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FOREWORD

This special publication is one in a series stemming from the National Nanotechnology Initiative (NNI) 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 dispersion procedure.

The current guideline presents a method to produce nanoparticle agglomerates of different sizes in cell culture medium that can be stable for at least two days. Importantly, this approach allows for different agglomerate size preparations with identical chemical components in the medium. 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 potential 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 key step to improve the reliability of cell toxicity measurements is the preparation of consistent ENM dispersions prior to toxicity testing. Different dispersion procedures may lead to variable results. In addition, ENM agglomeration can often occur quickly in cell medium as a result of the high ionic strength, thus posing a challenge for attaining reproducible cytotoxicity studies. To help understand the effect of agglomeration on toxicity, this protocol describes a simple, reliable method to produce stable ENM agglomerates of different sizes in cell media (4). For example, silver nanoparticles (AgNPs) with an initial mean unagglomerated size of ≈ 23 nm were used to produce agglomerates ranging in size from 43 nm to 1400 nm in a cell culture medium containing proteins (4). Importantly, we identified mixing rate, time between mixing steps, and ENM concentration as critical factors for achieving reproducible dispersions. In addition, we showed that this procedure is applicable to multiple ENMs: gold nanoparticles (AuNPs) (Figure A1), silver nanoparticles (Figure A2), cerium dioxide nanoparticles (Figure A3), and positively charged polystyrene particles (Figure A3).

2. Principles and Scope

This protocol, directly adapted from (4), is proposed for the reliable preparation of ENM dispersions with varying agglomerate sizes in cell culture media (Dulbecco's Modified Eagle Medium (DMEM) supplemented with bovine serum albumin (BSA)). Application of this procedure for ENM dispersions in environmental media (e.g., EPA moderately hard water) is beyond the scope of this protocol. The method described herein, if applied correctly, yields a reliable dispersion of ENM agglomerates with tightly controlled and reproducible sizes. These agglomerates generally retain their size for at least two days as demonstrated in a previously published study (4).

3. Terminology

This protocol complies with definitions relevant to nanotechnology as set forth in ASTM International E2456 *(5)* and is consistent with the draft standard ISO TS 80004-1:2010 *(6)*.

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 *(6)*.

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—assembly 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. Reagents, materials, and equipment

4.1 Reagents

4.1.1 Dulbecco's modified eagle medium with 4.5 g/L glucose and sodium pyruvate but without phenol red or L-glutamine

4.1.2 Bovine serum albumin in powdered form (e.g., Sigma Aldrich ≥ 96 % pure, essentially fatty acid free)

4.1.3 100 ug/mL Streptomycin and 100 U/mL Penicillin (e.g., Life Technologies Penicillin-Streptomycin (10000 U/mL))

4.1.4 Engineered nanomaterials dispersed in water (this procedure was tested with four ENMs as described in section 1 and is anticipated to work for the full range of ENMs)

4.2 Materials

4.2.1 Approximately 5 mL syringe with 0.2 µm polypropylene filter or a similar filter with other compatible material (All materials should be sterile and endotoxin free if the ENM dispersion is to be used for cytotoxicity testing)

Note—Pyrogens (also known as endotoxins) are shed from the outer membrane of Gram-negative bacteria during cell division or lysis. These toxins are relatively heat-stable and are not destroyed under typical sterilizing conditions. As a result, pyrogens are ubiquitous and can interfere with the accuracy of toxicity assays. To depyrogenize glassware, bake at 250 °C for 2 h or at 200 °C overnight.

Note—Limulus Amoebocyte Lysate (LAL) reagent grade pyrogen-free water can be obtained from commercial vendors.

Note—Sterility and absence of pyrogen contamination should be verified for all materials in contact with the dispersion. If using the LAL test for pyrogens, avoid using cellulose-based filters, as they can be a source of beta-glucan, which interferes with the LAL assay. An LAL test for use with dispersions containing ENM has been published by ISO (29701:2010) and is recommended (7).

4.2.2 15 mL polystyrene centrifuge tube

4.2.3 Calibrated pipettes and disposable tips

4.3 Equipment

4.3.1 Vortex mixer

4.3.2 Analytical balance

5. Preparation of ENM agglomerate dispersions in cell media

5.1 Preparation of stock DMEM and BSA solutions

5.1.1 Prepare a stock solution with DMEM and antibiotics (add 100 μg/mL and 100 U/mL for streptomycin and penicillin, respectively; e.g., this can be done by adding the pre-made solution in 4.1.3 at 1 % volume/volume). Add 4 % BSA (by mass) by weighing the appropriate mass of BSA on an analytical balance and adding it to the DMEM solution. All DMEM solutions described in subsequent steps have antibiotics added.
Note—If the dispersion is not intended for toxicological assessment, pyrogen-free conditions may not be necessary.

5.1.2 Filter the solution using a 0.2 μm polypropylene filter to remove large protein aggregates that may interfere with nanoparticle size measurements.

5.2 "0 second" mixing procedure (designed to yield smallest agglomerate size)

5.2.1 Add 0.8 mL DMEM and 1 mL DMEM with 4 % BSA to a 15 mL polystyrene centrifuge tube.

5.2.2 Add 0.2 mL of ENM stock suspension while vortexing. The speed of the vortexing may impact the size of the agglomerates obtained (see Figure A2 for additional information). This will reduce the final concentration of BSA in the solution to 2 %.

5.3 Mixing procedure to obtain "1 s" or "5 s" ENM agglomerates in cell media

5.3.1 Add 0.8 mL DMEM to a 15 mL polystyrene centrifuge tube.

5.3.2 Hold the sample in place on the vortexer using a foam holder and turn the vortexer to 115 rad/s (1100 rpm).

5.3.3 Add 0.2 mL of ENM dispersion and vortex at a speed of 115 rad/s (1100 rpm) for a predetermined period (e.g., 1 s or 5 s). This step allows the ENM dispersion to agglomerate for a controlled period.

5.3.4 After mixing for 1 s or 5 s, add 1 mL of DMEM with 4 % BSA to stop agglomeration. It is believed that BSA coats the agglomerates and thereby produces steric hindrance to prevent further agglomeration. This step will reduce the final concentration of BSA in the solution to 2 %.

5.4 Mixing procedure to obtain larger ENM agglomerates in cell media

5.4.1 Add 0.8 mL DMEM to a 15 mL polystyrene centrifuge tube.

5.4.2 Add 0.2 mL of ENM dispersion and vortex at a speed of 115 rad/s (1100 rpm) for 2 s.

5.4.3 Stop the mixer and allow the ENMs to agglomerate for a predetermined period.

5.4.4 To stop agglomeration, add 1 mL of DMEM with 4 % BSA while vortexing the sample. This will reduce the final concentration of BSA in the solution to 2 %.

5.5 Characterize the samples using a validated size quantification technique (e.g., dynamic light scattering (DLS) (8), transmission electron microscopy, etc.). It is important to monitor changes

during the time of the assay or other experiment in order to establish stability of formed agglomerates.

6 Abbreviations

AgNP	silver nanoparticle
AuNP	gold nanoparticle
BSA	bovine serum albumin
DLS	dynamic light scattering
DMEM	Dulbecco's modified eagles medium
ENM	engineered nanomaterial
LAL	Limulus Amoebocyte Lysate (LAL)
NP	nanoparticle

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We acknowledge the contributions of Laurie E. Locascio and Melissa D. Halter who contributed to manuscript (4) from which this protocol was adapted.

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Appendix

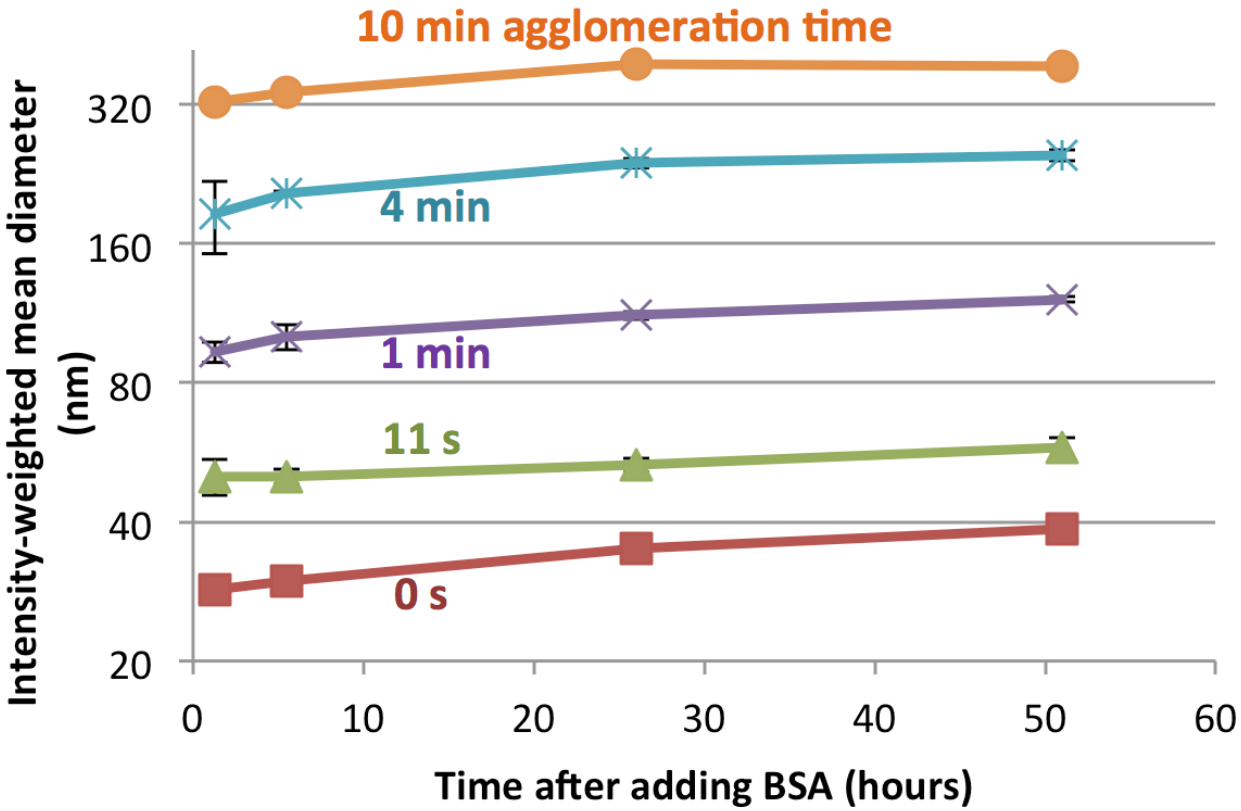

Figure A1: Stability of agglomerates of 30 nm AuNPs over 2 d at 37 ºC dispersed at 4.8 µg/mL in DMEM with 2 % BSA at five different mean agglomerate sizes. The times labeled inside the figure correspond to the times between adding the NPs to DMEM and adding BSA to quench the agglomeration process. The x-axis is the incubation time after adding BSA. The AuNPs were added to the media while vortexing rapidly at 1900 rpm. The intensity-weighted mean agglomerate sizes measured by DLS increase by between 5 % and 30 % over 50 h, calculated from DLS measurements; the DLS data were adjusted for vibration and rotation of agglomerates as described in (4). Error bars represent one standard deviation for three replicate samples, while error bars that are not visible are smaller than the data points.

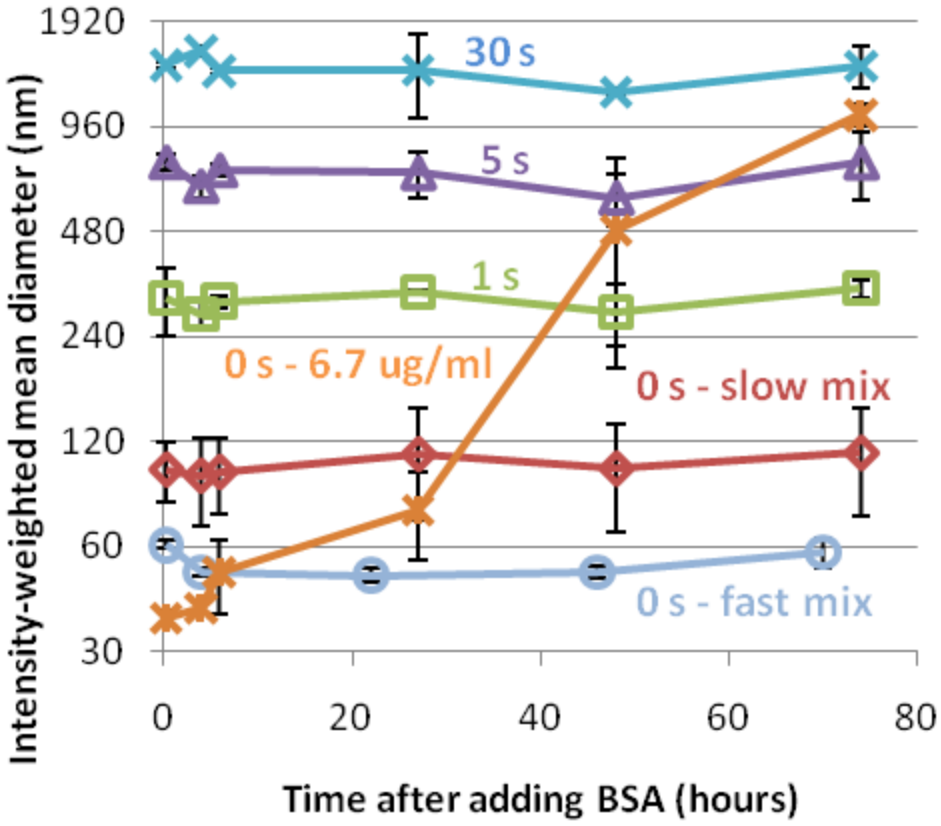

Figure A2: Stability of agglomerates of 23 nm AgNPs in DMEM and 2 % BSA over 3 d at 37 ºC dispersed at 110 µg/mL (black) at four different initial agglomerate sizes, corresponding to agglomeration in DMEM for approximately 0 s, 1 s, 5 s, or 30 s before adding DMEM with BSA. For the 0 s dispersions, the AgNPs were added to DMEM + 2 % BSA while vortexing slowly (at 1100 rpm) or quickly (at 1900 rpm). This demonstrates that mixing speed can influence the size of the agglomerate. In addition, the stability of AgNP agglomerates at 6.7 µg/mL (gray), with BSA added at 0 s while vortexing at 1900 rpm, is shown. Stability of the agglomerate size over time, as measured by the compensated intensity-weighted mean agglomerate size calculated from DLS. The times labeled inside the figure correspond to the agglomeration times between adding the NPs to DMEM and adding BSA to quench the agglomeration process. The x-axis is the incubation time after adding BSA. Error bars represent one standard deviation of three samples.

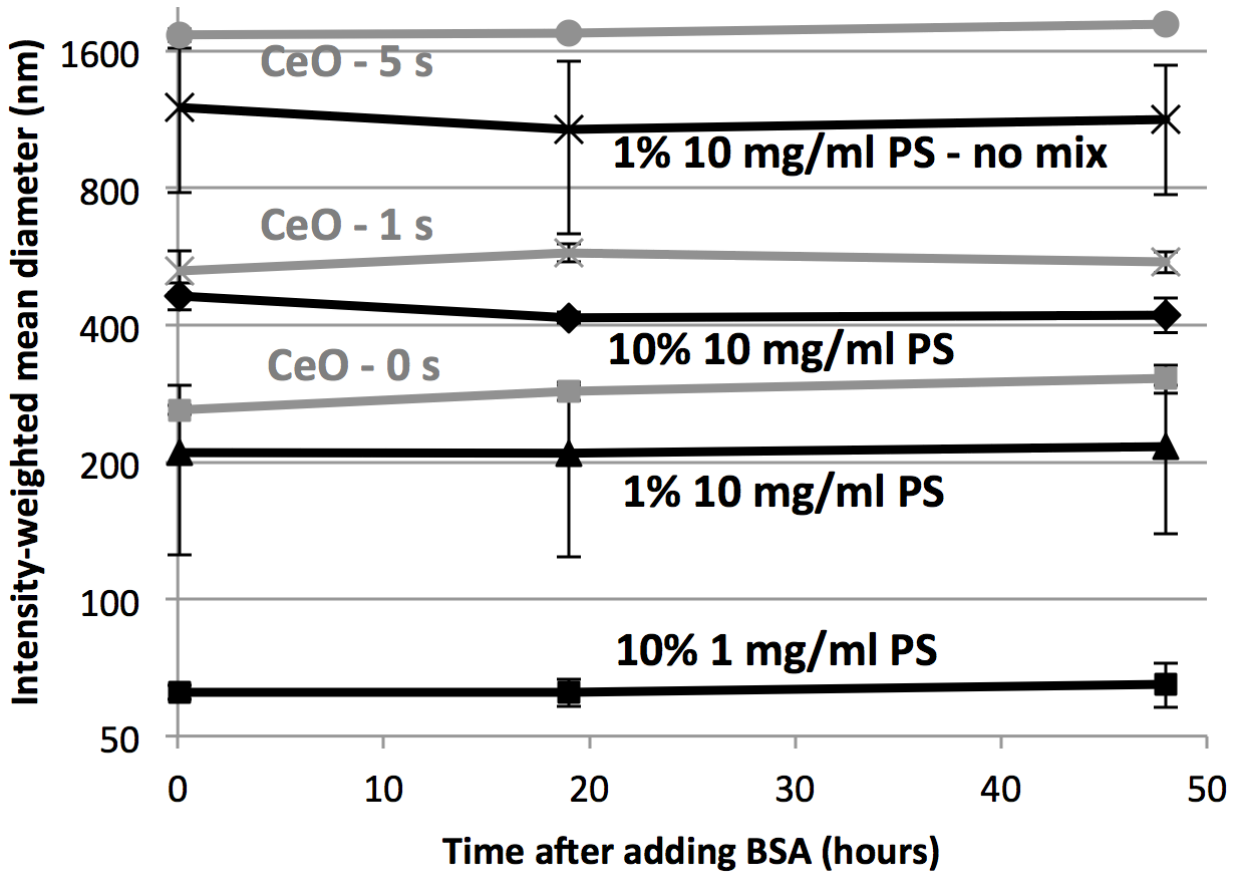

Figure A3: Controlled agglomeration and stability of ≈ 64 nm cerium oxide NPs (gray) and ≈ 57 nm positively-charged amine-modified polystyrene NPs (black) in DMEM + 2 % BSA over two days at 37 ºC. The cerium oxide NPs were dispersed at 1 mg/mL, and the times labeled inside the figure correspond to the agglomeration time between adding the NPs to DMEM and adding BSA to quench the agglomeration process. The agglomeration of the polystyrene NPs depended on the concentration of NPs both in the injected water solution and in the final dispersed suspension. Either 1 % or 10 % of a 1 mg/mL or 10 mg/mL suspension of polystyrene NPs dispersed in water was pipetted into a solution containing DMEM with 2 % BSA, demonstrating that the agglomeration state can depend on the precise order in which dilution series are carried out. In addition, if the NP dispersion was pipetted into a stagnant DMEM BSA solution without vortexing, then the size was very large and irreproducible. The x-axis is the incubation time after adding BSA. Error bars represent one standard deviation of three samples.