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Effective delivery of sonication energy to fast settling and agglomerating nanomaterial suspensions for cellular studies: Implications for stability, particle kinetics, dosimetry and toxicity

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Abstract

Typical *in vitro* assays used for high throughput toxicological screening and measuring nano-bio interactions are conducted by pipetting suspensions of engineered nanomaterials (ENMs) dispersed in nutrient-rich culture media directly onto cells. In order to achieve fairly monodisperse and stable suspensions of small agglomerates, ultrasonic energy is usually applied to break apart large agglomerates that can form upon suspension in liquid. Lack of standardized protocols and methods for delivering sonication energy can introduce variability in the ENM suspension properties (e.g. agglomerate size, polydispersity, suspension stability over time), and holds significant implications for *in vitro* dosimetry, toxicity, and other nano-bio interactions. Careful assessment of particle transformations during dispersion preparation and sonication is therefore critical for accurate interpretation of *in vitro* toxicity studies. In this short communication, the difficulties of preparing stable suspensions of rapidly settling ENMs are presented. Furthermore, methods to optimize the delivery of the critical sonication energy required to break large agglomerates and prepare stable, fairly monodispersed suspensions of fast settling ENMs are presented. A methodology for the efficient delivery of sonication energy in a discrete manner is presented and validated using various rapidly agglomerating and settling ENMs. The implications of continuous *vs.* discrete sonication on average hydrodynamic diameter, and polydispersity was also assessed for both fast and slow settling ENMs. For the rapidly agglomerating and settling ENMs (Ag15%/SiO₂, Ag and CeO₂), the proposed discrete sonication achieved a significant reduction in the agglomerate diameter and polydispersity. In contrast, the relatively slow agglomerating and settling Fe₂O₃ suspension did not exhibit statistically significant differences in average hydrodynamic diameter or polydispersity between the continuous and discrete sonication approaches. Our results highlight the importance of using the proposed material-specific discrete sonication method to effectively deliver the critical sonication energy necessary to reproducibly achieve stable and fairly monodispersed suspensions that are suitable for *in vitro* toxicity testing.

Graphical abstract

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Keywords

Engineered nanomaterials; dispersion preparation; sonication; agglomeration; nanotoxicology

1.0 Introduction¹

As the number and variety of engineered nanomaterials (ENMs) continues to increase across applications ranging from electronics, agriculture, medicine, cosmetics, printing, and food [1–12], there is a need for methods to quickly evaluate potential hazards and risks to human health and the environment [13–15]. Due to the relatively high cost and time commitment for animal toxicity studies, much nanotoxicology research to date has focused on developing high throughput *in vitro* screening methods for comparing the toxicity of large panels of ENMs [16–19].

Typically, *in vitro* assays used for high throughput screening and measuring nano-bio interactions are conducted by pipetting suspensions of nanoparticles dispersed in nutrient-rich media directly onto cells in culture. While some nanomaterials generated by wet methods exhibit a monodisperse size distribution by nature of their synthesis method and surface chemistry, ENMs in dry powder form are suspended in test medium at desired mass concentrations for toxicity testing [20–22]. Once suspended in physiologically relevant media, material- and media-specific parameters [23–24] (*e.g.* surface charge, surface chemistry, ionic strength, pH, protein content) can lead to particle flocculation, dissolution, and other interactions with serum components, oftentimes resulting in rapid and significant agglomeration [24–26].

The size, polydispersity and stability of ENM suspensions hold significant implications for *in vitro* dosimetry, toxicity, and other nano-bio interactions [27]. Large rapidly-settling agglomerates will result in i) a significantly higher dose of particles delivered to cells over time, ii) a lower available surface area (per mass of agglomerate) for interactions with cells as compared with smaller agglomerates, and iii) possible impacts on dissolution rates compared with well dispersed suspensions of stable small monodisperse agglomerates [28–29]. Furthermore, unstable and polydisperse suspensions are challenging to model in terms

¹Abbreviations: engineered nanomaterial (ENM); delivered sonication energy (DSE); critical delivered sonication energy (DSE_{cr}); reactive oxygen species (ROS); hydrodynamic diameter (dH); polydispersity index (PDI)

of their fate and transport *in-vitro* and makes the delivered to cell dose metrics as a function of time difficult to estimate [30]. Recent studies demonstrate that particle transformations in liquid media can greatly impact dosimetry (*i.e.* the time for particles to be delivered to cells), as well as the accurate interpretation of hazard rank order for large panels of materials [31]. Furthermore, various groups have shown that for a specific ENM, large agglomerates exhibit different cellular responses than small agglomerates [32–34].

In order to effectively break large agglomerates present in ENM powder form and achieve monodisperse and stable suspensions of small agglomerates, ultrasonic energy is usually applied. Sonication involves propagating sound waves into liquid media, resulting in the formation of small vacuum bubbles that collapse violently once they attain a critical volume [35]. Collisions between suspended agglomerates, as well as the collapse of cavitation bubbles formed on the agglomerates' surface, can lead to significant reductions in the average agglomerate diameter and the overall agglomerate size distribution. By these mechanisms, sonication has proven to be a relatively energy efficient method to rapidly break up large agglomerates into fairly monodisperse and stable suspensions of small agglomerates [35–36].

Several groups have recently developed and published standardized ENM dispersion protocols including the National Institute of Standards and Technology (NIST) [37], NANOGENOTOX team of the French agency for Food, Environmental and Occupational Safety [38], the National Institute of Environmental Health Sciences (NIEHS) [39], European Framework 7 project Risk Assessment of Engineered Nanoparticles (ENPRA) [40], Organisation for Economic Co-operation and Development (OECD) [41], as well as other academic research groups [42–43]. Key elements identified from these standardized protocols include: i) calorimetric calibration of sonication equipment to ensure accurate reporting of the sonication energy delivered to a sample [25,35]; ii) sonication of ENM powder in sterile deionized water to avoid reactive oxygen species (ROS) generation *via* sonolysis [44] and denaturation of serum proteins with potential subsequent effects on nanoparticle corona formation, agglomeration and surface reactivity [45–46]; iii) identification of the material-specific critical sonication energy required to achieve the smallest possible agglomerates that are stable over time [25, 43]; and iv) dilution of stock water dispersions into relevant cell culture test media at desired concentrations. Reports of ENM dispersion methods should also include details critical to sonication including dispersion volume, media and buffer formulations, pH, sonication times or energy input (in J/ml).

The delivered sonication energy (DSE) required to achieve fairly monodisperse aqueous ENM suspensions at the lowest agglomeration state that will result in agglomerates that remain stable over time when suspended in cell culture medium, is referred to as the critical DSE (DSE_{cr}) [25,43]. The DSE_{cr} is determined following the previously described method, whereby ENMs suspended in deionized water are sonicated at a short interval, characterized for agglomerate hydrodynamic diameter, vortexed, sonicated at a short interval, *etc.* This cycle is then repeated until the hydrodynamic diameter stabilizes within 5% of the smallest measured size and agglomerate diameters [43]. Once the DSE_{cr} is identified for a given

material, all future dispersions should be sonicated up to the DSE_{cr} to ensure stable suspensions when ENMs are mixed in cell culture medium and used in cellular studies.

When preparing suspensions of certain ENMs for cellular studies, vortexing a stock suspension of ENMs suspended in deionized water prior to sonication up to the DSE_{cr} is sufficient to achieve a homogenous distribution of particles throughout the sample. A homogenous distribution of particles throughout the sample ensures that the sonication energy is evenly distributed across the suspended particles and effectively delivered to break up the large agglomerates [25,29]. Under these circumstances the ENMs remain in suspension and sonication energy can be delivered to reach the DSE_{cr} . In these cases, continuously sonicating over that period of time up until the DSE_{cr} is reached is sufficient to break apart the agglomerates and achieve stable suspensions.

However, it has become apparent that some metal and metal oxide ENMs can rapidly flocculate forming large agglomerates up to the micron scale in size. These large agglomerates will then rapidly settle to the bottom of the sample container, sometimes within a matter of seconds. In such cases, particles are no longer homogeneously distributed throughout the sample container over the duration necessary to deliver the sonication energy required to achieve the previously determined DSE_{cr} and break the large agglomerates. As a result, for this class of rapidly agglomerating and fast settling ENMs, the widely used continuous sonication over a period of minutes does not effectively deliver the energy necessary to break up these large and rapidly settling agglomerates. The challenge then becomes how to effectively deliver the DSE_{cr} to such fast settling materials. This fast settling phenomenon was never addressed before in the literature.

In this short communication, we consider the difficulties of delivering the critical sonication energy required for preparing stable ENM suspensions for this class of rapidly settling ENMs. Specifically, we developed and evaluated methods to optimize the delivery of the critical sonication energy required to break large agglomerates and prepare stable, unimodal suspensions. The proposed and validated method for “discrete” sonication and delivery of ultrasonic energy to the class of rapidly agglomerating and settling ENMs is presented and validated in various case studies and compared with the widely used continuous delivery approach. This method provides a useful tool for additional investigations to be carried out by other investigators in the Nanotechnology Health Implications Research (NHIR) consortium and nanotoxicology research community to facilitate achieving stable and fairly monodisperse suspensions suitable for *in vitro* toxicity testing for this class of fast settling ENMs.

2.0 Materials and Methods

2.1 Materials

ENMs investigated are listed in Table 1. All materials were generated by the Administration and Research Coordination Core (ERCC) as part of the NHIR consortium efforts. Fast settling ENMs included composite material Ag15%/SiO₂ (d_{BET} : 6nm and 7nm for Ag and SiO₂, respectively), CeO₂ (d_{BET} : 5.3 nm), and Ag (d_{BET} : 18 nm). Representative slow-settling material Fe₂O₃ (d_{BET} : 10nm) was also investigated for comparison. All ENM

powders were generated in-house by flame spray pyrolysis using the Harvard Versatile Engineered Nanomaterial Generation System (VENGES) as previously described [47–49].

Specific surface area (SSA) was determined by the nitrogen adsorption/Brunauer-Emmett-Teller (BET) method using a Micrometrics Tristar 3000 (Micrometrics, Inc., Norcross, GA, USA). Equivalent primary particle diameter, d_{BET} was calculated, assuming spherical particles, as

$$d_{BET} = \frac{6}{SSA \times \rho_p}$$

where ρ_p is the particle density, which was obtained for each ENM in powder form using a pycnometer (Quantachrome Instruments, ULTRAPYC 1200e) through N_2 volume displacement and the volume-to-pressure relationship known as Boyle's Law.

Details on the synthesis and characterization of ENMs used in this study are presented by the authors elsewhere [25].

2.2 ENM Dispersal and Determination of Critical Sonication Energy (DSE_{cr})

For each ENM, the material-specific critical sonication energy (DSE_{cr}) was determined following the previously described methodology [43]. In brief, ENMs were dispersed at 500 µg/ml in 1 ml of deionized water in 50 ml, conical polyethylene tubes, by sonication with a cup horn Branson Sonifier S-450D (Branson Ultrasonics, Danbury, CT) (maximum power output of 400 W at 60 Hz, continuous mode, output level 3). The cup horn sonicator used was calibrated at these specific settings according to previously described methods [43], and the power delivered to the sample was determined to be 2.59 W. Following sonication at a brief interval (*e.g.* 78 J/ml, 155 J/ml, 233 J/ml, equivalent to 30 seconds, 60 seconds, and 90 seconds respectively using our sonicator with power output of 2.59 W and a 1 ml suspension), hydrodynamic diameter was measured by dynamic light scattering (DLS, Malvern Nanosizer, Worcestershire, UK). The mean and standard deviation for hydrodynamic diameter from DLS volume weighted size distribution and polydispersity index (PdI) across three replicate measurements for a given sample aliquot are reported in Table 1. The sonication interval was determined for each ENM by preparing fresh samples sonicated at a nominal DSE (*e.g.* 155 J/mL, or a 60 second sonication duration) and measuring agglomerate size at half the previously measured DSE. For example, if the agglomerate size stabilizes after 155 J/ml of sonication (*e.g.* 60 seconds), we prepared a fresh sample and measuring agglomerate size following 78 J/ml of sonication (*e.g.* 30 seconds), effectively reducing the DSE by half. Sonication and DLS measurement at the specified time intervals are then repeated until a stable suspension of monodisperse (*e.g.* polydispersity index <0.4) and small agglomerates is achieved. To determine the DSE_{cr}, DSE was plotted for each ENM to determine the DSE value resulting in a diameter within 5% of the observed minimum.

Stock suspensions sonicated to the DSE_{cr} were then diluted to 100 µg/ml RPMI cell culture media supplemented with 10% fetal bovine serum (FBS), a concentration on the high end of

those typically used for *in vitro* nanotoxicology studies. The stability of these small agglomerates suspended in cell culture media was then confirmed by making size measurements 24 hours post sonication. Suspensions in cell culture media were vortexed for 30 seconds immediately prior to DLS measurement at the 24 hour post-sonication time point.

2.3 Continuous vs. Discrete Sonication

In order to address the challenge of effectively delivering the required critical sonication energy to fast settling materials, the standard continuous delivery of sonication energy was modified to a “discrete sonication” approach as outlined in Figure 1. In brief, sonication energy is delivered in short time intervals, interspersed with periods of vortexing to ensure mixing and a homogenous distribution of particles throughout the sample during the sonication process. For fast settling materials, aqueous ENM suspensions at 500 µg/ml were sonicated at the same interval that was used to establish the asymptotic curve and determine the DSE_{cr} (e.g. 39 J/ml, 77 J/ml, or 158 J/ml, equivalent to 15 seconds, 30 seconds, or 60 seconds respectively) as described above. Following sonication at this pre-determined interval, samples were then vortexed for 30 seconds to ensure proper mixing of any large and fast settling agglomerates. This process is then repeated until the delivered energy reached the DSE_{cr} (see Figure 1 for details of the proposed revised discrete sonication protocol). The implications of continuous vs. discrete sonication on average hydrodynamic diameter and polydispersity were also investigated using DLS.

2.4 Statistical Methods

The data are presented as mean $\pm$ standard deviation of the mean. Statistical analysis between mean hydrodynamic diameter resulting from discrete sonication approach vs. continuous sonication approach was carried out using a Student's t-test. A p value of <0.05 was considered statistically significant.

3.0 Results and Discussion

3.1 ENM characterization and determination of DSE_{cr}

Table 1 summarizes ENM characterization data in dry powder form, and the DSE_{cr} for each material. It is important to note that each material required a different degree of energy to break apart large agglomerates in the aqueous suspension and achieve stable suspensions in culture medium of small agglomerates, ranging from 320 up to 630 J/ml, almost a twofold difference. These differences highlight the importance of identifying the material-specific DSE_{cr} to ensure ENM suspensions will be suitable for *in vitro* nanotoxicity testing.

Figure 2a shows a representative asymptotic curve used to determine the DSE_{cr} for Ag15%/SiO₂ in deionized water. Figures 2b through 2d demonstrate the implications of under- or over-sonication on ENM dispersion characteristics. For example, sonicating below the DSE_{cr} (under-sonication) resulted in a polydisperse suspension (PDI >0.4) of large agglomerates that were unstable over 24 hours in RPMI/10%FBS (see Figure 2a). Sonicating beyond the DSE_{cr} (over-sonication) resulted in an increase in agglomerate diameter and polydispersity and a decrease in stability over time (see Figure 2d). This is

likely due to an increase in the number of particle collisions, and an increased likelihood that such collisions result in re-agglomeration due to particle-particle surface interactions [35, 50]. As described above in Section 1, unstable and polydisperse dispersions are not desirable for the purposes of *in vitro* nanotoxicity testing.

3.2 Fast settling ENMs and continuous vs. discrete sonication

Table 1 also summarizes the impact of continuous vs. discrete sonication on dispersions of both fast and slow settling materials. Fast settling materials are defined as any ENM that does not exhibit monodisperse suspensions of small agglomerates following continuous sonication up to the material-specific DSE_{cr} . For each of the rapidly settling materials, discrete sonication achieves a significant improvement on the agglomerate size, polydispersity, and stability over time. For example, composite material Ag15%/SiO₂ exhibited rapid agglomeration in the stock deionized water suspension. Continuous sonication for approximately 3.1 minutes required to deliver the DSE_{cr} achieved polydisperse suspensions of relatively large agglomerates ($d_H = 528.2 \pm 120.6$; $PDI = 0.63 \pm 0.06$). The high polydispersity is a result of a failure to deliver the required critical sonication energy to the fast settling ENM agglomerates that settled rapidly to the bottom of the container and separated from the suspension. In contrast, discrete sonication of this ENM suspension in intervals of 30 seconds up to the DSE_{cr} reduced the hydrodynamic diameter and polydispersity index by roughly a factor of four and two respectively ($d_H = 135.2 \pm 2.0$; $PDI = 0.29 \pm 0.03$), indicative of the effective delivery of the required DSE_{cr} . Similar results were observed for the other rapidly agglomerating and settling ENMs such as CeO₂ and Ag (see Table 1), where discrete sonication up to the DSE_{cr} successfully broke the suspended agglomerates down to their smallest possible size in contrast to continuous sonication which did not successfully break down the larger agglomerates due to the fast separation of ENM agglomerates from the suspension.

Table 1 also outlines results for one slow settling ENM (Fe₂O₃). This ENM did not exhibit statistically significant differences in average hydrodynamic diameter or polydispersity between the continuous and discrete sonication approaches. It is also interesting to note that this material had the lowest DSE_{cr} of all ENMs reported (320 J/ml), an indication that less energy was required to break the Fe₂O₃ agglomerates down to their smallest possible size as compared with the other rapidly agglomerating and settling materials.

The mechanisms driving rapid agglomeration and settling for some ENMs tested (Ag15%/SiO₂, CeO₂) but not others (Fe₂O₃) require further consideration. DLVO (Derjaguin, Landau, Verwey and Overbeek) theory considers the balance of two opposing interfacial forces, electrostatic repulsion and van der Waals attraction, in relation to particle agglomeration and colloidal stability. For particles in suspension, electrostatic repulsion is a major force against particle agglomeration *via* the formation of an electrical double layer or zeta potential [51]. Typically, particles with a zeta potential of $< \pm 15$ mV are expected to readily agglomerate in suspension, while a zeta potential of $> \pm 30$ mV is considered the threshold beyond which the colloidal system stabilizes. Suspended particles between 15 and 30 mV may either agglomerate or stabilize in dispersion [52]. For the panel of ENMs investigated here, relatively weak electrostatic repulsive forces could possibly explain the

tendency for some but not all materials to rapidly agglomerate. For example, CeO₂ and Ag15%/SiO₂ exhibited a zeta potentials of 9 mV and 22 mV respectively, an indication that these aqueous ENM suspensions are likely to rapidly agglomerate and settle at the bottom of the container. In contrast, slowly agglomerating and settling ENM Fe₂O₃ exhibited a strongly positive zeta potential (31.9 mV), which may be at least partly responsible for the stability of this suspension. Other interfacial forces (magnetic, steric, electrosteric, depletion, solvation, *etc.*) can also impact agglomeration kinetics, and more research is necessary to better understand which ENMs can be expected to rapidly agglomerate and by what mechanisms.

3.3 Proposed Solution

As presented above, some ENMs will rapidly agglomerate and quickly settle while other ENMs are slowly agglomerating, making it difficult to deliver the required sonication energy for every ENM. Typically dispersion protocols use continuous sonication, which involves sonicating the suspension without stopping until the DSE_{cr} is reached. However, as we show in this study, a continuous approach does not deliver effectively the sonication energy for fast agglomerating and settling ENMs (see Figure 1a and b). Therefore, we propose applying the discrete sonication approach for all ENM suspensions to ensure appropriate delivery of sonication energy.

For the discrete sonication approach, sonication energy is delivered in short intervals, interspersed with periods of vortexing to ensure mixing and a homogenous distribution of particles throughout the sample. This process is then repeated until the delivered energy reaches the DSE_{cr} (see Figure 1a). In contrast, slow settling materials, for which the formed agglomerates are relatively easy to break as indicated by very low DSE_{cr} values, fewer DSE intervals are required to sufficiently break apart the large agglomerates (see Figure 1b).

4.0 Conclusion

Sonication is a critical step in the preparation of stable and reproducible ENM suspensions for *in vitro* toxicity testing. Standardized methods for characterizing and reporting sonication energy delivered to ENM suspensions improve control over critical particle parameters, interactions and transformations, with subsequent implications for accurate dosimetry and estimation of particle delivery to cells over time, as well as interpretation of the role that particle size may play in toxicity and other various nano-bio interactions. Up until now there has been limited discussion of challenges related to effectively delivering sonication energy for rapidly agglomerating and settling ENMs in suspension. For such materials, continuous sonication over a period of minutes does not effectively deliver the energy necessary to break up these large and rapidly settling agglomerates. As shown in this study, a discrete sonication approach is more suitable for rapidly agglomerating and settling ENMs (Ag15%/SiO₂, Ag and CeO₂), resulting in effective delivery of DSE_{cr} and significant reductions in the agglomerate size and polydispersity. Our results highlight the importance of utilizing the discrete sonication approach rather than the more commonly used continuous sonication to reproducibly achieve stable and fairly monodisperse suspensions that are suitable for *in vitro* toxicity testing.

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Highlights

- Standardized protocols for preparing engineered nanomaterial dispersions for *in vitro* nanotoxicology testing
- Critical sonication parameters for optimizing dispersion preparation
- Discrete sonication suitable for effective delivery of energy to rapidly agglomerating and fast settling materials

Figure 1. Overview of continuous vs. discrete sonication

a) Discrete sonication is the process whereby sonication energy is delivered in short intervals, interspersed with periods of vortexing to ensure mixing and a homogenous distribution of particles throughout the sample. This process is then repeated until the delivered energy reaches the DSE_{cr} . Continuous sonication involves sonicating the suspension, without stopping, until the DSE_{cr} is reached. Rapidly agglomerating materials will settle out of suspension, sonication energy will not effectively be delivered resulting in an unstable suspension of large agglomerates. Therefore such materials require the discrete sonication approach to effectively deliver the DSE_{cr} and achieve small uniform agglomerates. **b)** Slow settling materials, for which the formed agglomerates are relatively easy to break as indicated by very low DSE_{cr} values (blue squares), require fewer DSE intervals to sufficiently break apart the large agglomerates.

Figure 2. ENM-specific DSE_{cr} and Dispersion Stability

a) Determination of DSE_{cr} for 10nm Ag15%/SiO₂. b) average hydrodynamic diameter of suspension sonicated below DSE_{cr}; at time 0 and 24 hours post sonication; c) average hydrodynamic diameter of suspension sonicated at the DSE_{cr}; at time 0 and 24 hours post sonication; d) average hydrodynamic diameter of suspensions sonicated above DSE_{cr}; at time 0 and 24 hours post sonication.

Table 1

Colloidal properties following continuous vs. discrete sonication.

Material	SSA (m ² /g)	d _{BET} (nm)	DSEcr (J/ml)	ζ (mV)	Continuous sonication		Discrete sonication	
					dH (nm)	PDI	dH (nm)	PDI
Ag15%/SiO ₂	250.1±10.0	6.4 (Ag) 7.1±0.3 (SiO ₂)	480	22.2±1.5	528.2±120.6	0.63±0.06	135.2±2.0	0.29±0.03
CeO ₂	129.5±5.2	5.3±0.2	630	9.2±3.1	317.1±19.7	0.49±0.02	156.9±1.4	0.27±0.04
Ag	31.2±0.5	18.3±0.3	350	33.7±0.6	180.7±9.6	0.53±0.11	109.5±0.6	0.44±0.01
Fe ₂ O ₃	130.6±5.2	10.2±0.5	320	31.9±1.8	128.1±0.8	0.27±0.02	133.5±2.1	0.25±0.01

SSA (specific surface area) by nitrogen/Brunauer Teller (BET) method. d_{BET} : primary particle diameter determined from SSA; ζ: zeta potential, dH: mean hydrodynamic diameter from DLS volume weighted size distribution taken at t=0 post sonication and in DI water