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Preparation of Nanoscale TiO2 Dispersions in Biological Test Media for Toxicological Assessment

Version 1.1

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NanoEHS Protocols

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FOREWORD

This special publication is one in a series of protocols resulting from a collaborative research agreement between the National Institute of Standards and Technology (NIST) and Duke University's Center for the Environmental Implications of Nanotechnology (CEINT). The original version of this protocol (Ver. 1.0) was first posted on the CEINT web site (http://ceint.duke.edu) and it, along with any other previous version, is superseded by this updated special publication version. Updates to this protocol may be released in the future. Visit http://nist.gov/mml/np-measurement-protocols.cfm to check for revisions of this protocol or new protocols in the series.

NIST and CEINT are interested in soliciting feedback on this method. We value user comments and suggestions to improve or further validate this protocol. Please send your name, email address and comments/suggestions to nanoprotocols@nist.gov. We also encourage users to report citations to published work in which this protocol has been applied.

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. This protocol has 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

In this protocol, TiO_2 nanoparticle dispersions in relevant biological media are produced by following a series of steps applied to a stock TiO_2 aqueous nanoparticle dispersion. To prepare the stock TiO_2 aqueous dispersion, refer to the previous protocol in this series [12].

This protocol focuses on two representative media broadly used for *in vitro* assays: phosphate-buffered saline solution (PBS) and Dulbecco's Modified Eagle Medium containing 10 % fetal bovine serum (DMEM-FBS). In both cases, bovine serum albumin (BSA) is utilized as a stabilizing agent; series specific serum albumin has been demonstrated to function as a non-specific biocompatible stabilizer [9, 13, 14]. This protocol is proposed for the preparation of dispersions for generic acute bioassay applications; its use for chronic toxicity evaluations is beyond the scope of this work and should be validated by use of proper controls.

The method described herein, if applied correctly, yields 100 μg/mL monomodal nanoscale TiO_2 dispersions in PBS or DMEM-FBS, characterized by mean particle diameters of ≈ 75 nm (in PBS) and ≈ 83 nm (in DMEM-FBS) and pH values from 7.2 to 7.4 (in PBS) and ≈ 7.8 (in DMEM-FBS). The dispersions retain their particle size distribution for 48 h under relevant incubation conditions (in DMEM-FBS) and at room temperature (in PBS). Dispersions prepared following this protocol should be stored so as to minimize exposure to light (e.g., in amber vials).

[a] Information regarding SRM 1898 can be accessed at http://www.nist.gov/srm/.

3. Terminology

This protocol complies with definitions relevant to nanotechnology as set forth in the ASTM International 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 approximately 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).

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

4.1. Reagents

4.1.1. 10 mg/mL stock TiO_2 aqueous nanoparticle dispersion.

Note—Refer to [12] for materials and guidelines for the preparation of the 10 mg/mL TiO2 aqueous dispersion.

4.1.2. Type I biological grade de-ionized (DI) water (≥ 18 MΩ·cm resistivity); biological grade implies sterility and absence of endotoxin contamination.

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 not intended for toxicological assessment, pyrogen-free conditions may not be necessary.

4.1.3. 0.1 mol/L HCl (aq) and 0.1 mol/L NaOH (aq) reagent grade solutions

4.1.4. Bovine serum albumin (BSA), 99+ % high purity reagent grade (lipid and IgG free) powder (e.g., SeraCare Life Sciences, Code No. AP-4510-80)

4.1.5. *For dispersion in PBS*:

PBS (10x) solution with 0.067 mol/L (PO_4), calcium and magnesium free, passed through 0.1 µm sterile filter (e.g., HyClone Laboratories, Cat. No. SH30258.02)

4.1.6. *For dispersion in DMEM with 10 % FBS*:

DMEM, 4.5 g/L glucose and sodium pyruvate without L-glutamine & phenol red, Sterile (e.g., Mediatech, Inc., Cat. No. 17-205-CV), with: Penicillin-Streptomycin (5000 U/mL Penicillin and 5000 ug/mL streptomycin), (e.g. Invitrogen Cat. No. 15070-063), Fungizone Antimycotic (250 ug/ml amphotericin B and 205 ug/mL Sodium deoxycholate), (e.g. Invitrogen Cat No. 15290-018), and Glutimax Supplement (L-Alanyl- L-Glutamine, 200 mM), (e.g. Invitrogen, Cat. No. 35050-161)

The above preparation is referred to herein as DMEM

FBS (e.g., Gemini Bio-Products, Cat. No. 100-106)

4.2. Materials

4.2.1. 10 mL and 30 mL sterilized amber glass vials

4.2.2. Aluminum or polystyrene weighing dishes

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

4.3. Equipment

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.

Note—For details on the validation of the particle size distribution (PSD) of dispersions, as well as the optimization of the BSA-TiO2 mixing sequence prescribed in this protocol, refer to [11].

5.1. Dispersion in PBS

5.1.1. Prepare a 10 mg/mL stock TiO_2 aqueous nanoparticle dispersion, per *[12]*.

5.1.2. Prepare 50 mL of PBS (1x) by doing a 1:10 dilution of the PBS (10x) with DI water, i.e., add 5 mL of PBS (10x) to 45 mL of DI water. Measure the pH of the prepared PBS (1x).

5.1.3. Adjust the pH of the prepared PBS (1x) solution to a value in the 7.2 to 7.4 range by addition of 0.1 mol/L HCl (aq) and/or NaOH (aq) as needed. Mix thoroughly after each acid or base addition step to allow for proper homogenization and attainment of equilibrium pH.

Note—The pH range was selected as acceptable for typical biologically relevant assays. If other values are desired, the stability of the dispersions must be tested accordingly.

5.1.4. After addition of HCl (aq) and/or NaOH (aq) to achieve the desired pH, add an adequate amount of PBS (10x) to compensate for the dilution of the PBS (1x) solution by the water added with the acid or base solutions; e.g., if 2 mL of 0.1 mol/L HCl (aq) were added to the PBS (1x) to obtain the desired pH, then add 0.22 mL (2 x 0.11) PBS (10x). After pH adjustment and equilibration, verify that the pH of the obtained PBS (1x) is in the range from 7.2 to 7.4.

5.1.5. Weigh 0.8 g of BSA powder and transfer to a 10 mL amber glass vial. Add 10 mL of DI water to the vial with the BSA, seal and gently shake to allow for complete dissolution of the BSA while minimizing foam formation. Do not use the BSA solution until visible material is completely absent (allow approximately 1 h). The final product is a transparent 80 mg/mL BSA solution in DI water.

5.1.6. Add 300 µL of the 80 mg/mL aqueous BSA solution (5.1.5) into a clean 10 mL amber glass vial.

5.1.7. Add 150 µL of the 10 mg/mL stock TiO_2 aqueous dispersion (5.1.1) into the vial with 300 µL of BSA solution (5.1.6).

5.1.8. In a 30 mL amber glass vial, add 14.5 mL of the PBS (1x) prepared in 5.1.4 and 50 µL of PBS (10x).

5.1.9. Using a calibrated pipette, transfer 450 µL of the TiO_2/BSA/water mixture obtained in (5.1.7) into the 30 mL vial with 14.55 mL of PBS solution (5.1.8), to yield a dispersion containing 100 µg/mL TiO_2 and 1.6 mg/mL BSA in PBS (1x). The pH of the resulting dispersion should be comparable to that of the original buffer medium (5.1.4).

5.1.10. For toxicological assays, the user is advised to conduct a control for BSA in the test medium (1.6 mg/mL) without TiO_2.

5.1.11. The resulting dispersion, stored in the 30 mL amber vial, retains its PSD for at least 48 h at room temperature.

5.2. Dispersion in DMEM with 10 % FBS

5.2.1. Prepare a 10 mg/mL TiO_2 aqueous dispersion, per [12].

5.2.2. Prepare 50 mL of DMEM-FBS by mixing 5 mL of FBS with 45 mL of DMEM (1x). The resulting pH should be ≈ 7.8.

5.2.3. Weigh 0.8 g of BSA powder and transfer to a 10 mL amber glass vial. Add 10 mL of DI water to the vial with the BSA, seal and gently shake to allow for complete dissolution of the BSA. Do not use the BSA solution until visible material is completely absent (allow approximately 1 h). The final product is a transparent 80 mg/mL BSA solution in DI water.

5.2.4. Add 18.75 µL of the 80 mg/mL aqueous BSA solution (5.2.3) into a clean 10 mL amber glass vial.

5.2.5. Add 150 µL of the 10 mg/mL TiO_2 aqueous dispersion (5.2.1) into the vial with 18.75 µL of BSA solution (5.2.4).

5.2.6. Using a calibrated pipette, add 14.83 mL of the DMEM-FBS prepared in 5.2.2 to a 30 mL amber glass vial.

5.2.7. Transfer 168.75 µL of the TiO_2/BSA/water mixture obtained in (5.2.5) into the 30 mL vial with 14.83 mL of DMEM-FBS (5.2.6), to yield a 100 µg/mL (TiO_2), 100 µg/mL (BSA) dispersion in DMEM-FBS. The pH of the resulting dispersion should still be comparable to that of the original medium (5.2.2). This procedure results in a 1.1 % dilution of the DMEM-FBS by the water added with the TiO_2 and BSA stocks.

5.2.8. If used for toxicological assessments, the user is advised to conduct separate control tests for the BSA (100 µg/mL) in the medium and for the water-diluted medium (1.1 %), in the absence of TiO_2.

5.2.9. The resulting dispersion, stored in the 30 mL amber vial, retains its PSD and pH (± 0.1 units) for at least 48 h under relevant incubation conditions (37 ºC, 90 % humidity, 5 % CO_2 atmosphere).

6. Expected outcome

Note—The particle size distribution (PSD) of the resulting dispersions was monitored for 48 h, corresponding to the typical acute toxicity assay timeframe. Although beyond the scope of this protocol, dispersions may remain stable for longer periods of time. This behavior has been validated only in pure media, without the presence of cells or other added components.

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

6.1. Dispersion in PBS

6.1.1. The obtained dispersions should have a white but translucent appearance if prepared using SRM 1898 or commercial 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.

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

If measured using LDS:

$D_m \approx$ (71 to 79) nm

$D_{10} \approx$ (60 to 64) nm

$D_{90} \approx$ (80 to 96) nm

If measured using DLS:

$D_m \approx$ (122 to 149) nm

$D_{10} \approx$ (69 to 91) nm

$D_{90} \approx$ (160 to 200) nm

The expected size range 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.

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. Refer to [18] for details on PSD characterization and validation criteria, as well as representative PSD profiles.

6.1.3 The pH of dispersions should be comparable (± 0.1 pH units) to the pH of the originating medium.

6.2. Dispersion in DMEM with 10 % FBS

6.2.1. The obtained dispersions should have a white but translucent appearance if prepared using SRM 1898 or commercial 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.

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

If measured using LDS:

$D_m \approx$ (81 to 85) nm

$D_{10} \approx$ (61 to 69) nm

$D_{90} \approx$ (94 to 115) nm

If measured using DLS:

$D_m \approx$ (138 to 170) nm

[b] 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.

$D_{10} \approx$ (86 to 113) nm

$D_{90} \approx$ (162 to 337) 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.

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. Refer to [18] for details and discussions on PSD characterization and validation criteria, as well as illustrations of representative PSD profiles.

6.2.3. The pH of dispersions should be comparable (± 0.1 pH units) to the pH of the originating medium.

7. Abbreviations

BSA	bovine serum albumin
DI	de-ionized
DLS	dynamic light scattering
DMEM-FBS	Dulbecco's Modified Eagle Medium containing 10 % FBS
ENM	engineered nanomaterial
FBS	fetal bovine serum
HEPA	high-efficiency particulate air
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LDS	laser diffraction spectrometry
PBS	phosphate buffered saline
PSD	particle size distribution
SRM	Standard Reference Material (a registered trademark of the National Institute of Standards and Technology)

8. Acknowledgements

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Appendix

A.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:

$$\text{range} = \left(\boldsymbol{x} - \frac{\boldsymbol{t}.\boldsymbol{s}}{\sqrt{\boldsymbol{n}}} \text{ to } \boldsymbol{x} + \frac{\boldsymbol{t}.\boldsymbol{s}}{\sqrt{\boldsymbol{n}}}\right)$$

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