involve active matrix degradation by cell-secreted or cell-surface expressed enzymes- typically matrix metalloproteases (MMPs). Interestingly, there is a substantial body of literature focused on the use of model systems to show how biochemical, mechanical and topographical signals in the cell's environment (typically focusing on stem cells[3]) influence cell fate. The development of exactly such in vitro model systems is now gaining pace for cancer metastasis research.

Designed 3D matrices as model systems to study metastasis

Designed nanostructured materials with precisely tunable properties that mimic aspects of the extracellular environment have the potential to lead us to a better understanding of the role that the tumor microenvironment plays in triggering metastasis[4]. It is now well established that 3D models are more relevant to mimic the tumor/metastasis microenvironment *in vivo*[5]. Commonly used matrices are naturally derived, including commercially available 3D culture systems such as Matrigel™, collagen gels or fibroblast-derived matrices. These materials can be informative as model systems- for example, collagen scaffolds were used to study and identify MMP independent migration pathways relevant to metastatic invasion[6]. Recognizing that natural ECM possesses a highly complex 3D organization that dictates function (which is currently impossible to mimic), matrices have been prepared by decellularizing of various tissues in order to preserve the native integrity of ECM and explore its ability to influence metastasis[7]. While effective in certain contexts, these naturally derived materials are unlikely to reveal molecular level understanding of cell-matrix interactions, as natural systems are not fully defined, have variable compositions, cannot be easily tailored and often contain biologically active materials (e.g. growth factors).

A range of synthetic materials have therefore been developed that can serve as a 'blank canvas' upon which bioactive groups can be rationally introduced. Typically, 'base' materials are selected which have seen previous use in biomedical context, such as poly-ethylene glycol (PEG), poly(lactic-co-glycolic acid) (PLGA) and poly-ε-capronolactone. Synthetic peptide-based materials such as commercially available Puramatrix™ are simplistic mimics of the ECM, which allow for cell culture under well-defined conditions. A number of designs of such self-assembling systems have been developed over the years, typically involving building blocks of 8-20 amino acid residues that can be easily functionalized with bioactive peptides. More specifically for the three primary components necessary to study metastatic disease, we discuss the current state-of-the-art for each.

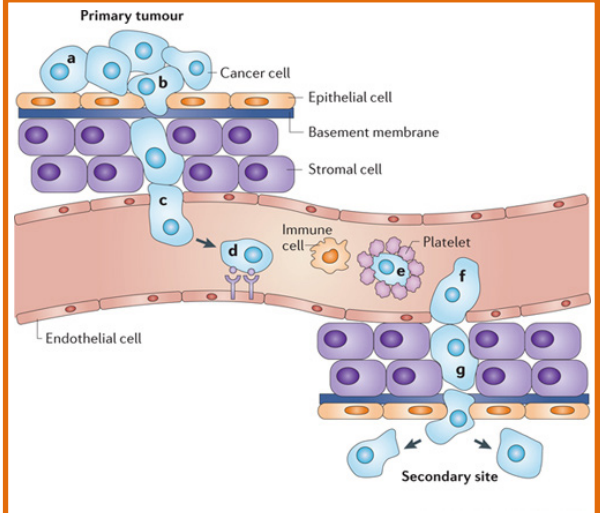

Figure 1. (*Reprinted with permission from Schroeder et al., 2012*)[2].

Adhesion

Adhesion typically involves integrins, the trans-membrane portion of focal adhesions that connect the cytoskeleton inside the cell to the extracellular matrix on the exterior. They bind to bioactive ligands in the surrounding matrix, such as the tri-peptide

RGD (arginine-glycine-aspartic acid). Introduction of RGD ligands into synthetic polymers is now straightforward using well-established polymerization techniques. There is much scope here for the inclusion of different ligands beyond RGD. For example, when using PEG-based hydrogels functionalized with adhesion peptides RGD and YIGSR (the integrin-adhesive regions of fibronectin and laminin, respectively) it was found that cancerous and non-cancerous mammary epithelial cells responded differentially to the adhesion cues[8]. Methods are now also available to introduce bioactive ligands and even entire proteins in precisely defined rations in self-assembled peptide materials[9].

In addition to the concentration of bioactive ligands, their presentation (spatial orientation, clustering) is critical. Questions about spatial organization can be addressed using precisely patterned ligands on surfaces, which may be achieved utilizing block copolymer micellar nanolithography. This approach has been used to demonstrate adhesion dependence with varying distance between RGD ligands, which in turn influenced melanoma cell fate[10]. While this is a 2D approach, the information that is obtained may be used to inform spacing of ligands in 3D constructs. In addition to static presentation of RGD ligands, a number of approaches are now available to dynamically regulate adhesion using switchable RGD ligands (by photolytic uncapping of protected precursors)[11]. These approaches have not yet been used in the context of metastasis and hold great promise in controlling temporal presentation of bioligands.

Migration

Cancer cell migration makes use of a combination of adhesion and enzymatic degradation, involving MMPs and hyaluronases (although non-enzymatic migration is also known[6]). The first designed PEG based gels crosslinked by MMP cleavable peptides were described over a decade ago[12]. Introduction of MMP cleavable linkers in PEG gels was recently used in a metastasis model. A PEG-heparin hydrogel was described that mimics the tumor angiogenesis microenvironment by incorporating RGD (adhesive), MMP-9 responsive (matrix degradation) and glycosaminoglycan (bioactive building block) motifs to take into account different metastasis characteristics[13].

Stiffness

Matrix stiffness is a known determinant of cell fate[3]. Methods are now available to tune this parameter precisely in PEG based materials as well as synthetic self-assembled peptide structures. An example is the use of collagen coated polyacrylamide hydrogel systems with tunable stiffness to study the metastatic potential through matrix stiffness induced epithelial to mesanchymal transition (indication of cancer cell invasiveness)[14]. The effects of

bio-adhesion and matrix mechanics could be investigated separately by varying either the cross-link density or ligand concentration in a gel that also included MMP degradable linkers. Results were shown to be similar to that observed in matrigel, demonstrating that key cell behaviors can be accurately mimicked in fully synthetic gels[15].

Future aspects and conclusions

We note that designed nanomaterials could be used in conjugation with microfluidics, providing access to confined environments while under flow[16]. This would enable (i) mimicry of extravasation[17]; (ii) development of structures for the efficient capture of circulating tumor cells (CTCs)[18] or (iii) study of the interactions of CTCs with endothelial barriers[19].

Tumors contain a variety of cell types (stromal, immune, in addition to tissue specific cells) so accurate mimicry of the microenvironment would require the presence of mixtures of cells. Key to fully understanding migration and invasion will be the development of microscopy techniques. This could include visualization of the invasive protrusions associated with metastasis e.g. using super-resolution (STED) microscopy. This could be combined with FRET approaches to monitor MMP activity and cell migration in real time.

Looking out 10 years, it is highly likely that researchers will be able to use this information in the clinical translation of nanomaterial based models to new materials based therapies.

Clearly, a wide range of synthetic and natural materials, processing and functionalization methods is currently available to create *ex vivo* models to study aspects of metastasis. What is missing, are fully designed model systems, that could mimic all critical aspects of the tumor microenvironment in a more controlled way, opening up opportunities to rationally and systematically vary environmental factors and discover which ones dominate. Not only are designed nanomaterials likely to provide new insights, they can also inform new therapies. There are tremendous opportunities for nanoscience to design artificial (synthetic) cell-compatible hydrogels as models to study metastatic cancer.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will be able to develop tunable scaffolds (stiffness, ligand incorporation, degradability) based on self-assembled structures as models to study each step of metastasis; biological findings

will inform materials design and, by close collaboration between cancer experts, chemists, materials scientists and engineers, new models should be developed to investigate specific aspects of metastatic disease; and superresolution fluorescence microscopy to visualize invasion. Looking further ahead over the next 5 years, researchers will be able to deliver specific, optimized matrices for establishment of secondary tumors; and a quantitative comparison of new *in vitro* models with current animal models. Looking out 10 years, it is highly likely that researchers will be able to use this information in the clinical translation of nanomaterial based models to new materials based therapies.

Microfluidic Models to Study Cell Extravasation and Metastasis

Roger Kamm, PhD
Biological and Mechanical Engineering
Massachusetts Institute of Technology, Cambridge, MA 02139

Introduction

Metastatic cancer remains the leading cause of mortality. While there have been considerable advances in the development of new approaches to the treatment of cancer, the control of metastasis is still one of the major challenges[20,21]. Despite its tremendous importance, a fundamental understanding of the processes that constitute the metastatic cascade remains elusive. As a result, there are few therapeutic approaches available to block the various steps of metastasis. Two factors contribute significantly to this glaring deficiency. First, modern animal models of metastatic disease[22,23,24], although responsible for much of what we have learned, provide inadequate insight into the disease process for lack of the ability to image the details of cancer progression, and because of the limited ability to control and monitor the local chemical and mechanical environments. In addition, there the inevitable questions regarding differences in behavior between cells from humans and those from test animals still exist. Second, the existing *in vitro* models using traditional cell culture methods such as well-plate systems and transwell assays[25], are unable to capture many of the key features that regulate the various stages of metastasis. The gap between *in vitro* and *in vivo* models is considerable, and both have severe limitations.

Further contributing to this knowledge gap is the enormous complexity of the metastatic cascade, which consists of multiple steps: local invasion of cells from the primary tumor into the surrounding tissue, entry into the circulation by intravasation, survival and transport via circulation to a remote site, extravasation into the metastatic site, and finally, recolonization (**Figure 2**)[26]. The challenges to producing a realistic *in vitro* model of any of these steps are enormous, yet recent progress in the development of microfluidic assays capable of 3D culture of multiple cell types, some with an intact endothelial monolayer, has given rise to optimism.

In the past several years, considerable progress has been made. This is largely due to projects funded through the new emphasis by the NCI on assay development and the physical aspects of cancer growth and invasion. And, although we are still at the early stages, advances have been impressive.

Current capabilities

Recent progress has resulted from new capabilities in several strategic areas, and advances in microfluidic technologies have enabled many of these. New approaches and models have appeared within the past decade, both in the context of primary tumor and metastasis[25], although for this chapter, we focus attention exclusively on the latter, with an emphasis on extravasation. Microfluidic assays typically consist of multiple channels or regions containing hydrogels with spatial arrangement and dimensions that facilitate chemical and mechanical signaling among various cell types seeded within the interconnected compartments. The goal of these devices is in creating a local microenvironment among the cellular compoents that replicates many aspects of *in vivo* interaction[25]. For some time, it has been possible to culture cells in 3D microenvironments, simulating the extracellular matrix of tissues[27]. Progress in 3D culture subsequently led to numerous studies in cell migration[28] and the culture of tumor spheroids with microvessels[29]. Studies have examined the role of various cytokines, including spatial concentration gradients, on the initiation of dispersion from a tumor, in some cases documenting the cells' transition from an epithelial to mesenchymal state (EMT)[30]. The capability to suspend cells in 3D and to generate gradients of either chemoattractants or hydrostatic pressure across matrix-containing regions has facilitated new studies on 3D migration[31], and the effects of matrix properties[32], other interacting cell types within the matrix[33], and interstitial flows such as exist at the tumor margin or in the vicinity of blood or lymphatic vessels[34].

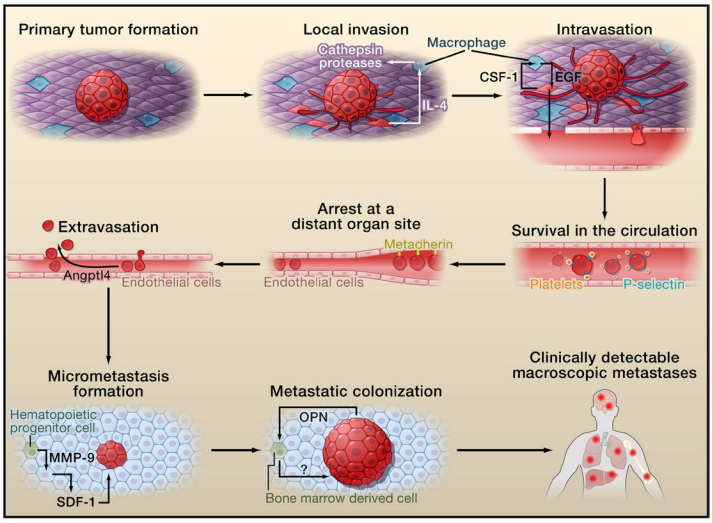

Figure 2. The metastatic cascade. From primary tumor to clinically observable metastases (*Reprinted with permission from Valastyan and Weinberg, 2011*)[26].

When one or more of the channels is lined with an endothelial monolayer, a model for intravasation can be produced by inducing cells seeded into the adjacent matrix to transmigrate into the channel[33]. Similarly, tumor cells introduced into the channel can adhere to the endothelium and transmigrate into the adjacent gel region, mimicking the process of extravasation into the remote host tissue[35]. In some cases, a microvascular network has been established within the gel region that can be perfused with a tumor cell-containing medium, leading to even greater realism in that the tumor cells can then either adhere to or become lodged in the smaller vessels, as they

would in the capillaries of the target organ[36]. Recent studies have also begun to introduce certain organ-specific cells into the matrix, demonstrating that the different rates of extravasation of a particular type of cancer can be replicated within relatively simple *in vitro* systems[37,38].

Future challenges

The use of microfluidics to model metastasis has been rapidly accelerating, but many barriers remain. One of the greatest challenges is to progressively improve the realism of the model while at the same time, keeping it sufficiently simple to use so that these methods remain accessible to the broader cancer research community. In the case of the primary tumor microenvironment, the introduction of cancer associated fibroblasts and tumor associated macrophages, along with the cells of the local microvessels will further enhance the realism of the models. Similarly, the addition of organ-specific stromal cells to models of the remote, metastatic organ will be an important step. Aside from the cellular environment, the matrix properties also need to be carefully considered, since the current choice of type 1 collagen, fibrin or even Matrigel has a significant influence on behavior. Most researchers currently use cell lines, but these should eventually give way to patient-derived tumor cells, and even to the potential for patient-derived induced pluripotent stem (iPS) cells for the creation of more realistic models.

Development of patient-specific models holds the potential for direct clinical application of microfluidics.

One of the greatest current limitations of microfluidics is that the cell numbers and volumes are small, thus making it difficult to employ many of the traditional biochemical or genetic analyses to probe cell function. Methods need to be developed for improved interrogation of the systems (e.g., protein analysis, RNA-seq) including the capability of real-time monitoring of signaling factors or cell function, beyond what can currently be accomplished by imaging.

As researchers expand to model other tissue types, new challenges will emerge. The difficulties in generating a realistic model of the blood-brain barrier are well recognized. Creating models of other organs such as those with high cell densities and intricate internal structural organization – liver, kidney, pancreas – will remain one of the most difficult problems to overcome.

Clinical potential

Development of patient-specific models holds the potential for direct clinical application of microfluidics. Use of iPS cell based systems, patient-derived explants, circulating tumor cells extracted from patient blood, or other similar models will eventually lead to the ability to screen for a therapeutic protocol that is optimized for each patient. In the context of metastasis, this implies an approach that would reduce the tendencies for the primary cancer to spread and recolonize. In addition, improvements in usability and increases in throughput will ultimately facilitate the transition into the clinic, and enable moderate to high throughput screening for combination therapies.

Milestones to address these critical areas that researchers should be able to be achieve over the next 3-10 year time frame include many aspects. In the next 3 years, researchers will have been able to develop many more organ-specific models of metastasis; and patient-specific assays for drug selection based on surgical or biopsy specimens. Looking further ahead over the next 5 years, researchers will be able to deliver multiple organ models on a single chip; high-throughput drug screening platforms; and potentially metastatic cancer-on-a-chip. Looking out 10 years, it is highly likely that researchers will be able to deliver iPS cell based models for patient specific drug screening in the clinic as well as, the really important milestone of, point-of-care assays for diagnosis and treatment planning.

In Vitro Models of the Blood-Brain Barrier

Peter Searson, PhD
Department of Materials Science and Engineering, School of Medicine, and Institute for NanoBioTechnology
Johns Hopkins University, Baltimore, MD 21218

Introduction

The blood-brain barrier (BBB), or neurovascular unit, is a complex dynamic system responsible for providing nutrients and essential molecules to power the brain while at the same time ensuring that signaling in the brain is not disrupted by fluctuations in chemistry, inflammation, or the entry of toxins or pathogens[39,40]. The blood-brain barrier maintains homeostasis by transducing signals from the vascular system and the brain, and comprises the brain microvascular endothelial cells (BMECs) that form the 600 km of capillaries, the basement membrane, and surrounding pericytes, astrocytes, and neurons. For example, the brain regulates oxygen supply by signaling via astrocytes, which have end-feet that completely surround the capillaries.

The highly specialized endothelial cells that form the lumen of microvessels and capillaries in the brain are characterized by high transendothelial electrical resistance (TEER > 1000 Ω cm^2), low permeability, expression of tight junction proteins (e.g. claudin-5 and occludin), transporters (e.g. LAT-1), and broad spectrum efflux pumps (e.g. P-gp). The two main components of the blood-brain barrier security system are the tight junctions and the efflux pumps. The formation of tight junctions at the boundaries between endothelial cells almost completely prevents paracellular transport into the brain. The array of broad-spectrum efflux pumps, primarily on the luminal surface, returns almost all non-essential small molecules back into circulation. Notable exceptions are caffeine, alcohol, and anesthetics. A consequence of this security system is that it is extremely difficult to deliver drugs to the brain following oral or intravenous administration. More than 98% of small molecule drugs and 100% of large molecule drugs do not cross the blood-brain barrier[41]. As a result, there are many diseases of the brain for which there are no drug treatments. Treatable brain disorders are limited to depression, schizophrenia, chronic pain, and epilepsy.

Recently it has become recognized that many diseases of the brain are associated with disruption of the blood-brain barrier[40]. While the details of these disruptions are not well understood, they most likely result in local increases in permeability that can lead to the disruption of signaling.

Current state of In Vitro BBB Models for Translational Development.

In the pharmaceutical industry and in academic research, the initial screening of drugs for treatment of central nervous system (CNS) diseases is performed using the transwell assay where the permeability of a drug is determined from the amount that crosses a monolayer of type II Madin-Darby Canine Kidney cells (MDCK.II)[42]. These are dog kidney epithelial cells and not human brain endothelial cells although this represents state-of-the-art in the field of pharmaceutical development for CNS drug therapies. MDCK cells transfected to express different efflux pumps can be used to assess whether molecules are substrates for these pumps. In many cases permeability coefficients obtained from the transwell assay are in reasonable agreement with brain perfusion studies in animal models, although the correlation to humans is not well understood. The transendothelial resistance and hence paracellular transport can be decreased by seeding astrocytes and pericytes, or astrocyte extract, in the basolateral compartment of the transwell chamber, highlighting the importance of these cells in the neurovascular unit[43].

A fundamental problem in BBB research is that animal-derived cell lines and immortalized human BMECs do not fully recapitulate the characteristics of human BMECs. For example,

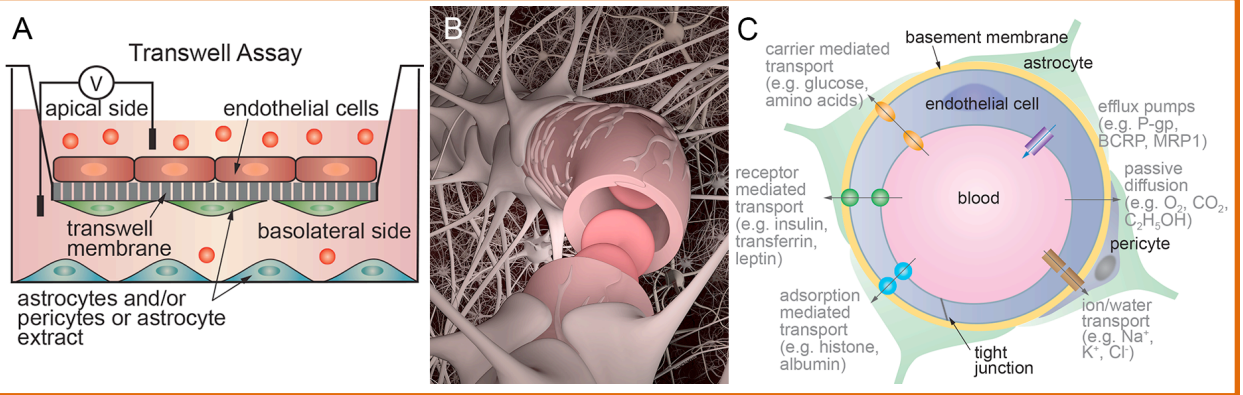

Figure 3. (A) The transwell assay is the standard in vitro tool for determining the permeability of a solute across the blood brain barrier. MDCK cells are widely used since they express tight junction proteins. Paracellular transport can be minimized by seeding astrocytes and/or pericytes, or astrocyte extract in the basolateral chamber. (B) The blood-brain barrier is modulated by functional interactions between brain microvascular endothelial cells, astrocytes, pericytes, and neurons, mediated by the 3D extracellular matrix and basement membrane. Shear flow in the microvessels and the high curvature also play a role in upregulating the blood-brain barrier phenotype. (C) The highly specialized endothelial cells in the brain are characterized by tight junctions that effectively limit paracellular transport, transporters that supply nutrients and other essential molecules, and an array of efflux pumps that return most solutes that cross the luminal membrane back into circulation.

the TEER values of MDCK monolayers are typically around 200 Ω cm^2, almost an order of magnitude lower than physiological values for the brain microvasculature (≈ 2,000 Ω cm^2). The disadvantages of primary hBMECs are that they are not readily available and lose some of their characteristics when cultured *in vitro*. Similarly, the distribution of efflux pump expression varies across species resulting in very different concentrations in the brain. Therefore the lack of physiologically relevant cell lines is a major limitation to advancing the field[44].

The traditional *in vitro* approach to screening drugs for cancer therapy is to assess efficacy by incubating the drug with the relevant cancer cells in culture, and then to assess permeability and brain penetration using the transwell assay (**Figure 3**). In recent work, the transwell assay has been modified to screen drugs for cancer therapy by seeding patient-derived glioma cells in the basolateral compartment and using a live/dead assay to assess efficacy. This approach mimics the pharmacokinetics by exposing the glioma cells to a concentration of the drug that is modulated by blood-brain barrier transport[45].

Recent developments suggest that stem cell engineering may be a solution to the lack of physiological endothelial cells for blood-brain barrier research.

Recent developments suggest that stem cell engineering may be a solution to the lack of physiological endothelial cells for blood-brain barrier research. Human brain microvascular endothelial cells have been derived from induced pluripotent stem cells[46,47]. The derived cells express relevant tight junction proteins, transporters, and efflux pumps, and treatment with retinoic acid results in TEER values in excess of 2,000 Ω cm^2. While more extensive characterization of these derived cells remains to be accomplished, these results could revolutionize the field.

Future of In Vitro BBB Models in Research and Development

The transwell assay provides a relatively high throughput assessment of blood-brain barrier transport, but does not capture the 3D cylindrical geometry of microvessels, the shear stress on the endothelium resulting from blood flow, or the local microenvironment. Engineered microvessel platforms using human cell lines that recapitulate the physiological blood-brain barrier have the potential to rapidly accelerate scientific discovery and the development of new therapies for diseases such as malignant brain cancer[48].

Further advances in stem cell engineering are likely to provide readily available human cell lines for blood-brain barrier research. Methods to harvest patient-derived cells will also be key in developing patient-specific therapies.

The blood-brain barrier remains a major roadblock in delivering drugs to the brain. New strategies for delivering drugs to the brain may include cell penetrating peptides, highjacking transporters (so-called Trojan horse approaches), or transiently increasing the permeability of the blood-brain barrier (e.g. vasomodulators, focused ultrasound, etc.).

The nature of disease-associated disruptions in modulating the local permeability of the blood-brain barrier and their role in disease remain important challenges that will be crucial to developing therapies for many diseases of the central nervous system.

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Section V: Tools and Resources to Accelerate Clinical Translation

Pre-Clinical Characterization of Nanomaterials

Rebecca Crist, PhD, and Scott McNeil, PhD
Nanotechnology Characterization Laboratory
Cancer Research Technology Program, Leidos Biomedical Research, Inc.
Frederick National Laboratory for Cancer Research, Frederick, MD 21702

The biggest challenge in preclinical characterization of nanomaterials is the diverse array of skills and knowledge required for a complete understanding of the formulation (**Figure 1**). A multidisciplinary team of experts including chemistry, immunology, toxicology, pharmacokinetics, pathology, and more is often required for an advanced evaluation of a nanomedicine, even and especially at the preclinical stage. Every data analysis and result depends on knowing exactly what the test material comprises. There have been numerous reported cases where toxicity was incorrectly assigned to a nanomaterial when in fact the toxicity stemmed from residual excipients, synthetic byproducts, biological impurities, undetected particle instability, or other anomaly[1–6].

The Nanotechnology Characterization Lab (NCL) was set up in 2004 as part of the NCI's Alliance for Nanotechnology in Cancer program to provide preclinical characterization services to oncology nanomedicine developers around the globe. The NCL staffs experts in a variety of fields who provide critical insight to organizations pursuing nanomedicine translation, but may not have the wide-ranging expertise or resources required for translational advancement. Having characterized more than 650 nanomaterial samples from nearly 100 different organizations, the NCL has had a unique opportunity to observe nanomaterial characterization challenges, including how the field has progressed over the years and insight into what lies ahead.

Challenges in Chemistry

It has been widely established that a nanomaterial's physical and chemical properties directly influence a variety of biological performances, including biodistribution, clearance, and immunotoxicity[7–10]. Therefore, a thorough characterization of these parameters is paramount to ensuring safe *in vivo* administration of the material. With this realization, the depth of routine physicochemical characterization performed on nanomaterials has increased dramatically. The recognition of the unequivocal importance of characterization and consistency is arguably the most significant advancement in this field.

The challenges associated with nanomaterial physicochemical characterization have shifted over the last decade. Initially, researchers grappled with proper ways to assess size, charge, or composition, including which measurement technique was most suited and what the most appropriate measurement conditions were. Now it is well accepted that materials should be analyzed by multiple orthogonal analytical techniques and under the appropriate biologically relevant conditions. However, with the evolution of more advanced nanotechnologies, new challenges in characterization are arising. One challenge at the forefront of physicochemical characterization of nanomaterials is surface analysis. It is imperative to know whether the surface ligands are covalently attached or simply physisorbed, which would allow their premature dissociation from the formulation. Furthermore, the density / coverage of the surface and the orientation and accessibility of the ligand(s) can also be important biological factors. As the number of surface modifications increases, so will the complexity in characterization. This is a particularly challenging area because techniques developed for one type of nanomaterial (e.g., liposomes) will not necessarily work for others (e.g., metals). Having realized the importance of surface properties for biological performance, there will be considerable advancements in tools to evaluate surface properties over the next few years[11]. Our laboratories and others have already begun to invest significant resources into this area.

Resources for scale-up and GMP manufacture of nanomedicines remain as another critical area of need for future development. The NCL is continually asked for advice on where to go for scale-up and / or GMP production services. There are limited establishments with the capabilities to meet this increasing demand for late-stage preclinical synthesis of complex nanomedicines. National efforts are underway now to address this critical gap in translation.

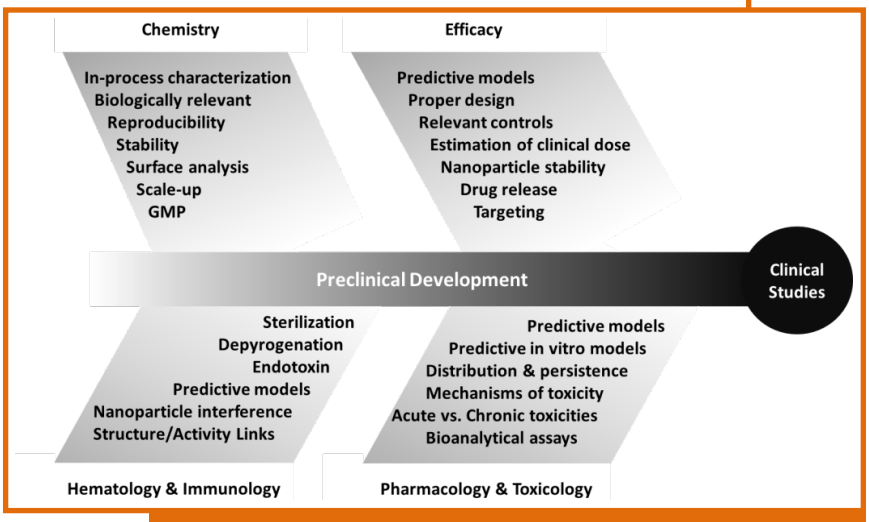

Figure 1. Challenges in Preclinical Characterization of Nanomedicines. Preclinical characterization of nanomedicines requires analysis in a variety of fields, each of which has their own set of challenges. Some of the most significant challenges associated with chemistry, immunology, efficacy and pharmacology/toxicology are noted.

Challenges in Immunology

Although, there has been increasingly more effort put into the early immunological evaluation of nanomaterials, immunology continues to be an underappreciated area during the preclinical stage. Structure-activity relationship studies have been an integral part of the early understanding of nanoparticle immunological influences. The association of nanoparticle physicochemical traits to immunotoxicities has afforded a significant knowledgebase to which the field needs to continue to build upon. However, many challenges associated with immunological evaluation of nanomaterials still remain, including sterility, sterilization, depyrogenation, biological contaminants (e.g., endotoxin and b-glucan), and accuracy and predictability of *in vitro* and *in vivo* methods.

Endotoxin detection and quantification is an area many researchers continue to struggle with. Nanoparticles are notorious for interfering with many of the traditional immunology assays, especially endotoxin quantification assays. A significant amount of research has been published on identifying and circumventing this interference, particularly as related to endotoxin, but educational efforts in this area need to continue[12–18]. Many researchers often avoid endotoxin evaluation until late in their preclinical development. This can be a costly oversight. Not only can the identification and elimination of the contamination source be expensive and time consuming, high endotoxin levels could adversely affect data interpretation.

Predictive *in vitro* and *in vivo* models for evaluating immunotoxicology continue to be one of the most important aspects of nanoparticle immunological characterization. Common immunological and hematological reactions to nanoparticles include hemolysis, complement activation, thrombogenicity, and cytokine storm. Many of these toxicities can be detected using *in vitro* assays, some of which are known to be predictive of corresponding *in vivo* toxicities. For example, a 5% hemolysis rate *in vitro* has been shown to correlate to hematocrit and hemoglobin changes *in vivo*[19]. Other hematotoxic effects, (e.g., myelosuppression) can also be studied *in vitro*, but knowledge of the *in vivo* nanoparticle biodistribution is needed for accurate data interpretation. In such situations, a systematic approach combining both *in vitro* and *in vivo* data is proven to be the most reliable characterization approach.

Future work in the immunological evaluation of nanomaterials will require monitoring the long-term effects of nanoparticles on the immune system. Delayed type reactions are triggered by nanoparticle influences of immune cell function and are often very complex, frequently involving many different cell types. Although specialized *in vitro* immune function tests have been developed and shown to be predictive of *in vivo* toxicities for small

molecules, applicability of these to nanoparticles is challenged by a distinct biodistribution profile and mode of transport across biological barriers. Many of these challenges have been reviewed in detail[20].

Challenges in Efficacy

Without question, the biggest challenge in preclinical assessment of efficacy is the availability of appropriate and predictive animal models. Most efficacy studies are conducted using human cancer cell lines in immune-deficient mouse strains that compromise the plausible interaction between immune cells and nanomaterials *in vivo*. Additionally, these xenograft models are unable to adequately recapitulate the tumor stroma, which plays an important role in tumor progression and can impede drug delivery.

There has been significant progress in the development of more suitable *in vivo* cancer models with the sequencing of cancer genomes and improved molecular biology tools. Several genetically engineered mouse models (GEMMs) have been generated to evaluate tumor growth and progression by utilizing noninvasive imaging modalities. Histopathological analysis of genetically engineered mouse tumors at different stages of disease progression has shown reasonable similarities to human disease. In addition to GEMMs, another focus has been on patient derived xenografts (PDX). PDX models implant human tumor cells in a mouse, providing a more relevant tumor microenvironment and genetic complexity that can better predict clinical outcomes. Future progress in this area will require further refinement of existing tumor models using improved understanding of cancer initiation and progression (e.g., most common genetic predictors of disease progression, signaling pathways, role of tumor stroma).

Experimental design issues also often plague *in vivo* efficacy analysis. Because of the cost of *in vivo* animal studies, it is not uncommon for researchers to forego some needed controls or preliminary analyses. For example, it may be necessary to run several small scale preliminary experiments to gain a better understanding of the maximum tolerated dose (MTD), nanoparticle stability, or drug release *in vivo*. Lack of the adequate controls is another common omission. A good efficacy evaluation should test materials at their respective MTDs and include controls of the platform, current standard of care, and the non-targeted particle where applicable.

Challenges in Pharmacology & Toxicology

Similar challenges exist for preclinical pharmacology and toxicology testing as with preclinical efficacy studies—the availability of appropriate models and proper experimental design. Development of predictive *in vitro* and *in vivo* models of toxicity would be big advancements

in the pharmacological and toxicological understanding of nanomaterials. There are differences in the mononuclear phagocytic system (MPS) between the animal species utilized that could affect accurate prediction of pharmacology and toxicology in humans. There have already been significant improvements in the development of bioanalytical assays in this area. For example, novel methods for analysis of drug release in biological matrix have allowed for a better understanding of nanoparticle stability, tendency for aggregation, drug release, and quantification of encapsulated and unencapsulated drug fractions.

Acute toxicities of nanomaterials are being well studied now; however, long-term chronic toxicities associated with nanomaterials should be further explored and will be an area of future development for this field. A better understanding of the mechanisms of nanomaterial toxicity (e.g., oxidative-stress, lysosomal dysfunction, inflammation) will aid these efforts, and research is ongoing now towards this goal. Additionally, bioanalytical challenges such as determination of dose linearity; estimation of clinical dose; and distribution and persistence of nanoparticles in tissues will be critical for the translation nanomedicine.

Preclinical characterization of nanomaterials has shown considerable advancement over the last decade.

Conclusion

Preclinical characterization of nanomaterials has shown considerable advancement over the last decade. Methods are being continually developed and optimized to meet the needs of the evolving complexity of nanomedicines. Detailed nanoparticle surface characterization, predictive immunotoxicity assays, and quantitative evaluation of the encapsulated vs. free drug fractions highlight the growth of this field. Continuing to pursue new methods development as well as conducting research directed at understanding the nano-bio interface will uncover additional relationships between nanoparticle structure and biological activity. This information will be invaluable for devising new strategies for using nanotechnology to improve upon existing pharmaceuticals and deliver novel therapies in the future.

Pharmacokinetics and Pharmacodynamics Characterization of Nanotherapeutics

William C. Zamboni, PharmD, PhD
Eshelman School of Pharmacy
University of North Carolina, Chapel Hill, NC 27599

Introduction: Complex Pharmacology of Nanoparticles

Major advances in nanoparticles (NPs) have revolutionized drug delivery capabilities over the past decade. They provide numerous advantages, such as greater solubility, duration of exposure, less toxicity and delivery to the site of action over their small molecule counterparts, nevertheless NPs display substantial variability in systemic clearance and distribution, tumor delivery, and pharmacologic effects (efficacy and toxicity)[21]. NP research has historically focused on the development of NP formulations with less emphasis on evaluating the complex pharmacology and biology of NPs, which significantly influences the successful translation of these agents. This report is an overview of factors that affect the pharmacokinetics (PK) and pharmacodynamics (PD) of NPs in preclinical models and patients.

Major advances in nanoparticles have revolutionized drug delivery capabilities over the past decade.

The disposition of NPs is dependent upon the carrier, not the therapeutic entity, until the drug gets released from the carrier[22]. The nomenclature used to describe PK of NPs includes: encapsulated (the drug within or bound to the carrier), released (active drug that gets released from the carrier), and sum total (encapsulated drug plus released drug). After the drug is released from its carrier it is pharmacologically active (unless the released form is a prodrug) and subject to the same routes of metabolism and clearance as the non-carrier form of the drug. The pharmacology of NPs is complex and thus comprehensive PK studies must be performed in order to assess the disposition of encapsulated or released forms of the drug in plasma, tumor and tissues[23]. Considerable inter-patient variability exists in the PK/PD of NPs and appears to be associated with variability in the function of the mononuclear phagocyte system (MPS), which is the primary clearance pathway for NPs[24]. It is difficult to evaluate the factors that affect the PK and PD of NPs in animals and human patients, due to the fact that they are different and thus animal models may not be predictive of the effects displayed in patients[25].

NPs may be taken up by a wide variety of cells in the blood and in tissues; however, it has been discovered that NPs are primarily taken up by circulating monocytes and dendritic cells (DC) in blood, Kupffer cells in the liver, DC in the lymph nodes, and macrophages in the spleen all of which are components of the MPS[26,27]. Uptake mechanisms may occur through different pathways and are often facilitated by the adsorption of opsonins to the NP surface and subsequent phagocytosis by MPS cells. Although, the uptake of NPs by the MPS does appear to be the predominant factor that affects the clearance of NPs from the blood as well as the distribution of NPs to tissue and possibly even the tumor itself. Yet, it is currently unclear if the distribution of NPs from the blood and into tumor and/or tissues occurs by capture (i.e., the NP enters the tissue and then is taken up by the MPS cell) or hijacking (i.e., the MPS cell takes up the NP in the blood and carries it to the tissue)[28]. This complex issue complicates the optimal design of NPs and, moreover, the evaluation of the primary factors that alter NP delivery to solid tumors. **Figure 2** illustrates the complex interaction between NPs and the MPS. The following two sections will discuss, in more detail, these factors with respect to NP PK/PD and subsequent delivery to solid tumors.

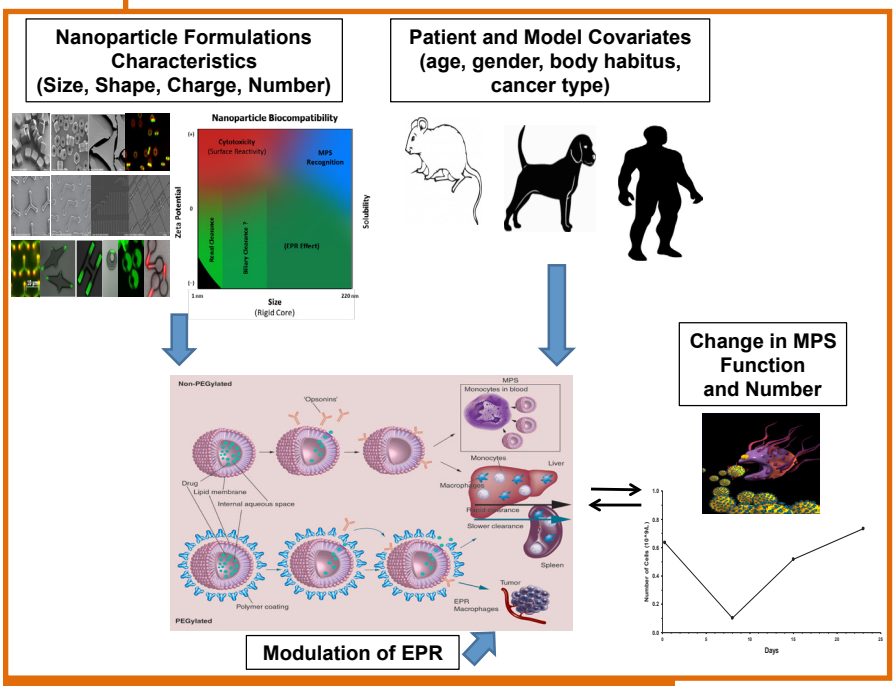

Figure 2. Summary of the complex bi-directional interaction between NPs and MPS. The factors affecting the PK and PD of NPs consist of the interactions between the characteristics of the NP carrier and host related factors. The NP characteristics consist of the size, shape, surface modifications, surface charge, and number of NPs administered. Several mediators (e.g., chemokines) and factors (e.g., age, gender, body habitus, tumor type and location, other drugs) have been reported to alter the PK and PD of NPs in animal models and in patients. The uptake of NPs by the MPS cells may also alter the function and number of MPS cells.

Factors Affecting the PK and PD of Nanoparticles

The factors affecting the PK and PD of NPs consist of the interactions between the characteristics of the NP carrier and host related factors. The NP characteristics consist of the size, shape, surface modifications, surface

charge, and number of NPs administered[22]. In an attempt to minimize opsonization and the subsequent uptake by the MPS, a commonly used strategy, although this is dependent upon the NP material type used, is to conjugate polyethylene glycol (PEG) onto the surface of the NPs. However, the optimal length, amount, and configuration of PEG or other surface coatings is unclear and is unique to each NP carrier[29,30]. There also may be hidden complications of PEGylating NPs. While PEGylation does prolong the circulation of NPs in blood compared to non-PEGylated NPs, the addition of PEG may increase the interpatient variability in the clearance of NPs[31]. Moreover, the number of NPs administered per dose significantly affects the clearance and distribution of NPs[32]. This affect is most likely due the non-linear or saturable uptake of NPs by the MPS.

Several mediators (e.g., chemokines) and factors (e.g., age, gender, body habitus, tumor type and location, other drugs) have been reported to alter the PK and PD of NPs in animal models and in patients[22]. One of the more clinically relevant issues to consider is that the type and location of the tumor may alter the PK of NPs and thus it may not be optimal to administer the same dose of a nanotherapeutic to patients with different types of tumors. The mechanisms of these interactions appear to all involve the MPS. MPS is highly promiscuous and thus takes up all types of particles (e.g., drug carriers, virus, antibodies, bacteria), but appears to have only a limited capacity to take up these particles. Thus, the presence of other natural or man-made particles in the body may alter the PK and PD of NPs. There also appears to be significant differences in the MPS function and PK of NPs across species and across different strains within a species[25,33]. Moreover, the PK and interaction of NPs with the MPS after repeated doses of NPs is opposite in some animal models compared to that of human patients[34,35].

Factors Affecting the Delivery of Nanoparticles to Solid Tumors

While conventional drugs encounter numerous obstacles *en route* to their target, in theory NPs can take advantage of tumor's leaky vasculature to extravasate into tissue via the enhanced permeability and retention effect (EPR)[36]. Furthermore, the poor lymphatic drainage in tumors leads to accumulation of the NPs for prolonged duration, allowing them to release the drug in tumor cells over time. Passive NP targeting exploits the classic features of tumor biology in order to increase exposure of NPs in the tumor.

In theory, EPR is the primary route of NP delivery to tumors (even for active, targeted nanotherapies), but heterogeneity of EPR between tumor types, location of the tumor (e.g., primary versus metastatic, organ, intracranial versus extracranial) and the inability to ensure homogeneous delivery to all regions of the tumor is forcing the need to understand the more fundamental aspects of EPR[37]. Variations in the distribution of blood flow, in vessel

permeability, in microenvironment density, and specific interactions of MPS cells within the tumor may all play an important role in the distribution and penetration of NPs to tumor[38]. It has been reported that the EPR effect is directly influenced by physiologic contributions such as vascular pore dimensions, vascular structure, surrounding stroma[36]. In addition, there appear to be interactions between macrophages and others immune system cells that influence tumor microenvironment factors[28].

In theory, active targeting of NPs may further improve tumor delivery and activity by allowing the NPs to bind to specific cells in tumors using surface-attached ligands capable of recognizing and binding to cells of interest[21]. Targeting strategies have consisted of the use of antibodies, nucleic acids, carbohydrates, peptides, aptamers, and vitamins. It is currently unclear if active targeting of NPs to factors on tumor cells can overcome the inherent barriers associated with the tumor matrix. With the notable exception in the treatment of hematological malignancies, whose use of active targeting strategies would, of course, avoid these issues and barriers[39].

While NPs are able to deliver more drug to solid tumors compared to small molecule drugs, the efficiency (e.g., % of drug) of NPs to penetrate from blood and into the tumor matrix is significantly less than small molecule drugs[38]. Thus, better and more effective NPs that exploit EPR are needed as well as employing methods to evaluate and address the structural and functional hindrances in the tumor microenvironment[40]. However, a major limitation to addressing these issues remains the lack of detailed studies comparing the EPR effect and NP delivery to tumors in preclinical tumor models and human patients.

Future Directions for Understanding PK/PD in Nanotherapeutics

The pharmacology of NPs is highly complex and the factors that alter the PK and PD of NPs, especially the clearance and delivery to solid tumors are highly variable and multifaceted. Future studies need to develop novel *in vivo* and high-throughput screening methods as well as experimental designs that can successfully evaluate how NP PK and PD are affected by the variable nanotherapy schemes, the MPS, and other immunologic factors and conditions. In addition, studies are needed to evaluate the factors influencing and inhibiting the efficient delivery of NPs to tumors as well as how these factors can be overcome[40]. However, before any of these issues can be addressed, we first need to identify and profile these factors in animal models and in patients to identify which preclinical model(s) optimally predict these effects in patients.

Preclinical Animal Models for NP PK and PD

It is currently unclear which animal model most accurately predicts the PK and PD (efficacy and toxicity) of NPs, especially after repeated dosing, in patients. For example, after repeated dosing of some NPs in animal models (e.g., dogs) there is higher clearance of NP after subsequent doses (accelerated blood clearance (ABC)); whereas, in patients the clearance of NPs is reduced after repeated dosing which results in accumulation of drug[34,35]. These differences may be due to differences in MPS function of animal models versus humans. However, the disconnect between ABC in animals and reduced clearance of NPs in human patients does not occur for all NP agents. The lack of consistent changes in clearance after repeated dosing of NPs in animal models and patients further complicates the determination of the optimal models and study design for all NPs. As the type and location of the tumor may also influence the PK and PD of NPs, studies in non-tumor bearing animals may not be as predictive as needed.

Nanoparticle Formulation Characteristics

Theoretical changes made to formulations to enhance or alter the PK and PD of NPs may not readily translate to changes *in vivo* and thus comprehensive *in vivo* studies are needed to evaluate these effects. The optimal size, shape and number of NPs dosed are currently unclear[21,22]. Studies suggest that smaller NPs may be better than larger NPs as a means to overcome potential barriers in solid tumors. However, the specifics of this parameter needs to be defined. Information from other carrier-mediated agents (polymer conjugates; antibody drug conjugates (ADC)) may be used to better define the size parameter of NPs. As the number of NPs dosed appears to be a critical parameter affecting NP PK this suggests that the dose of NPs should be based on the number of NPs administered instead of the mg of drug inside of the NP. It is also unclear if the optimal NP characteristics for the treatment of one type of cancer will be the same for other types of cancers.

Analytical and Biodistribution Studies

Based on the complexity and high variability in the PK of NPs, detailed methods and studies are needed to evaluate the PK of NPs in blood, tumor and tissues[22]. It is critically important to evaluate the PK of the NP encapsulated and released form of NP drugs. This has been evaluated for some NPs in plasma; however, these studies need to be extended to evaluate encapsulated and released drug in tumor and tissues in order to be of any relevance within acute and long-term PK studies. In addition, it may be important to distinguish the exposure of NPs in various cell types within tumor and tissues. It is also becoming apparent that circulating cells in the blood (e.g., MPS cells) act as a depot site for NP agents and thus

NPs may be detectable in circulating MPS cells for a longer period of time than in plasma. Understanding how the uptake of NPs by circulating cells in the blood influences the distribution of NPs to the tumor, liver and spleen, is also important. The ability to measure intracellular exposures (e.g., lysosome or nucleus) of the NP carrier and active-anticancer agent is also critically important for all NPs, but especially important for actively-targeted NPs[41]. In parallel to analytical PK studies, we also need to evaluate the biodistribution of NPs using imaging technologies, as this will be critical to comparing EPR and tumor delivery in animal models and in patients[40].

Interaction Between NPs and the MPS

Studies suggest that there is a bi-directional interaction between the immune system, especially the MPS, and NPs[28]. MPS cells are the primary pathway responsible for the uptake and removal of NPs from blood or plasma. In addition, the interaction or uptake of NPs by the MPS may alter the function of MPS cells and even be cytotoxic to the MPS. However, this bi-directional interaction is highly variable and is dependent upon the characteristics of the NPs and factors that affect MPS function in animal models and in patients[26,27]. The type of tumor, tumor burden and location of the tumor may alter MPS function and the PK and PD of NPs and thus the appropriate dose of NP may not be the same for all malignancies. As a result studies need to be performed to profile the sequence of events and interaction between NPs and the MPS (e.g., subject covariates, opsonization, complement activation, MPS recognition, phagocytic uptake by MPS, NP PK and PD, change in MPS function, cytotoxicity to MPS) after administration of single and repeated doses of NPs in animal models and in patients.

Tumor Delivery of NPs

There is a fundamental need for preclinical tumor models to accurately represent the types of tumors seen in patients in order to conduct informative profiling and developmental studies of NPs. It is thought that metastatic, orthotopic, and GEMM are better options for NP studies than flank tumor xenografts. However, systematic studies of several types of NPs in each tumor model have not been reported and are desperately needed to advance the field of NPs in the treatment of solid tumors. In addition, studies suggest that primary and metastatic intracranial tumors have enhanced delivery of NPs compared with small molecule anticancer agents. It is unclear if the mechanism(s) of the enhanced delivery NPs to intracranial tumors is the same as non-intracranial tumors. Studies of NPs should use valid preclinical tumor models of intracranial and non-intracranial solid tumors in patients to address these issues[22,36].

Historically, investigators have predominantly tried to improve the tumor delivery of NPs by altering the characteristics of the NP carrier. One potential NP factor that needs to be further evaluated is the potential for smaller NPs to achieve greater delivery and distribution throughout the tumor matrix[42,43]. However, changes to the NP carrier may only achieve incremental improvements in the delivery of NPs to tumors due to the inherent barriers within the tumor matrix. Thus, there is a need to develop treatment strategies, regimens, methods and devices to overcome or alter the tumor barriers. These plans could include pharmacological agents or non-invasive treatment modalities. For example, recent approaches to normalize both tumor vasculature and physical forces surrounding vessels have been explored[44]. Co-medications that effect stroma and blood pressure are also known to influence EPR effect. The use of non-invasive methods that apply external beams that alter tumor barriers also holds significant potential benefits[45]. Another fundamental problem with NPs is that, even when they are able to penetrate into tumors, the release of drug from the carrier is relatively low and highly variable[23]. Thus, there is a need to develop treatment strategies to increase the release of drug from the NP and into the tumor matrix.

...researchers could individualize treatment with NPs based on selection of tumors with high EPR, tumor targets and patient specific doses.

Milestones to address these critical areas that researchers should be able to be achieve over the next 5-15 year time frame include many aspects. In the next 5 years, researchers will identify animal models that predict the PK and PD (toxicity and efficacy) of NP agents; identify the factors affecting the tumor delivery and distribution of NPs in intracranial and non-intracranial models; and develop novel analytical methods and platforms to characteristic the pharmacology of NPs as part of high throughput screens, *in vivo* models and in patients. Looking further ahead over the next 10 years, researchers will define the bi-directional interaction between NPs and the MPS, as well as other parts of the immune system, in preclinical models and in patients; optimize NP carrier characteristics to avoid delivery to normal tissues and enhance delivery to intracranial and non-intracranial tumors; and develop treatment strategies, regimens, methods and devices to overcome or alter the tumor barriers to enhance the delivery of NPs to tumors. Looking further ahead over the next 15 years, researchers could individualize treatment with NPs based on selection of tumors with high EPR, tumor targets and patient specific doses.

Informative Assessment on Novel Oncology Therapeutics in Preclinical Cancer Models

Serguei Kozlov, PhD
Center for Advanced Preclinical Research, Laboratory of Animal Studies
Leidos Biomedical Research, Inc.
Frederick National Laboratory for Cancer Research, Frederick, MD 21702

Introduction

It was not until the most recent decade that the tremendous complexity and diversity of molecular mechanisms, which underlie malignant transformation and cancer growth, became recognized. This new found knowledge fueling advanced efforts to dissect the cancerous pathways, pinpoint predictive biomarkers and promising drug targets and propose novel more efficacious therapeutic strategies to rein in the cancer disease[46]. As a significant component of the '*bench-to-bedside*' translational research arsenal, animal models of cancer occupy a capstone position and have become a broadly recognized mainstay in support of the preclinical phase for drug development's critical path[47,48]. In particular, mouse models have been constructed – either entirely surgically, by engrafting tumor cells/fragments into a judiciously chosen type of rodent recipients, or by using more 'cutting-edge' technologies via molecular engineering to edit the mouse genome in order to program selected sets of endogenous murine cells for oncogenic transformation (e.g., for the purpose of developing cancerous lesions of specific nature in pre-determined organs or anatomic locations). Presently, these models, which are reviewed in further details below, are broadly employed within a variety of experimental paradigms. The bulk, of which, are aimed at interrogating candidate therapeutics relative to their bioavailability, toxicity, mechanisms of systemic distribution, excretion and therapeutic action, as well as to their anti-tumor efficacy prior to moving these compounds into costly clinical testing workflows[49–51]. Such step-wise strategy has proven itself advantageous in preserving strained resources available to drug developers, while increasing scale and throughput of therapeutic testing; avoiding costly mistakes while mitigating the emotional burden of treating cancer patients; and, ultimately, accruing invaluable data to informatively guide clinical decisions in cancer disease management.

Patient-Derived Xenograft Models

Recognizing the heterogeneity and cellular complexity of cancer and the concomitant ability to reproduce the individual aspects of diverse malignancies in animal models is of critical importance for directing an informative preclinical assessment. This is of particular importance for evaluation of targeted and pathway-specific therapeutics, which display

efficacy only within a limited subset of the cancer patient population (e.g., that feature the appropriate molecular signature(s) of disease). Furthermore, individual (and not infrequently highly similar histo-morphologically) tumors may display acquired drug resistance to standard-of-care and first-line therapeutics; which mandates further evaluation of molecular content of the resistant disease's portion, followed by application of advanced next generation cancer therapeutics and/or combinatorial treatment regimens. With the purpose of attacking multiple components of the pro-oncogenic environment, which triggered the acquired resistance to mono-therapeutic intervention, in the first place. Last, many particularly aggressive tumor types reveal the notorious intra-tumoral heterogeneity, as evidenced by the presence in the same tumor mass of distinct sub-populations of transformed cells, all driven by divergent combinations of oncogenic drivers. This heterogeneity represents yet another tremendous challenge for selection of the most efficacious and durable therapeutic treatment available.

As such, patient-derived xenograft (PDX) models are constructed by grafting freshly dissected cancerous tissue (e.g., gained during tumor de-bulking surgeries or via diagnostic biopsies) either subcutaneously or orthotopically into carefully selected immunocompromised recipient mice. These can be reliably generated with a high take rate from a variety of tumor types[52–54]. Moreover, recent advances in the PDX modeling field have afforded preclinical drug developers the ability to derive models from metastatic or relapsed cancerous lesions as well as cancerous cells that have been deposited via tumor exfoliation or invasive growth into either ascitic fluid or blood circulation (e.g., circulating tumor cells)[55,56].

Among the myriad of substantial benefits PDX models' offer for preclinical

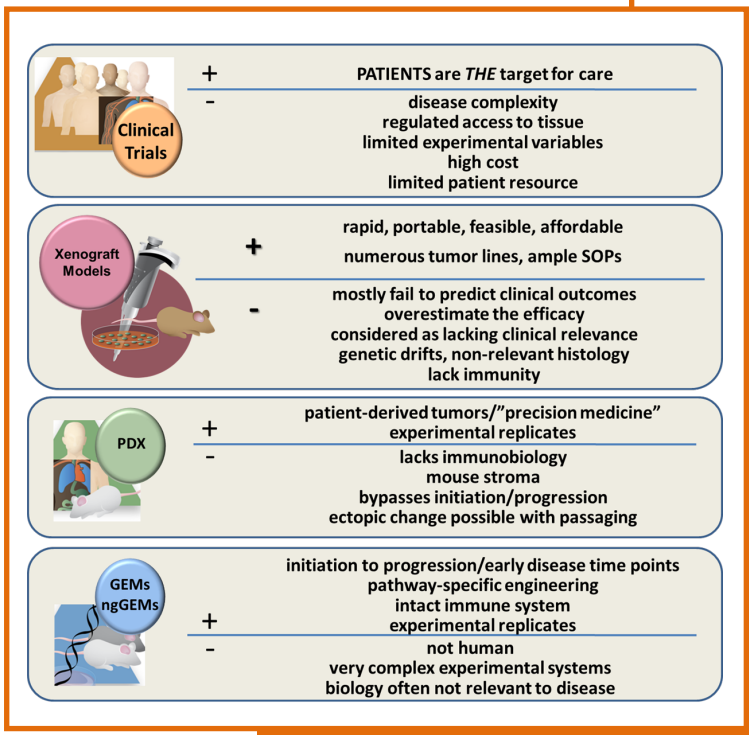

Figure 3. Comparative summary of cancer model types currently employed in preclinical evaluation vs. the clinical trials framework for oncology drug assessment. Various human-in-mouse grafted, mouse-in-mouse grafted and autochthonous/*de novo* models offer benefits for translational experimentation. All the while, featuring drawbacks limiting their applications and justifying integrated options of preclinical assessment in multiple relevant models.

assessment that should be highlighted when compared to the conventional established cell line-derived xenografts include, better preservation of original tumors' mutagenomes; the ability to mimic minimal residual and metastatic disease phases; and a faithful resemblance of therapeutic responses *vis-a-vis* those observed in parental tumors. Furthermore, the PDX models reveal histopathologic patterns and biomarker expression signatures closely approximating those of donor tumors. Also, they allow interactions between stroma or other tumor microenvironment components and the transformed tumor cells to be observed. Despite these advantages in employing PDX models for preclinical evaluation, several shortcomings should be mentioned limiting application of these models for broader use as a uniform testing platform. Mice bearing primary grafts of clinically obtained tissue specimens are immunocompromised – albeit efforts are underway in multiple organizations to reconstitute PDX recipient mice with a functional human immune system – thus largely excluding applications of PDX animals in the assessment of therapeutic strategies pursuing anti-tumor vaccination or activation of tumor immune surveillance mechanisms (e.g., immunomodulatory therapies). Furthermore, gradual passaging of PDX tumors, required to expand the pool of graft-bearing animals available for preclinical experimentation, is prone to substantial genetic and epigenetic drift, which is documented for several types of clinical malignancies. This is due to the fact that, although initially abundant at early passages, human stroma undergoes gradual replacement by its murine counterpart. This has the effect of disrupting the physiologic integrity of the tumor-stroma interaction and/or attenuating the signaling mechanisms required for sustained proliferation. The end result for the model is a misinterpretation of drug efficacy. Despite these challenges, as evidenced by rapidly growing interest and investments from multiple drug development organizations, PDX models have proven themselves as a superior predictive preclinical testing resource and are expected to gain further attention within the community of preclinical oncology experts.

Genetically Engineered Mouse Models

Genetically engineered mouse models (GEMMs), in the context of testing scientific hypotheses, have been extensively vetted as a strategy to elucidate a variety of biological mysteries, which range from developmental biology to mechanistic foundation of clinically challenging ailments. Albeit, it was not until recently when the GEMMs of oncogenic maladies started earning a widespread recognition as a predictive platform for assessment of cancer treatment options and discovery of novel diagnostic signatures, disease biomarkers, and promising drug targets. This could perhaps be best justified by the inherent complexity of cancer GEMMs, not infrequently requiring management of multi-allelic mouse inter-crosses and/or entailing implementation of tedious technologically complex workflows (e.g., inducing carcinogenesis by surgical application of infectious agents, monitoring tumor progression *in situ* via sophisticated imaging techniques, or statistically assessing

the whole gamut of disease histo-pathologic, cellular and molecular outcomes). However, once characterized and validated, the advantages of employing cancer-specific GEMMs for preclinical assessment are numerous. GEMMs provide virtually the only available experimental setting for cancer modeling that affords the cancer biologist and oncologists to monitor dynamics of autochthonous tumors from initiation through to late stage progression and metastatic spread. All the while, simultaneously capturing the disease's stochastic nature, molecular heterogeneity, and tumor-microenvironment interactions. Pending successful humanization of PDX models, the GEMM is, so far, the only experimental system featuring the presence of the fully intact immune system, an indispensable prerequisite for testing immunomodulatory therapies and anti-cancer vaccination strategies. Such models can be precisely engineered to activate a selected set of oncogenic drivers in a predefined cell sub-population or type, in the desired anatomic location. Finally, GEMMs could mimic important facets of cancer such as acquired drug resistance, incidence of minimal residual or metastatic disease, genomic instability, and heterogeneity. Although serving as a platform for numerous variables and multiple preclinical testing paradigms, genetically engineered mice remain undoubtedly the most laborious and expertise demanding preclinical asset. Of which, the application of GEMMs can be further limited by inconsistency in disease appearance, replicability, penetrance and latency, availability of robust colony management infrastructure, and the particular high-throughput options for genotyping and *in vivo* imaging. As a result, several dedicated and integrated Centers have been established. These Centers are tasked with developing optimized tractable strategies for preclinical assessment in GEMMs aimed at addressing these and other challenges impeding the broad application of GEMMs for preclinical drug development in oncology and other fields (e.g., autoimmune and neurodegenerative disorders). Such organizations are, not only expected to act as pivotal points of preclinical expertise, but are structured to offer contractual or partnership support to third parties as well as to be the hubs that disseminate best practices, optimized SOP's, and other resources. With the end goal of facilitating the application of cancer GEMMs for basic and translational purposes.

Non-Germline GEM and Syngeneic GEM-Derived Allograft Models

Despite the undeniable advantages GEMMs present for the preclinical drug evaluation arena; reaching the experimental throughput to match demand of drug developers and cancer translational biologists remains a formidable challenge. This is further amplified, today, by an almost exponential expansion of drug discovery pipelines propelling the demand for more robust preclinical assessment. This is particularly true for multiple promising and physiologically relevant models that display prolonged latency (e.g., in excess of one year from cancer disease initiation to detectable tumor), low penetrance, or significant attrition due to inconsistent or ectopic cancer incidence. A collection of

novel experimental approaches to model cancer disease in a more expedient, practical, flexible, standardized and ultimately cost-conscious way, designated non-germline GEMMs (ngGEMMs), has recently emerged and is gaining rapid adoption in both reputable academic labs and drug development organizations[57]. For example in one of the ngGEMM techniques, conventional GEMMs are bred to obtain preimplantation embryos that are converted into pluripotent embryonic stem (ES) cells, *ex vivo*, which contain the complete combination of desired oncogenic alleles (usually engineered as inducible mutations)[58]. The resultant ES cells undergo extensive genetic and karyotypic characterization prior to being employed for the production of chimeric animals according to well-established embryologic procedures. Such strategies afford the scalable, low cost maintenance of very broad portfolios of GEMMs to enable large synchronized experimental cohorts while simultaneously eliminating the need for costly step-wise interbreeding of multiple alleles and concomitant high volume genotyping. The end result is the models' improved clinical relevance[59]. Furthermore, in chimeric – but not in conventionally bred – models, a progeny of ES cells, genetically programmed for cancerous transformation, are intercalated into the hosts' embryo-derived tissue that lacks genetic alteration. Accordingly, this develops into non-pathogenic surrounding anatomic structures. This is to the contrary of oncogenic processes happening in tissues of conventionally bred animals, by which broad activation of oncogenic events in the entire target cellular subset or even whole tissue (e.g., the genetic field effect) result in either multiple "coalescing" lesions, not amenable to consistent longitudinal monitoring, or gives rise to overly aggressive tumors, limiting the therapeutic window beyond practicality. Some recently employed strategies utilizing modified ES-based chimeric ngGEMMs, have been used to rapidly assess systemically (i.e., in the context of the actual cancer disease) the biologic impact(s) of potential disease modifiers or putative drug target genes via targeted alteration of its expression in ES cells (e.g., using RNAi or CRISPR/Cas9 technologies) and subsequent tests of carcinogenicity *in vivo*[60]. The chimeric ngGEMM production technique carries only a few potential pitfalls that stem from intrinsic epigenetic instability of the pluripotent stem cells, risks of acquiring additional ectopic mutagenesis events, or undergoing loss of pluripotency in the course of ES passaging.

Yet another type of ngGEMM preclinical resource is referred to as mouse-in-mouse transplantation, or GEM-derived allograft (GDA), models. Construction of GDA animals entails dissection of cancerous tissues (either primary tumor or metastatic lesions, or even isolation of bloodborne CTC cells from murine circulation) and subsequent re-introduction of these cells – either as a dissociated single cell suspension, or as subcutaneously or orthotopically tissue fragments, – into a recipient mouse of identical genetic background[61,62]. Such syngeneic host animals, similar to conventional genetically engineered mice, harbor a fully intact immune system and thus are applicable for both investigation of

the immuno-oncology interface in cancer as well as testing of relevant IMT therapeutics. These GDA mice are generally characterized by a higher consistency and associated reproducibility in tumor appearance and histology, as well as shortened timeframe from implantation to development of enrollment-grade tumors ready for preclinical experimentation[63,64]. The dissociated cells derived from primary lesions can furthermore be genetically manipulated *ex vivo*, by established transfection or transduction techniques to, for example, visualize the grafted tumor or its derivative secondary metastatic lesions via expression of tracer markers such as fluorescent GFP/RFP proteins. Similar elegant approaches could be further extended to rapidly interrogate the functional implications of a suspected tumor modifier or candidate drugs' target genes with respect to their carcinogenic potential and/or sensitivity vs. resistance to pharmacologic challenges. This would be simply achieved via manipulating their expression level in tumor cells that will be subsequently tested in the GDA mice *in vivo*. **Figure 3** summarizes several of the aforementioned model types, also comparing them to conventional cell line-based xenograft models in a "strengths-weaknesses" format.

...efforts are underway in several organizations to assemble the proficient resources to advance the preclinical arena towards consolidated expertise in cancer disease modeling.

Conclusions and Future Directions: Integrated Strategies for Informative Preclinical Assessment in Predictive Animal Models

A common belief shared by a majority of the mouse modeling experts suggests that there is no "ideal" or "perfect", one-size-fits-all cancer model type. Or more specifically, that no single strategy of engineering the oncologic disease in mice will allow unambiguous and adequately granular recapitulation of all aspects of clinical malignances to facilitate straightforward predictions of disease progression path or deduction of unequivocally failure-proof treatment plans. To the contrary, an integrated multidisciplinary approach enabling simultaneous assessment of multi-dimensional data sets gathered from different cancer models that are subject to a battery of experimental assays presents itself as the most promising avenue in guiding clinical development and is strongly advocated for by preclinical science professionals. Although challenges still persist in identifying the best-fit robust, while sufficiently reproducible and portable, experimental frameworks. And more importantly, frameworks satisfying the unmet need criteria of the oncology field and attuned to current rigorous trends in precision medicine. Luckily, efforts are underway in several

organizations to assemble the proficient resources to advance the preclinical arena towards consolidated expertise in cancer disease modeling. The ultimate package of deliverables from such coordinated activities (e.g., pursued at the NCI Center for Advanced Preclinical Research, see https://ccr.cancer.gov/capr-about for further information) is anticipated to include collections of best practices and standard operating procedures; information on optimized materials, reagents, instrumental base, partnership business models and intellectual property mechanisms; and access to integrated enterprise quality information systems designed to accumulate, warehouse, evaluate, share and disseminate the full spectrum of preclinical data from multiple sources. But above all, such initiatives will offer tutelage and access (and whenever applicable or justified, sponsorship) to experimentally validated portfolios of preclinical modeling resources. Resources, of which, have been carefully selected to support flexible testing for the variety of novel diagnostic approaches, disease outcome monitoring and assessment methodologies, or improved oncology therapeutics. It is also both reasonable and enticing to argue that the current and projected progress in application of translational cancer models for preclinical drug development will galvanize and pave the way for collinear efforts in other clinical arenas – such as neurodegenerative or cardiovascular diseases, inflammation, and autoimmunity – to produce a similar toolkit of methodologies that explore relevant preclinical murine models for devising better treatment options.

...such initiatives will offer tutelage and access to experimentally validated portfolios of preclinical modeling resources.

Multiscale Modeling and Simulation to Guide Rational Nanomaterials Design

Paolo Decuzzi, PhD
Houston Methodist Hospital Research Institute, Houston, TX 77030

Over the last decade, new nanomaterials, devices and systems have been developed for the diagnosis, imaging and treatment of multiple malignancies[21,65,66]. Nanoparticles with different geometrical and physico-chemical properties have been engineered, loaded with multiple agents, and systemically administered for the detection and treatment of primary and metastatic tumors[67,68]; nano/micro-fluidic chips have been presented for the rapid screening of potential medications and for the identification of cancer biomarkers[69,70]; and miniaturized devices have been designed for molecular imaging on patient-derived histological samples[71]. Although most of these nano-systems are developed following rather empirical approaches, mathematical modeling and computer simulation, over multiple biophysical scales, are crucial in understanding their *in vivo* behavior and optimizing their performance for clinical translation. As computational sciences have already had a profound impact across multiple disciplines of science and technology development, 'Computational Nanomedicine' could have an equally pervasive impact in our ability to rationally engineer novel and more efficient nanostructures, nanodevices, and nanomaterials for biomedical applications. Current efforts and future perspective in this field are discussed briefly below and in order of biophysical scale, from large to small.

Whole-animal scale modeling.

Multi-compartment mathematical models are now extensively used to understand, predict and compare, the *in vivo* pharmacokinetics (PK) of therapeutic and imaging agents[72]. In particular, based on anatomical and biological information, these models divide the whole-body in multiple compartments, which are interconnected via specific transport and adsorption parameters. Since PK models have been successfully applied for estimating the organ-specific absorption, distribution, and excretion of systemically injected small molecules; similar approaches are now being established for the biodistribution of nanoparticles (NPs). However, the predictive power of these PK models is still quite limited by empiricism and the lack of mechanistic information on the organ-specific deposition and sequestration of NPs.

Most recently, compartment-based models have been adopted for predicting the blood concentration of cancer biomarkers[73]. These models are extremely relevant to early cancer detection and aim at elucidating the correlation between blood biomarker concentration

and tumor size. Unfortunately, clinical data are not generally available to address such a question, thus this is an area where mathematical modeling can be helpful. Specifically, using a one-compartment model integrated with a conventional tumor growth law, it was possible to estimate the blood concentration of tumor biomarkers over time (**Figure 4**). Based on published data on ovarian carcinoma and considering CA125 as a tumor biomarker, the model computed that 8 years are required in order to detect a continuously growing malignant mass with the currently available clinical tools. These computational models clearly emphasize the need for developing more sensitive detection techniques, but also imply that increases to the blood concentration of biomarkers for facilitating earlier detection are necessitated[74].

Tumor and single-organ scale modeling.

Sophisticated multi-scale and multi-physics computational models have been developed for predicting the response of malignant masses to different treatments, including molecular and nano-based therapies as well as radiation and thermal ablation interventions[75]. These models have similarly been used for understanding and optimizing the vascular transport and tumor accumulation of NPs[76,77]. In particular, using an immersed finite element method, the vascular distribution of NPs was studied in whole blood (**Figure 5**). These computer simulations, supported by experimental intravital microscopy data, demonstrated that small NPs (≤ 100 nm) tend to distribute quite randomly within capillaries without interacting with red blood cells. Inversely, large NPs (> 500 nm) preferentially accumulate next to the vessel walls, in a size-dependent manner. This data suggests that sub-micron particles could be more efficiently employed for targeting the diseased vasculature as compared to conventional 100 nm NPs, whose tumor accumulation is primarily driven by the Enhanced Permeability and Retention (EPR) effect. Still focusing on the vascular deposition of NPs, computational models have been developed to predict the accumulation of systemically injected NPs in the tumor neovasculature[77]. By combining a mesoscale model for the vascular adhesion of NPs with a multi-dimensional tumor growth model, it was predicted that the fraction of NPs accumulating in the malignant tissue depends only on the vascularity. Additionally, it was observed that a moderate NP affinity for the tumor endothelium provided the optimal balance between spatial distribution and absolute tumoritropic accumulation. Clearly, this is another example where multi-scale

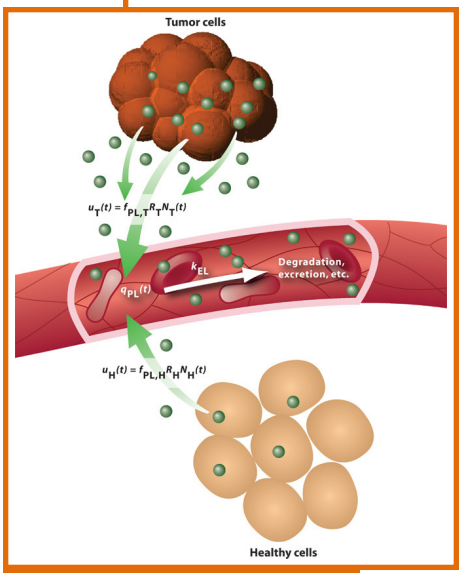

Figure 4. One-compartment model for plasma biomarker kinetics (*Reprinted with permission from Hori and Gambhir, 2011*)[73].

and multi-physics mathematical modeling provides input for rationally engineering NPs with enhanced tumoritropic accumulation.

Computational models can also be used to directly compare the therapeutic efficacy of a single bolus injection of drug molecules with an equivalent dose administered via NPs[78]. By modeling the interplay between mass transport in the microvasculature and blood perfusion in the extravascular volume, computer simulation allowed prediction of interstitial drug concentrations, rates of metabolization, and fractions of cell killing over time. These studies concluded that, for an equivalent injected dose, nano-based treatments ensure higher intratumor drug accumulation and longer exposure times as compared to single bolus injections, thus resulting in higher apoptotic indexes.

Cell and single nanoparticle scale models

Mathematical modeling has been fundamental in elucidating the biophysical mechanisms regulating NP transport dynamics within the vasculature and via internalization into cells[80]. For instance in vascular adhesion, numerical simulations suggested that oblate spheroidal particles would more avidly adhere to the vessel walls as compared to spherical particles of identical volume[81]. Also, mathematical models demonstrated that NP size and shape play a crucial role in modulating cellular endocytosis[82,83]. More recently, computational models for NP cell uptake and drug release were developed to characterize the multi-drug resistance in cancer cells[84]. Supported by experimental evidence, these models revealed that NP-mediated delivery increases both the total concentration and temporal exposure of chemotherapeutic molecules to the target cells. As a consequence, the respective IC_{50} values were improved upon as compared to free drug molecules.

Mathematical models can also be directly used to improve the performance of nanomaterials. For instance, by using molecular dynamics simulation, the diffusion of molecules within nanoporous structures, around nanoparticles, and proteins can be studied (**Figure 6**). Following this approach, the magnetic resonance imaging performance of mesoporous particles loaded with iron oxide NPs and Gd-macromolecules was predicted and optimized for future clinical use[79].

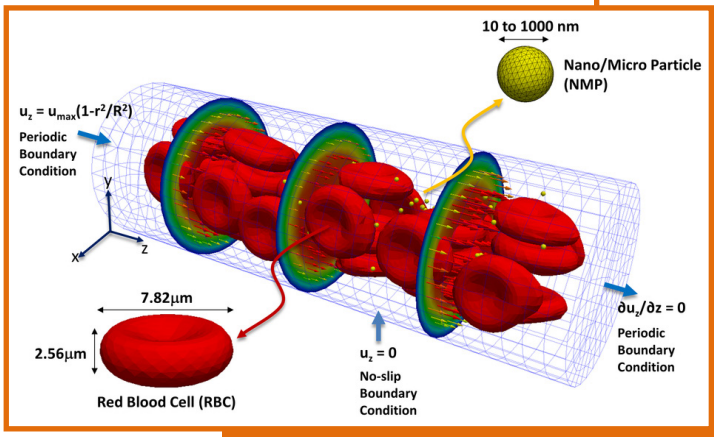

Figure 5. Modeling the transport of NPs into whole blood (*Reprinted with permission from Lee et al, 2013*)[76].

Future perspectives

'*Computational Nanomedicine*' could play a major role in facilitating and accelerating the clinical translation of nanotechnologies and in enabling what is often referred to as precision medicine. At the individual NP level, molecular dynamics simulation can be used to engineer NPs with new architectures enhancing the loading efficiency of drug molecules and contrast agents. This will allow us to reduce the injected doses and limit potential side effects; to improve upon imaging contrast agents for early disease detection; and enable combination therapies (i.e., polypharmacy) to be more rapidly correlated to efficacy. At the cell scale, mathematical models are needed to elucidate the role of thermal ablation therapies and mechanical stresses on cell proliferation and drug resistance. At the organ level, more sophisticated models of tumor growth. Those which account for the spatio-temporal heterogeneity of malignancies, occurrence of *de novo* and acquired drug resistance, presence of tumor initiating cells, and tissue deformability, known to modulate cell growth and migration, will have to be developed. The integration of cell scale and tumor growth models will help us designing new intervention strategies, where diseased cells and tumor microenvironment are coupled for synergistic and efficient targeting. Finally, more efforts should be devoted in developing truly multi-physics and multi-scale computational PK models for predicting patient-specific biodistribution of NPs. These mechanistic PK models should be derived by the hierarchical integration of cell/organ level mesoscopic models with conventional schemes for pharmacokinetic analyses. In this effort, the contribution of multi-modal imaging data will be crucial in the validation phase as well as in the actual clinical utilization for acquiring patient-specific information to be fed back into the computational models. In a near future, mechanistic PK models will help doctors to identify *a priori* the optimal *4S* – size, shape, surface properties and mechanical stiffness – NP properties for maximizing tumor accumulation; and the proper combination of therapeutic agents for eradicating the disease in each individual patient, allowing for eventual realization of 'precision medicine.'

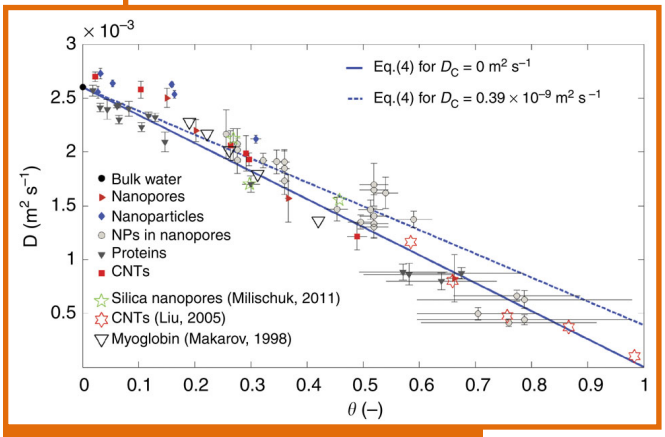

Figure 6. Molecular dynamics representation of a silicon nanopore containing iron oxide nanoparticles, a single walled carbon nanotube, a green fluorescence protein (top). Correlation between the diffusion coefficient of water molecules *D* and a geometrical parameter *Θ* (*Reprinted with permission from Chiavazzo et al, 2014*)[79].

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Section VI: Commercialization of Nano-Products for Cancer

Commercialization of Cancer Nanomedicines: Opportunity and Challenges

Lawrence Tamarkin, PhD
CytImmune, Rockville, MD 20850

Chemotherapeutics in Cancer Therapy

The treatment of cancer remains an ever-growing problem. In developed countries, the most common approach to treating solid tumors, in particular, starts with surgical resection followed by chemotherapy and/or radiotherapy. Such a clinical treatment strategy, requiring sophisticated hospitals with sophisticated staff, equipment and supplies, which are quite costly. For the developing nations of the world, this approach may be an insurmountable economic challenge. And, the efficacy of this approach has not resulted in a dramatic improvement in overall survival rates for most cancers[1].

Using nanoparticles to deliver potent anti-cancer agents to solid tumors, which represent 85% of all cancers reported annually, has the potential to change this paradigm, and potentially change patient outcomes. As solid tumors grow, whether primary or metastatic cancer, new blood vessels grow to support that growth. These new blood vessels are leaky with fenestrations ranging in size from 0.2-1.2 μm^2. This unique biology provides an ideal opportunity for systemically administered nanoparticle-based medicines (nanomedicines), ranging in size from 10-100 nm, to target tumors by exiting the circulation through these fenestrations, potentially resulting in improved biodistribution, bioavailability, safety and efficacy. In effect, the leaky tumor neovasculature argues that solid tumors should only be treated, prior to surgery, *in situ* with nanomedicines, taking advantage of this unique biology and potentially improving the therapeutic index of potent anti-cancer drugs. Recognizing this therapeutic opportunity is the clinical rationale for changing the current cancer treatment paradigm for the vast majority of solid tumors from a surgery first protocol, to medical treatment first.

If nanomedicines are effective in significantly reducing or eliminating cancers, making subsequent surgeries less complex or unnecessary, then this treatment regimen is a clear opportunity for the pharmaceutical industry to help reduce healthcare costs worldwide. Such a public health strategy might effectively improve patient outcomes for the largest number of cancer patients. And, the potential role nanomedicines might play in this paradigm shift, worldwide, represents a major motivating factor for biotechnology

and pharmaceutical companies to seriously explore the clinical development of cancer nanomedicines.

Since the tumor neovasculature is inherently leaky, irrespective of cancer type or disease stage, this biology may be used again and again in its treatment. So, from the perspective of biotechnology and pharmaceutical companies, treating cancer as a chronic medical disease that requires periodic nanomedicine treatments to control/suppress recurrent disease is an added economic incentive to develop nanoparticle-based cancer medicines.

Design of Cancer Nanomedicines

However, the leaky tumor neovasculature is both an opportunity and a challenge for nanoparticle-based medicines. As noted above, the opportunity exists for nanomedicines smaller than 100 nm to passively exit the circulation and remain in the tumor interstitial space, the "enhanced permeability and retention" (EPR) effect. But, is the EPR effect sufficient for the delivery of cancer killing drugs? Comparative data have shown that inclusion of a tumor targeting ligand that binds to a cell surface receptor reduces the time for a nanomedicine to reach a solid tumor from hours to minutes[3]. Consequently, in the design of new nanomedicines for commercialization having a tumor-targeting ligand needs to be considered.

...the opportunity exists for nanomedicines smaller than 100 nm to passively exit the circulation and remain in the tumor interstitial space...

Conversely, a challenge that the leaky tumor neovasculature creates for systemically administered cancer therapeutics, including nanomedicines, is that other similar or smaller-sized blood components also leak into the tumor interstitial space, creating an interstitial pressure gradient in tumors, where the fluid pressure inside the tumor is greater than it is outside the tumor[4]. This high interstitial fluid pressure (IFP) creates a physical barrier, preventing systemic cancer treatments, such as nanomedicines, from reaching their target, the cancer cells.

Clinically, the effect of destroying the high tumor IFP has been most dramatically seen in patients with in-transit melanoma or sarcoma[5]. Using hyperthermic limb perfusion to locally treat these patients first with a vascular disrupting agent, which destroys the high tumor IFP, followed by chemotherapy, has, on average, been reported to result in an 85% complete local response. In effect, this regional limb perfusion protocol eliminates this physical barrier, enabling follow-on chemotherapy to reach its target and kill the cancer cells.

By design, if a nanomedicine is able to destroy tumor blood vessels, then, using the tumor targeting mechanisms noted above, the systemic administration of a nanomedicine to a cancer patient prior to surgery could eliminate the high tumor IFP. With this added mechanism of action, such nanomedicines might have the greatest potential of achieving the high response rates seen with regional limb perfusion. Consequently, incorporating an agent capable of destroying the high tumor IFP should also be considered when creating cancer nanomedicines for systemic treatment of solid tumors.

Looking to the future of creating commercializable cancer nanomedicines, some critical first steps in design and manufacture need to be considered. For example, translation of a nanotechnology-based research concept into a commercial nanomedicine product requires that thought be given to the biocompatibility of the material comprising the nanomedicine platform, the therapeutic payload (ideally a new drug entity), the immunogenicity of the resultant nanomedicine, the ability to actively target tumors and attack cancer cells, the metabolism and elimination of the material comprising the nanomedicine platform, and the ability to scale-up the nanomedicine manufacturing process to commercial lot sizes in a current good manufacturing process (cGMP) facility. And, the resultant product must be stable, with a two-year shelf life at a minimum. Without a clear understanding of these issues, as well as patent protection of the accompanying intellectual property, the translation of a nanotechnology-based drug concept into a nanomedicine product might never be achieved.

Regulatory and Financial Hurdles to Commercialization

Many of the issues noted above must be satisfactorily addressed in the *Investigative New Drug (IND) application* that is required by the Food and Drug Administration (FDA) to initiate human clinical testing. And for nanomedicines specifically, the Chemistry, Manufacturing and Controls (CMC) section of the IND is quite critical in that the Sponsor must fully explain the composition of the new drug, how the nanomedicine is formulated, its stability under various conditions that might approximate its use, and the analytical tests used to interrogate the final drug product and its components. Providing this critical data is a challenge for new nanomedicines, and being sure that the data meet the requirements of the FDA for new product registration and sale is not guaranteed. And, such uncertainty is often perceived as a risk for pharmaceutical companies and for investors, such as venture capital companies that oftentimes provide the necessary capital to develop new technologies.

Such uncertainty stems in part from the fact that the FDA has not issued specific guidance or analytical benchmarks that all nanomedicines must achieve. In fact, the FDA has

maintained that the current procedures for new drug testing and evaluation sufficiently cover the development of nanomedicines[6]. In addition, current FDA policy states that each nanomedicine should be reviewed and evaluated on a case-by-case basis, similar to other drugs in clinical development.

Herein lies the conundrum for the development of new nanomedicines. Developers of nanomedicines typically want as few regulatory hurdles as possible to allow for maximum creativity and flexibility, while large pharmaceutical companies, who usually have the expertise and resources for later stage drug development and commercialization, want as much specificity as possible about the regulatory requirements for final drug product approval to better estimate their financial commitment/exposure in bringing a new nanomedicine to market.

Each element needs to be optimized to create a new nanomedicine product formulation that can be commercialized.

To help overcome this obstacle, nanomedicine stakeholders need to create a nanomedicine development matrix to streamline optimization of the final drug product. For example, to create the ideal ratio of each nanomedicine component to insure that the new formulation has all the functionality needed for optimal safety and efficacy may require that each new nanomedicine formulation be tested directly *in vivo* for pharmacokinetics and biodistribution, looking for longer half-life of the therapeutic payload and specific organ/tissue targeting, respectively, initially skipping over both *in vitro* and *ex vivo* testing. By going from new formulation to in vivo testing, back and forth, might provide the quickest, most cost-effective strategy to define a successful nanomedicine formulation.

The Opportunity

Therefore, to truly improve the outcome of patients with solid tumors, as an example, the ideal cancer nanomedicine needs to: avoid immediate immune detection by the mononuclear phagocyte system (MPS); carry a novel active pharmaceutical ingredient (API), not re-package an already approved drug; target tumors by both passive (EPR) and active (receptor binding) mechanisms; disrupt the high IFP in tumors; and be manufactured using a scalable, robust, reproducible, and cost-effective process. Each element needs to be optimized to create a new nanomedicine product formulation that can be commercialized. And, commercialization most likely requires that patents be issued domestically and internationally to protect the composition of the final drug product, its method of production and its use.

Academia and industry need to seize the opportunity that nanotechnology-based medicines present for changing the cancer treatment paradigm and the outcome for patients with solid tumors; not focusing on perceived challenges and risks, but on the potential to dramatically impact cancer care for the world's population by treating cancer patients with safe and effective cancer nanomedicines prior to surgery, even for resectable tumors.

Manufacturing Challenges of Nano-Products

Mark Mitchnick, MD and Robert W. Lee, PhD
Particle Sciences, Inc., 3894 Courtney Street, Bethlehem, PA 18017

Why Bother with a Nanoparticle?

This brief chapter will survey the field of Nano-product manufacturing. First, the term "nano-product" implies that there is some similarity between all things "nano". Outside of the obvious shared dimensional quality, nano-products are actually widely divergent. For this review we will limit ourselves to discussing oncology related nano-particulates and not consider devices fabricated at the nano-scale. Such particles range from simple nano-particulates of pure drug to highly structured multicomponent particles and delivery systems. The term includes solid structures, liquid phases and systems that incorporate small and/or large molecules. Further, "nano" is really nothing new and, on a commercial level, we have been manipulating nanostructures for a very long time. The difference is that now we are more conscious of it and have a much greater ability to measure both what we are doing and its impact.

Because of the many possible nanoparticle structures, they can serve a host of roles in oncology therapeutics and vaccines. On a mechanical level, nano-structures can be biomimetic and engineered to be site selective. Chemically, behaviors such as solubility, reactivity and affinity can be manipulated. Further, nanoparticles can be co-formulated with other technologies imparting even greater flexibility. Ultimately, nanoparticle drug constructs can provide a variety of performance benefits that increase effectiveness: improved pharmacokinetics, improved safety profiles, improved stability, and targeted delivery.

As an indication of the activity in this space, in a Jan 17, 2013 article[7] on nanomedicine products that are approved or in various stages of clinical study by the European Medicines Evaluation Agency were summarized. Of the 247 products noted, there were a total of 33 approved drugs at the time of the study. In the oncology space, **Table 1** gives a list of approved nanotechnology-based oncology products from a publication on cancer nanomedicines[8].

Table 1: Nanotechnology Oncology Products Approved as of 2014				
Product	***Nanoplatform/ agent***	***Indication***	***Status***	***Company***
Doxil	PEGylated liposome/ doxorubicin HCl	Ovarian cancer	Approved 11/17/1995 FDA50718	Ortho Biotech (acquired by JNJ)
Myocet	Non-PEGylated liposome/ doxorubicin HCl	Metastatic breast cancer	Approved in Europe and Canada, in combination with cyclophosphamide	Teva Pharma B.V.
DaunoXome	Lipid encapsulation of daunorubicin citrate	First-line treatment for advanced HIV-associated Kaposi's sarcoma	Approved in USA	Galen Ltd
ThermoDox	Heat activated liposomal encapsulation of doxorubicin	Breast cancer, primary liver cancer	In Phase III in USA	Celsion
Abraxane	Nanoparticulate albumin/paclitaxel	Various cancers	Approved 1/7/2005 FDA21660	Celgene
Rexin-G	Targeting protein tagged phospholipid/ microRNA122	Sarcoma, osteosarcoma, pancreatic cancer, and other solid tumors	Fully approved in Philippines in 2007, Phase III Fast Track Designation, Orphan Drug Status Acquired in USA	Epeius Biotechnologies Corp
Oncaspar	PEGylated asparaginase	Acute lymphoblastic leukemia	Approved 6/24/2006	Sigma-Tau Pharmaceuticals
Resovist	Iron oxide nanoparticles coated with carboxydextran	Liver/spleen lesion imaging	Approved 2001 for European market	Bayer Schering Pharma AG
Feridex	Iron oxide nanoparticles coated with dextran	Liver/spleen lesion imaging	Approved in 1996 by FDA	Berlex Laboratories
Endorem	Iron Oxide nanoparticles coated with dextran	Liver/spleen lesion imaging	Approved in Europe	Guerbet
DepoCyt	Liposome/ cytarabine	Lymphomatous meningitis	Approved in USA	Sigma-Tau Pharmaceuticals

Scale Up Principles

The progression of a formulation manufacturing process from the benchtop to GMP is a critical step for all pharmaceuticals – it is also often very challenging. It involves the simultaneous increase in scale and the maturation of the various unit operations. Even if a formulation is very effective biologically, if it can't be reproducibly scaled to commercially relevant quantities, it is of questionable value. Therefore, from the beginning of the product

development process one needs to keep in mind eventual commercialization, i.e., using off-the-shelf manufacturing equipment if possible, using excipients that are available in the appropriate grade and generally recognized as safe (GRAS), and using processes that have a high probability of being scaled. Deviations from these are of course possible and are, in fact, quite common but their impact needs to be evaluated in real-time. In addition to safety, efficacy and quality, cost needs to be considered. Clearly, the lower the cost the greater number of people that can be potentially helped although subsidies of one kind or another can mitigate even truly expensive therapies. Also one needs to keep in mind that the infrastructure to handle highly potent compounds, as are typically required for oncology agents, is relatively scarce and that this, coupled with the need for GMP and special expertise around nanoparticles, limits the number of available commercial resources. So, early identification and involvement of a scaling partner is key. For academic groups this typically means partnering with a commercial CDMO. For commercial developers, recruitment of internal resources or an appropriate sub-contractor is needed. Either way, early transfer of the product production function will speed development and greatly enhance later chances of success.

The QBD[9] (quality by design) approach is the organizing framework under which the pharmaceutical industry now operates. A review of QBD is not appropriate here but, in brief, it is a proactive scientific approach to pharmaceutical development that pivots around the desired product attributes and provides for the establishment of well-defined processes that result in a reproducible product. During the QBD process, CQA's (critical quality attributes) are defined. CQA's are product properties that are key to safe and effective performance - the amount of drug per dose, the rate of dissolution or the sterility of an injectable are typical examples. Operating by QBD principles and using tools such as DOE (design of experiments), a well-run scale up program will progress in scale generally by increments of 10 fold. Going from mg to grams for instance or 100 mL to the liter scale. Scale up not only considers drug product production, but material acquisition, training, filling, packaging, storage, and administration. As one progresses in scale, greater attention should be paid to the equipment and processes and each weighed against their respective commercial viability.

The QBD approach is the organizing framework under which the pharmaceutical industry now operates.

Production methods and product attributes are intimately linked. Two methods of particle size reduction can yield similar size distributions but different polymorphs as a simple example. All data generated in a drug product development effort is potentially part of

the regulatory submission. This includes details on both active pharmaceutical ingredient (API) and drug product production. Some of the performance data, mainly toxicological, is required and is performed under GLP's (Good Laboratory Procedures). The purpose of this requirement is of course to insure, or at least to be able to assess the risk to, the safety of the clinical trial participants. Thus, the product used in that testing absolutely needs to be identical, in all of its CQAs, to the clinical trial materials. For a product composed purely of API, the manufacturing process used for that API is less important since equivalency of the API from one process to another can be established with some certainty. For complex nanoparticles, the situation is less clear-cut. CQA's are sometimes difficult to define early in development and thus the impact of a manufacturing variation likewise becomes difficult to quantify. For this reason, optimally, by the time legally mandated testing is being performed the manufacturing process should be essentially the same as that which will be used for clinical trial material production. In practical terms, generally speaking, this means that the process should be scaled to a clinically relevant degree no less than 12 months from the estimated first-in-human trial. To accomplish this, process rationalization should start, as a rule of thumb, at least two years prior to the first-in-human target date and, ideally, as early as possible. The more complex the product, the earlier rationalization should begin.

While each product will present its own set of challenges, there are some recurring themes. Perhaps the most frequent shortcoming manufacturers encounter in the advancement of therapeutic nanoparticles is a lack of thorough characterization of the product and the identification, to the extent possible, of the CQA's. This requires, among other things, an early emphasis on the appropriate analytical methods, which is something that is frequently neglected. Other common errors include advancing very low yield processes, failure to identify GMP sources of materials, advancing products based on single batch results, using non-scalable production methods, failure to involve regulatory expertise early on, and inadequate consideration of intellectual property constraints.

Characterization

After a therapeutic nanoparticle is identified, the qualities that enable its benefits should be well understood. Scaling a poorly characterized product is a waste of time. Basic properties should all be well documented and can include, among others, particle size, zeta potential, pH, viscosity, encapsulation efficiency, API assay and related substances, dissolution, solid state, binding efficiency and batch-to-batch variability (i.e., reproducibility). As a rule, one should have a basic idea of stability and use different lots of raw materials, if available, to test potential impact, if any. Raw materials that are themselves variable should be evaluated to establish if that variation impacts product success.

Yield

While many if not most newly developed products will have low yields, a commercially viable product must at least have the promise of adequate yields. At first this can be a paper exercise but should become a focus early on.

Sourcing

All materials used in production of products for human use will be required to be made under cGMPs or, in rare instances where GMP materials are not available and the need is compelling, be controlled to a degree that simulates GMP quality. In development, when possible, all materials used should be from GMP suppliers. This does not mean that the materials need be of GMP quality only that equivalent GMP supplies are available. By their nature however, nano-therapeutics will often incorporate unique excipients that are not available under GMP's. While not inherently bad, and potentially necessary, any such material adds a very significant cost, time and regulatory burden to the drug product development path. Educated assumptions as to their impact should be incorporated into the plan so that rational decisions as to their relative value can be made.

...efficacy should ideally be demonstrated multiple times using multiple batches of the therapeutic with proper controls.

Proof-of-Concept

While not actually a scale up issue, advancing thinly documented therapies wastes finite resources. Great scientific advances don't always make great drug products. Prior to dedicating resources to scale up, efficacy should ideally be demonstrated multiple times using multiple batches of the therapeutic with proper controls. As above, characterization is key.

Processes

After initial proof-of-concept, efforts towards using commercially viable processes should be made whenever possible. At the nano-scale, changes in process invariably result in product changes and these may or may not impact performance in a predictable way. In addition to process driven attribute changes, production methods are evaluated as to practicality. As an example, using a precipitation process at 0.1% solids would mean that for every kg of product one would produce 1,000 kg of waste. For a nanoparticle that might only contain

5% of API that translates to 1 kg of API generating 20,000 kg of waste. While potentially possible, this is certainly less than attractive. Early efforts at practical processes are vital.

Regulatory

This encompasses many aspects including, among others, toxicology and manufacturing conditions. Early developers will benefit from having access to regulatory advice to provide an understanding of the regulatory path for the various kinds of products. As an example, for a sterile product, knowledge of the relative overhead of a terminally sterilized product vs. one aseptically produced will greatly aid the developer in their process choices.

Intellectual Property

As of this writing, the US Patent Office is issuing patents with numbers approaching 9 million. Assessing one's own invention against this pool is hard enough but when one also needs to consider API patents, method of use claims and various manufacturing techniques as part of the intellectual property pool to be considered, the job becomes truly daunting. As a practical matter, developers need to be current at least in their field's literature. When approaching advanced preclinical development, involving an IP professional is advisable if the developer is financially capable of doing so.

Manufacturing

As above, nanoparticles encompass a wide variety of structures so there is no one manufacturing system to review. In general, the caveats for manufacturing include those under scale up with the addition of the necessary Quality and cGMP overhead. Independent of the nuances of a specific nano-product, the steps common to all manufacturing efforts include: technology transfer, analytic method validation and process validation. Each of these involve literally dozens of steps themselves and are intimately linked to each other. Listing them as separate efforts is purely for organizational purposes.

...developers need to be current at least in their field's literature.

Technology transfer involves moving the process from the innovators' lab to the manufacturing site. In this author's experience, this is best done during preclinical development. This allows the manufacturer to gain experience with the process and help it mature along a commercially viable path. Usual practice is that decisions around process improvement, packaging, specifications, labeling and final sourcing have not been made at the time of transfer. In the scheme presented in this chapter much of the process development effort is

effectively shifted to the CDMO making that partnering choice even more important. When possible, it is most efficient to have the same partner do both scale up and manufacturing. This saves time and a great deal of money as transferring methods is costly. A good manufacturer will also help insure that the background information needed in regulatory filings is properly assembled and ready for presentation.

Analytical methods evolve from basic-to-advanced following along with the product itself. The term "phase appropriate" is often used to describe this maturation process. The analytical methods insure the quality of the drug product, its consistent behavior, and ultimately its safety. For in-human studies the analytical methods need to be robust and, most developers will state, validated. Certain methods, sterile filtering, do not vary by development stage and needed to be fully validated even for a Phase I. This is for obvious safety reasons: a microbial contaminate in an injection could have catastrophic results. Clarity on analytical method, stage and purpose is critical. As an example, "stability" has a specific meaning from a regulatory perspective: the product has the same physicochemical properties, within predetermined limits, at some time post-manufacture as it did at the time of manufacture. On the other hand, an innovator often views stability as meaning that the product still works (i.e., has the desired biological activity, after some period of time). Both definitions are valuable and awareness of each is needed for an efficient development process.

Pharmaceutical manufacturing is a unique discipline but should not be separated from the development process.

Once the manufacturing process is locked, each unit operation needs to be refined to the point that the manufacturer has confidence in its repeatability. Ideally there is some way to monitor each unit-op to assess its function in real-time although this, referred to Process Analytic Technology (PAT) in QBD terms, is often not feasible in early stage clinical manufacturing. At a minimum, the process as a whole is demonstrated through engineering runs to produce the desired product, meeting the predetermined specifications. Invariably, because deep product production experience is lacking by definition, early clinical production relies heavily on post-production quality testing. Again, this points to the importance of the proper development of analytical methods. For certain types of products various unit operations are actually validated. This is most evident in sterile processes where the product is either produced under aseptic conditions or terminally sterilized. For aseptic production media fills are required. A media fill is a dry run of the entire process in the clean room with thorough microbial sampling of staff, product and facility to demonstrate the processes ability to

produce a sterile product. For terminally sterilized products, as above, the sterilizing process itself is fully validated.

Future Direction for Manufacturing

Pharmaceutical manufacturing is a unique discipline but should not be separated from the development process. Rather, discovery-to-commercialization should be viewed as a continuum with the handoff from one group to another taking place in phases. The basics of nano-based manufacturing are here and established today. The next 5 to 10 years will see incremental improvement in processing capabilities mostly, we believe, in the areas of aseptic handling and throughput. Why? Simply because that is where the acute need is. Along with this will come standardization and dissemination of procedural operations, again driven by regulatory mandates, not the result of any real innovation. The innovation opportunity lies in the emergence of a disruptive change, not to the nano-products themselves but to the method of manufacture. Among other properties, such a manufacturing advance will be …"cheaper, simpler, smaller and …… more convenient to use"[10] and, if history is any indication, it will be the smaller more nimble companies that champion this change and its adoption.

Regulatory Evaluation of Nanotechnology in Diagnostics for Human Use*

Kevin Lorick, PhD and Kim Sapsford, PhD
Office of In Vitro Diagnostics and Radiologic Health
U.S. Food and Drug Administration, Silver Spring, MD 20892

Background

Nanotechnology is a rapidly evolving field that has tremendous potential to advance human health and medicine. Nanomaterials have already been integrated into medical products designed to treat and diagnose serious and life threatening disease[11]. However, as often is the case, people assume that new is better; or what works well in the laboratory will work well, without modification, in a clinical setting. The zealousness to bring the latest and greatest to market, or be the first to publish on a particular topic can be at the expense of generating a high quality, well characterized, final product, which in the case of medical applications risks injury to the end user, i.e., the patient. It is the role of medical product regulation and regulatory agencies worldwide to both protect and promote the public health. United States Law, in the form of the **Federal Food, Drug, and Cosmetic Act of 1938** (the Act) and the **Public Health Service Act of 1944** (the PHS Act) give primary authority to regulate medical products to FDA.

Introduction to Diagnostic Device Regulation

FDA protects the public health by insuring that medical products are safe and effective for their Intended Use. They promote the public health by guaranteeing that the best and most innovative medical products are available to the public.

Products intended to diagnose a disease or condition, whether implantable (such a heart monitor within a pace maker), *in vivo* (such as an electroencephalogram used on a living person) or *in vitro* (using materials collected from a living person such as blood and urine tests) are considered medical devices. Devices are regulated by FDA's Center for Devices and Radiologic Health (CDRH), with a few exceptions[12]. *In Vitro* diagnostic devices (IVDs) are a special category of device with specific labeling requirements[13]. Whether a product is safe and effective is determined partially by risk classification. Depending upon the classification, an appropriate level of review of the scientific, clinical and manufacturing data for the product is applied[14,15].

While exceptions to each rule exist, generally: *Class I* devices are considered low risk and are therefore exempt from FDA review prior to being placed on the market. Manufacturers of these devices are still required to follow several procedures, referred to as General Controls. These include registration of the company with FDA; listing of all medical products the company sells; following current Good Manufacturing Practices (cGMP, known as the Quality System Regulations for devices); establishing a system for handling customer complaints, establishing a system for preventative actions, corrections and corrective actions (CAPA); performing corrections and removals as necessary (recalls); and providing labeling that is complete, truthful and accurate.

Such nanotechnology-containing devices may still be determined to be substantially equivalent to legally marketed devices or exempted from future premarket notifications and FDA review.

Manufacturers of *Class II* (moderate risk) devices are subject to the same General Control procedures as a Class I product, as well as additional Special Control procedures. The Special Controls are procedures designed to mitigate the moderate risks identified with the device. Special Controls include a submission of pre-market notification for FDA review. This procedure is described in FDA guidance documents and under section 510(k) of the Act. *Such applications are referred to by FDA and industry as, a 510(k) submission*. Review is based on a demonstration of substantial equivalence to another legally marketed Class II device, referred to as the predicate. The idea being that if the clinical value of the predicate is established, the manufacturer of a similar device only needs to show that their device is analytically and technically the same as the predicate. Clinical data is generally not required. If the new is found to be substantially equivalent to the predicate device, the 510(k) device is "cleared" for marketing. Manufacturing facilities are inspected after the device has been cleared.

Class III devices are considered the highest risk. Manufacturers of these devices are required to obtain pre-market approval (PMA). Approval of a PMA application generally requires a clinical study and inspection of both the clinical study sites and the site of manufacturing prior to the device coming on the market. Companies are also required to report all changes to device design or manufacturing[14].

Regulation of New Technologies - Nanotechnology

The Agency does not recognize a formal definition for nanotechnology[16,17], but we ask the same question of any new technology that comes into the Agency: Does it affect the safety or effectiveness of the device for its intended use? In general, the presence of a material that has not previously been used in a medical product may raise additional questions/ concerns from regulators. That said, simply adding nanotechnology to a medical device does not necessarily cause it to fall into a different classification than similar marketed Class I or II devices. Such nanotechnology-containing devices may still be determined to be substantially equivalent to legally marketed devices or exempted from future premarket notifications and FDA review.

FDA regulation has evolved over the years and will continue to do so to accommodate new emerging technologies...

If the nanotechnology enables a device to function through different principals than the predicate device, it likely would not be considered substantially equivalent, but the risk of using the new device may still not be considered high. When any new technological characteristic creates a unique device, FDA's *de novo* classification process provides a pathway for a device to be put into Class I or Class II for which general controls or general and special controls provide a reasonable assurance of safety and effectiveness, but for which there is no legally marketed predicate device. For example, special controls for a nanotechnology may reasonably include requirements for well-done physical and physiological characterizations of the new material. Once the nanotechnology-enabled device is classified as Class I or II through the de novo process, similar devices could come to market as exempt devices or by use of the 510(k) pathway, rather than premarket approval.

Combination Products

It has long been a goal of visionaries in the field of nanotechnology to generate a nanomachine that could diagnose, treat and ultimately cure a patient on the cellular level[18,19]. Moving towards such goals, nanotechnology has enabled medical products to develop beyond single mode of action devices into multifunctional platforms performing several functions – such as nanotheranostics that combines therapeutics with diagnostics. Medical products are regulated according to their primary mode of action (PMOA). In the case of products with multiple modes of action, so called combination products, it falls to the FDA's Office of Combination Products to determine whether a product achieves its primary therapeutic benefit from its action as a drug, a biologic product, or a medical device.

Once this determination is made, the regulation of the product will be assigned to the appropriate Center, either CDRH, the Centers for Drug Evaluation and Research (CDER) or Biologics Evaluation and Research (CBER). The Center(s) who have expertise in the additional parts of the combination product are consulted in the review process to insure consistency. For example, contrast agents for MRI are regulated as drugs by CDER while IVD's intended to screen the blood supply are regulated as biologics by CBER. Review of these products may reasonably include consults to MRI and IVD specialists, respectively, and hence involve CDRH. If we envision a potential nanotheranostics product for ex vivo therapy, where tissue may be removed from a patient, manipulated outside of the body, and the re-introduced to the patient, the regulatory framework would likely be related to both the ex vivo biology (regulated by CBER) and the diagnostic device (regulated by CDRH) and potentially CDER depending on the nature of the therapy.

Future Scientific and Clinical Developments

The current regulations, as they stand, provide a sound framework upon which to develop medical products that incorporate nanotechnology. That said, two major factors are found to influence future regulations:

1. The introduction of new technologies in to the medical products realm. FDA has had to deal with smartphones, genetic engineering, personalized medicine and other paradigm shifts in medicine that were precipitated by new scientific discoveries.

2. The behavior of entities marketing medical products. Major shifts in Food and Drug law have occurred because of findings of fraud, corruption, poor quality, false or off-label advertising. These findings, unfortunately, do not usually come to light until after tragedy has struck.

FDA regulation has evolved over the years and will continue to do so to accommodate new emerging technologies, such as nanotechnology, that have the potential to significantly benefit human health and medicine.

Regulatory Evaluation of Nanotechnology in Drug Products*

Katherine Tyner, PhD, Kim E. Sapsford, PhD, Subhas Malghan, PhD, and Anil K. Patri, PhD
Nanotechnology Task Force
U.S. Food and Drug Administration, Silver Spring, MD 20892

In recent years, there has been an increased focus on developing novel drug delivery systems, targeted therapies, and medical devices, including *in vitro* diagnostics, through the use of nanotechnology and nanomaterials. Such focus is translating to an increasing number of submissions for drug products and medical devices to the United States Food and Drug Administration (FDA). Although subject to the same regulatory standards and pathways as any drug or device, unique properties that arise from the small size and large surface area of nanomaterials may lead to additional scientific considerations when following current FDA guidelines and practices.

FDA has not defined the term "nanotechnology" or related terms, given the wide diversity the Agency has seen with these products. FDA has, however, published general guidance on products involving the use of nanotechnology[20]. According to this guidance, when considering whether an FDA-regulated product involves the application of nanotechnology, FDA will ask:

1. Whether a material or end product is engineered to have at least one external dimension, or an internal or surface structure, in the nanoscale range (approximately 1 nm to 100 nm), and

2. Whether a material or end product is engineered to exhibit properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to one micrometer (1,000 nm).

History of Nanotechnology in Drugs and Devices

The Center for Drug Evaluation and Research (CDER) is responsible for reviewing applications for new and generic drugs, new indications for already approved products, and active ingredients and labeling for over-the-counter drugs. CDER reviews each drug product application on its merits, regardless of the presence (or absence) of nanomaterials. CDER has a long history of approving drug products that contain nanomaterials (**Table 2**)[21]. In

recent years, the number of applications to CDER has increased, with over 350 individual applications submitted to date.

Table 2: Representative drug products involving the application of nanotechnology

Platform/Type	*Example*		
	Name	*NDA Approval Year*	*Indication*
Liposome	DOXIL® (Doxorubicin)	1995[a]	Ovarian cancer; AIDS-related Kaposi's Sarcoma; Multiple Myeloma
Inorganic nanoparticle	FERRLECIT® (Sodium ferric gluconate complex)	1999[b]	Iron deficiency anemia in patients with chronic kidney disease (CKD).
Protein nanoparticle	ABRAXANE® (Paclitaxel)	2005	Metastatic breast cancer; Locally advanced or metastatic non-small cell lung cancer (NSCLC); Metastatic adenocarcinoma of the pancreas
Polymer nanoparticle	MACUGEN® (Pegaptanib sodium)	2004	Neovascular (wet) age-related macular degeneration.
Emulsion	RESTASIS® (Cyclosporine)	2002	To increase tear production
Lipid complex	AMPHOTEC® (Amphotericin B)	1996	Invasive aspergillosis
Nanotube	SOMATULINE DEPOT® (Lanreotide acetate)	2007	Acromegalic patients who have had an inadequate response to or cannot be treated with surgery and/or radiotherapy
Nanocrystal	TRICOR® (Fenofibrate) 48mg/145mg tabs	2004[c]	Primary hypercholesterolemia or mixed dyslipidemia; Severe hypertriglyceridemia.
Micelle	TAXOTERE® (Docetaxel)	1996	Breast Cancer; Non-Small Cell Lung Cancer; Hormone Refractory Prostate Cancer; Gastric Adenocarcinoma; Squamous Cell Carcinoma of the Head and Neck Cancer

[a] First ANDA approval in 2013.
[b] First ANDA approval in 2011.
[c] First ANDA approval in 2012.

Nanotechnology was first exploited in "first generation" products of nanocrystals or liposomes, where the drug products were typically reformulations of previously known, often poorly water soluble, drug substances. Nanotechnology was used to increase bioavailability, alter biodistribution, or both. In recent years, a "second generation" of products has begun to emerge, which incorporates more complex structures and functions into the drug formulation (example: drug delivery systems with targeting capabilities).

Medical devices are regulated by FDA's Center for Devices and Radiologic Health (CDRH). Products intended to diagnose a disease or condition, whether implantable *in vivo* (such as

a heart monitor within a pace maker), external *in vivo* (such as an electroencephalogram used on a living person) or *in vitro* (using materials collected from a living person such as blood and urine tests) are considered medical devices. CDRH reviews each medical device application, regardless of the presence (or absence) of nanomaterials, by asking the same question: Is this product safe and effective for its Intended Use. Under the Federal Food, Drug and Cosmetic Act, Code of Federal Regulations (CFR) title 21, 860.3, medical devices are classified into three categories based on risk: class I, class II and class III, often referred to as low, moderate and high risk, respectively. Device classification determines the regulatory pathway and the types of controls to which a medical device may be subject. Although CDRH does not have a long history of clearing/approving medical products that contain nanotechnology, there are a limited number of *in vitro* diagnostics that have been cleared/approved and the current regulations, as they stand, provide a sound framework upon which to regulate such devices.

Review Considerations for Drug Products and Devices Containing Nanomaterials

FDA has multiple guidance's for products involving the application of nanotechnology. These guidance's may be Agency-wide, Center-specific, or even product-specific. **Table 3** lists several of the relevant FDA guidance's involving nanotechnology.

Nanotechnology was used to increase bioavailability, alter biodistribution, or both.

In general, drug product applications contain the following information:

- Description and composition
- Physicochemical characterization
- Description of the manufacturing process and packaging
- Specifications needed for product release
- Analytical methods and validation of these methods used to characterize the drug product
- Stability studies to support an expiration date, or shelf life, and in-use conditions.

Table 3: FDA Guidance on Nanotechnology		
Guidance Category	***Name***	***Weblink***
NANOTECHNOLOGY		
General and cross-cutting topics	Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology	http://www.fda.gov/regulatoryinformation/guidances/ucm257698.htm
Food	Assessing the Effects of Significant Manufacturing Process Changes, Including Emerging Technologies, on the Safety and Regulatory Status of Food Ingredients and Food Contact Substances, Including Food Ingredients that are Color Additives	http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm300661.htm
Cosmetics	Safety of Nanomaterials in Cosmetic Products	http://www.fda.gov/Cosmetics/GuidanceRegulation/GuidanceDocuments/ucm300886.htm
Animal & Veterinary	Draft Guidance for Industry: Use of Nanomaterials in Food for Animals	http://www.fda.gov/Cosmetics/GuidanceRegulation/GuidanceDocuments/ucm300886.htm
Chemistry, Manufacturing, and Controls (CMC)	Draft Guidance for Industry: Liposome Drug Products Chemistry, Manufacturing and Controls; Human Pharmacokinetics and Bioavailability; and Labelling Documentation	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070570.pdf
GENERIC DRUG PRODUCTS		
Bioequivalence Recommendations	Draft Guidance on Doxorubicin Hydrochloride	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM199635.pdf
Bioequivalence Recommendations	Draft Guidance on Amphotericin B	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM384094.pdf
Bioequivalence Recommendations	Draft Guidance on Verteporfin	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM384173.pdf
Bioequivalence Recommendations	Draft Guidance on Paclitaxel	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM320015.pdf
Bioequivalence Recommendations	Draft Guidance on Sodium Ferric Gluconate Complex	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM358142.pdf
Bioequivalence Recommendations	Draft Guidance on Ferumoxytol	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM333051.pdf
Bioequivalence Recommendations	Draft Guidance on Iron Sucrose	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM297630.pdf
Bioequivalence Recommendations	Draft Guidance on Sirolimus	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM089640.pdf
Bioequivalence Recommendations	Draft Guidance on Paliperidone Palmitate	http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM270384.pdf

The presence of nanomaterials, due to their unique properties, may warrant emphasis on different portions of the review of the drug product. There is a great diversity in drug products containing nanomaterials, ranging from metal colloids to polymeric micelles. Such diversity can make it difficult to apply generalities to all drug products containing nanomaterials. Despite the diversity, some common attributes exist when considering the quality of drug products containing nanomaterials. These include:

- Size and size distribution
- Nanomaterial composition
- Three dimensional structure
- API to nanomaterial ratio
- State of API (e.g., encapsulated, bound, etc.)
- Surface functionalization and state of the surface ligands (if any)
- Surface coating quantitation, density and polydispersity
- Zeta potential or surface charge

In addition, how the characterization of these quality attributes is conducted may vary greatly from one application to another, and is generally more involved than technologies or methods that have been traditionally used for other drug products. Finally, it is generally recognized that orthogonal or complementary methods are needed for key quality attributes of drug products containing nanomaterials due to the high impact of these critical physicochemical properties on the ultimate product performance.

Nanotechnology in medical diagnostics and devices

In general, the presence of a material that has not previously been used in a diagnostic medical device may raise additional questions or concerns from regulators. However, simply adding nanotechnology to a medical device does not necessarily cause it to fall into a different classification than similar marketed Class I or II devices that do not incorporate nanotechnology. Such nanotechnology-containing devices may still be determined to be substantially equivalent to legally marketed devices (called a predicate device) or exempted from future premarket notifications and FDA review.

Nanotechnology may enable medical products to develop beyond a single mode of action into multi-functional platforms performing several functions...

If the nanotechnology enables a device to function through a different principle than the predicate device, it likely would not be considered substantially equivalent to a predicate, but the risk of using the new device may still not be considered high. In such cases, FDA's *de novo* classification process provides a pathway for the device to be put into Class I or Class II. For devices, for which there is no legally marketed predicate device, general controls or general and special controls provide a reasonable assurance of safety and effectiveness. For example, special controls for a nanotechnology may reasonably include requirements for well-done physical and physiological characterizations of the new material. Once the nanotechnology-enabled device is classified as Class I or II through the *de novo* process, it can be used as a predicate for similar devices and these could come to market as exempt devices or by use of the 510(k) pathway, rather than premarket approval (PMA).

Nanotechnology may enable medical products to develop beyond a single mode of action into multi-functional platforms performing several functions – such as nanotheranostics that combines therapeutics with diagnostics. In the case of products with multiple modes of action, so called combination products, it falls to the FDA's Office of Combination Products to determine the primary mode of action (PMOA) of a product. Once this determination is made, the regulation of the product will be assigned to the appropriate Center, either CDRH, CDER or Biologics Evaluation and Research (CBER). The Center(s) who have expertise in the additional parts of the combination product are consulted in the review process to ensure consistency.

Future Regulatory Outlook

The number and complexity of submissions of drug and medical device products containing nanomaterials is expected to increase in the next 5-10 years as the potential of nanotechnology within the medical field is fully realized. Although not treated differently within the regulatory pathway, these drug and medical device products often have different emphasis on parts of the review process due to the specialized properties of the nanomaterials and the product's intended performance (drugs) or use (devices). In either case, an understanding of the scientific basis of the functioning of the nanomaterial within the product, as well as the instrumentation used to characterize it, will assist both applicants and reviewers alike in speeding these products to market.

*Disclaimer: The views presented in these articles do not necessarily reflect those of the Food and Drug Administration.

Section VI: References

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