NIST - NCL Joint Assay Protocol, PCC-7

Measuring the Size of Nanoparticles Using Transmission Electron Microscopy (TEM)

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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

Transmission electron microscopy (TEM) has been found to be an excellent tool for characterizing the size of nanoparticles. This assay protocol outlines procedures for sample preparation and the determination of mean nanoparticle size (in projection) using TEM. Although the projected particle size is the primary determinant of the measured particle diameter, other parameters can impact these measurements and influence the measured size. Therefore, guidelines for making successful size measurements in the nanometer-size range are provided, as well as a discussion of relevant standards and data analysis. This protocol can be applied to any suitable TEM instrument with image recording capability.

The specimen preparation protocol has been specifically tailored for application to NIST Reference Materials RM8011, RM8012 and RM8013, which consist of negatively charged citrate-stabilized gold nanoparticles in aqueous solution at a mass concentration nominally 50 μg/g. The procedures should work with other nanoparticles that carry a negative surface charge or zeta potential. Moreover, these procedures can also be applied to positively charged or neutral nanoparticles with some modification. Each procedure may require some optimization by the user in order to obtain satisfactory deposition density and to minimize artifacts such as aggregate formation on the substrate or build up of organic films resulting from additives that might be present in the solution phase. This protocol is intended to be an evolving document; addenda that address material-class-specific sample preparation and analysis issues will be appended as available. Some suggested references for TEM analysis are provided at the end of this document **(1-3)**.

2. Sample Preparation Procedures

2.1 Wear appropriate personal protective gear (e.g., gloves, lab coat, goggles, respirator, etc.) and take appropriate precautions when handling a nanomaterial.

2.2 TEM sample grids with a continuous silicon oxide film that is electron transparent are preferred. Grids of silicon monoxide (on formvar film supported by copper

mesh), or silicon dioxide thin film membrane grids (such as SmartGirds™ from Dune Sciences[1]) are also suitable.

2.3 Glassware and apparatus used for sample preparation should be cleaned with filtered, demineralized water and stored dry. If commercial cleaning agents are used, care should be taken to remove all traces of the cleaning detergent as this may leave residues on the nanomaterial. If available, store apparatus under HEPA filtered air (e.g., in a clean bench).

2.4 The apparatus used for dispersion and deposition of particles consists of a small glass vial with a screw-on cap, a teflon pillar about 10 mm high that may be inserted into the vial, a petri dish, and a teflon block about 40 mm by 40 mm square, see Figure 1. The vial and pillar are used to functionalize the TEM sample grid, and the petri dish and block are used to deposit the nanoparticles onto the functionalized grid.

Figure 1. Apparatus used to functionalize and capture nanoparticles on TEM grids. Shown are the small glass vial with Teflon pedestal in the inverted cap, Teflon block with two test chips under cover, beakers used for dip rinsing, and a box of pipette tips.

[1] Commercial materials are identified in the protocol to adequately specify the measurement procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials identified are necessarily the best available for the purpose.

2.5 Derivitization of the TEM sample grid is accomplished by exposing the silicon oxide to a small amount (about 10 μL) of amino-propyl-dimethyl-ethoxy-silane (APDMES, NH2 – (CH2)3 – Si – (CH3) 2 (C2H5O)) solution. This solution serves to attach well-separated positive charges onto the oxide surface which will attract and bind to the negatively charged nanoparticles.

2.5.1 The small glass vial is used as the sealable treatment chamber to minimize the air volume, as well as to prevent moisture from entering and APDMES vapors from escaping. The teflon pedestal is used to elevate the grid and provide a non-wetting surface for the APDMES solution.

2.5.2 Place the pedestal into the inverted vial cap. Place the grid with the silicon oxide surface upwards onto the pedestal.

2.5.3 Cover the grid with a 10 μL drop of APDMES; it is not a problem if some APDMES solution gets under the grid.

2.5.4 Carefully screw the glass cover onto the cap and let stand at room temperature for the chosen Derivatization Time (DT); in most cases, one hour is sufficient.

2.5.5 Open the vial enclosure, and, holding the grid with tweezers, dip rinse the grid thoroughly with ethanol. Wick the excess liquid off the grid using filter paper. Place the dried grid onto the clean Teflon block.

2.5.6 Clean the glass vial enclosure with ethanol immediately to minimize dimerization of the APDMES residue due to ambient moisture exposure.

2.6 The nanoparticles in this protocol are citrate-stabilized and therefore possess a negative charge; they will be attracted to the positively charged TEM grid surface.

2.6.1 Place the APDMES treated grid face up onto the cleaned teflon block.

2.6.2 Place a 10 μL drop of the nanoparticle solution onto the grid. It is not a problem if some of the solution goes under the grid provided that the support surface and the underside of the grid are clean.

2.6.3 Cover the grid with a petri dish lid and let stand at room temperature for the chosen Capture Time (CT), typically from 5 to 20 min.

2.6.4 Carefully dip-rinse the grid with demineralized water, followed by dip rinsing in ethanol and wicking dry with filter paper.

2.7 When cleaning up, follow appropriate and acceptable waste disposal procedures for the specific nanomaterial.

3. Measurement Procedures

For the purposes of this protocol, the TEM measures the projected images of particles deposited onto an electron-transparent substrate. Internal structure, as well as surface morphology, may contribute to the image appearance.

3.1 A well-aligned and stable TEM is operated at a fixed magnification that allows a large number of nanoparticles to be visible within the micrograph field of view, while ensuring that each individual nanoparticle is recorded with a large number of image pixels. As an example, a nanomaterial with roughly 50 nm particles, imaged at nominal 50,000 times magnification, recorded on a CCD camera with pixel dimensions of 14 μm (square pixels) would consist of nanoparticles approximately 180 pixels in diameter. A CCD camera with 2048 by 2048 pixels would contain a maximum of about 120 particles within the field of view of a single micrograph.

3.2 Record enough micrographs to image a minimum of 200 nanoparticles per sample per grid square from a minimum of 2 widely separated regions of the grid. Image recording times should be sufficiently long so that the average grey-scale ratio of particle to background is at least 5:1. Recording times also should be kept short (1 to 2 s) to minimize contributions from stage drift.

4. Precautions and Guidelines

4.1 Nanoparticle Number Density

The ideal TEM sample for nanoparticle size analysis is one with a large number of individual nanoparticles within the desired TEM micrograph field of view, but without excessive agglomeration or bunching of nanoparticles. Two factors may influence the nanoparticle number density on the TEM grid: the efficiency of the derivitization process, and the concentration of nanoparticles in solution. In general, the derivitization process for silicon monoxide films will

result in fewer positive charges on the TEM grid than silicon dioxide films. Likewise, dilute nanoparticle solution concentrations will require longer capture times to obtain a suitable number of nanoparticles than more concentrated solutions. Some experimental iteration may be required to optimize the TEM specimen preparation technique.

4.2 Handling of TEM Sample Grids

TEM grids (especially thin film membranes) are very fragile and must be held by their edges with fine tweezers so as not to damage or crack the membrane. It is also important when dip-cleaning or rinsing the grids that they are inserted vertically (edge first) into liquids. Any side-to-side motion of the grids may rupture the support film. Likewise, drying of the grids should be accomplished by wicking away liquid using filter paper. In the case where liquid is retained between the tweezer tips, a small piece of filter paper may be inserted near the tip to wick away excess liquid.

The use of pressurized air canisters for drying should be avoided as air bursts may damage the grids. Exposure of TEM grids to the ambient environment should be minimized to reduce the likelihood of dust contamination. Grids should be stored in suitable boxes in dust-free or desiccating cabinets.

4.3 TEM Alignments

A detailed description of the TEM alignment will not be given here; however, it is important to note that a well-aligned TEM is essential to obtain accurate particle size results. In addition to typical alignments that are routinely done for bright-field TEM, such as well-aligned illumination, high voltage centering, beam tilt purity and coma, and astigmatism, other factors will influence image quality and resolution. These factors include distortions (e.g., spiral and pincushion), aberrations, instabilities, hysteresis and thermal drift of the electron optics, objective lens excitation (focus), specimen stage drift characteristics, and sample charging or alteration (damage) due to interactions with the electron beam. Detailed procedures on alignment should be described in the TEM instrument manual.

Once the appropriate imaging conditions and magnification are achieved in the TEM, no further changes should be made to the electron optical conditions. Micrographs of particles within the field view should be recorded, and then the microscope stage translated to a new region. The sample grid should be flat with no stage tilts applied. The objective lens excitation should not be significantly changed to focus an image after the first field of view is captured; rather, the height of specimen stage may be mechanically adjusted with fine controls.

4.4 Measurements

A minimum of 200 discrete particles should be measured from each of at least two widely separated regions of the sample (that is, different grid squares or membrane regions). Obviously, larger numbers of particles should be counted to minimize errors and provide higher levels of confidence. Foreign debris in a given image (e.g., dust particles or residues from the rinsing and drying process) should be avoided.

5. Performance Verification and Data Analysis

5.1 Standards and Performance Verification

Typical TEMs are capable of a wide range of magnification and have myriad operational modes, with the result that the indicated magnification of the TEM instrument may differ from the actual magnification by as much as 10 %. Therefore, TEM instruments must be calibrated in order to relate particular electron optical conditions to a known length scale. Moreover, it is recommended to periodically run standards to provide qualification (i.e., verification) of correct instrument operation within manufacturer specifications and to validate measurement procedures.

For TEM magnification calibration purposes, latex size standards down to nominally 20 nm (NIST-traceable polystyrene spheres) are available from a number of commercial suppliers. Below 20 nm, colloidal gold is commercially available in a wide range of sizes down to nominally 2 nm. Crystals of catalase enzyme may be used for this purpose. Grating samples (either linear or cross-grating with nominal pitch of 2160 lines per mm) may be used to evaluate the

performance and assess the distortions in the microscope projector lens system. If the TEM is capable of point resolutions below 0.2 nm, properly oriented thin foil specimens of single crystals such as silicon may be used for calibration purposes by resolving the periodic spacings of the well-known crystalline lattice. Certified reference materials are also available from NIST (https://srmors.nist.gov/), including SRMs 1963a and 1964 (100 nm and 60 nm polystyrene lattices) and RMs 8011, 8012 and 8013 (10 nm, 30 nm, and 60 nm gold nanoparticles). All water-dispersed standards are subject to instabilities over time and shelf-life limitations. Check that your standard of choice has not exceeded the stated expiration date.

As discussed in ISO 13322-1:2004 the measurement of particle-size distributions by microscopy methods is apparently simple, but due to the limited amount of sample examined, considerable care must be exercised in order to ensure that the analysis is representative of the entire sample **(4)**. ISO 13322-1:2004 recommends splitting the original sample and making measurements on three or more parts. Statistical analysis of the data, for example using the Student's t-test, will reveal whether the samples are truly representative of the whole.

Particle size results obtained from TEM measurements may not coincide with those obtained from other techniques (e.g., dynamic light scattering). This is due in part to differences in the weighted averages determined in each case (e.g., number for TEM versus intensity for dynamic light scattering), as well as differences in the physical property that is actually measured (e.g., projected area versus hydrodynamic diffusion area). TEM is sensitive to the size of primary particles, whereas dynamic light scattering is influenced by the presence of small quantities of large particles or clusters of smaller particles.

5.2 Data Analysis and Measurement Parameters

A detailed description of the data analysis will not be given here. There are many methods available to analyze the size distribution, and these methods will process the data differently using different inherent assumptions. It is left for the reader to identify the appropriate data analysis algorithm or to seek 3rd party software solutions. Widely used software includes ImageJ (rsb.info.nih.gov/ij/) and IgorPro (www.wavemetrics.com).

The basic process in determining the particle size distribution is to transform a digitized micrograph from a grey-scale image into a binary image consisting of discrete particles and background. Classification of image pixel values may be accomplished by means of thresholding the image, and then tabulating the number of pixels for each particle. Image thresholding operations are subject to user bias and ideally should be automated as much as possible. In cases where the background grey values are not uniform across the image (for example, the presence of dust particles on the grid or residues from an incomplete cleaning process), automated routines may fail requiring the user to manually select appropriate threshold values for multiple sub-regions.

Once the proper binary image is obtained, further classification of image pixels can be performed. A nanoparticle will consist of a large number of contiguous pixels that meet the threshold criteria. However, some pixels from the background also will have grey values sufficient to satisfy the nanoparticle threshold. The background pixels typically will be singular or small numbers of contiguous pixels, therefore a minimum size criterion may be applied to the binary image. Likewise, a given nanoparticle may possess individual or a small number of pixel “holes” found completely contained within the nanoparticle; these holes may be “filled” to eliminate them from the analysis.

From the binary image, the nanoparticle size will be determined by measuring the number of contiguous pixels that meet the classification criteria for a particular micrograph. The number of pixels (or rather the area) is then converted into the equivalent circular diameter assuming a hard, uniformly spherical particle. Therefore, the particle size does not consider any pronounced faceting.

The nanoparticle circularity value, defined as $4\pi A/P^2$ (where A is the particle area and P is its perimeter), approaches 1 for an ideal circle. Particles with circularities of 0.25 or below may be considered as aggregates or artifacts in the size analysis. The use of overlays of particles found on the original micrograph is recommended to assess the presence of these artifacts in the analysis.

6. Reporting Particle Size Data

ISO 13322-1:2004 gives specific recommendations for reporting of test results derived from microscopy methods. At a minimum, report the mean particle size and variance based on the measurements as well as the number of particles measured. If a size distribution analysis algorithm is applied, then it should be identified along with any key parameter values used in the analysis. Other critical information that should be reported includes: TEM instrument make and model, operating voltage, beam current settings, objective lens excitation, and diffraction aperture size (if used). Additional information that can be helpful to include in a report are: a typical micrograph at the measurements conditions, frame size, pixel dimensions, image acquisition time per micrograph, and mean signal-to-noise ratio between background and particles.

7. References

1. K.Y. Jung, B.C. Park, W.Y. Song, B.-H. O, and T.B. Eom, *Measurement of 100-nm polystyrene sphere by transmission electron microscope*, Powder Technology **126** (2002) 255.
2. D. Ozkaya, *Particle Size Analysis of Supported Platinum Catalysts by TEM,* Platinum Metals Review **52** (2008) 61.
3. G.C. Claver and W.H. Farnham, *Polymer particle damage in the electron microscope—a practical problem*, Powder Technology **6** (1972) 313.
4. ISO 13322-1:2004. Particle size analysis -- Image analysis methods -- Part 1: Static image analysis methods - available for electronic purchase and download at http://www.iso.org/.

8. Glossary of Terms

This section contains a limited glossary of terms that may be helpful to those reading and using this document.

binary image: Digitized image consisting of an array of pixels, each of which has a value of 0 or 1, whose values are normally represented by dark and bright regions on the display screen or by the use of two distinct colors.

capture time: The amount of time the functionalized silicon oxide film is exposed to the nanoparticle solution. Longer times provide more opportunity for nanoparticles to approach and bind to the functionalized grid. Excessive capture times, however, may result in agglomeration of nanoparticles leading to complications in the size analysis.

derivitization time: The amount of time the silicon oxide film is exposed to the APDMES solution treatment. Longer times will provide a larger number of positive point charges on the film.

equivalent circular diameter: The diameter of a circle having the same area as the projected image of the particle.

field view: Field which is viewed by a device, e.g. transmission electron microscope photographic film plate or CCD camera.

frame view: Sub-field in a field view in which particles are counted for image analysis

grey-scale image: An image where each pixel is permitted multiple grey level values.

nanosize: For purposes of this protocol, implies that all physical dimensions are smaller than 100 nm (1 nm = 10^{-9} m).

pixel or picture element: Individual sample in a digital image that has been formed by uniform sampling in both the horizontal and vertical directions.

qualification: Proof through measurement with reference material that an instrument is operating in agreement with the manufacturer's specifications.

threshold: Grey level value which is set to discriminate objects of interest from the background.

validation: Proof through use of a reference material that a measurement procedure is acceptable for all elements of its scope.

9. Abbreviations

APDMES	aminopropyldimethylethoxysilane
CCD	charge-coupled device
CT	capture time
DT	derivitization time
HEPA	high-efficiency particulate air
ISO	International Organization for Standardization
m	minute
min	minute
mm	millimeter
μL	microliter
μm	micrometer
NIST	National Institute of Standards and Technology
nm	nanometer
RM	reference material
s	second
SRM	standard reference material®
TEM	transmission electron microscopy