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FOREWORD

This special publication is one in a series stemming from the National Nanotechnology Initiative (NNI) nanotechnology environmental health and safety (Nano-EHS) Research Strategy which identified Nanomaterial Measurement Infrastructure as one of the essential areas of research needed in order to develop an effective risk assessment and management plan regarding various aspects of nanotechnology in consumer products as it pertains to human health, exposure and the environment. The National Institute of Standards and Technology (NIST) was identified as a lead agency in the development of measurement strategies for the robust development to assess the potential effects of engineered nanomaterials and their fate in the environment. One important factor in these measurements is a reliable method to measure changes to engineered nanomaterials, such as particle dissolution, in biologically and environmentally relevant matrices.

The current protocol presents a method to measure dissolution of silver nanoparticles in biological and environmental matrices by measuring changes to the dispersions using ultraviolet-visible (UV-vis) absorbance measurements. Updates to this protocol may be released in the future. Visit http://nist.gov/mml/np-measurement-protocols.cfm to check for revisions of this protocol, or new protocols in the series. We also encourage users to report citations to published work in which this protocol has been applied.

1. Introduction

Making accurate measurements of the environmental fate and biological effects of engineered nanomaterials (ENMs) is critical for reliable risk assessment of these materials. However, results from toxicological studies, such as measurements of their cell toxicity, often differ substantially among laboratories and have questionable reliability (1-3). One important factor that can lead to variability in these measurements is that nanomaterials may undergo substantial changes such as agglomeration and dissolution in test media used to make toxicological measurements. If the changes to the nanomaterials are not accurately measured, this can lead to inaccurate dosing metrics (3). Silver nanoparticles (AgNPs), for example, can undergo numerous dynamic changes during nanoEHS studies such as agglomeration and dissolution (3-5). In addition, dissolved silver ions can form silver chloride nanoparticles in test media containing chloride (3, 4, 6), and thus measurements of dissolved silver may not fully captured the extent of AgNP dissolution during an experiment. One challenge in measuring the changes to AgNPs in these systems is differentiating between AgNPs and AgCl particles. If measurements of total silver and dissolved silver are made by using techniques such as inductively-coupled plasma mass spectrometry before and after ultrafiltration, it is possible to differentiate between silver particles and dissolved silver. However, ultrafiltration may not distinguish between AgNPs and AgCl particles if they have similar sedimentation coefficients.

In this document, we describe a protocol, directly adapted from (4), to measure changes in the amount of silver remaining in the AgNP form for AgNPs with constant agglomeration states by quantifying the localized surface plasmon resonance (LSPR) using ultraviolet-visible (UV/vis) absorbance spectrum measurements. While AgNPs, for a given particle diameter, will give a consistent LSPR absorbance spectrum in the absence of agglomeration or dissolution (7), AgCl particles do not have an associated LSPR absorbance. AgCl particles may produce some light scattering at lower wavelengths, but this scattering can usually be subtracted since its spectrum is sufficiently different from the AgNP LSPR absorbance spectrum. Importantly, this technique also does not require the separation of the AgNPs from dissolved ions. When using the area under the absorbance peak from 340 nm to 650 nm, the bias (2 x standard deviation) of the relationship between dissolution measured by inductively-coupled plasma-mass spectrometry (ICP-MS) and UV/vis spectroscopy with unagglomerated citrate-coated AgNPs that dissolved between 20 % and 70 % was -0.5 %±5.4 %. The relationship between the integrated absorbance peak from 340 nm to 650 nm and the fractional dissolution from ICP-MS is shown in Figure A1.

2. Principles and Scope

This protocol is proposed for the measurement of AgNPs remaining in biological and environmental solutions using UV-vis absorbance spectroscopy as long as agglomeration of the AgNPs does not occur. Additional measurements are needed to quantify dissolved silver ions (i.e., ultracentrifugation), and this information along with those made in this protocol can provide information about the chemical speciation of silver in test media.

3. Terminology

This protocol complies with definitions relevant to nanotechnology as set forth in ASTM International E2456 *(8)* and is consistent with the draft standard ISO TS 80004-1:2010 *(9)*.

nanoparticle—sub-classification of ultrafine particle that is characterized by dimensions in the nanoscale (i.e., between approximately 1 nm and 100 nm) in at least two dimensions; also referred to as "nano-object" in ISO TS 80004-1:2010 *(9)*.

primary particle — the smallest discrete identifiable entity associated with a particle system; in this context, larger particle structures (e.g., aggregates and agglomerates) may be composed of primary particles.

aggregate — a discrete assemblage of primary particles strongly bonded together (i.e., fused, sintered, or metallically bonded).

> *Note—The adjective "primary", when used in conjunction with the term aggregate, is employed in the present context to indicate the smallest achievable dispersed particle entity.*

agglomerate—assemblage of particles (including primary particles and/or smaller aggregates) held together by relatively weak forces (e.g., van der Waals, capillary, or electrostatic), that may break apart into smaller particles upon further processing.

> *Note—Although we define them as distinct entities, the terms aggregate and agglomerate have often been used interchangeably to denote particle assemblies*.

dispersion—used in the present context to denote a liquid (aqueous) in which particles are homogeneously suspended, or the process of creating a suspension in which discrete particles are homogeneously distributed throughout a continuous fluid phase; implies the intention to break down agglomerates into their principal components (i.e., primary particles and/or aggregates).

4. Materials and equipment

4.1 Reagents

4.1.1 High-purity nitric acid

4.1.2 AgNPs dispersion in water (this protocol is for AgNPs already dispersed in water; the dispersion protocol for AgNPs in water and the synthesis of AgNPs are beyond the scope of this protocol)

4.1.3 Test media (e.g., de-ionized water, EPA moderately hard water with humic acid, or cell culture media Dulbecco's modified eagle medium (DMEM) with 4.5 g/L glucose and sodium pyruvate but without phenol red or L-glutamine and with 2 % bovine serum albumin added)

4.2 Materials

4.2.1 Centrifugation vials (e.g., Fisherbrand™ Snap-Cap™ Microcentrifuge Tubes: Flat Top 1.5 mL)

4.2.2 Autosampler plasticware (e.g. 15 mL round or conical polypropylene vials) for making ICP-MS measurements

4.2.3 Calibrated pipettes and disposable tips (e.g**.,** Eppendorf™ Research™ Plus Pipetters and Fisherbrand™ Standard Pipette Tips)

4.2.4 1-cm quartz cuvette, or any cuvette that allows UV/vis measurements between 300 nm and 1100 nm

4.2.5 Disposable semi-micro cuvettes for DLS measurements (e.g. Malvern 12mm Square Polystyrene Cuvettes (DTS0012))

4.3 Equipment

4.3.1 Inductively-coupled plasma mass spectrometer that is capable of making quantitative measurements of silver at concentrations at least one hundred times lower than the AgNP concentration to be tested

4.3.2 UV/vis absorbance spectrophotometer (single pass) capable of measuring between 300 nm and 1100 nm

4.3.3 Dynamic light scattering instrument or any other instrument for quantifying the AgNP size distribution

4.3.4 Centrifuge capable of spinning the samples at a sufficiently fast concentration to separate the AgNPs and AgCl particles from ions (e.g., 20800 g for 30 min as described in 5.2.1)

5. Measuring of changes in the AgNP concentration in complex media

5.1 UV/vis absorbance measurements

5.1.1 Calibrate or validate the UV/vis spectrometer in the range 300 nm to 1100 nm. This can be accomplished using, for example, NIST standard reference material 931g (liquid absorbance filters, UV-VIS) or 2031b (metal-on-fused-silica neutral density filters).

5.1.2 Measure the absorbance from 300 nm to 1100 nm of the test media in which AgNPs will be dispersed (without the AgNPs) relative to deionized water. The test media should be diluted with deionized water in the same way as the AgNP solutions in 5.1.3.

5.1.3 Measure the absorbance from 300 nm to 1100 nm of the initial AgNP samples. The AgNPs should be diluted with deionized water to approximately 5 ug/mL or whatever concentration results in peak absorbance values between 0.5 and 1.0. All AgNP samples should be vortexed immediately prior to measurement here and in 5.1.4 in order to avoid artifacts from sedimentation of agglomerates.

5.1.4 Measure the absorbance from 300 nm to 1100 nm of the AgNP samples in the test media at different time points. It is important to also measure AgNP agglomeration (e.g., using dynamic light scattering as described in (10)) as agglomeration will impact the absorbance peak and could result in an incorrect estimation of the AgNP dissolution.

5.1.5 Subtract the absorbance of the media from that of the AgNP samples.

5.1.6 To minimize effects of slight changes in the baseline, the baseline (mean absorbance between 900 nm and 1,100 nm) can be subtracted from each curve (4). For samples with Cl present (this will be known for test media commercially purchased or prepared in the laboratory), subtract the absorbance of AgCl NPs from the spectrum using the equation $Abs_{AgCl} = (Abs_{325}-0.055xAbs_{400}) \times (\lambda/325)^{-4}$. To develop this equation (4), scattering due to AgCl NPs was approximated by assuming Rayleigh scattering (absorbance is proportional to λ^{-4}) and setting the absorbance at 325 nm (Abs_{325}) to 5.5 % of the peak absorbance (Abs_{400}); unagglomerated AgNPs are known to have an LSPR near 400 nm. For example, the absorbance reported at 400 nm by the spectrophotometer may be the absorbance between 399.5 and 400.5 if it reports values at 1 nm increments. Note that different sizes of AgNPs may have peak absorbances that differ from 400 nm, and in this case the actual peak absorbance value should be used. The absorbance at 325 nm was

chosen for fitting because unagglomerated AgNPs have a minimum at 325 nm, and 5.5% was used because Abs_{325} is 5.5% of Abs_{400} for unagglomerated AgNPs. This correction factor will be most accurate when AgCl NPs are small, because deviations from Rayleigh scattering become significant when the NPs are not much smaller than the wavelength of light.

5.1.7 Integrate the peak from 340 nm to 650 nm. This can be accomplished by adding the absorbance values between 340 nm and 650 nm using software such as Microsoft Excel. For example, if the spectrometer measures absorbance at increments of 1 nm, add all 311 absorbance measurements between 340 nm and 650 nm. The percentage decrease in the integrated peak area will give you an estimate of the AgNP dissolution for a sample without agglomeration at whatever time points are measured as described in 5.1.4.

5.2 Measuring AgCl NP formation

5.2.1 Measure the formation of silver ions in the AgNP samples (e.g., centrifugation at a high speed (20800 g for 30 min) followed by elemental analysis of the supernatant (e.g., using ICP-MS as described in (4))). The accuracy of different measurement methods for quantifying silver ion dissolution is beyond the scope of this protocol.

5.2.2 Subtract the amount of total AgNP dissolution (determined in step 5.1.7) from the total mass of dissolved silver (determined in step 5.2.1); to convert from the dissolved silver concentration to the total mass of dissolved silver, multiply the dissolved silver concentration by the volume of solution.

6 Abbreviations

AgNP	silver nanoparticle
AgCl	silver chloride
DLS	dynamic light scattering
DMEM	Dulbecco's modified eagle medium
ENM	engineered nanomaterial
EPA	environmental protection agency
ICP-MS	inductively-coupled plasma-mass spectrometry
LSPR	localized surface plasmon resonance
NP	nanoparticle
UV/vis	ultraviolet-visible

7 Acknowledgements

We acknowledge the contributions of Danielle Cleveland and Carly Lay A. Geronimo who contributed to manuscript (4) from which this protocol was adapted.

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Appendix

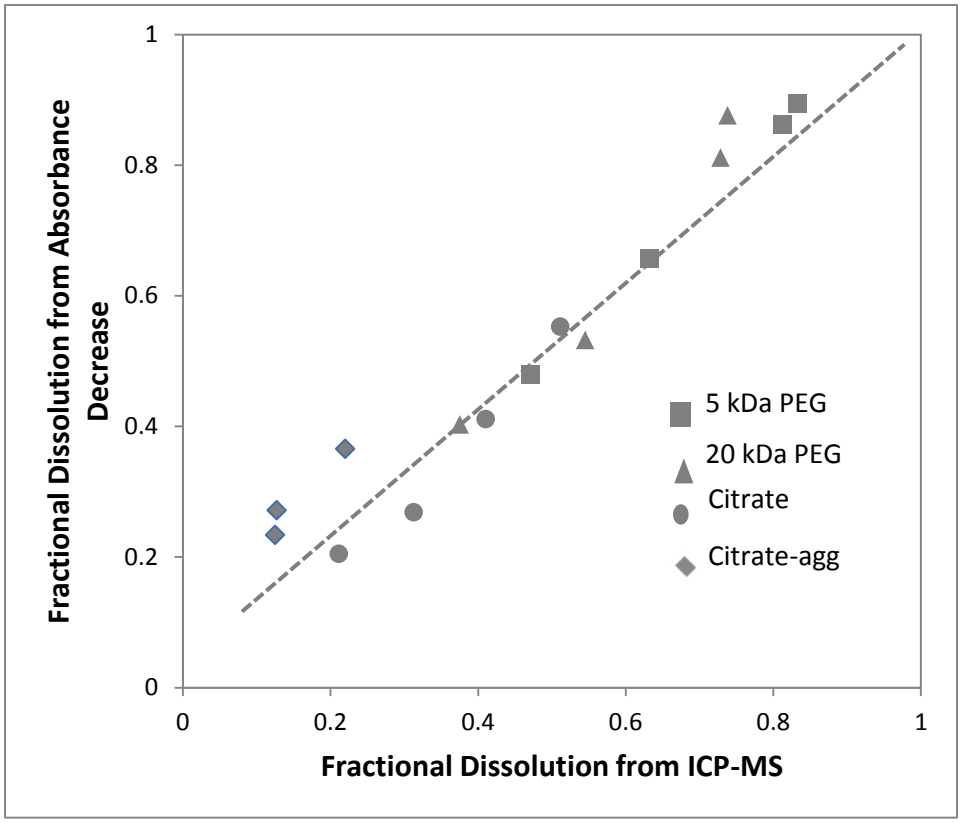

Figure A1 Comparison of fractional dissolution of AgNPs measured by decrease in absorbance to dissolution measured by ICP-MS. The decrease in absorbance was calculated from the integrated area under the absorbance curve. Dissolution was measured for AgNPs coated with (1) 5 kDa PEG-thiol (squares) and (2) 20 kDa PEG-thiol (triangles) in 100 mmol/L HNO3, (3) citrate-coated AgNPs dispersed as primary particles in 150 mmol/L KNO3+2% BSA (circles), and (4) citrate-coated AgNPs dispersed as agglomerates that grow over 5 days from 230 nm to 780 nm (DLS intensity-weighted mean diameters) in 7.5 mmol/L KNO3+0.1% BSA (diamonds). The diagonal dotted grey line indicates the 1:1 correlation expected from Beer's law. Standard deviations for replicated absorbance and ICP-MS measurements of the same sample are smaller than the symbols.