NCL Method PCC-19

Asymmetric-Flow Field-Flow Fractionation

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

Nanomaterials are inherently polydispersed. Traditional techniques, such as the widely used batch-mode dynamic light scattering (DLS) analysis, are not ideal nor thoroughly descriptive enough to define the full complexity of these materials. Asymmetric-flow field-flow fractionation (AF4) with various in-line detectors, such as ultraviolet–visible (UV-Vis), multi-angle light scattering (MALS), refractive index (RI) and dynamic light scattering (DLS) is an alternative technique that can provide flow-mode analysis of not only size distribution, but also shape, drug release/stability, and protein binding [1-3].

2. Principle

The technique of field-flow fractionation (FFF) was first developed by Giddings in 1966 and further advanced into a class of flexible analytical fractionating techniques with unique capabilities to separate analytes ranging from ~1 nm to ~100 μm in size [4]. The separating device is usually a thin, flat channel with a parabolic laminar flow passing through, which carries the sample forward from the inlet to the outlet. Perpendicular to the forward channel flow, an external physical field is applied to the channel to drive the accumulation of sample at the bottom wall. It is a broad family of separation methods based on the nature of the physical field applied to generate separation. The identity of this applied external physical field defines the type of FFF; for example, centrifugal field generated by centrifugation (CFFF), a temperature difference (TFFF), and electric field (EFFF) [5-7].

Asymmetric-flow field-flow fractionation (AF4) is the most widely used type of FFF. In AF4, the bottom wall is a semi-permissive membrane with a specific cutoff size, allowing the penetration of solvent and small molecules below the cutoff size but retaining the sample components larger than the cutoff size, as shown in Figure 1. The physical driving force is a cross flow – perpendicular to the forward channel, for sample accumulation at the bottom membrane. The combination of the two forces applied eventually results in the separation of the sample compound according to their respective diffusion coefficients (i.e. their hydrodynamic radius or molar mass, respectively). With adjustable cross flow, this makes AF4 a powerful fractionating technique with great flexibility to accommodate samples with different size ranges [8,9].

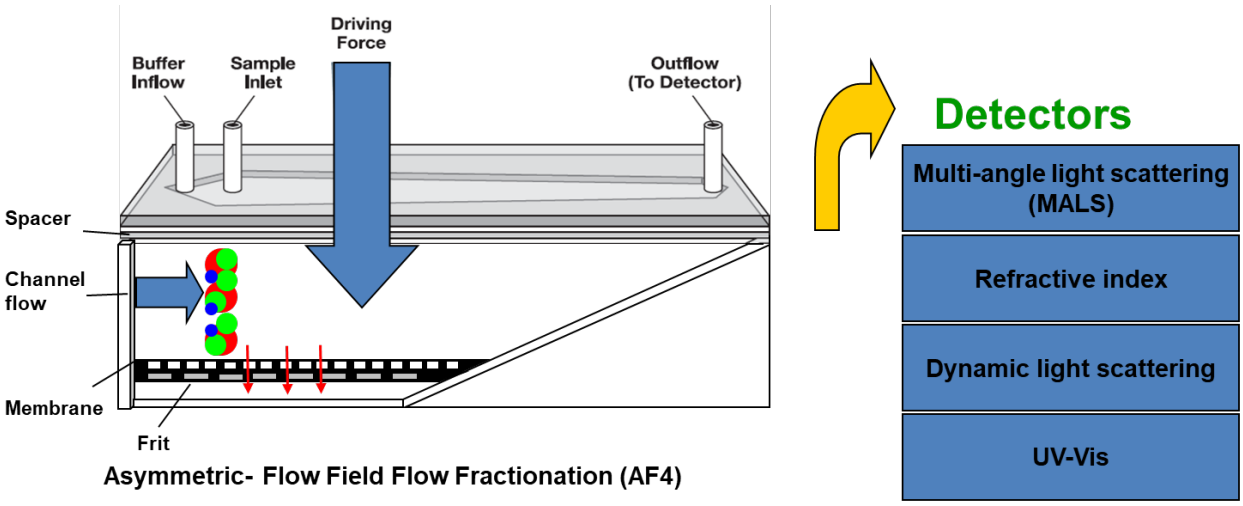

Figure 1. Working principle of asymmetric-flow field-flow fractionation (AF4) separation.

Owing to its great compatibility with different buffers, AF4 has been widely utilized to characterize biological and non-biological analytes, such as proteins, polymers, liposomes, virus-like particles, etc. It allows the separation of molecules in solution and particles in the same separation run. The separation takes place without the use of a stationary phase as in column chromatography. Typically, when coupled with downstream detectors such as UV-Vis, RI, MALS and DLS, purity, radius of gyration, and hydrodynamic size of the fractionated sample can be measured.

In addition, the collected sample fractions can be further analyzed with methods not suitable for in-line (downstream) detection, such as transmission electron microscopy (TEM), reversed phase high performance liquid chromatography (RP-HPLC) with either UV-Vis or fluorescence detection, and resistive pulse sensing to name a few.

This protocol will not discuss the data analysis in detail; instead, this protocol focuses on the AF4 technical aspect. Many aspects of AF4 analysis are nanoparticle and application specific and will be up to the reader to adapt for each application.

3. Equipment and Reagents

Note: The NCL does not endorse any of the suppliers listed below; their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted.

3.1 Equipment

3.1.1 Isocratic pump (G1310B, Agilent)

3.1.2 Well-plate autosampler (G1329A, Agilent)

3.1.3 AF4 separation channel (Eclipse AF4 or Eclipse DualTec, Wyatt Technology)

3.1.4 MALS detector (DAWN HELEOS II, Wyatt Technology)

3.1.5 UV-Vis detector (G1315B, Agilent)

3.1.6 Refractive index detector (Optilab, Wyatt Technology)

3.1.7 DLS instrument (Zetasizer Nano ZS, Malvern).

The separation channel had a length of 275 mm (long channel) and a 350 μm spacer. A 10 kDa regenerated cellulose membrane was used for all separations. Other channel lengths, spacers and membranes are available; these have worked best for the nanoparticles tested at NCL.

3.2 Reagents

3.2.1 Bovine serum albumin monomer (BSA) (Sigma-Aldrich, A1900).

3.2.2 Phosphate buffered saline (PBS, pH 7.4) - recommended buffer for measurements of NPs

3.2.3 Human plasma: For plasma incubation studies, human plasma was collected from healthy volunteer donors under National Cancer Institute (NCI) at Frederick Protocol OH99-C-N046.

4. Experimental Procedure

4.1 System Setup

Note: This is specific for the Wyatt system; user manual procedure from Wyatt is referenced [10, 11] in this protocol and modified according to NCL application.

4.1.1 Launch software (ChemStation in this protocol)

Double-click ChemStation software and navigate to the Eclipse Manual Control window from top menus, select “Instrument” > “More Wyatt Eclipse” > “Control”

4.1.2 Purge System

Begin with slow flow rates, set a 1 mL/min detector flow and a 1 mL/min cross flow first in “Focus Mode” for a few minutes then switch to “Elution Mode” for a few more minutes.

4.1.3 Switch to a new membrane

- Fresh membrane should be used every time when running a new sample or switching to a different solvent.
- Replace the membrane inside the long channel following the procedure in the Wyatt user guide.
- Reconnect the channel to the Eclipse chassis, following the order “Sample” → “Inlet” → “Crossflow”, do not connect the outlet.
- Adjust detector flow rate to 3 mL/min in “elution + inject” mode to flush the surface of the membrane to remove any bubbles and particulates on the new membrane.
- To remove any air trapped under the membrane, disconnect the crossflow waste tubing from the Eclipse chassis and place this end into a beaker. Switch from “elution” mode to “focus” mode and apply a focus flow of 1.5 mL/min for 15 minutes.
- Reconnect all the tubing to the Eclipse chassis, set detector flow rate to 1 mL/min in “elution + inject” mode, wait until channel pressure and cross flow pressure are constant (may take up to 15 min).

4.1.4 Conditioning a new membrane

- To minimize the non-specific binding onto the membrane surface and improve the mass recovery, condition injections are made such that the sample (or a sample of similar chemical composition) is overloaded onto the channel in order to saturate any active sites on the new membrane surface with a defined covering layer.
- For aqueous system, the manufacturer (Wyatt) recommends using bovine serum albumin (BSA) with a mobile phase solution of PBS. Typically, two 100 µL of 5 mg/mL BSA solution are run.
- Setup BSA method

- Select “Instrument” > “Setup Wyatt Eclipse” to open the “Eclipse method editor” window
- Input the necessary information including Separation device, Device properties, Flow setting and Focus valve position. Set the detector flow rate to 1.0 mL/min, inject flow to 0.2 mL/min and focus flow rate to 3.0 mL/min. An example of BSA Eclipse timetable is as follows:

Table 1. Recommended Eclipse method timetable for BSA injection (long channel)

Start Time (min)	End Time (min)	Duration (min)	Flow mode	Vx Start (mL/min)	Vx End (mL/min)
0.00	2.00	2.00	Elution	3.00	3.00
2.00	4.00	2.00	Focus	-	-
4.00	9.00	5.00	Focus + Inject	-	-
9.00	11.00	2.00	Focus	-	-
11.00	26.00	15.00	Elution	3.00	3.00
26.00	28.00	2.00	Elution	3.00	0.00
28.00	31.00	3.00	Elution	0.00	0.00

- After two runs of BSA sample, the second set of data can be used to set up the necessary system parameters in the ASTRA software, including band broadening, alignment and normalization (refer to the ASTRA user guide).
- BSA running results including expected elution time, consistent fractograms and target molecular weight of monomer, dimer, etc. can be used as a quick check for system readiness. Afterwards the membrane can be used with full performance.

4.2 AF4 Method Optimization

Based on the AF4 separation theory, there are several key parameters that are critical for high-resolution separation, including cross flow, channel height, sample focusing, type of membrane and the amount of loaded sample. These

factors collectively determine fractionation quality and changing one parameter usually influences the other factors on resolution power. Testing different combinations of these factors, however, can be expensive, time consuming and labor intensive, and thus can be impractical. Thus, understanding the working principles of AF4 and determining the complexity of the analyzed samples (e.g., prior examination by batch mode DLS, electron microscopy, etc.) will be useful in guiding the method development process.

4.2.1 Cross flow

Based on the AF4 theory, cross flow is the driving force counteracting the Brownian motion of particles to separate particles with different hydrodynamic sizes at different channel flow laminae at steady state (Equation 1). Thus, cross flow is a defining factor in AF4 fractionation quality. To determine the optimal cross flow for sample fractionation, it is necessary to evaluate various cross flow rate ($\dot{V}_{cross}$)/ channel flow rate ($\dot{V}_{channel}$) settings. We advise to start with varying the $\dot{V}_{cross}/\dot{V}_{channel}$ ratio in constant gradient based on the particle sizes and channel pressure. A rule of thumb is to start by analyzing an unknown sample using a gradient elution profile, from a cross flow which allows sufficient retention of early components of interest (usually from literature) and gradually then decrease it to 0 mL/min.

$$t_r = \frac{\pi \eta d w^2}{2kT} \cdot \frac{\dot{V}_{Cross}}{\dot{V}_{Channel}}$$

Equation 1 [12].

Where t_r = retention time, w = channel thickness, $\dot{V}_{cross}$ = cross flow rate, $\dot{V}_{channel}$ = channel flow rate, d = Stokes diameter, η = solvent viscosity, k = Boltzmann's constant, T = absolute temperature.

4.2.2 Channel

4.2.2.1 Channel height

According to the working principle of AF4 (Equation 1), the channel's geometry, especially its height, is critical to high quality fractionation. The channel height is generally the thickness of the

channel, determined by the spacer utilized in between the upper wall and the bottom accumulation membrane. A series of spacers with different thicknesses (250, 350, 490, and 600 µm) are provided by Wyatt. Different channel heights give different parabolic laminar flow rate profiles as well as different system pressures and thus affect separation resolutions. Also, a thicker channel allows the analysis of larger sample amounts. In this protocol, to recover sufficient amount of sample for downstream analysis, we used spacer with a thickness of 350 µm.

4.2.2.2 Focus

Focus point can directly affect total sample inject amount. We used a 100-µL sample loop in our instrument for sample loading. During the focus step, a flow opposing the channel forward flow was introduced from the outlet, and, together with the channel flow, it focuses the sample into a narrow band close to the injection port. The focusing flow rate and focusing time determine focusing efficiency. It is recommended to perform focus position tests, using the flow methods listed below:

Table 2. Recommended focus test timetable (long channel, 350 µm wide spacer, recommended by Wyatt [13])

Start Time (min)	End Time (min)	Duration (min)	Flow mode	Vx Start (mL/min)	Vx End (mL/min)
0.00	1.00	1.00	Elution	3.00	3.00
1.00	2.00	1.00	Focus	-	-
2.00	3.00	1.00	Focus + Inject	-	-
3.00	5.00	2.00	Focus	-	-

Channel spacer also comes with wide and narrow taper widths to be used at different focus point.

4.2.2.3 Frit-inlet channel

For less stable or easy to aggregate samples, we recommend using frit-inlet channel to avoid the focusing step by conventional AF4 technique. Using a frit-inlet channel, the sample is injected in a relatively low flow rate (typically 0.2 mL/min) through the channel inlet, while a much higher flow (1 – 3 mL/min recommended) is introduced into the channel through the inlet frit. Thus, the sample is driven in direction of the accumulation wall by the frit flow, resulting in a relaxation of the sample components within the inlet frit area.

4.2.3 Membrane

In AF4, a semi-permissive membrane is placed at the bottom of the channel with a specific cutoff size, allowing the penetration of solvent and small molecules below the cutoff size but retaining sample components of interest. Because the sample fractionation is performed close to the membrane, sample may nonspecifically bind to the membrane surface and cause sample loss during the separation. Thus, it is important to choose a compatible membrane material with the specific sample. There are two types of membrane materials provided by the manufacturer (Wyatt) in precut form:

Table 3. Precut membranes for Eclipse from Wyatt

Material Active Layer	MWCO[1] [kDa]	Specific Flux[2] [L m^{-2} bar^{-1} h^{-1}]	Membrane Moisture Expansion[3] (H_2O) [µm]	Temperature Range [°C]	pH Range
Regenerated Cellulose (RC)	10	8.0	101	5 - 80	2 - 10
	30	10.3	129		
Polyether Sulfone (PES)	5	6.2	68	5 - 90	3 – 13
	10	9.6	88		
	30	8.2	67		

Note:

1. Molecular weight cutoff (in kDa); the molar mass exclusion value determined with water soluble polymers
2. Specific Flux is the liquid flow rate through the membrane in liters per square meter of membrane surface area in one hour at 1 bar pressure
3. Determined by measuring the elution time of BSA from a short channel containing a 490 µm tall, wide taper spacer

Smaller MWCO will affect overall system pressure which means cross flow range is less and therefore may lead to poor resolution. In this protocol, we selected the RC membrane with 10 kDa cutoff for our studies.

4.2.4 Sample Loading

Once the key fractionation parameters are determined, the next step is to choose right amount of sample to be injected into the AF4 system. The minimal amount of material required for AF4 is mainly determined by the sensitivity limit of the in-line detectors, i.e., the UV, MALS, DLS and RI signals and the fraction needed for downstream analysis. The signal/noise (S/N) ratio must be adequate for accurate data collection and interpretation. It is also important to carefully avoid overloading the channel. The maximal amount of material is determined by the required

resolution of fractionation, which depends on the purpose of the experiment and the complexity of the sample. At very high concentrations, the mutual interference between NPs near the accumulation wall may result in steric effects, reversing the fractionation order and perturbing the shape (broadening/widening of the peaks) of the fractogram.

4.3 Sample Preparation

For normal, non-plasma incubated samples, stock sample solutions are usually diluted 100-fold with PBS. This dilution procedure is specific to the nanoparticle and its light scattering properties so as not to saturate detectors or overload membrane. For plasma incubation studies, we typically use the same final dilution as the non-plasma sample, for example, 10 μL of nanoformulation was incubated with 100 μL human plasma at 37°C for 2 hr. Prior to injection, 890 μL PBS was added to make the final injection solution 100-fold dilution in 10% (v/v) plasma and a 100-fold diluted sample.

5. Method Application/Examples

AF4 can be used to gain a plethora of information about nanoparticles, including their size distribution, the chemical makeup/composition across this size distribution, or particle amounts across the distribution. Several manuscripts of these applications offer example analyses [3,14,15].

5.1 Size Distribution

AF4 elution profiles can be optimized to provide a clearer picture of the size distribution of a nanoparticle. For example, a single peak that appears in batch-mode DLS can actually be a mixture of two or more discrete populations or a heterogenous mixture of a range of sizes. An optimized AF4 elution profile can aid in exposing the true size distribution. The elution profile presented in Table 4 is offered as a good starting point for polydispersed nanoparticles with broad size ranges from 50 – 300 nm.

In the example presented by Hu et al, a polymeric micelle that showed a single peak in batch-mode DLS was resolved into two distinct peaks using AF4

with UV and DLS detection. By either coupling additional in-line detectors, or collecting fractions for off-line analysis, the relative abundance of each population, as well as the chemical makeup of each, can also be measured.

Table 4. AF4 elution profile for the analysis of polydispersed nanoparticles, 50-300 nm.

Start Time (min)	End Time (min)	Duration (min)	Flow mode	Vx Start (mL/min)	Vx End (mL/min)
0.00	2.00	2.00	Elution	1.00	1.00
2.00	4.00	2.00	Focus	-	-
4.00	9.00	5.00	Focus + Inject	-	-
9.00	19.00	10.00	Focus	-	-
19.00	29.00	10.00	Elution	1.00	1.00
29.00	31.00	2.00	Elution	1.00	0.50
31.00	41.00	10.00	Elution	0.50	0.00
41.00	61.00	20.00	Elution	0.00	0.00
61.00	63.00	2.00	Elution + Inject	0.00	0.00
63.00	66.00	3.00	Elution	0.00	0.00

5.2 Stability

AF4 can also be used as a quick screening tool to assess the stability of a nanoparticle, i.e. to approximate rapid drug release in a biological setting. It is recommended to incubate nanoparticles in the presence of human plasma prior to AF4 analysis; however, for users without access to human plasma, BSA-coated membranes also work well for assessing nanoparticle stability. AF4 membranes are often passivated with BSA, offering insight into how a nanoparticle will behave in the presence of protein in biological matrix. Specifically, this technique is used to assess whether the drug is rapidly released from the nanoparticle and partitions to protein in the plasma or the BSA-passivated membrane, or whether it is retained with the nanoparticle.

Following AF4 separation, fractions can be collected either automatically or manually, then assayed for drug content in each of the collected fractions. It has been shown that nanoparticles which show no or very little drug recovery also show a rapid release of drug in biological matrix. These results can be confirmed using other, more quantitative, assays, such as the stable isotope tracer ultrafiltration assay [16,17].

A representative AF4 elution timetable for smaller nanoparticles (approx. 40-50 nm range) is shown in Table 5. A representative elution profile for larger nanoparticles (approx. 100 nm) is shown in Table 6. The software SCOUT DPS (Wyatt Technology) is another tool to identify a starting elution profile. It uses first-principles physics to simulate the separation process and thus optimize the channel flow rate, cross-flow rate gradients, spacer height and membrane porosity for AF4 method development. More details about the software can be found on the company website [18].

Table 5. AF4 timetable for the analysis of approx. 40-50 nm nanoformulations

Start Time (min)	End Time (min)	Duration (min)	Flow mode	Vx Start (mL/min)	Vx End (mL/min)
0.00	2.00	2.00	Elution	1.00	1.00
2.00	4.00	2.00	Focus	-	-
4.00	9.00	5.00	Focus + Inject	-	-
9.00	19.00	10.00	Focus	-	-
19.00	29.00	10.00	Elution	1.00	0.00
29.00	44.00	15.00	Elution	1.00	0.00
44.00	59.00	15.00	Elution	0.00	0.00
59.00	64.00	5.00	Elution + Inject	0.00	0.00
64.00	69.00	5.00	Elution	0.00	0.00

Table 6. AF4 timetable for the analysis of approx. 100 nm nanoformulations

Start Time (min)	End Time (min)	Duration (min)	Flow mode	Vx Start (mL/min)	Vx End (mL/min)
0.00	2.00	2.00	Elution	1.00	1.00
2.00	4.00	2.00	Focus	-	-
4.00	9.00	5.00	Focus + Inject	-	-
9.00	19.00	10.00	Focus	-	-
19.00	29.00	10.00	Elution	1.00	1.00
29.00	31.00	2.00	Elution	1.00	0.50
31.00	81.00	50.00	Elution	0.50	0.50
81.00	83.00	2.00	Elution	0.50	0.00
83.00	85.00	2.00	Elution	0.00	0.00
85.00	87.00	2.00	Elution + Inject	0.00	0.00
87.00	92.00	5.00	Elution	0.00	0.00

5.3 Protein Binding

AF4 combined with plasma incubation studies is also useful for assessing protein binding. Nanoparticles are incubated in human plasma, then diluted prior to injection into the AF4. Shape factors (ratio of radius of gyration to hydrodynamic radius) before and after plasma incubation are calculated and compared to give a qualitative assessment of the protein binding.

This is demonstrated in the manuscript by Hu et al [3], whereby three commercially available liposomes with different degrees of PEGylation were studied: Doxil, Onivyde and AmBisome. The elution profile in Table 6 was used for these samples and was optimized to separate free plasma proteins from ~100 nm nanoparticles. The shape factors for each of these formulation pre- and post-incubation in plasma are shown in Table 7. Doxil, which has the highest amount of PEG on the surface (~5%), showed no significant shift or change in the shape factor post-incubation. Onivyde, which has a lower degree of PEGylation, showed a slight increase in the shape factor. AmBisome, which does not contain PEG on

the surface, showed a significant increase in the shape factor, more than 38%. The increased shape factor indicates a mass distribution shift to the surface of the liposomes, suggesting protein binding on the surface.

Table 7. Shape factors of Doxil, Onivyde and AmBisome before and after plasma incubation

	Composition (molar ratio)	Shape factor before plasma incubation	Shape factor after plasma incubation
Doxil	HSPC : Chol : mPEG-DSPE 55 : 40 : 5	$\rho = 0.80$	$\rho = 0.77$
Onivyde	DSPC : Chol : mPEG-DSPE 59.8 : 39.9 : 0.3	$\rho = 0.86$	$\rho = 0.96$
Ambisome	HSPC : Chol : DSPG 53 : 26 : 21 (no PEGylation)	$\rho = 0.77$	$\rho = 1.13$

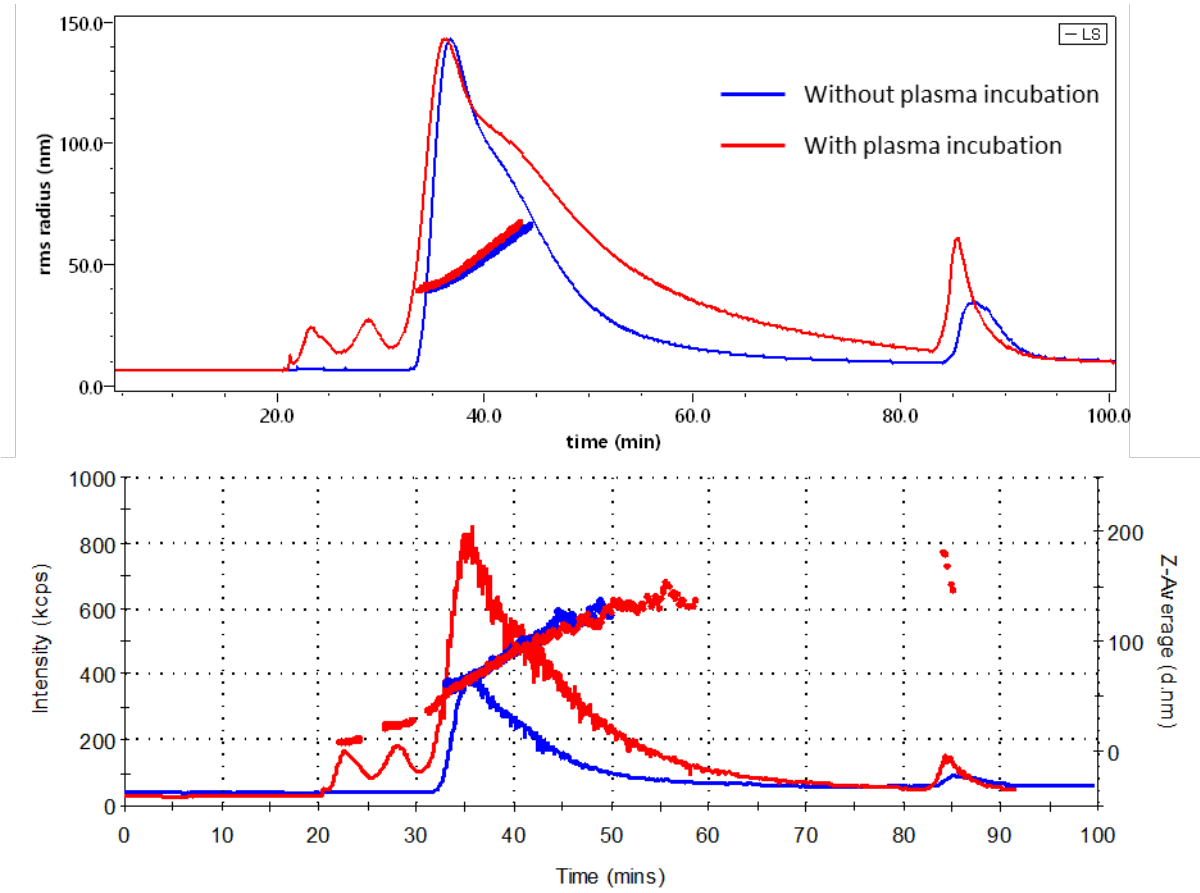

Figure 2. Assessment of protein binding. Flow-mode AF4 separation of Ambisome using both MALS (upper panel) and DLS (lower panel) detection. The blue traces represent nanoparticles without plasma incubation. The red traces represent nanoparticles with plasma incubation. The intensity threshold for with plasma incubation was >120 kcps and for with plasma incubation was >100 kcps.

6. References

1. Albanese, A., Tang, P. S., Chan, W. C. The Effect of Nanoparticle Size, Shape, and Surface Chemistry on Biological Systems. Annu Rev Biomed Eng. 14, 1–16 (2012).
2. Desai, N. Challenges in Development of Nanoparticle-Based Therapeutics. AAPS J. 14, 282–295 (2012).
3. Hu, Y., Crist, R. M., & Clogston, J. D. The utility of asymmetric flow field-flow fractionation for preclinical characterization of nanomedicines. Anal Bioanal Chem. 412, 425–438 (2020).

4. Giddings, C. J. A new concept based on a coupling of concentration and flow nonuniformities. Sep Sci. 1, 123–125 (1966).

5. Yang, F. J. F., Myers, M. N. & Giddings, J. C. Programmed sedimentation field-flow fractionation. Anal Chem. 46, 1924–1930 (1974).

6. Giddings, J. C., Martin, M. & Myers, M. N. High-speed polymer separations by thermal field-flow fractionation. J Chromatogr A. 158, 419–435 (1978).

7. Caldwell, K. D. & Gao, Y. S. Electrical field-flow fractionation in particle separation. 1. Monodisperse standards. Anal. Chem. 65, 1764–1772 (1993).8. Fraunhofer, W. & Winter, G. The use of asymmetrical flow field-flow fractionation in pharmaceutics and biopharmaceutics. Eur J Pharm Biopharm. 58, 369–383 (2004).

9. Yohannes, G., Jussila, M., Hartonen, K. & Riekkola, M. L. Asymmetrical flow field-flow fractionation technique for separation and characterization of biopolymers and bioparticles. J Chromatogr A. 1218, 4104–4116 (2011).

10. TN6000, Eclipse AF4 with separation channel quick start guide. Wyatt Technology Corporation.

11. User's manual: Eclipse separation system AF4. Wyatt Technology Corporation.

12. Light Scattering University. FFF Practical Applications. Wyatt Technology Corporation (2018).

13. MC, SC and LC User Manual Rev.1.0. Separation Channel User Manual. Wyatt Technology Corporation.

14. Caputo, F., Arnould, A., Bacia, M., Ling, W. L., Rustique, E., Texier, I., Mello, A. P., & Couffin, A. C. Measuring Particle Size Distribution by Asymmetric Flow Field Flow Fractionation: A Powerful Method for the Preclinical Characterization of Lipid Based Nanoparticles. Mol Pharm 16(2): 756–767 (2019).

15. Ansar, S.M., Mudalige, T. Characterization of doxorubicin liposomal formulations for size-based distribution of drug and excipients using asymmetric-flow field-flow fractionation (AF4) and liquid chromatography-mass spectrometry (LC-MS). Int J Pharm. 574, 118906 (2020)

16. Skoczen, S., McNeil, S. E., Stern, S. T. Stable isotope method to measure drug release from nanomedicines. J Control Release. 220, 169–174 (2015).

17. Skoczen, S., Snapp, K.S., Crist, R.M., Kozak, D., Jiang, X., Liu, H., & Stern, S. T. Distinguishing Pharmacokinetics of Marketed Nanomedicine Formulations Using a Stable Isotope Tracer Assay. *ACS Pharm Transl Sci.* DOI: 10.1021/acsptsci.0c00011 (2020).

18. https://www.wyatt.com/products/software/vision.html#vision-2. Wyatt Technology Corporation.