CEINT/NIST PROTOCOL

PREPARATION OF NANOSCALE TiO_2 DISPERSIONS IN AN ENVIRONMENTAL MATRIX FOR ECO-TOXICOLOGICAL ASSESSMENT

Ver. 1.0

February 02, 2012

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 assessment are key elements in the evaluation of the environmental, health and safety risks of engineered nanomaterials (ENMs). While significant effort and resources have been devoted to the toxicological evaluation of many ENMs, including nanoscale TiO_2 *(1-4)*, obtaining conclusive and reproducible results continues to be 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)*. In order to address these issues, the National Institute of Standards and Technology (NIST) jointly with the Center for the Environmental Implications of Nanotechnology (CEINT) have developed a series of standardized and validated protocols for the dispersion of ENMs from a powdered material source for both human health and environmental testing applications. These protocols have been developed and validated using NIST Standard Reference Material (SRM) 1898[a]. SRM 1898 consists of a widely studied and industrially relevant TiO_2 nanomaterial with broad commercial penetration and a production history dating back several decades *(3, 8-10)*.

While the procedures detailed in this series focus on the dispersion of SRM 1898 in specific aqueous media, it is believed that the adopted characterization, optimization and validation approaches can be more generally applied to the preparation of ENM dispersions in any relevant matrix. For this reason, and to allow for broader applicability, experimental details and discussions regarding the characterization, process optimization and validation steps adopted for the development of the dispersion method are detailed in a separate publication *(11)*.

2. Principles and scope

This protocol is proposed for the preparation of dispersions for generic acute eco-toxicity applications; its use for chronic toxicity or other environmental studies, while potentially efficacious, is beyond the scope of the present work and should be validated by use of proper controls.

In this protocol, a TiO_2 nanoparticle dispersion in a relevant environmental matrix is produced by following a series of steps applied to a TiO_2 aqueous nanoparticle stock. Following the Organization for Economic Cooperation and Development (OECD) Guideline 202 for testing of chemicals *(12)*, the environmental matrix used in this work is reconstituted from four stock solutions defined in ISO 6341 *(13)*, yielding a calculated hardness of ≈ 170 mg/kg (ppm) as $CaCO_3$.

In this protocol, humic acid (HA) is utilized as a stabilizing agent to disperse SRM 1898 in the selected test matrix. Natural organic matter – and humic acid in particular – has been demonstrated to function as a non-specific stabilizer in environmental matrices *(14-16)*.

The method described herein, if applied correctly, yields 15 mL of a 100 μg/mL monomodal nanoscale TiO_2 dispersion in the selected test matrix, characterized by a mean particle diameter of ≈ 75 nm and pH values in the (7.0 to 7.7) range, without the need for pH adjustment steps. The tested TiO_2 concentration was adopted from the limit test concentration recommended in

[a] SRM 1898 was in production as of the publication of this protocol, with a target date for release in early 2012. The NIST SRM inventory can be accessed at http://www.nist.gov/srm/.

OECD Guideline 202, while the dispersion's pH was validated to fall within the Guideline's recommended pH range. The dispersion retains its particle size distribution and pH at room temperature for up to 96 h, which is the maximum duration for acute toxicity assays *(17)*). Dispersions prepared following this protocol should be stored in darkness or in amber glass vials, as TiO_2 is photoactive.

3. Terminology

This protocol complies with definitions relevant to nanotechnology as set forth in the ASTM International standard E2456 *(18)* and is consistent with the draft standard ISO TS 80004-1 *(19)*. Additional guidance is derived from recommendations of the International Union of Pure and Applied Chemistry *(20)*.

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 *(19)*.

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

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

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

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

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

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

4. Reagents, materials and equipment[b]

4.1. Reagents

4.1.1. 200 μg/mL stock TiO_2 aqueous nanoparticle dispersion.

4.1.2. Type I biological grade de-ionized (DI) water (≥ 18 MΩ·cm resistivity); biological grade implies sterile and pyrogen-free water.

[b] The identification of any commercial product or trade name does not imply endorsement or recommendation by the National Institute of Standards and Technology.

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

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

Note—Sterility and absence of pyrogen contamination should be verified for all materials in contact with the dispersion. If using the LAL test for pyrogens, avoid using cellulose-based filters, as they can be a source of beta-glucan, which interferes with the LAL assay.

Note—If the dispersion is <u>not</u> intended for toxicological assessment, pyrogen-free conditions may not be necessary.

4.1.3. Humic acid (HA) powder (e.g., Suwanee River Humic Acid Standard II, International Humic Substances Society, MN, USA)

4.1.4. ISO Test Water 6341 aqueous stocks (in order of increasing ionic strength):

Stock A: 3 mmol potassium chloride: 0.23 g of potassium chloride, KCl, in 1 L of DI water

Stock B: 31 mmol sodium bicarbonate: 2.59 g of sodium bicarbonate, $NaHCO_3$, in 1 L of DI water

Stock C: 20 mmol magnesium sulfate heptahydrate: 4.93 g of magnesium sulfate heptahydrate, $MgSO_4 \cdot 7H_2O$, in 1 L of DI water

Stock D: 80 mmol calcium chloride dihydrate: 11.76 g of calcium chloride dihydrate, $CaCl_2 \cdot 2H_2O$, in 1 L of DI water

<u>4.2. Materials</u>

4.2.1. 20 mL and 30 mL sterilized and pyrogen-free glass vials

4.2.2. aluminum or polystyrene weighing dishes

4.3.2. calibrated pipettes and sterile and pyrogen-free disposable tips covering a (0.020 to 5.000) mL range; adjustable volume pipettes are most convenient

<u>4.3. Equipment</u>

4.3.1. analytical balance with readability to 0.1 mg

For verification of expected outcome:

4.3.3. pH meter

4.3.4. Laser Diffraction Spectrometer (LDS), ***or***

4.3.5. Dynamic Light Scattering (DLS) instrument

5. Preparation of TiO_2 nanoparticle dispersions

Note—To avoid contamination, all glassware in contact with the media or suspensions should be meticulously cleaned, rinsed with ethanol, and dried prior to use. Glassware can be sterilized using an autoclave, by exposure to hot dry air (130 °C to 170 °C) for 2 h to 4 h in an oven, or by prolonged contact with alcohol. Avoid detergents if possible; if detergents are used, rinse with copious amounts of DI water prior to rinsing with ethanol and drying. Store and work in high-efficiency particulate air (HEPA) filtered clean bench if available; if not, containers should be capped or sealed with thermoplastic (e.g., Parafilm).

Note—Use clean sterile pipette tips and sterile procedures.

5.1.1. Prepare 20 mL of 100 mg/L HA (aq.) solution, by adding 0.002 g of HA and 20 mL of DI water in a 20 mL glass vial. After adding both components, allow the covered solution to equilibrate for 48 h. The solution should have a pH of 4.0 ± 0.2 after equilibration. Proceed to the following steps after equilibration of the HA solution (i.e., 48 h after preparation of the solution).

5.1.2. Prepare 50 mL of 200 μg/mL TiO_2 aqueous nanoparticle dispersion, by adding 0.01 g of SRM 1898 or equivalent into 50 mL of water, and following the sonication conditions prescribed in*(21)*.

5.1.3. In a 30 mL amber glass vial, add 3 mL of the HA solution (5.1.1)

5.1.4. Add 7.5 mL of the TiO_2 stock prepared in 5.1.2. into the vial with the HA solution (5.1.3)

5.1.5. Add 3.46 mL of water into the above mixture (5.1.4)

5.1.6. Add 0.26 mL of ISO stock solution A into the above mixture (5.1.5)

5.1.7. Add 0.26 mL of ISO stock solution B into the above mixture (5.1.6)

5.1.8. Add 0.26 mL of ISO stock solution C into the above mixture (5.1.7)

5.1.9. Add 0.26 mL of ISO stock solution D into the above mixture (5.1.8)

5.1.10. This procedure will yield a dispersion containing 100 μg/mL TiO_2 and 20 mg/L HA in OECD compliant hard water with a hardness of ≈ 170 mg/L (ppm) as $CaCO_3$.

Note—The above mentioned hardness value is that calculated from the amount of Ca and Mg added with the starting stock solutions.

5.1.11. If intended for toxicological assessment, the user is advised to conduct separate control tests for HA (20 mg/L) in the test medium (in the absence of TiO_2).

6. Expected Outcome

Note—The particle size distribution (PSD) of the resulting dispersions was monitored for 96 h, corresponding to recommended acute toxicity assay timeframes ranging from 24 h to 96h (17). Although beyond the scope of this protocol, dispersions may remain stable for longer periods of time. This behavior has been validated without the presence of cells or other added components in the test media.

Note—Serial dilutions may cause agglomeration, this should be tested accordingly.

6.1. The resulting TiO_2 dispersions should have a white but translucent appearance if prepared using SRM 1898 or commercial P25.

Note—If source powders other than SRM 1898 or P25are used, the appearance may vary depending on the final particle size, particle concentration, and other factors.

6.2. The particle size distribution (PSD) of the P25 dispersion should be monomodal, with the following volume-based mean particle diameter (D_m), D_{10} and D_{90} values:[c]

If measured using LDS:

$D_m \approx$ (70 to 81) nm;

$D_{10} \approx$ (59 to 63) nm

$D_{90} \approx$ (79 to 102) nm

If measured using DLS:

$D_m \approx$ (120 to 127) nm;

$D_{10} \approx$ (68 to 86) nm

$D_{90} \approx$ (146 to 163) nm

The expected range for size parameters was calculated from three independent replicates obtained following the prescribed procedure. Refer to the Appendix for details on the calculation of the expected size parameter ranges, and illustrations of representative PSD profiles. Refer to *(11)* for details and discussions on PSD characterization and validation criteria.

The volume-based mean particle diameter, as well as the D_{10} and D_{90} values for aqueous P25 dispersions prepared following the protocol should be reported by the user to allow for comparison with the values specified herein.

[c] D_{10} and D_{90} 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 instruments. They may or may not be obtainable directly from commercial DLS instruments, depending on the manufacturer.

6.3. The pH of dispersions after preparation should be ≈ 7.0. After (24 to 96) h, dispersions may experience a slight increase in pH ranging from 0.3 to 0.7 units. The dispersions should remain well within the OECD recommended pH range of 6 to 9 *(12)* during the studied timeframe (24 to 96) h.

7. Abbreviations

DI	de-ionized
DLS	dynamic light scattering
ENM	engineered nanomaterial
HA	humic acid
HEPA	high-efficiency particulate air
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LAL	Limulus Amoebocyte Lysate
LDS	laser diffraction spectrometry
OECD	Organization for Economic Cooperation and Development
PSD	particle size distribution
SRM	Standard Reference Material (a registered trademark of the National Institute of Standards & Technology)

8. Acknowledgements

We thank Michelle Twyla Blickley, NIST Surface and Microanalysis Science Division, for providing the ISO stock solutions used in the development of this protocol.

9. 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. *J. Adv. Mater.*, **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, Pulmonary 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 Lett.*, **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. *Nature Nanotech.*, **2010**. *13*(7): p. 2675-2687.
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*, **2011**. *5*(4): p. 711-729.
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, A standardized approach for the dispersion of titanium dioxide nanoparticles in biological media. *Nanotoxicology*, **2011**. - *submitted*.
12. OECD Guideline 202 for Testing of Chemicals, Daphnia sp., Acute Immobilisation Test. **2004**, Organization for Economic Cooperation and Development.
13. ISO 6341: Water quality - Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) - Acute toxicity test, Third Edition. **1996**, International Standards Organization, Geneva, Switzerland.
14. Thio, B.J.R., D.X. Zhou, and A.A. Keller, Influence of natural organic matter on the aggregation and deposition of titanium dioxide nanoparticles. *J. Hazard. Mater.*, **2011**. *189*(1-2): p. 556-563.
15. Keller, A.A., H.T. Wang, D.X. Zhou, H.S. Lenihan, G. Cherr, B.J. Cardinale, R. Miller, and Z.X. Ji, Stability and aggregation of metal oxide nanoparticles in natural aqueous matrices. *Environ. Sci. Technol.*, **2010**. *44*(6): p. 1962-1967.
16. Gao, J., S. Youn, A. Hovsepyan, V.L. Llaneza, Y. Wang, G. Bitton, and J.C.J. Bonzongo, Dispersion and toxicity of selected manufactured nanomaterials in natural river water samples: Effects of water chemical composition. *Environ. Sci. Technol.*, **2009**. *43*(9): p. 3322-3328.
17. EPA-821-R-02-012: Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, Fifth edition. **2002**, U.S. Environmental Protection Agency.
18. E2456-06: Standard terminology relating to nanotechnology. **2006**, ASTM International

19. ISO TS 80004-1: Nanotechnologies - Vocabulary - Part 1: Core terms. **2007**, International Standards Organization, Geneva, Switzerland.
20. Version 2009-09-07, Release 2.1.5: Compendium of chemical terminology. **2009**, International Union of Pure and Applied Chemistry (IUPAC).
21. Taurozzi, J.S., V.A. Hackley, and M.R. Wiesner, Protocol for the preparation of a nanoscale TiO2 aqueous dispersion for toxicological or environmental testing. *CEINT/NIST Protocol (http://ceint.duke.edu/allprotocols)*, **2011**.

10. Appendix

10.1. Calculation of expected particle size parameters

The expected range for D_m, D_{10} and D_{90} values was obtained using the following equation:

$$\bar{x} \pm \frac{t \cdot s}{\sqrt{n}}$$

Where ***x*** and ***s*** are the average and standard deviation, respectively, of the measured size parameter from three independent replicates, ***t*** is the student test parameter for a 95 % confidence interval and two degrees of freedom (***t*** = 4.30), and ***n*** is the number of tested samples (***n*** = 3).

10.2. Representative PSD profiles

The following figure illustrates representative LSD and DLS PSD profiles obtained for dispersions prepared following the procedure described in this protocol.

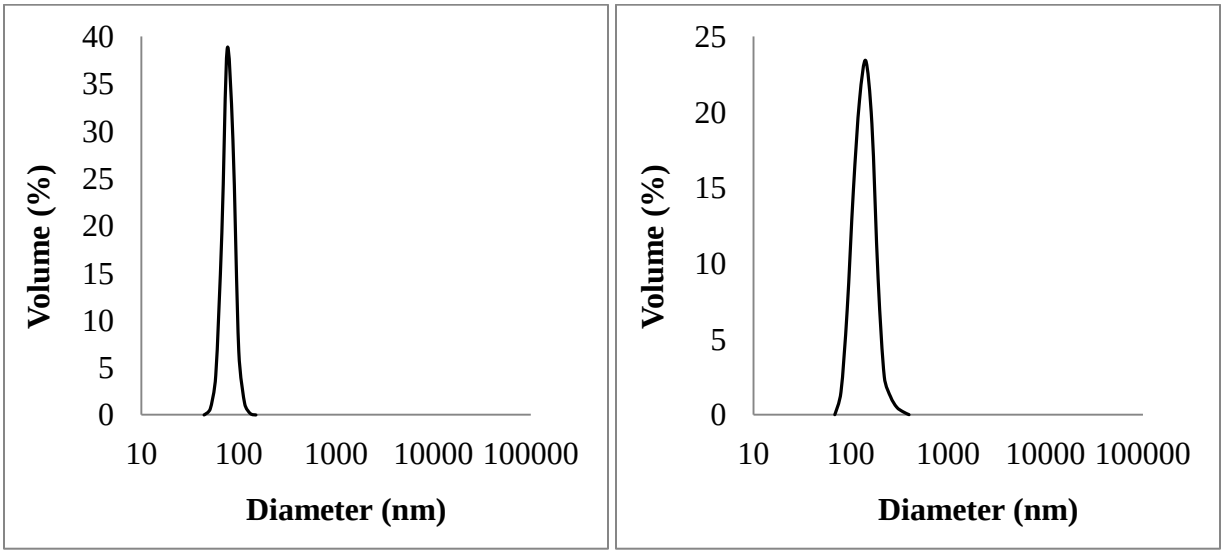

Figure 1. LDS (left) and DLS (right) volume-based PSD profiles of SRM 1898 dispersions prepared following the procedure described in this protocol. Measurements were performed using the dispersion medium in the measurement cell. The x-axis is shown on a log scale.