

Sonication energy for the preparation of aqueous nanoparticle dispersions

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ABSTRACT

The size of nanoparticle (NP) agglomerates significantly affects the dose to organisms and observed effects when evaluating the fate and toxicity of NPs. Stable NP dispersions are made by using ultrasonic waves to break apart large agglomerates, and several standard sonication protocols have been proposed to improve data reproducibility and dispersion consistency. A review of 56 recent nanotoxicology studies revealed that sonication practices vary greatly in the type of ultrasonicator used, total energy input, and reporting of associated metadata. To facilitate comparison across studies, we demonstrate a method to deliver equivalent energy to NP dispersions using three different ultrasonicator systems: probe, cup horn, and bath. Calorimetric calibration was performed to determine the energy delivered by each system, which took into account effects of energy dissipation through media and the geometry of each type of sonicator. The power input was varied while maintaining an equivalent energy input of 8400 J. Our sonication protocol was applied to CeO₂ and TiO₂ NPs of similar primary particle size dispersed in ultrapure water, 0.1 mM KCl, and simulated freshwater. The hydrodynamic diameter (HDD) was measured using dynamic light scattering to assess agglomeration. We found that when energy was held constant, HDD was not significantly different between ultrasonication systems or power inputs for a given material and dispersion medium. To determine the effects of energy input, we varied the delivered sonication energy (840-84000 J) for NP dispersions in ultrapure water. The HDD of CeO₂ NPs decreased with increasing energy, but TiO₂ NPs did not have energy dependent agglomeration behavior, demonstrating that optimal energy input for stable NP dispersions is material specific. Our work here provides a standardized method to deliver equivalent sonication energy, even when employing different ultrasonication systems and power settings. We recommend that future studies implement these calibration methods and routinely report sonication energy, dispersion medium, NP composition details, and HDD to better contextualize NP exposure for comparative and regulatory purposes.

Keywords—nanoparticles, nanoparticle agglomeration, nanotoxicology, ultrasonication

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I. INTRODUCTION

Studies which evaluate the aquatic fate and toxicity of NPs are often difficult to reproduce and this may be due, in part, to variations in methods used to prepare NP dispersions [1]. To evaluate the behavior and toxicity of nanoparticles (NPs) in biological systems, NPs are first dispersed in relevant media. Once placed in liquid, nanomaterials often form large agglomerates due to attractive van der Waals forces, which can affect environmental interactions and cause NPs to rapidly settle [2], [3]. Many studies utilize ultrasonication (commonly referred to as “sonication”), which applies acoustic energy (>20kHz) to break apart large agglomerates

in order to form suspensions of particles in the nanometer size range [1]. For many NPs without a surface stabilizer, no amount of sonication energy can completely break apart agglomerates and form dispersions of primary particles [4]–[6]. The goal of sonication is therefore to minimize NP agglomerate size and form relatively stable and monodisperse suspensions [7], [8].

The manner in which suspensions are sonicated greatly affects the agglomeration state and resulting NP surface area, and can potentially alter the surface chemistry of NPs [9], [10]. An interlaboratory study which compared the size and surface charge of prepared gold, polystyrene, silica, and ceria NPs pointed to ultrasonication practices as

a major culprit for variability in dispersion stability, particularly for dispersions prepared from dry nanopowder [11]. The effects of sonication have also been shown to result in differences in toxicity. Kang et al. and Sager et al. compared the uptake and toxicity of NPs with and without sonication and found that well dispersed exposures resulted in higher rates of lung deposition and toxicity in mammals [12], [13]. A recent meta-analysis of *Daphnia magna* nanotoxicity experiments found that inconsistencies in observed toxicity among studies were primarily explained by differences in dispersion techniques, including sonication methods [14].

Reproducible methods of sonication are particularly important for surface reactive materials and NPs that dissolve and release toxic metal ions. During sonication, the formation and collapse of cavitation bubbles produces local areas of extremely high temperature and pressure (approximately 5000 K and 50000 kPa), which can lead to the formation of reactive oxygen species (ROS) [15]–[17]. ROS can alter the NP surface by oxidation, and has been shown to modify or degrade commonly used organic surface coatings [1], [18]. The same effect could potentially lead to the disintegration of carbon based nanomaterials, such as fullerenes or carbon nanotubes [19]. The interaction of ROS with media components may also have implications for toxicity. Sonication with a common surfactant was found to cause the production of toxic degradation byproducts, and cell viability decreased with increasing sonication time [20].

Energy input during sonication can enhance the dissolution rate of soluble species. Sonication has been shown to increase the rate of ion release from Cu and Mn NPs, which can potentially increase the observed toxicity [9], [10], [21]. A comparison of different sonication methods revealed a direct effect on the acute toxicity of Ag and CuO NPs to *Daphnia magna*, and this was found to be the result of increased dissolution associated with longer sonication times [22]. The ability of sonication to alter not only the agglomeration state of NPs, but also NP surface and dispersion medium highlights the need for uniform sonication practices for dispersion, particularly for toxicity evaluation.

1.1 Ultrasonication systems

Ultrasonication systems function to disperse nanoparticle suspensions by propagating acoustic waves through the medium which results in high energy cavitation that acts to break apart agglomerates. Several types of sonication systems are available for NP dispersion preparation and are classified by manner of energy delivery as either direct or indirect methods (Fig. 1). Direct ultrasonication involves immersing a probe directly

into the suspension, which allows for high intensity energy delivery. This method, also defined as probe sonication, is generally recommended for the disruption of agglomerated NP dispersions [23]–[28]. Indirect methods include cup horn and bath ultrasonication, where energy must travel through water (or some other liquid) to the sample. A cup horn sonicator is considered a high intensity ultrasonic bath and is typically used for cell disruption, protein extraction, and releasing DNA and RNA from cells [29]. The high energy delivered during sonication can lead to a temperature increase of the sample, and to minimize this, probe and cup horn sonicators can be operated in pulse mode as opposed to continuous sonication. In addition, probe sonication is often performed in an ice bath and cup horn sonicators are commonly operated in a thermostat configuration designed to maintain a constant bath temperature. A bath sonicator delivers lower power and does not circulate water, but can accommodate larger sample volumes than a cup horn. Indirect methods are not recommended in standard protocols for dispersing NPs, but are often chosen to maintain sterile exposure conditions and avoid sample contamination by the probe or to avoid the release of titanium from the probe surface into NP suspensions.

1.2 Amplitude

For the probe and cup horn configurations, the programmed amplitude, often reflected as percent (%) of the maximum, refers to the displacement of the probe tip as it vibrates. For example, the maximum amplitude for a 13 mm probe using a 750 W Sonics system (Fig. 1) is 114 μm , and the %

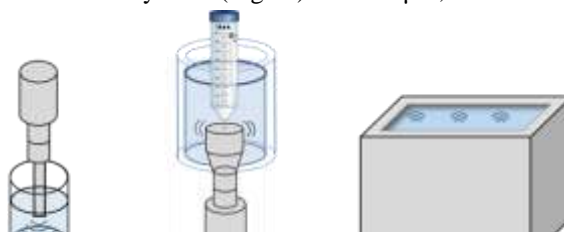


Figure 1. Schematic of a probe ultrasonicator (direct), cup horn ultrasonicator (indirect) and bath ultrasonicator (indirect).

amplitude is the fraction of that length traveled. This energy at the tip of the probe is dissipated through the liquid and causes alternating high and low pressure waves. A higher amplitude is accompanied by greater power and higher intensity of cavitation [30]. During sonication, the programmed amplitude is held constant and the power is varied in response to resistance to movement of the probe, which can be affected by the viscosity of the medium, temperature, and NP concentration.

1.3 Standard protocols

Several protocols have been published to standardize the preparation of NP dispersions [23]–[25], [27],

[28]. Recently, the Organization for Economic Cooperation and Development (OECD) updated guidelines which are consistent with most other protocols and is among the most detailed [31]. In brief, this guideline recommends preparing a NP stock concentration of 0.5 to 5×10^{12} particles/L in ultrapure water at a final volume of 125 mL. Concentrations based on particle count are difficult to determine when preparing stocks from nanopowder, and a volume of 125 mL may cause excessive NP waste when only smaller quantities are needed for experimentation. The standard recommended sonication system is a probe sonicator and protocols call for sonicating at a power of 40 W for 10 minutes. Although probe sonicators are known to deliver the highest intensity, submerging the probe directly in suspensions can cause contamination and leaching of titanium from the probe surface. Additionally, specific reporting requirements are outlined, which include detailing information about the volume of sample and solvent, sonication time or energy input, and characteristics of the dispersion media such as pH, ionic strength, and organic matter content [27].

1.4 Calibration of sonicators

To account for differences in sonication systems, standard protocols require calibrating the probe sonicator at all power settings to determine delivered energy. The sonication energy reported in studies often refers to the electrical output from the instrument; however, this energy is transformed to mechanical energy and does not accurately represent the acoustic energy actually delivered to the sample [32]. Efficiency depends on the specific instrument, the characteristics of the NP dispersion (medium, volume, and particle concentration), temperature, and time. Calibration provides a measurement of how much energy is absorbed by the system and can account for aging of the piezoelectric crystals inside the converter which, over time, can affect the amplitude of vibrations and therefore the power delivered [29]. Calorimetry and chemical dosimetry, including the Frick reaction and KI oxidation have been used to calibrate individual sonication systems [32], [33]. Although chemical methodologies can be useful, calorimetry is the most widely used because it is simple, requires few materials, and is not sensitive to the initial temperature of the NP suspension. Utilizing the calorimetric approach, the delivered power is determined by measuring the change in temperature of the medium during sonication with the assumption that mechanical energy is converted to heat. Recent studies outline calorimetric methods for calibrating probe sonicators that were applied to deliver equivalent energy densities to SiO₂ and TiO₂ NP suspensions across different laboratories [34]–[36].

1.5 Review of sonication practices in nanotoxicology

Despite standardized sonication guidelines, methods used in practice vary greatly among published nanotoxicology studies. We performed a literature review of 56 recent nanotoxicology studies (2007-2018) which sonicated NP stocks prior to exposure using Google Scholar and applying the search terms nano/nanoparticles/nanomaterials, sonicate/sonication and toxicity (Table S1). Of the 56 studies reviewed, most (51%) reported using an ultrasonic bath, but others used a probe, cup horn sonicator, or did not report the type of sonicator used (Fig 2A). As we previously described, the type of sonicator greatly impacts the intensity and power delivered to the sample.

The sonication energies reported ranged across orders of magnitude (approx. $5 \times 10^{-1} - 2 \times 10^3$ J) for those studies that either explicitly reported energy or provided sufficient information about power and time. More than half of studies did not report energy. Energy should be reported in context of the sample volume, because volume can affect the disruption effect for a given energy input. Portions of the sample in closer proximity to the probe/horn are likely to experience a greater disruptive effect, so sonicating large volumes provides a lower energy density; whereas, in smaller volumes the entire sample might be in close contact with the probe. The majority of studies (79%) fail to report sample volume used during sonication. The size of the probe also affects the intensity of energy delivered, and although many studies specify the sonication instrument used, only four of the studies surveyed here explicitly reported the probe diameter.

The concentration of NP stocks were generally reported on a mass basis and varied among studies across many orders of magnitude, ranging from 10 µg/mL to 40×10^3 µg/mL (Table S1). NP concentration affects the rate of particle collisions during sonication, which can act to either break apart agglomerates or in some cases induce further agglomeration [26]. Although existing protocols specify a narrow range of NP concentrations for sonication in an effort to limit variability in particle collisions across studies, these are provided as particle concentrations. Most studies continue to report NP concentrations on a mass basis, and this may be due to limited availability of instrumentation required to measure accurate particle counts at the nanoscale across a range of particle concentrations. This could make compliance with standard protocols difficult, and be responsible for the large discrepancies in concentrations used.

The dispersion medium was generally reported to be ultrapure water, a buffer solution, or exposure medium, such as cell culture or simulated natural waters (Table S1). Ions in dispersion media are

known to directly affect suspension stability and particle agglomeration by compressing the electric double layer and increasing agglomeration. Organic matter can coat the NP surface and provide a stabilizing affect that prevents agglomeration [4]. Components of the medium can also influence agglomeration behavior by affecting how sonication energy is delivered. Changes in viscosity can alter the resistance to movement of the probe and affect the power input. Ionic strength and density can affect how the medium dissipates the delivered energy [1]. For environmentally relevant media, the impact is likely to be small; however, media properties can act to augment the potential effect of sonication energy on NP surface reactivity. Proteins in biological media, for example, have been shown to promote dissolution of metal and metal oxide NPs during sonication [9], [10].

We ranked the reviewed studies based on quality and completeness of the reported metadata described above (Fig 2B). Studies which reported all relevant details that would be required for replication (sonicator type, energy, time, NP composition, volume, medium, concentration) were given a rating of “7.” For every missing piece of information, one point was subtracted. Studies who received a “1” rating typically only reported the NP material and stated that sonication was performed. Metadata should be sufficiently detailed to establish meaningful trends among studies, and such numerical frameworks have been proposed to improve data quality for nanomaterial regulation [37], [38].

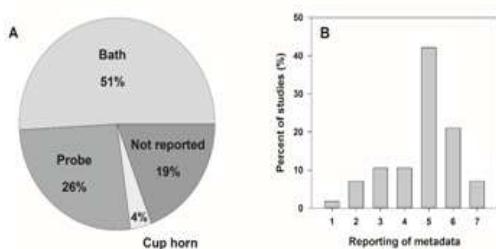


Figure 2. A) Types of sonicators used B) histogram of studies rated by quality and completeness of reported metadata based on a review of 56 nanotoxicology studies.

In this study, our objective was to update standard NP dispersion protocols in order to allow for reproducible data regardless of the equipment being utilized in any given laboratory, and to highlight the discrepancies in current practices reported in the literature. We hypothesize that agglomerate size is dependent on the total energy input by the ultrasonicator, regardless of what sonicator type or power setting is used. We aimed to produce similar CeO₂ and TiO₂ NP dispersions using

three different sonicator systems: probe, cup horn, and bath. CeO₂ and TiO₂ were selected due to their widespread use, limited dissolution, and known propensity to agglomerate in solution. We applied our calibration procedure across different programmed amplitudes and validated this method in three relevant dispersion media.

II. MATERIALS AND METHODS

2.1 Sonicator calibration

Calorimetric calibration was performed for a probe and cup horn ultrasonicator (Vibra Cell 750, 20 kHz, Sonics & Materials, Inc., Newtown, CT) and an ultrasonic bath (Fisher Scientific, 1.9 L, 70 W, 40 kHz). A thermocouple was used to measure the temperature of water as a function of time for programmed amplitudes of 20%, 30%, and 40% on the probe and cup horn sonicators. 40% was selected because it is the highest allowable amplitude for the cup horn configuration. The ultrasonic bath does not have an option to program different powers, so calibration was only performed at one power. The sonicators were insulated and it was assumed that no heat was lost to the environment and all mechanical energy was converted to thermal energy. Water in the cup horn was not circulated during calibration. The delivered acoustic power was calibrated by performing a linear regression of temperature as a function of time and solving for power (1):

$$P = mC_p \left(\frac{dT}{dt} \right) \quad (1)$$

where P is the delivered acoustic power (W), C_p is the specific heat of water (4.2 J/g°C) and m is the mass of water (g). The delivered power was used to calculate sonication time for a given energy (2):

$$t = \frac{E}{P} \quad (2)$$

where t is sonication time (s), E is energy (J), and P is delivered acoustic power (W). The energy was held constant at 8400 J and the time was varied to evaluate the impacts of different delivered power.

2.2 Nanoparticle stock preparation

CeO₂ and TiO₂ (anatase) were purchased from Sigma Aldrich (St. Louis, MO) and had a similar average primary particle size of 25 nm. Stock suspensions (1000 mg/L) were prepared by dispersing dry nanopowder in ultrapure water (Milli-Q 18.2 Ω resistivity), 0.1 mM KCl, or simulated freshwater (FW) comprised of 15 mM NaCl, 0.5 mM KCl, 1 mM CaCl₂·2H₂O, 0.15 mM KH₂PO₄, 0.05 mM Na₂HPO₄ and 1 mM MgSO₄·7H₂O and adjusted to a pH of 7.0 [39]. All chemicals were reagent grade. Suspensions were vortexed, then diluted to a concentration of 50 mg/L in a volume of 10 mL, except for the probe sonication group which

was prepared in a volume of 40 mL to accommodate the probe tip.

2.3 Sonication

Sonication was performed with a 750 W, 20 kHz Vibra-Cell ultrasonic processor (Sonics & Materials, Inc., Newtown, CT) equipped with a 13 mm diameter probe (probe sonication) and a 51 mm diameter probe equipped with a cup horn attachment with continuously flowing cooling water (cup horn). The programmed amplitude was set for 20%, 30%, and 40% for the probe and cup horn sonicators and the time needed to achieve equivalent energy was determined by calibration (Table 1). The ultrasonic bath (1.9 L, 70 W, 40 kHz, Fisher Scientific, Pittsburgh, PA) does not allow the user to adjust the amplitude and was used at the single factory power setting. Samples prepared using the cup horn and ultrasonic bath configuration were 10 mL NP stock in a 15 mL plastic conical tube. Samples prepared with the probe sonicator were 40 mL NP stock in a 50 mL tube to accommodate volume displacement by the probe and avoid contact of the probe with the wall of the tube. To prevent a significant increase in temperature for all sonicator types, the probe samples were placed in an ice bath, cooling water was continuously circulated through the cup horn, and the water in the ultrasonic bath was refreshed before each trial.

Programmed Amplitude	Instrument Reported Power (W)	Calibrated Power (W)	Sonication Time (s)
Probe			
20%	33.8	10.0	35
30%	48.8	15.4	23
40%	78.8	19.6	18
Cup Horn			
20%	27.5	22.2	379
30%	52.0	27.9	301
40%	77.3	40.1	209
Bath	70	15.0	564

Table 1. Sonication reported power, calibrated power and estimated times to deliver 8400 J total energy to dispersions.

For all powers and sonicators, the energy was held constant at 8400 J by altering the duration of sonication (Table 1). For dispersions in ultrapure water, the energy was further varied from 840-84000 J to evaluate the effect of total energy on agglomeration.

2.4 Dynamic Light Scattering

Hydrodynamic diameter (HDD) was measured using dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) immediately

following sonication. Sonicated NP dispersions (1.5 mL) were placed in a disposable cuvette prior to measurement. Temperature was held constant at 25 °C. Control dispersions were prepared in all three media in which samples were vortexed but not sonicated prior to measurement. The z-average was used as a measure of agglomerate size.

2.5 Statistics

SigmaPlot version 13.0 (Systat Software, San Jose, CA, USA) was used to perform all statistical analyses. All experiments were performed in triplicate. Differences among sonication type and power inputs for each NP and each dispersion media were compared using analysis of variance (ANOVA) with Holm-Sidak post-hoc analysis. Differences were considered statistically significant when $p \leq 0.05$. Error bars represent standard error of the mean. Linear regression was performed for sonication calibration curves to determine the increase in temperature per unit time. The decrease in HDD as a function of sonication energy was fit to a first order exponential decay model.

III. RESULTS

Delivered acoustic power was calibrated for the ultrasonic bath and for the probe and cup horn ultrasonicators at different programmed amplitudes (20-40%) (Fig. 3). A linear relationship between temperature and time was observed for all systems ($R^2 \geq 0.94$). The calibration curve for the probe sonicator had a steeper slope than the indirect systems at all amplitudes and temperatures reached 70°C within five minutes.

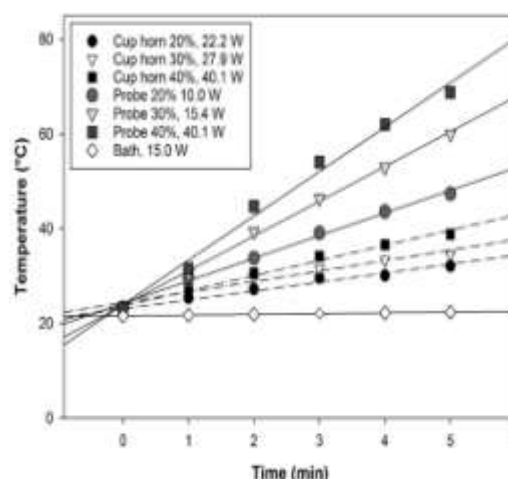


Figure 2. Calibration curve of probe, cup horn, and probe sonicators. A linear regression was performed for each to determine delivered acoustic power.

3.1 Determination of sonication times

The total energy delivered was held constant at 8400 J, which can be achieved by all

three systems in less than 10 minutes. The ultrasonic bath delivered an average power of 15.0 W. The power delivered by the cup horn sonicator was calibrated to be 22.2, 27.9, and 40.2 W for programmed amplitudes of 20%, 30%, and 40%, respectively. The power delivered to the probe sonicator was lower (10.0, 15.4, and 19.6 W, respectively) due to the small diameter of the probe. The equivalent energy was therefore normalized by probe surface area.

3.2 Agglomerate Size

The HDD was measured immediately after sonication to evaluate the efficacy of sonication on NP dispersion. For both CeO₂ and TiO₂, the HDD was significantly lower after sonication relative to the unsonicated control in all three dispersion media (Fig. 4). Agglomerate size did not vary significantly among sonicator types or amplitudes when an equivalent energy was input. TiO₂ agglomerates were generally larger than CeO₂ in ultrapure water and 0.1 mM KCl, but the HDD was similar to CeO₂ in FW. TiO₂ also had higher polydispersity and higher variance among technical replicates.

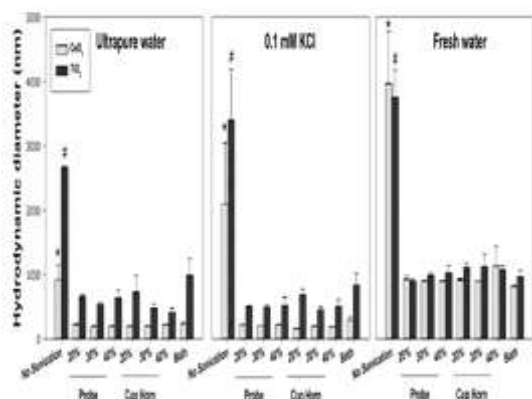


Figure 3. Hydrodynamic diameter of CeO₂ and TiO₂ NPs after probe and cup horn ultrasonication (20%, 30%, and 40%) and bath sonication in ultrapure water, 0.1 mM KCl, and FW. There was no significant difference in HDD among sonication conditions. The HDD of all sonicated groups was statically different from the no sonication control ($p < 0.05$).

3.3 Energy dependence

Energy input was varied from approximately 840-84000 J (0.1 – 10x energy/unit surface area) by altering the sonication time for NP suspensions in ultrapure water to evaluate the effect of energy delivered on HDD. For programmed power amplitudes of 30%, the HDD of CeO₂ decreased with increasing energy and HDD reached a minimum of approximately 120 nm (Fig. 5). The HDD of TiO₂ NPs decreased and reached a minimum of approximately 600 nm at 8400 J while

higher sonication inputs resulted in increased variance and a slightly higher average HDD.

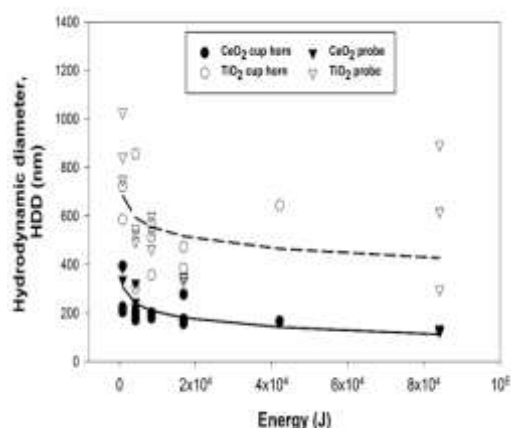


Figure 4. HDD of CeO₂ and TiO₂ NPs in ultrapure water after probe and cup horn ultrasonication at 30% amplitude with delivered energy of 8400 J, 0.1x energy (840 J), 0.5x energy (4200 J), 2x energy (16800 J), and 10x energy (84000 J). HDD as a function of energy was fit to a first order exponential decay.

IV. DISCUSSION

This study assessed current practices of ultrasonically nanoparticle dispersions for toxicity testing. A review of published studies found that significant discrepancies exist among studies and protocols vary greatly in instrumentation and energy input. Additionally, most studies do not provide sufficient detail and do not report pertinent information about energy, medium, sample volume, or concentration, all of which are necessary for reproducibility. These metadata significantly contribute to the impact of acoustic energy on NP dispersion. The lack of standardized practices results in exposure variance that can impact our understanding of NP-biological interactions and influence the results of toxicity testing.

We successfully calibrated three major types of sonicators to deliver equivalent acoustic energy. Importantly, this provides a means for research groups with different equipment to adapt existing protocols and increase uniformity in NP dispersions across studies. The times and powers used in this study produced NP dispersions of similar hydrodynamic diameters for all three sonicator types and power inputs. This suggests that consistent dispersions can be prepared with equipment available to each laboratory if sufficiently characterized and calibrated.

Although calorimetric calibration of individual sonicators is recommended by standard guidelines and used in a limited number of studies, it is clearly not widely implemented in preparation of NP dispersions for toxicity testing. The present study

extends current calibration methods to include cup horn and bath sonication for direct comparison independent of instrumentation. We also demonstrate that smaller volumes and concentrations can be used for sonication, which reduces NP waste. Current standard protocols exclusively recommend high intensity probe sonication for NP disruption, but many studies use bath or cup horn sonication in practice to avoid contamination of the sample. This may also be due to the availability of instrumentation, as bath ultrasonicators are common and less expensive than other options. Some studies working with controlled biological conditions intentionally opt for indirect methods to prevent contamination of the NP exposure by the sonication probe [40].

Among the nanotoxicology studies we reviewed, ultrasonic bath was the most commonly used type of sonicator for NP dispersion. Protocols only outline specific user guidelines for a probe sonicator, so dispersion techniques with a bath system were not consistent, and sonication times ranged from 10 minutes to 6 hours. Standard dispersion protocols should be modified to include guidelines on bath sonication in order to standardize practices across research groups. We calibrated the ultrasonic bath using calorimetry and insulated the top to the best of our ability to minimize heat loss. A power of 15 W was delivered to the system, which is significantly lower than the reported instrument output power of 70 W. The manufacturer reported power does not account for energy loss as electrical energy is converted to mechanical energy, thus it does not accurately represent the delivered acoustic power.

Probe and cup horn sonication was performed using the same ultrasonic power supply equipped with different probe attachments. The NP sample volume was 10 mL for indirect methods but for was increased to 40 ml in a 50 mL conical tube for direct sonication to submerge the probe without allowing direct contact with the sides of the tube. Volume determines the energy density, so at a given concentration, sonication system, and NP material, lower volumes can cause a greater disruptive effect [24]. However, the proximity of NP sample to the probe also determines the disruptive effect and despite the higher volume used for probe sonication, the majority of the NP sample is in close proximity to or in direct contact with the probe. Standard protocols recommend a volume of 125 ml in a glass beaker but we demonstrated that smaller volumes can be used, which reduces the amount of NP needed, as well as the amount of NP waste generated.

For the calibration of power delivered by the cup horn, the mass term in Eq. 1 was determined using density of water (1 g/mL) and the volume of

both the NP sample and the water bath through which the energy travels. Much of the delivered sonication energy is dissipated through the water in the cup horn, and this was observed during calibration by a lower temperature change despite a higher power output from the instrument. The delivered power is used to heat a larger total volume. The cup horn was more difficult to insulate so the delivered power may be an underestimate. The lower power delivered by the probe also indicates that less overall power was required to vibrate the probe at a certain amplitude. The lower surface area of the probe (1.3 cm² vs. 31.7 cm²) causes the power to be concentrated at the tip and produce a higher intensity energy, thus we normalized sonication energy by probe surface area instead of overall energy. Manufacturer recommendations suggest that for a given amplitude, the cup horn sonication time should be 4 times that of the probe time to achieve a similar NP dispersion. However, our calculations called for times of approximately 12 times longer for cup horn than the probe, depending on the amplitude, and we conclude that this rule should not be generally applied. Our results emphasize that surface area of the probe should be reported and taken into account when selecting a sonication protocol. Many sizes are available, ranging in diameter from 2 mm to 25 mm, which result in a wide range of delivered intensities. The power determined by calibration was lower than the instrument reported power for all three sonication systems (Table 1). This could be due, in part, to difficulties in insulating the system during calorimetric calibration. The difference was greater for the probe sonication system than the cup horn, with the calibrated power approximately 70% lower on average than the instrument reported power. For the cup horn, the difference was less significant at a programmed amplitude of 20%. This may indicate that at higher amplitudes there is more energy loss, making the instrument readout less accurate. The larger temperature changes during calibration for higher powers may result in more uncertainty due to heat loss from the system, a trend which could potentially be minimized by performing calibration curves over shorter periods of time. The amplitudes selected here were chosen because 20% is the minimum allowable on the instrument and 40% is the maximum for cup horn and microtip attachments.

The calibrated energy produced comparable dispersions using different sonicators and power settings, but differences were still observed between CeO₂ and TiO₂ NPs, despite having a similar primary particle size. The HDD of TiO₂ was larger than CeO₂ in ultrapure water and 0.1 mM KCl, which is consistent with what other studies have observed [41], [42]. The variance of measured HDD was generally higher for TiO₂ NPs, which may be

attributed to the higher polydispersity index (PDI) values. PDI values (Table S2) were higher for TiO₂, indicating that TiO₂ suspensions had a broader size distribution than CeO₂. Normalizing for sonication energy and parameters allows for interpretation of material specific agglomeration behavior attributed to the NP surface and properties of the exposure media.

The effect of sonication type and programmed amplitude was evaluated in three different dispersion media: ultrapure water, 0.1 mM KCl, and freshwater. Ultrapure water is recommended in OECD guidelines for stock preparation. KCl was selected here as a model weak salt solution consisting of monovalent cations, and simulated freshwater (FW) was used here to represent testing conditions for freshwater toxicity tests. FW has a higher ionic strength and contains divalent cations, which have been shown to form ionic bridges between adjacent particles and increase agglomeration [43]. Higher ionic strength affects the particle surface charge and can compress the electrical double layer, causing a weaker electrostatic repulsive force [44], [45]. Agglomeration is most significant at the isoelectric point (IEP), the pH at which the repulsive surface charge of NPs is neutralized and the suspension becomes unstable. The IEP of TiO₂ (anatase) has been shown to be a function of primary particle size, and is approximately pH 5.2 for 26 nm [46]. The IEP of CeO₂ NPs has been reported to range from pH 6 to pH 8 [47], [48]. The pH values of the media used in this study were all adjusted to 7. The isoelectric point can shift in high ionic strengths and in the case of TiO₂ (anatase) shifts up to possibly become less stable at neutral pH [49]. As expected, both NPs had significantly larger HDD in FW than in ultrapure water or KCl.

The total energy was varied for the probe and cup horn sonicators by changing sonication time. For CeO₂ in ultrapure water prepared with both types of sonicators, an increase in energy led to a decrease in HDD. The HDD reached a minimum of approximately 120 nm (Fig 5)., The HDD of TiO₂ NPs decreased with increasing energy until 8400 J, when the variance increased and the average HDD appeared to increase. This is consistent with previous studies which have observed that increasing sonication can actually lead to the re-agglomeration of NPs due to increased particle collisions [50]. One value for sonication energy is therefore not suitable to minimize HDD for all nanomaterials meaning that energy needs to be independently optimized for each material. Studies have proposed the use of a critical delivered sonication energy to systematically determine the energy required to minimize HDD for each specific NP suspension [7], [42]. The work

shown here is a necessary prerequisite for the wide implementation of such approaches.

V. CONCLUSION

This study illustrates that sonication energy is most important when considering reproducibility of NP dispersion protocols, especially when evaluating toxicity. We found that despite many published protocols to standardize dispersion preparation, significant discrepancies exist in sonication procedures and reporting in nanotoxicology studies. Calorimetric calibration uses simple techniques that can be used to report equivalent energy across sonicator types and power input. We modified existing calibration techniques to account for differences in probe surface area, and conclude that this simple approach should be applied in future studies improve compliance in standard methods and significantly improve reproducibility. The results shown here suggest that delivered energy, not sonicator type, is the determining factor for agglomeration state of NPs in a given dispersion medium. Future studies should also evaluate the effects of sonication on particle toxicity. For standardization across nanotoxicology studies, we recommend reporting appropriate metadata (concentration, volume, dispersion medium) in addition to energy and agglomerate size to best characterize NP exposure in relevant media.

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