CEINT/NIST PROTOCOL FOR

PREPARATION OF A NANOSCALE TiO_2 AQUEOUS DISPERSION FOR TOXICOLOGICAL OR ENVIRONMENTAL TESTING

Ver. 1

January 24, 2011

J. S. Taurozzi[1], V. A. Hackley[1], M. R. Wiesner[2]

[1] National Institute of Standards and Technology, Material Measurement Laboratory, Gaithersburg, MD 20899-8520, USA

[2] Duke University, Department of Civil and Environmental Engineering, Durham, NC 27708, USA

1. Introduction

Toxicity and fate assessments are key elements in the evaluation of the environmental, health and safety risks of engineered nanomaterials (ENMs). While significant efforts and resources have been devoted to the toxicological evaluation of many ENMs, and in particular nanoscale TiO_2 [1-4], obtaining conclusive and reproducible results remains a challenge [5]. This can be traced in part to the lack of standardized dispersion protocols and the inconsistent application of dispersion procedures in relevant biological and environmental matrices [6, 7].

This protocol was developed and validated using National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1898,[a] which consists of an industrially relevant and widely studied commercial TiO_2 nanomaterial with a production history dating back several decades [3, 8-10]. The protocol provides a validated method for the preparation of aTiO_2 nanoparticle dispersion in high purity de-ionized water, as a stock preparation compatible with biological and environmental matrices. Furthermore, this protocol can be used in combination with matrix-specific protocols for the preparation of dispersions in more complex biological or environmental media. Matrix-specific protocols are currently under preparation and will be issued in the near future.

While the procedures detailed in this document focus on the dispersion of SRM 1898 in high purity water, it is believed that the adopted characterization, validation and optimization approaches can be more generally applied to the preparation of aqueous dispersions for a wide range of ENMs that are available in the *dry powder form*. For this reason, and to allow for a broader applicability of this work, experimental details and discussions regarding the characterization, validation and process optimization adopted for the development of the dispersion method are offered as appendices to this protocol.

2. Principles and Scope

In this protocol, an aqueous TiO_2 nanoscale dispersion is produced, starting with a dry powder and by application of ultrasound (a process referred to here as sonication). For guidelines on reporting relevant conditions and critical parameters relating to the ultrasonic dispersion of ENMs, consult reference [11]. For additional relevant and general considerations on the application of ultrasound to prepare ENP dispersions, refer to [7, 12].

The feedstock for the test material, SRM 1898, is a commercial TiO_2 nanomaterial commonly referred to in the literature and within the scientific community as "Degussa P25" or simply "P25".[b] This product is used in a variety of applications, such as a surrogate for the class of untreated TiO_2 products used in sunscreen formulations, in food formulations and personal care products, and as an additive to protect cosmetics and medicinal formulations from sunlight [13].

---

[a] SRM 1898 was in production as of the publication of this protocol, with a target date for release in 2011. The NIST SRM inventory can be accessed at http://www.nist.gov/srm/.

[b] Certain commercial equipment, instruments, or materials are identified in this document in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology or the Center for the Environmental Implications of Nanotechnology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

It has also served as a benchmark in photocatalysis research and development [14] and, more recently, P25 has been utilized as a test material and benchmark for the toxicological assessment of ENMs, including the Organization for Economic Cooperation and Development (OECD) Sponsorship Program for the Testing of Manufactured Nanomaterials [3, 9, 10].

The method described herein, if applied correctly, yields a monomodal P25 dispersion in water, characterized by a mean particle diameter of ≈ 70 nm and a pH between 3.7 and 4.9. The method is validated for the preparation of dispersions in the (0.5 to 20) mg/mL concentration range. Dispersions prepared following this protocol should be stored so as to minimize exposure to light (e.g., in amber vials), at room temperature, and used within 24 h of preparation.

3. Terminology

This protocol complies with definitions relevant to nanotechnology as set forth in the ASTM standard E2456 [15] and is consistent with the draft standard ISO TS 80004-1 [16]. Additional guidance is derived from recommendations of the International Union of Pure and Applied Chemistry [17].

nanoparticle - sub-classification of ultrafine particle that is characterized by dimensions in the nanoscale (i.e., between 1 nm and 100 nm) in at least two dimensions; also referred to as "nano-object" in ISO TS 80004-1 [16].

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), which are not easily broken apart.

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

agglomerate - assemblages 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 are often used interchangeably to denote particle assemblies*.

dispersion - used in the present context to denote a liquid (aqueous) in which particles are 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. AEROXIDE® TiO_2 P25 (Evonik Degussa GmbH, Germany); available from distributors world-wide or obtained as a certified reference material from NIST (SRM 1898; in production, not yet available at time of publication).

4.1.2. 18.2 MΩ·cm resistivity Type I biological grade de-ionized (DI) water; biological grade implies sterility and absence of endotoxin contamination.

Note: Sterility and absence of endotoxin contamination should be verified for all materials in contact with the dispersion.

4.2. Materials [c]

4.2.1. 100 mL, ≈ 5 cm diameter cylindrical glass beaker

4.2.2. 125 mm wide x 65 mm deep crystallizing glass dish

4.2.3. 50 mL borosilicate volumetric flask or pipette

4.2.4. Aluminum or polystyrene weighing dish

4.2.5. Clamps or other locking device for holding beaker in place

4.2.6. Chopped ice

4.3. Equipment

For the preparation of the aqueous dispersion:

4.3.1. Analytical balance

4.3.2. Probe-type sonicator with a standard ½ inch (1.3 cm) diameter titanium horn fitted with a removable flat tip (or similar ultrasonic device)

For verification of acceptance criteria:

4.3.3. pH meter and electrode

4.3.4. Laser diffraction spectrometer (LDS), ***or***

4.3.5. Dynamic light scattering (DLS) instrument, ***or***

4.3.6. X-ray disc centrifuge (XDC) particle size analyzer

5. Preparation of aqueous TiO_2 nanoparticle dispersion

5.1. Using an analytical balance and an aluminum or polystyrene weighing dish, weigh an adequate mass of dry TiO_2 powder to achieve the desired concentration in a 50 mL water volume. For guidance on mass-concentration relationships, refer to the table below.

Mass (g)	Concentration in 50 mL volume (mg/mL)
0.025	0.5
0.05	1
0.5	10
1	20

[c] Glassware in contact with liquids or samples should be cleaned, sterilized, and dried prior to use. Avoid detergents if possible; if detergents are used, rinse with copious quantities of DI water before drying. Glassware can be sterilized using an autoclave, by exposure to hot dry air (130 °C-170 °C) for 2-4 h in an oven, or by prolonged contact with alcohol. Containers should be capped or sealed (e.g., with thermoplastic such as Parafilm) or stored in a HEPA filtered clean bench.

Note: This protocol has been validated for SRM 1898 mass quantities in the range of (0.05 to 1) g in 50 mL of water, yielding concentrations in the (0.5 to 20) mg/mL range, respectively. If other concentrations or volumes are used, the reader is advised to verify the applicability of this protocol. For greater accuracy and reproducibility with respect to mass concentration, the TiO_2 powder should be dried overnight at an elevated temperature between (105 and 150) ºC and then allowed to cool to room temperature in a desiccator prior to use.

5.2. Add the weighed mass of powder to a 100 mL, ≈ 5 cm diameter cylindrical glass beaker. Add 50 mL of DI water to the beaker with the powder.

5.3. Place the beaker inside the 125 x 65 mm glass dish and secure the beaker in the center of the dish by use of clamps or other locking device to ensure that the beaker remains in place during sonication.

5.4. Fill the 125 x 65 mm glass dish with enough water and chopped ice to allow for the ice water bath level to encase the beaker to approximately the level of the water contained in the beaker.

5.5. Immerse the sonicator horn into the liquid in the beaker down to about 2.5 cm below the liquid level in the beaker. Center the horn in the beaker; the horn should not touch the sides or the bottom of the beaker, as this could cause the beaker to shatter during sonication.

5.6. Select a sonicator setting that yields a delivered power of approximately 50 W.

Note: Refer to [12] for details on the recommended calibration procedure for the determination of the sonicator's delivered power.

5.7. Operate the sonicator at this delivered power level for 15 min, using an 80 % pulsed operation mode (e.g., 80 % on / 20 % off during each second of operation time), or similar on/off time sequence.

Note: Refer to Appendix C for details on the optimization of the sonication sequence.

5.8. After sonication, transfer the aqueous dispersion to an amber borosilicate glass container and store at ambient temperature until further use. Do not refrigerate.

Note: If an amber glass container is not used, store the stock dispersion in a cabinet or other container such that exposure to light is minimized. It is not established whether or not exposure to typical laboratory light levels is consequential with respect to dispersion properties or biological behavior of the TiO_2 nanoparticles.

Note: The stability of dispersions prepared according to this protocol has been validated up to 48 h; however, since this protocol is intended as a preliminary step for the preparation of dispersions in relevant media, the resulting dispersions should be used as soon as possible following preparation and in accordance with directions prescribed in medium-specific protocols (currently under preparation).

6. Acceptance criteria

6.1. The obtained TiO_2 aqueous dispersion should have an opaque white appearance if prepared using P25.

Note: If source powders other than P25 are used, the appearance may vary depending on the final particle size, particle concentration and other factors. Refer to section A.3 for further details on the applicability and repeatability of the procedure for different P25 sources.

6.2. The particle size distribution (PSD) of P25 dispersions prepared following this protocol should be monomodal, with a volume-based mean particle diameter of:

≈ 75 nm, if measured using XDC;

≈ 70 nm, if measured using LDS;

≈ 110 nm, if measured using DLS.

The volume-based mean particle diameter, as well as the D_{10} and D_{90} values,[d] for aqueous P25 dispersions prepared following the protocol should be reported by the user to allow for comparison with the values specified herein. Refer to Appendix A for details on PSD characterization and validation criteria, as well as the determination of the mean diameter, D_{10} and D_{90} values.

6.3. The pH of P25 aqueous dispersions prepared using this protocol should be between 3.7 and 4.9, with lower pH values obtained for higher P25 concentrations. Refer to Appendix B for details on pH and stability measurements for P25 dispersions at varying concentrations.

Note: The successful implementation of this protocol for ENMs other than P25, including other sources of TiO_2 or other metal oxide ENMs, is dependent on knowing the isoelectric point (IEP) or zero point of charge of the ENM; in order to obtain a stable dispersion for ENMs that are electrostatically stabilized (i.e., that do not contain a sterically stabilizing capping agent), the final pH should be at least 2 to 3 pH units below or above the IEP. This may require the addition of a compatible acid or base, and should be evaluated as part of the optimization process (see Appendix C). Multiple test or practice runs may be required in order to determine the required quantity and concentration of acid or base to add; pH adjustment should, optimally, be performed after step 5.2 and prior to sonication. If dispersions are to be used for toxicological or environmental tests, additives for adjusting the pH should be selected based on their biocompatibility and relevance to the test matrix, and considering potential sonication-induced physicochemical changes[7].

[d] D10 and D90 refer to characteristic percentile size values associated with the cumulative volume or mass less than 10 % and 90 %, respectively, of the total volume or mass within the distribution. These parameters are routinely reported by LDS and XDC instruments. They may or may not be obtainable directly from commercial DLS instruments, depending on the manufacturer.

APPENDICES

Appendix A. Procedure to Validate Dispersion State

A.1 Characterization of the Particle Size Distribution (PSD)

Laser diffraction spectrometry (LDS), dynamic light scattering (DLS), and X-ray disc centrifugation (XDC) were used as size characterization techniques for the validation of the protocol with respect to producing a monomodal dispersion in the nanoscale size range. These techniques were selected as they allowed for *in situ* measurements with minimal sample transformations, ensuring that the measured PSD profiles reflected the actual state of the "as produced" dispersions rather than technique specific or sample preparation induced artifacts in size (e.g., drying induced agglomeration or probe-sample interaction artifacts commonly observed when using SEM, TEM or AFM). Furthermore, while LDS and DLS allowed for the measurement of PSD profiles based on light scattering properties, XDC provided an additional degree of validation as an orthogonal technique that relies on fundamentally different principles to determine size (i.e., centrifugal sedimentation and X-ray absorption).

To validate the size distribution of dispersions obtained by the prescribed protocol, a triplicate set of 10 mg/mL SRM 1898 aqueous dispersions were prepared following the procedure prescribed in the protocol, and their PSDs measured using LDS, DLS and XDC. In addition to SRM 1898, the procedure was validated across several different P25 commercial lots (section A.3).

LDS measurements were performed using a Partica LA-950 V2 (Horiba Instruments Inc., Irvine CA, USA), equipped with an 87 detector, high-resolution silicon photodiode array (75 detectors for forward/low-angle light scattering and 12 detectors for high-angle and backscatter light scattering); operating with a 5 mW 650 nm red laser and a 3 mW 405 nm blue light emitting diode. Measurements were conducted by introducing the sample into a stirred 15 mL glass cell containing DI water, until an adequate blue line transmittance level was attained (between 70 % and 90 % transmittance). For all LDS measurements, the instrument was zeroed using DI water as a blank. Volumetric PSDs were determined by application of the Mie scattering model, with a particle refractive index of 2.5.

DLS measurements were performed using a Zetasizer Nano ZS (Malvern Instruments Inc., Westborough MA, USA) in backscatter configuration ($\theta = 173^{o}$) at a laser wavelength of $\lambda_0 =$ 633 nm. Samples were measured in 1.5 mL disposable cuvettes by dilution in DI water (up to 1 mL total volume) to achieve an appropriate light attenuation level. Measurement protocols used in this method are described elsewhere [18]. For each sample, measurements were performed in triplicate, with the number and duration of sub-measurements for each run determined automatically by the instrument's software. A non-negatively constrained least squares inversion algorithm was used to generate the PSD. A regularization parameter of 0.01 was selected, with data parsed over 70 bins. For conversion from intensity-weighted to volumetric PSDs, the Mie scattering model was applied using a particle refractive index of 2.5.

XDC measurements were performed using a BI-XDCW X-ray disc centrifuge system (Brookhaven Instruments Corp, Holtsville NY, USA) equipped with a standard PMMA 10 cm disc. For XDC measurements, 25 mL of a 10 mg/mL sample was loaded into the disc and spun at 3500 rpm (58.3 Hz) for 32 min. The particle size distribution was calculated using the radial scanning mode and the Stokes equation for laminar flow particle settling under a centrifugal field.

Unless otherwise noted, all PSD profiles are shown on a volume basis, and are thus equivalent to a mass weighted distribution. To allow for a quantitative and normalized comparison between different PSD profiles, the following two characteristic size parameters were used:

Mean Diameter (D_m): The arithmetic mean diameter of the sample's PSD, obtained by:

$$D_m = \frac{\sum q(j)X(j)}{\sum q(j)} \qquad (1)$$

Where *j* is the particle diameter division of the *volume (%) vs. diameter* frequency distribution, *q(j)* is the frequency value (%) for diameter division *j*, and *X(j)* is the central particle diameter for the diameter division *j*.

Mode Diameter (D_M): The size corresponding to the peak maximum in a PSD profile.

Diameters representing cumulative 10^{th} (D_{10}) and 90^{th} (D_{90}) percentiles: These diameters are used as a measure of the polydispersity or *spread* of a given size distribution; the parameters indicate that 10 % or 90 % (by mass or volume) of the particles in the evaluated size fraction have a diameter equal to or less than the D_{10} or D_{90} values, respectively. Percentile diameters can be obtained directly from a cumulative plot of the volumetric PSD (see e.g., [19]), and are usually available as output from commercial LDS and XDC instruments.

Uncertainties associated with reported characteristic size values are based on a Type A uncertainty analysis. The sample standard deviation (or standard uncertainty) was first calculated for replicate measurement results obtained under repeatability conditions. The standard deviation was then multiplied by *k*, where *k*=2 is the coverage factor approximating a 95 % confidence interval. The resulting uncertainty interval is referred to as the expanded uncertainty. [20]

Representative PSD profiles and characteristic dimensions for the prepared 10 mg/mL SRM 1898 dispersions, measured using the three selected techniques are shown in Figure A1 and Table A1.

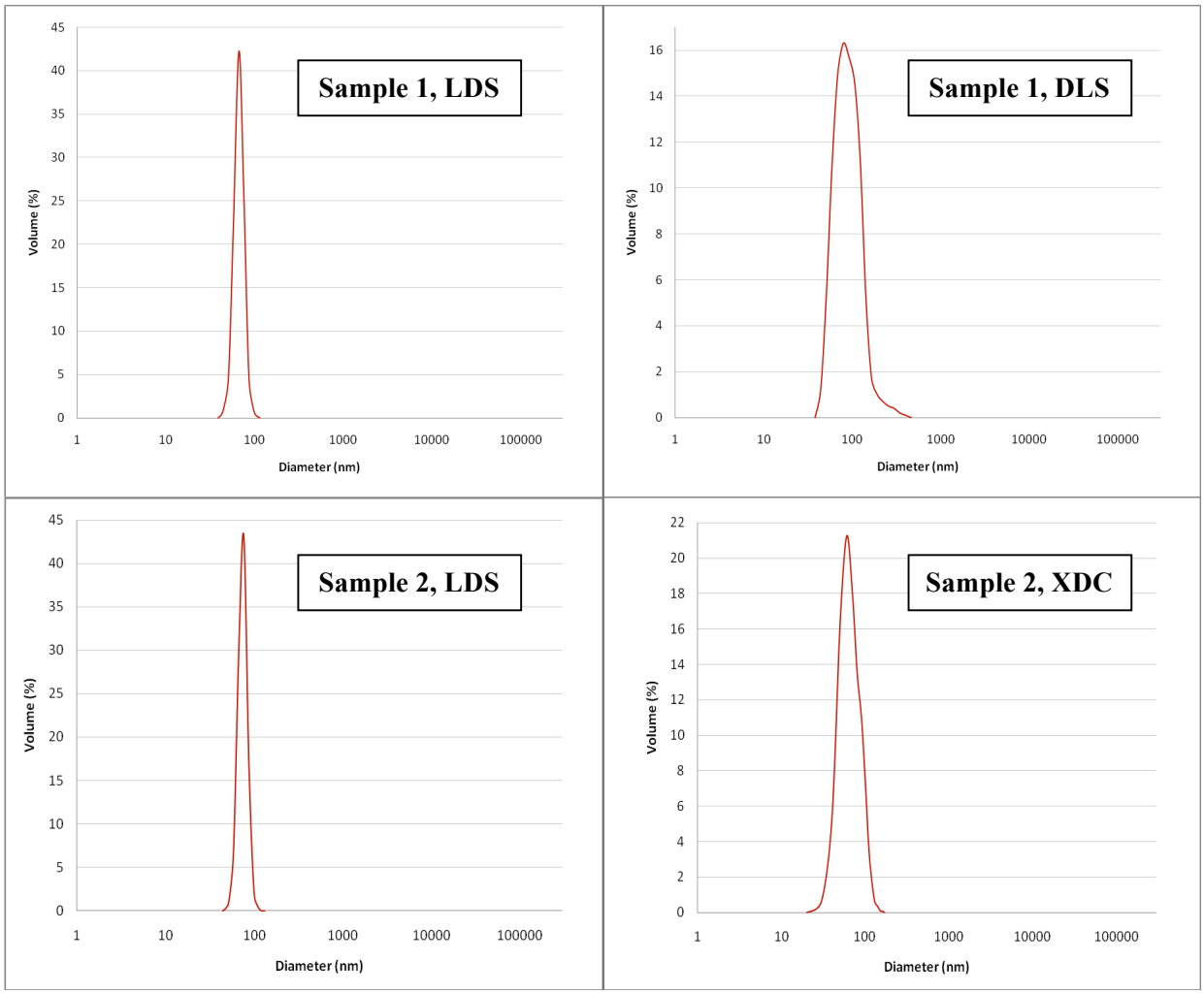

Figure A1. Representative PSDs obtained for 10 mg/mL SRM 1898 dispersions. Results from LDS (top, left) and DLS (top, right) for "Sample 1", and LDS (bottom, left) and XDC (bottom, right) for "Sample 2". All dispersions were obtained using the optimized sonication sequence. Sample labels are arbitrary.

Table A1. Averages and uncertainties (95 % confidence interval) for mean diameter (D_m), mode diameter (D_M), D_{10} and D_{90} values of SRM 1898 10 mg/mL dispersions measured using LDS, XDC and DLS. Averages and uncertainties were obtained from the measurement of three independent dispersions with each technique. All dispersions were obtained following the optimized sonication sequence.

Technique	D_m (nm)	D_M (nm)	D_{10} (nm)	D_{90} (nm)
LDS	71.1 ± 3.9	70.9 ± 2.7	59.3 ± 1.6	84.3 ± 4.8
XDC	77.0 ± 6.6	54.3 ± 5.7	31.7 ± 22.4	119.3 ± 22.5
DLS	112.4 ± 4.0	113.8 ± 5.1	67.8 ± 8.0	151.1 ± 3.5

As shown in Figure A1 and Table A1, LDS and XDC yielded a good statistical match (with respect to the confidence intervals) for the sample mean diameters, and a reasonable consistency

with respect to the other characteristic percentiles. By contrast, DLS exhibited larger mean diameter and D_{90} values, which reflects, in part, the known sensitivity of DLS to the presence of small quantities of large particles.[e] Additionally, DLS measures the equivalent spherical hydrodynamic envelope defined by the primary aggregate's displaced volume, including entrained solution and a hydration shell; thus, DLS hydrodynamic size is typically larger than a hard core based size. As XDC and LDS are orthogonal in nature, and less sensitive to artifacts that commonly impact DLS measurements, these two methods in combination provide validation of the characteristic PSD profile of dispersions prepared following the prescribed protocol. Since LDS requires substantially less sample mass to perform measurements, compared with XDC, it was therefore adopted as the principal size characterization technique for all further validation tests.

The obtained results show a high repeatability for the prescribed protocol in terms of yielding consistent PSDs for dispersions obtained from a single P25 production lot (NIST SRM 1898) and at a single particle concentration of 10 mg/mL, as indicated by the relatively low variation in the measured size parameters, based on LDS. It must be noted that the higher relative uncertainty for the XDC D_{10} value is likely due to the fact that it is close to the lower detection limit of the instrument (≈ 10 nm). As shown in the following sections, further tests were performed to assess the repeatability of the protocol for the preparation of dispersions at different particle concentrations and using different P25 production lots.

A.2 Repeatability of dispersion protocol at different particle concentrations

To evaluate the repeatability of the protocol with respect to producing consistent PSDs at different particle concentrations, NIST SRM 1898 was dispersed at concentrations of (0.5, 1, 10 and 20) mg/mL, in 50 mL of DI water. PSD profiles of the resulting dispersions were measured using LDS, showing comparable distributions in all cases (Figure A2, Table A2).

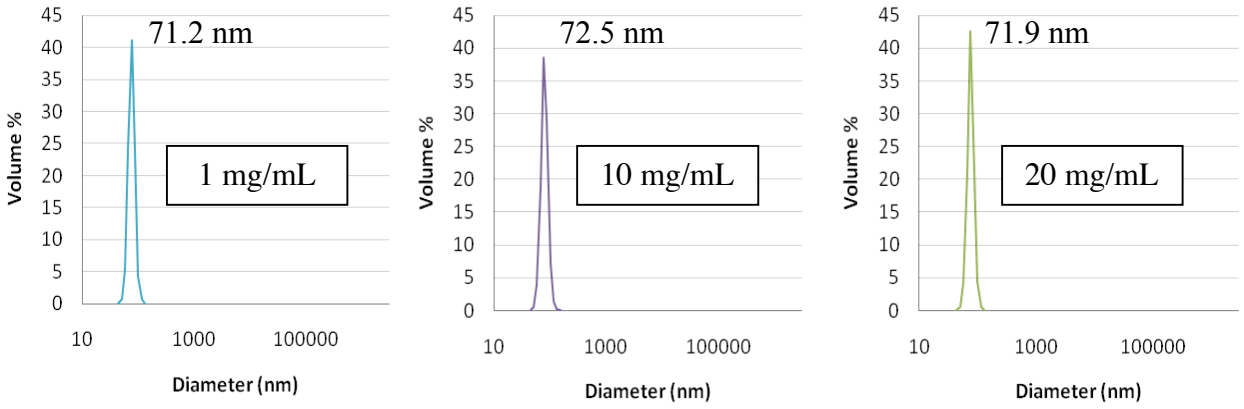

Figure A2. LDS PSD profiles of SRM 1898 dispersions at concentrations of 1 mg/mL (left), 10 mg/mL (middle) and 20 mg/mL (right). Peak values are shown in each plot. PSDs of 0.5 mg/mL dispersions are shown in Figure A3.

[e] In the DLS analysis, conversion from an intensity-based to a volumetric-based PSD necessarily involves greater uncertainty, compared with LDS and XDC, due to the inherent low resolution associated with photon correlation based analysis and the reliance on a single scattering angle.

Table A2. Mean diameter (D_m), mode diameter (D_M), D_{10} and D_{90} values of (0.5, 1, 10 and 20) mg/mL SRM 1898 dispersions prepared following the prescribed protocol.

P25 Conc. (mg/mL)	D_m (nm)	D_M (nm)	D_{10} (nm)	D_{90} (nm)
0.5	69.5	69.8	58.7	82.2
1	71.6	71.2	59.4	84.8
10	74.0	72.5	60.4	86.9
20	72.4	71.9	60.1	85.1

As shown in Table A3, all of the measured D_m, D_{10} and D_{90} values of dispersions prepared at different particle concentrations were within the 95 % confidence intervals obtained from the PSD validation tests, shown in Appendix A, Table A2 - LDS.

A.3 Repeatability of dispersion protocol for different P25 sources

The repeatability of the protocol for the preparation of dispersions with consistent PSDs using different P25 production lots was tested using material from SRM 1898 (P25 control no. 4166091998, produced in 2006) and two additional P25 lots: CN 4168112198, produced in 2008 and used in the OECD testing Program, and CN 11B1, produced in 2001. The prescribed protocol was applied to all three powder production lots, at a concentration of 0.5 mg/mL. The PSDs of the obtained dispersions were measured using LSD. Results are shown in Figure A3 and Table A3.

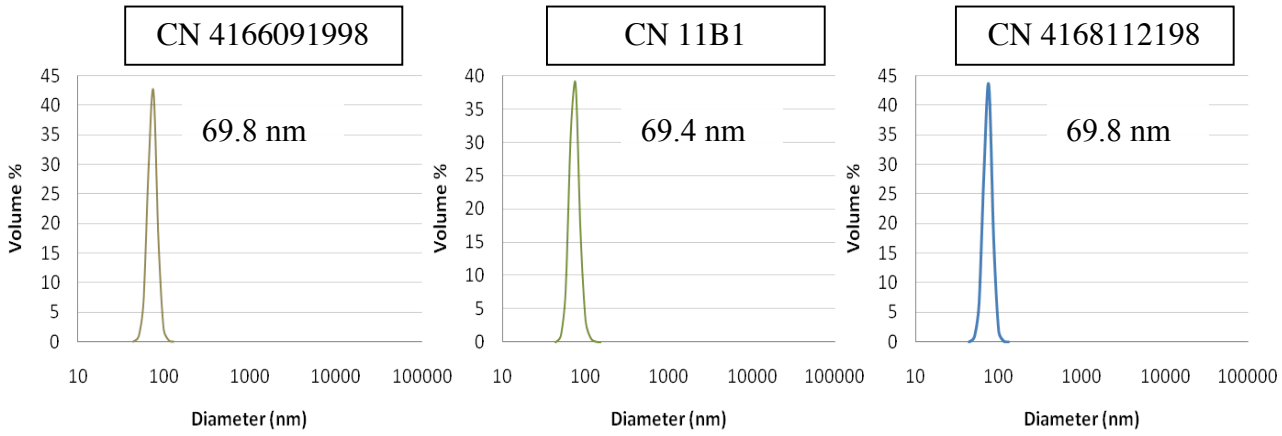

Figure A3. LDS derived PSD profiles for 0.5 mg/mL dispersions obtained using different P25 sources (control numbers for each lot indicated above each plot). Mode diameters (i.e., most frequent value) are indicated in each size plot.

Table A3. Mean diameter (D_m), mode diameter (D_M), D_{10} and D_{90} values for P25 dispersions prepared from different production lots, by applying the prescribed protocol.

P25 Source	Production year	Dm (nm)	D_M (nm)	D_{10} (nm)	D_{90} (nm)
CN 11B1	2001	69.5	69.4	58.4	83.1
CN 4166091998	2006	69.5	69.8	58.7	82.2
CN 4168112198	2008	69.4	69.8	58.8	81.9

As shown in Figure A3 and Table A4, the protocol produced statistically identical results (relative to the 95 % confidence interval) with narrow PSDs and a single mode centered near 70 nm, independent of the material source. All of the measured D_m, D_{10} and D_{90} values for the dispersions prepared from different P25 production lots were within the 95 % confidence intervals obtained from the PSD validation tests (Appendix A, Table A2 – LDS).

It must be noted that the protocol was also tested for the dispersion of two additional P25 lots manufactured more than 15 years ago. These two powder sources could not be fully dispersed, showing a minor microscale size mode upon application of the prescribed procedure. This deviation may be related to actual differences in the production process relative to more recently produced material, or to aging-induced physicochemical changes in these powder sources, which possibly manifest in the strengthening of agglomerate/aggregate bonding over time. Nonetheless, further optimization tests were carried out to evaluate the possibility of dispersing sources affected by aging. For this purpose, the oldest available production lot (with a production date around 1988) was subject to further optimization tests. By increasing the sonication time and power (sonication time increased by 5 min and power by ≈ 30 W, with respect to protocol conditions), this powder was successfully dispersed to a monomodal ≈ 70 nm peak fraction with an absence of microscale agglomerates. While we do not intend to prescribe specific dispersion procedures for compromised P25 sources, it is worth noting that production date, aging and/or other factors (such as inadequate storing conditions) may result in an incomplete dispersion of the powder, even if the protocol is applied properly. However, based on our findings, it is believed that further optimization steps can potentially allow for the attainment of complete dispersion to a reproducible and single mode PSD for any P25 source material. The reader is advised to consult [7] and Appendix C for optimization guidelines and procedures.

Appendix B. pH and Stability of Dispersions

B.1 IEP determination

To better understand the stability of dispersions, in light of their intended use for the preparation of dispersions in complex biological or environmental media, the isoelectric point (IEP) of SRM 1898 dispersed according to the prescribed protocol was evaluated by determining the zeta potential (derived from electrophoretic mobility measurements) over an appropriate range of pH values.

Zeta potential measurements were performed using a Zetasizer Nano ZS (Malvern Instruments Inc., MA, USA), operating in the monomodal analysis mode and using a palladium electrode dip cell. Zeta potential values were calculated from the measured electrophoretic mobility using the Smoluchowski relationship, which assumes thin double layer conditions and may not be strictly applicable to particles with a relatively thick electrical double layer and small diameter; however, the absolute magnitude of the zeta potential is not critical for the determination of the IEP, and the Smoluchowski value is commonly reported in the literature and is a default setting on many commercial instruments. Therefore, Smoluchowski-derived values are reported here for comparative purposes only.

pH measurements were conducted using an Orion 3-Star pH meter (Thermo Electron Corp., MA, USA) , outfitted with an InLab Semi-Micro polymer electrolyte pH electrode (Mettler Toledo, OH, USA) and an Orion 927006MD Automatic Temperature Compensation probe (Thermo Electron Corp., MA, USA).

The SRM 1898 dispersion used for these tests was prepared following the protocol at a concentration of 0.5 mg/mL. After sonication, two sets of zeta potential versus pH measurements were obtained; one set starting from the basic pH end and titrating towards acid pH values, and another set with the reverse titration trend. For the basic-to-acid titration, 1.25 mL of the sonicated SRM 1898 suspension was transferred into 50 mL of a 10^{-3} mol/L NaOH solution (for a starting pH near 11) in a 60 mL plastic beaker with a magnetic stir bar. While stirring, the pH was lowered by addition of HCl (aq), and zeta potential measurements obtained at selected pH values down to ≈ pH 3. At each relevant pH value, an aliquot of 1 mL was taken from the suspension and used to measure the zeta potential. A similar procedure was followed for the reverse (acid-to-basic) titration set, in this case using 50 mL of a 10^{-3} mol/L HCl solution for a starting pH near 3, and titrating with NaOH (aq) to ≈ pH 11.

Since the pH, zeta potential and IEP are used here within a qualitative context, uncertainties are not reported for these values.

The titrations as described above converged to an IEP near pH 7, as shown in Figure B1. For pH values above 7, the basic to acid titration values are shown, while for pH values below 7, the acid-to-basic titration values are shown.

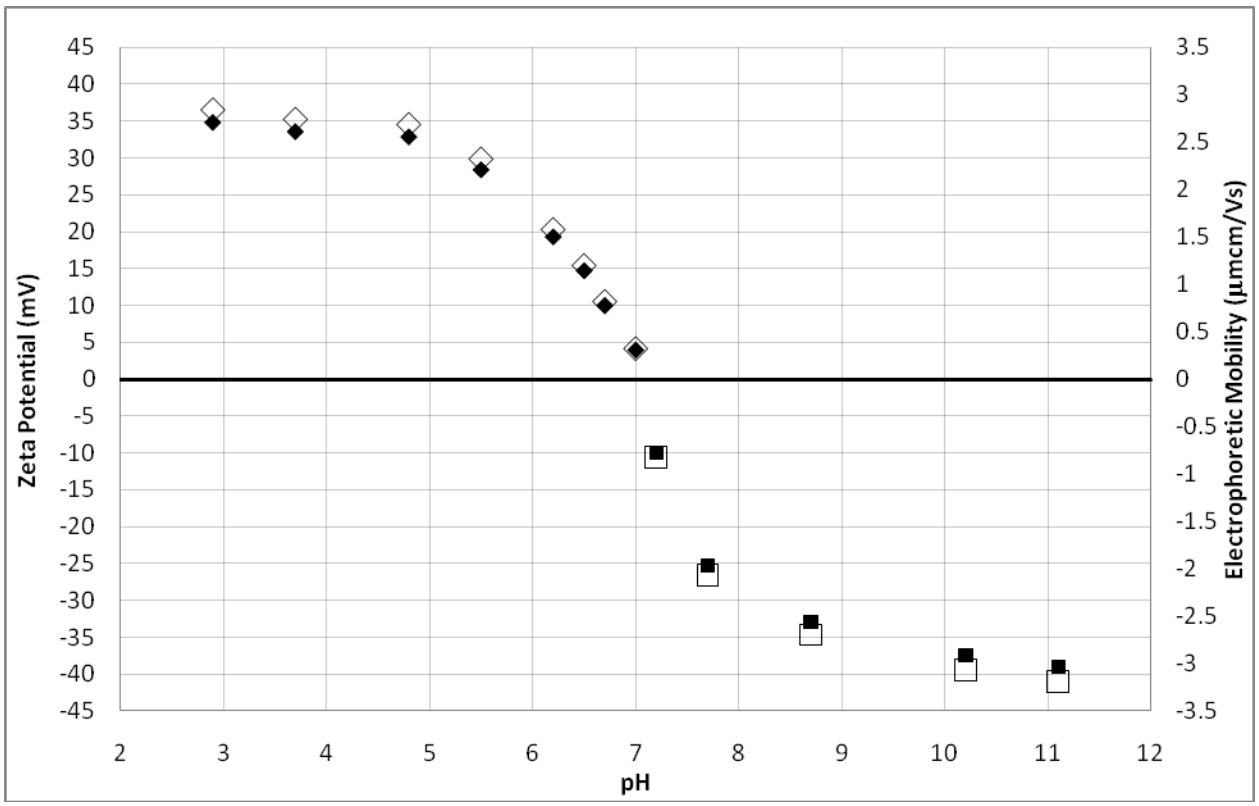

Figure B1. Acid-to-basic (diamonds) and basic-to-acid (squares) titration sets for electrophoretic mobility (filled markers) and zeta potential (hollow markers) as a function of pH for SRM 1898 prepared following the prescribed protocol; titrations converge to an IEP at ≈ pH 7.

The obtained IEP suggests that the suspended nanoparticles are most susceptible to agglomeration at biologically and environmentally relevant pH values (i.e., near neutral pH). This finding stresses the importance of measuring the stability of the particles in relevant media and adopting measures, such as using relevant stabilizing agents (e.g., proteins), to control agglomeration that can potentially result from changes in pH, which are likely to manifest upon inoculation in relevant media.

B.2 pH of dispersions at different concentrations

The prepared dispersions showed pH values in the (3.7 to 4.9) range, with dispersions at high particle concentrations showing lower pH values than dispersions at low concentrations (see Figure B2 for representative dispersion pHs at different particle concentrations).

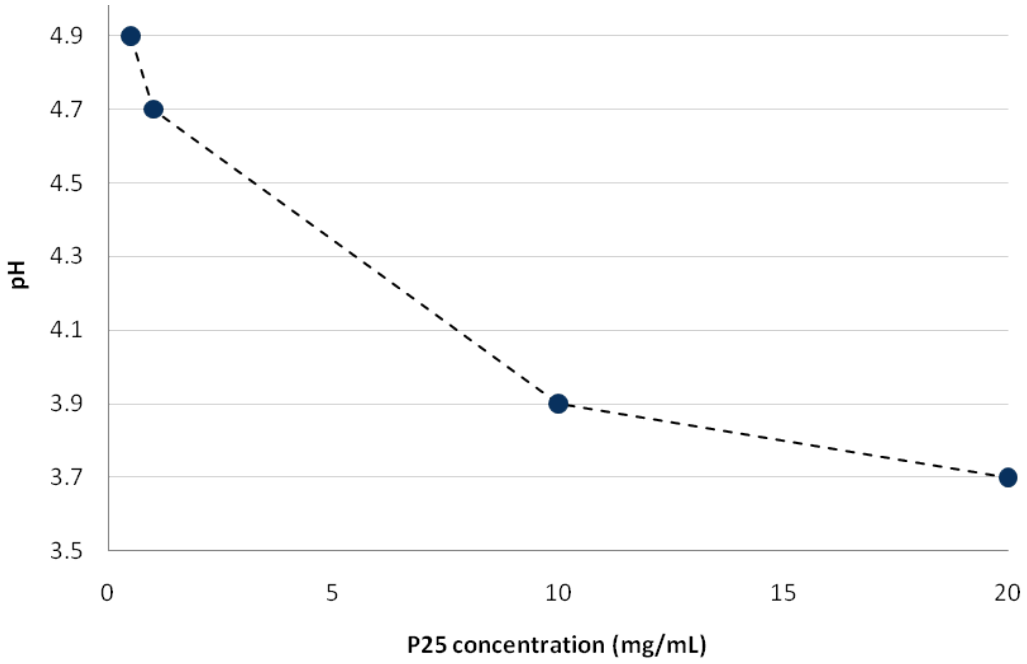

Figure B2. Representative pH values for aqueous dispersions of SRM 1898 at different concentrations.

The measured acidic pH values of the as-prepared dispersions are consistent with the manufacturer reported pH values for aqueous P25 dispersions (pH range of 3 to 5) [13] and are explained by the release of protons into the water from residual HCl in the P25 dry powder. The residual HCl is a byproduct of the gas phase pyrogenic hydrolysis of titanium tetrachloride, which is the basis of the AEROSIL process by which P25 is produced. Accordingly, and as experimentally confirmed, higher P25 concentrations yield lower pH values due to a greater quantity of protons released into the same volume of medium.

While the aqueous dispersions obtained in this work are not intended for use "as is" in toxicological or environmental tests - but instead to be employed as stock solutions that are applied towards the preparation of dispersions in relevant biological or environmental media - the measured acidic pH for SRM 1898 aqueous dispersions points to the need for pH measurements of TiO_2 (and other metal oxide) dispersions in relevant media, to ensure that their pH meets the desired value for the intended test. The stability of the SRM 1898 aqueous dispersions prepared following the optimized sonication sequence was measured in terms of the conservation of the PSD profile over 48 h.

All of the prepared dispersions at the tested particle concentrations were stored in capped amber glass vials at room temperature and retained their PSD over at least 24 h, with the 10 mg/mL and 20 mg/mL dispersions retaining their PSD for more than 48 h. If dispersions prepared following the protocol prescribed herein are intended for use over longer periods of time, stability should be validated accordingly.

Appendix C. Optimization of Dispersion Procedure

As mentioned previously, the technique adopted to obtain nanoparticles from the ENM source powder was direct (probe) sonication. This technique is a simple operation that can achieve a

higher degree of powder fragmentation, at constant specific energy, than other conventional dispersion techniques [7, 21-23].

Sonication parameters (power, time and operation mode) were optimized to achieve the smallest possible nanoparticle dimensions and a monomodal size distribution. A Branson 450 analog horn sonicator (Branson Ultrasonics Corp., CT, USA) was used for all dispersion tests.

Uncertainties associated with reported sonication power values are based on a Type A uncertainty analysis. The sample standard deviation (or standard uncertainty) was first calculated under repeatability conditions for replicate measurement results. The standard deviation was then multiplied by k, where k=2 is the coverage factor approximating a 95 % confidence interval. The resulting uncertainty value is referred to as the expanded uncertainty. [20]

LDS was selected as the size characterization technique for dispersion optimization studies. For a detailed discussion on the selection and suitability of this technique over other conventional techniques (such as DLS) for the measurement of size profiles in dispersion optimization studies, please refer to [19, 24].

C.1.Sonication power

Prior to the evaluation of the dispersion effect of sonication power, the probe sonicator used for the optimization tests was calibrated in order to correlate different sonicator settings to quantifiable power values. The calibration was performed as prescribed in [12]. Table C1 summarizes representative power values for different sonicator settings.

Table C1. Calorimetrically measured delivered sonication power corresponding to different sonicator settings (see [12] for calibration details). Averages and associated uncertainties correspond to a 95 % confidence interval for triplicate measurements.

Setting	**Power (W)**
1	9.25 ± 1.15
3	32.2 ± 2.3
5	52.34 ± 0.96
8	96.95 ± 4.35
10	123.3 ± 12.6

For power optimization tests, 0.025 g of SRM 1898 was added to a 100 mL, 5 cm diameter glass beaker. Then, 50 mL of DI water was added to the beaker with the powder to yield a 0.5 mg/mL P25 concentration. The beaker was immersed in an ice water bath that covered the beaker just above the water level contained within the beaker. The sonicator horn was fitted with a standard 0.5 inch (1.3 cm) flat tip and immersed in the beaker at an immersion depth of ≈ 2.5 cm below the water level in the beaker. Sonicator settings from 1 to 10 were then applied, operating in

continuous mode for 1 min. Representative PSD profiles and characteristic size parameters obtained for dispersions prepared under different power levels are shown in Figures C1 and C2.

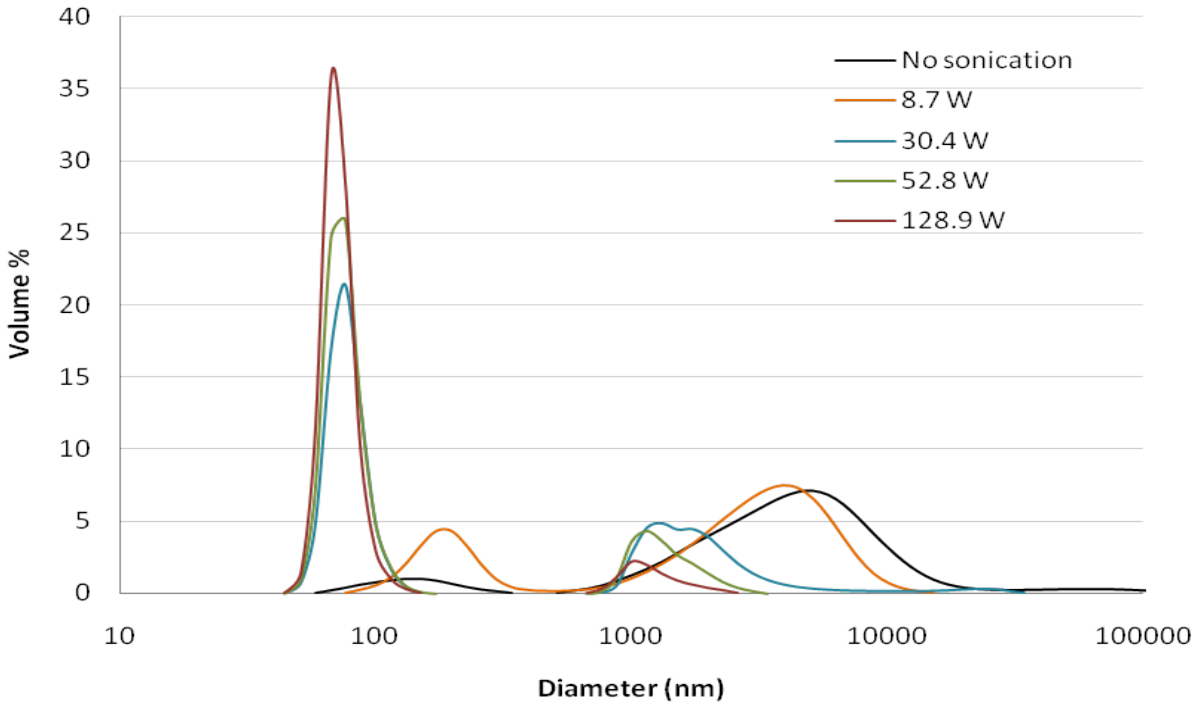

Figure C1. PSD profiles obtained using LSD for SRM 1898 dispersed in DI water at different sonicator power levels (sonication time 1 min, continuous mode).

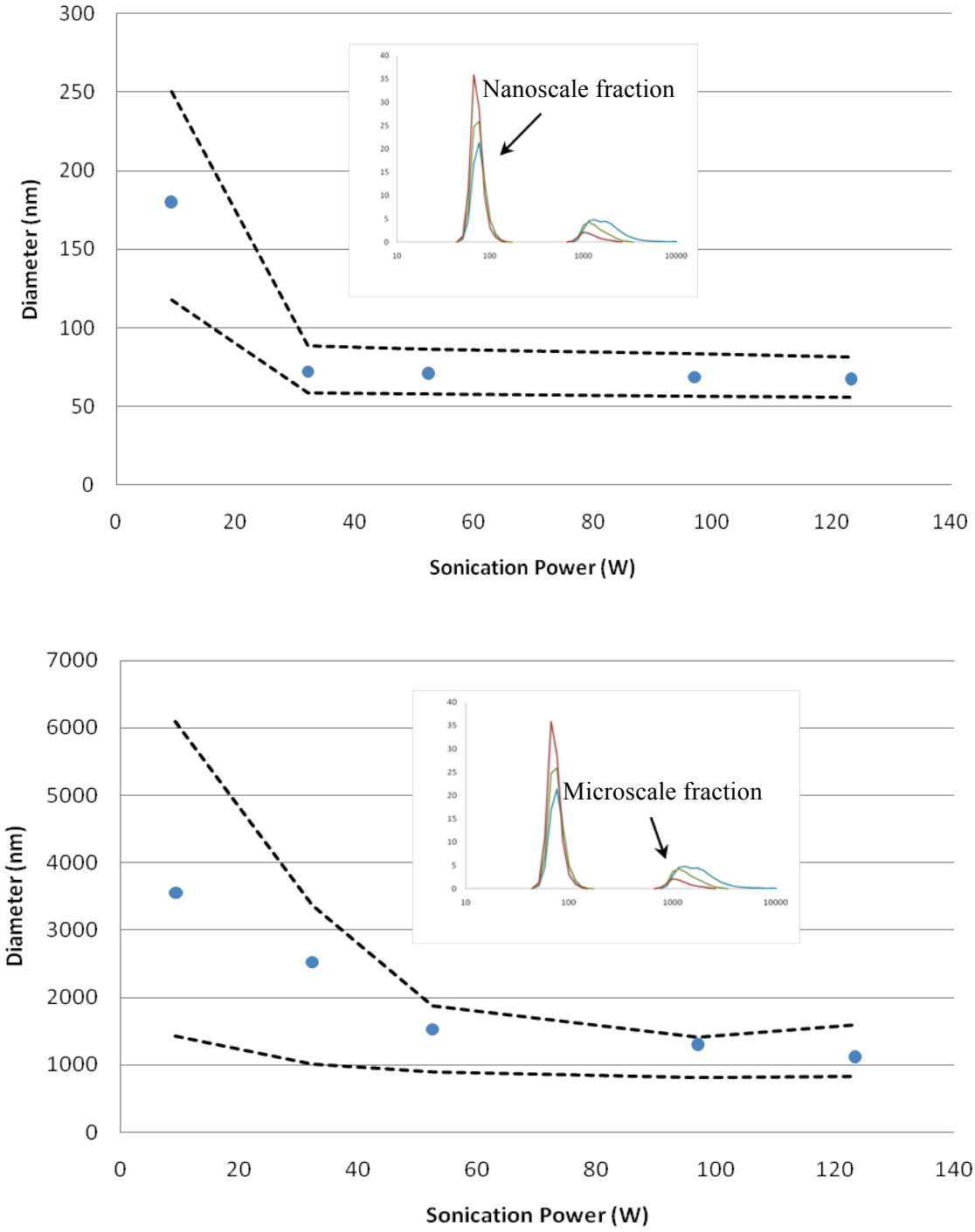

Figure C2. Mean diameters (dots), D_{10} (lower dashed line) and D_{90} (upper dashed line) values for each of the two size fractions (nanoscale -top and microscale -bottom) observed in the bimodal PSDs of SRM 1898 aqueous suspensions sonicated at different power levels (sonication time 1 min, continuous mode).

As shown in Figure C2, the unsonicated dispersion shows a highly polydisperse and multimodal PSD predominantly in the (1 to 10) μm range, with a major fraction (93 % of the total particulate volume) centered at around 6 μm and a minor fraction of particles with a peak at around 130 nm. As the powder in suspension undergoes sonication induced fragmentation, the original PSD profile shifts to a well-defined bimodal distribution, showing a microscale fraction with a peak at (1 to 3) μm and a nanoscale fraction with a peak at (50 to 80) nm. As sonication power is increased, the relative predominance of the microscale fraction decreases, going down to 20 % of the total particulate volume after sonication at 50 W (from the original 93 % for the non-sonicated suspension). These trends (size fraction characteristic peak values and decrease of the microscale fraction) are consistently observed as sonication power increases and, in particular, at powers at or above ≈ 50 W, wherein two consistent size fractions develop, one with a peak approaching ≈ 1 μm and a nanoscale fraction with a peak at ≈ 70 nm (Figure C1). These two peaks remain relatively unaltered at power values above 50 W. The observed behavior suggests two configurations for P25 aggregates/agglomerates, wherein the 70 nm peak likely indicates primary aggregates (i.e., small assemblies consisting of fused primary crystallites), while the 1 μm peak suggests that the 70 nm aggregates associate, likely through weaker Van der Waals attractive forces or a small number of cleavable contact points, forming larger *metastable* agglomerates that tend towards a specific size mode (i.e., at around ≈ 1 μm).

C.2. Sonication time and operation mode

As no significant difference in the bimodal distribution peak sizes or fraction spreads were observed beyond a power of ≈ 50 W (see section C.1) and higher power settings could further result in excessive sample heating and undesirable side effects under longer sonication times, a sonicator power setting of 5 (≈ 50 W) was chosen for further time optimization steps (setting is specific to the device used in this study; other device and probe combinations would require calibration according to reference 12).

Under otherwise similar conditions to those explained in section C.1, and operating at power setting 5 in continuous mode, different sonication times (30 s, 1 min and 5 min) were tested. As shown in Figure C3, increasing sonication time resulted in a significant decrease in the microscale fraction relative volume, while the nanoscale peak position remained relatively unaltered. This trend further confirms that the micrometer scale peak corresponds to weakly bound agglomerates that can be further fragmented if sufficient energy is delivered, while the 70 nm peak corresponds to hard “primary aggregates” consisting of strongly bound primary crystallites that cannot be further disrupted via sonication at the tested delivered energies.

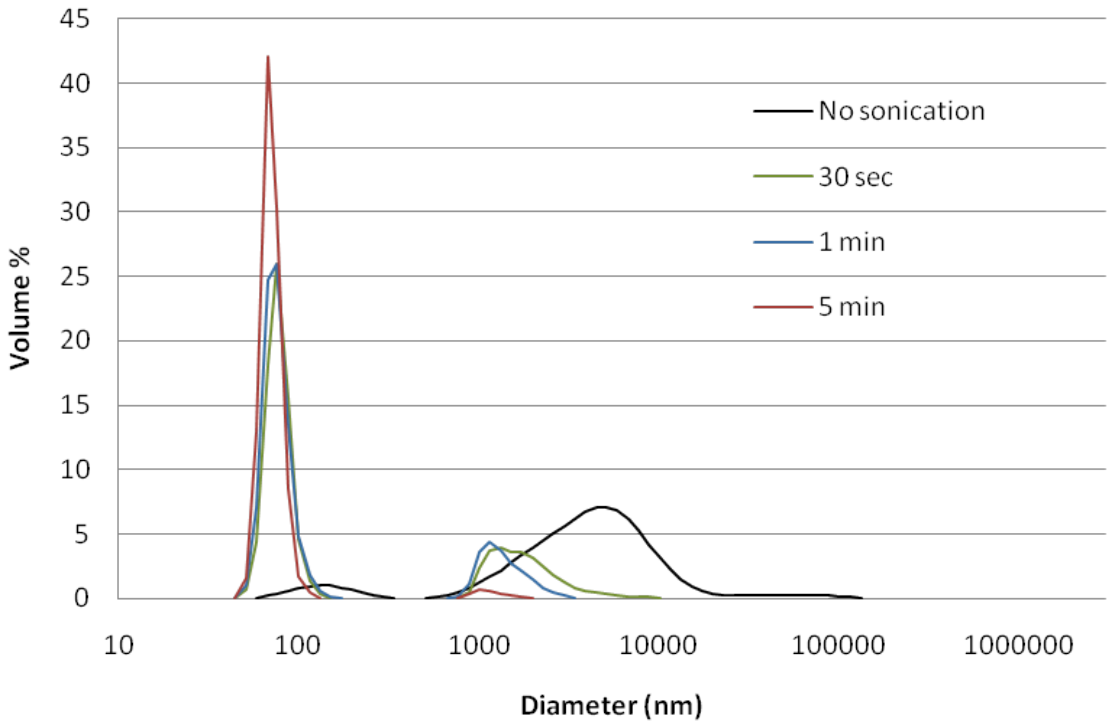

Figure C3. PSD profiles for SRM 1898 in DI water at different sonication times (sonication power setting 5, continuous mode).

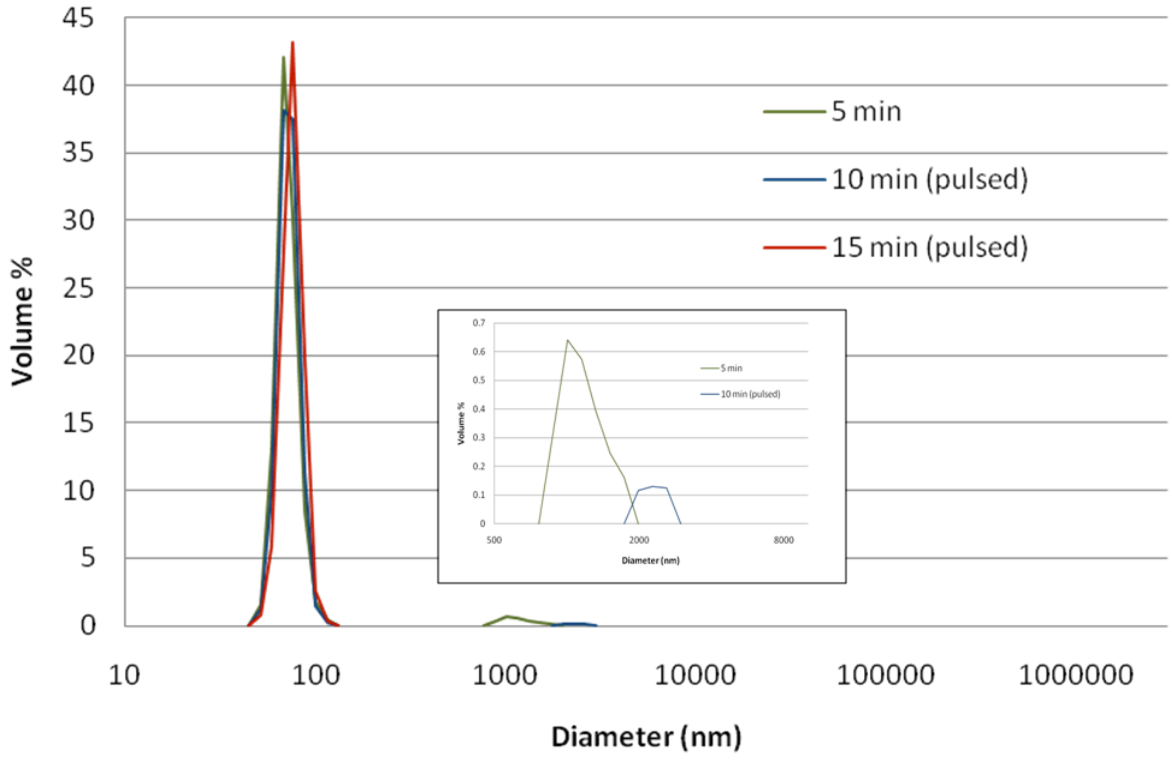

Figure C4. PSD profiles for SRM 1898 in DI water at different sonication times (sonication power setting 5, continuous -5 min- and pulsed modes -10 min and 15 min); inset shows an expanded view of the microscale size range.

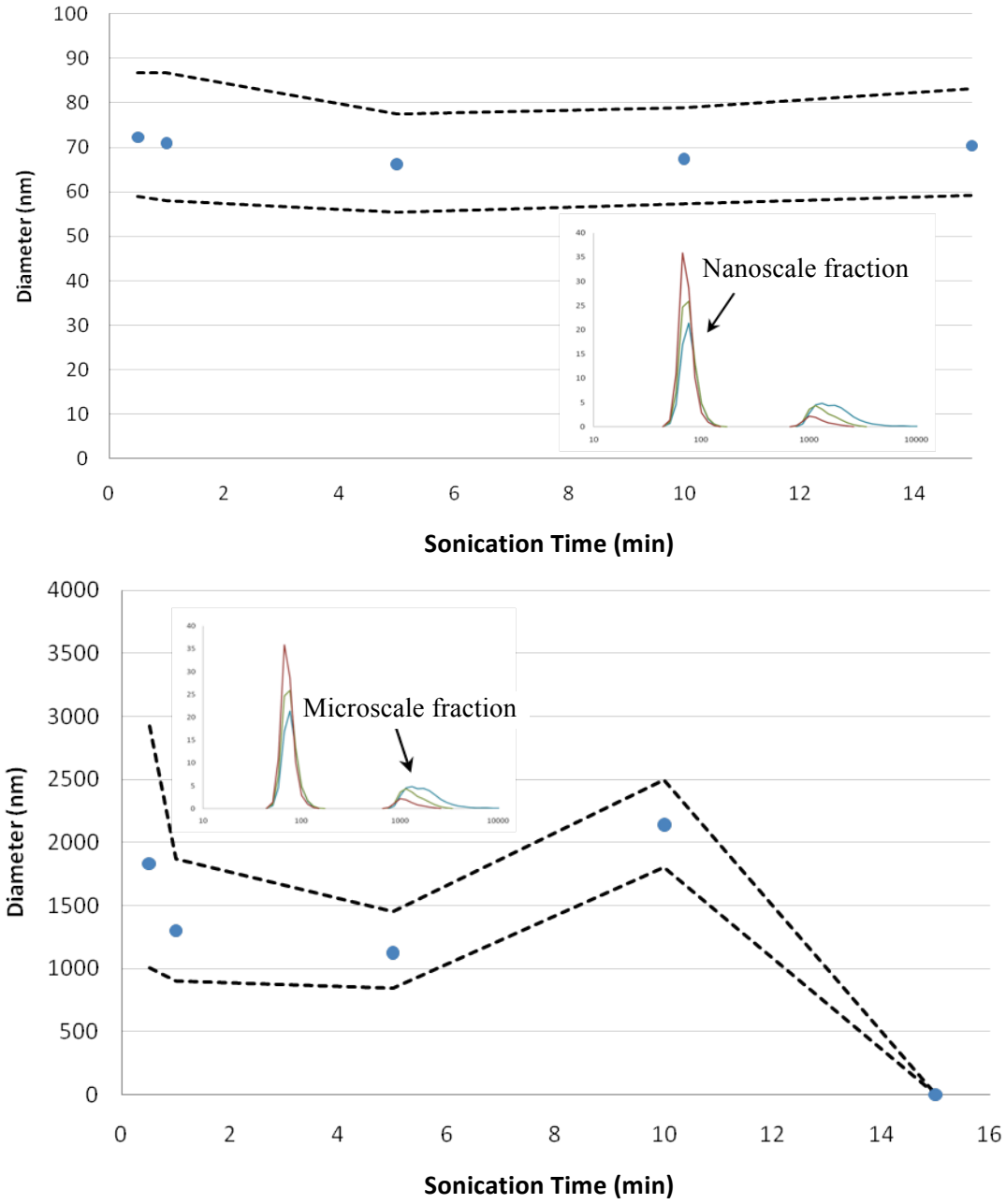

Figure C5. Mean diameters (filled circles), D_{10} (lower dashed line) and D_{90} (upper dashed line) values for each of the two size fractions (nanoscale shown in top graph and microscale on bottom) observed in the bimodal PSDs for SRM 1898 aqueous suspensions sonicated at different times and operation modes (sonication power setting 5, continuous up to 5 min- and pulsed modes -10 min and 15 min).

At sonication times above 5 min in continuous mode, the heating of the suspension was appreciable, even when immersed in the ice water bath. For this reason, pulsed operation was employed for further time optimization tests. Sonication times where then tested under an 80 % pulsation regime (0.8 s on, 0.2 s off). As shown in Figures C4 and C5, at 10 min of sonication there was still an identifiable - though minor - microscale peak, while after 15 min of sonication

the microscale peak was fully eliminated. Therefore, a sonication time of 15 min with an 80 % pulsation mode were selected for further tests.

From the above tests, an optimal set of sonication conditions was obtained: sonication power of ≈ 50 W and sonication time of 15 min at an 80 % pulsation mode for a TiO_2 concentration of 0.5 mg/mL in 50 mL of DI water using a flat tip at a water immersion depth of 2.5 cm in a cylindrical 100 mL, 5 cm diameter glass beaker. These sonication conditions yielded a narrowly dispersed, monomodal ≈ 70 nm mean particle diameter aqueous dispersion with no measurable microscale size fraction. A detailed, step by step description of the preparation procedure under optimized conditions is given in section 5 of the protocol. Validation tests for the optimized procedure prescribed herein are given in Appendix A.

Abbreviations

DI	de-ionized
DLS	dynamic light scattering
ENM	engineered nanomaterial
IEP	isoelectric point
ISO	International Organization for Standardization
LDS	laser diffraction spectrometry
NIST	National Institute of Standards and Technology
OECD	Organization for Economic Cooperation and Development
PSD	particle size distribution
SRM	Standard Reference Material
XDC	X-ray disc centrifugation

References

1. Landsiedel, R. L. Ma-Hock, A. Kroll, D. Hahn, J. Schnekenburger, K. Wiench and W. Wohlleben, *Testing Metal-Oxide Nanomaterials for Human Safety.* Advanced Materials, 2010. **22**(24): p. 2601-2627.
2. Pichat, P., *A Brief Survey of the Potential Health Risks of TiO2 Particles and TiO2-Containing Photocatalytic or Non-Photocatalytic Materials.* J Adv Oxid Technol, 2010. **13**(3): p. 238-246.
3. Warheit, D.B., T. R. Webb, K. L. Reed, S. Frerichs and C. M. Sayes, P*ulmonary toxicity study in rats with three forms of ultrafine-TiO2 particles: Differential responses related to surface properties.* Toxicology, 2007. **230**(1): p. 90-104.
4. Wu, J.H., W. Liu, C. B. Xue, S. C. Zhou, F. L. Lan, L. Bi, H. B. Xu, X. L. Yang and F. D. Zeng, *Toxicity and penetration of TiO2 nanoparticles in hairless mice and porcine skin after subchronic dermal exposure.* Toxicology Letters, 2009. **191**(1): p. 1-8.
5. *Titanium Dioxide (TiO2) IARC Monograph 93.* International Agency for Research on Cancer, 2006.
6. Roebben, G., S. H. Ramirez-Garcia, V.A., M. Roesslein, F. Klaessig, V. Kestens, I. Lynch, C. M. Garner, A. Rawle, A. Elder, V. Colvin, W. Kreyling, H. F. Krug, Z. Lewicka, S. McNeil, A. Nel, A. Patri, P. Wick, M. R. Wiesner, T. Xia, G. Oberdörster and K. Dawson, *Interlaboratory reproducibility of size and surface charge measurements on nanoparticles prior to biological impact assessment.* 2010. submitted to J. Nanopart. Res.
7. Taurozzi, J.S., V.A. Hackley, and M.R. Wiesner, *Ultrasonic dispersion of nanoparticles for environmental, health and safety assessment – Issues and recommendations.* Nanotoxicology, 2010. doi 10.3109/17435390.2010.528846.
8. *Standard Reference Material 1898 (in production), National Institute of Standards and Technology, Gaithersburg, MD, http://www.nist.gov/srm/*.
9. *List of Manufactured Nanomaterials and List of Endpoints for Phase One of the OECD's Testing Program, Series on the safety of manufactured nanomaterials, Number 6*. 2008, Environmental Diractorate, Organization for Economic Co-operation and Development. Paris, France.

10. Porter, D., K. Sriram, M. Wolfarth, A. Jefferson, D. Schwegler-Berry, M. Andrew and V. Castranova, *A biocompatible medium for nanoparticle dispersion.* Nanotoxicology, 2008. **2**(3): p. 144-154.
11. Taurozzi, J.S., V.A. Hackley, and M.R. Wiesner, *Reporting guidelines for the preparation of nanoparticle dispersions from dry materials.* CEINT/NIST Protocol (http://ceint.duke.edu/allprotocols), 2010.
12. Taurozzi, J.S., V.A. Hackley, and M.R. Wiesner (2010) *Protocol for the preparation of nanoparticle dispersions from powdered material using ultrasonic disruption*. CEINT/NIST Protocol (http://ceint.duke.edu/allprotocols).
13. *Highly dispersed metallic oxides produced by the Aerosil process.* **Technical Bulletin 56, Degussa**.
14. Ohno, T., K. Sarukawa, K. Tokieda and M. Matsumura, *Morphology of a TiO2 photocatalyst (Degussa, P-25) consisting of anatase and rutile crystalline phases.* J. Catal., 2001. **203**(1): p. 82-86.
15. *Standard terminology relating to nanotechnology*, in *E2456-06*. 2006, ASTM International West Conshohocken, PA.
16. *ISO TS 80004-1. Nanotechnologies - Vocabulary - Part 1: Core terms*. 2007, International Standards Organization, Geneva, Switzerland.
17. IUPAC, *Compendium of chemical terminology*, in *Version 2009-09-07: Release 2.1.5*. 2009.
18. Hackley, V.A. and J.D. Clogston, *Measuring the size of nanoparticles in aqueous media using batch-mode dynamic light scattering.* NIST - NCL Joint Assay Protocol PCC-1 (http://ncl.cancer.gov/working_assay-cascade.asp), 2007.
19. Allen, T., *Particle size measurement - Volume 1: Powder sampling and particle size measurement*. 5th ed. 1996: Chapman & Hall.
20. Taylor, B.N. and C.E. Kuyatt, *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results, NIST Technical Note 1297.* National Institute of Standards and Technology, U. S. Department of Commerce (http://physics.nist.gov/Pubs/guidelines/TN1297/tn1297s.pdf), 1994.
21. Hielscher, T., *Ultrasonic production of nano-sized dispersions and emulsions (http://arxiv.org/ftp/arxiv/papers/o708/0708.1831.pdf)*, in *Dans European Nano Systems Workshop*. 2005: Paris, France.
22. Mandzy, N., E. Grulke, and T. Druffel, *Breakage of TiO2 agglomerates in electrostatically stabilized aqueous dispersions.* Powder Technol., 2005. **160**(2): p. 121-126.
23. Park, B.S., D.M. Smith, and S.G. Thoma, *Determination of agglomerate strength distributions .4. Analysis of multimodal particle size distributions.* Powder Technol., 1993. **76**(2): p. 125-133.
24. Taurozzi, J.S., V.A. Hackley, and M.R. Wiesner, *Preparation of nanosclae TiO2 dispersions in biological media for toxicological testing.* CEINT/NIST Protocol (http://ceint.duke.edu/allprotocols), 2011. In preparation.