Preparation of Nanoscale TiO$_2$ Dispersions in an Environmental Matrix for Eco-Toxicological Assessment

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J. S. Taurozzi
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J. S. Taurozzi
V. A. Hackley
Material Measurement Laboratory
Ceramics Division

M. R. Wiesner
Duke University
Durham, NC

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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1.0) was first posted on the CEINT web site (http://ceint.duke.edu) and it, along with any other previous version, is superseded by this updated special publication version.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)*. The appendix contains a summary of validation results characterizing the dispersed state of the suspensions resulting from application of the protocol in both OECD and EPA standardized water using humic acid (HA) as the dispersing agent; however, the OECD medium was the principal focus for protocol development.

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 $\approx$ 170 mg/kg (ppm) as $CaCO_3$.

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[a] SRM 1898 is available for purchase through the NIST SRM program office. Information, including the certificate of analysis, can be accessed at http://www.nist.gov/srm/.

In this protocol, HA is utilized as a stabilizing agent to disperse SRM 1898 in the selected test matrix. Natural organic matter – and HA 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 OECD Guideline 202, while the pH of the dispersion 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 approximately 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

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 ($\geq$ 18 MΩ·cm resistivity); biological grade implies sterile and pyrogen-free water.

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

4.2. Materials

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

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.

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 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:[b]

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.

6.3.The pH of dispersions after preparation should be $\approx$ 7.0. After (24 – 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
EPA	U.S. Environmental Protection Agency
HA	humic acid
HEPA	high-efficiency particulate air
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry

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[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.

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

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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:

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

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 ($n = 3$).

A.2. Validation data for replicate sample preparations using OECD hard water

LDS and DLS were used as size characterization techniques to validate the protocol with respect to yielding stable monomodal dispersions 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. Additionally, LDS measurements were confirmed previously for P25 using an x-ray disc centrifuge (see Ref.11) that utilizes an orthogonal measurement basis.

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 quartz cell. First, the system was blanked against a solution consisting of 12 mL of a HA blanking solution. The blanking solution was prepared by diluting 3 mL of stock HA solution into 14 mL of DI water, so as to yield a final HA concentration of approximately 20 mg/L. Then 1.5 mL of the test suspension was added to the cell containing the blanking solution, and the measurement was initiated. An appropriate blue line transmittance level was attained (between 70 % and 90 % transmittance) for all samples under these conditions. Volumetric PSDs were calculated 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 ($\theta = 173°$) at a laser wavelength of 633 nm. Samples were measured in 1.5 mL disposable cuvettes by dilution into DI water (20 µL of test suspension was diluted into 1 mL of DI water) to achieve an appropriate light scattering level. For each test sample, measurements were performed in triplicate, with the number and duration of sub-runs for each measurement determined automatically by the instrument 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.

For validation purposes, three independent replicate test suspensions were prepared following the prescribed protocol, and the PSD of each sample was measured using LDS and DLS shortly after

preparation. Afterwards, LDS measurements were performed on each test suspension at 48 h, 5 d, 7 d and 9 d following preparation. Figure A1 compares representative LSD and DLS PSD profiles obtained for the freshly prepared suspensions, while Tables A1 and A2 summarize the size values obtained for each replicate after the initial preparation as determined by LDS and DLS, respectively.
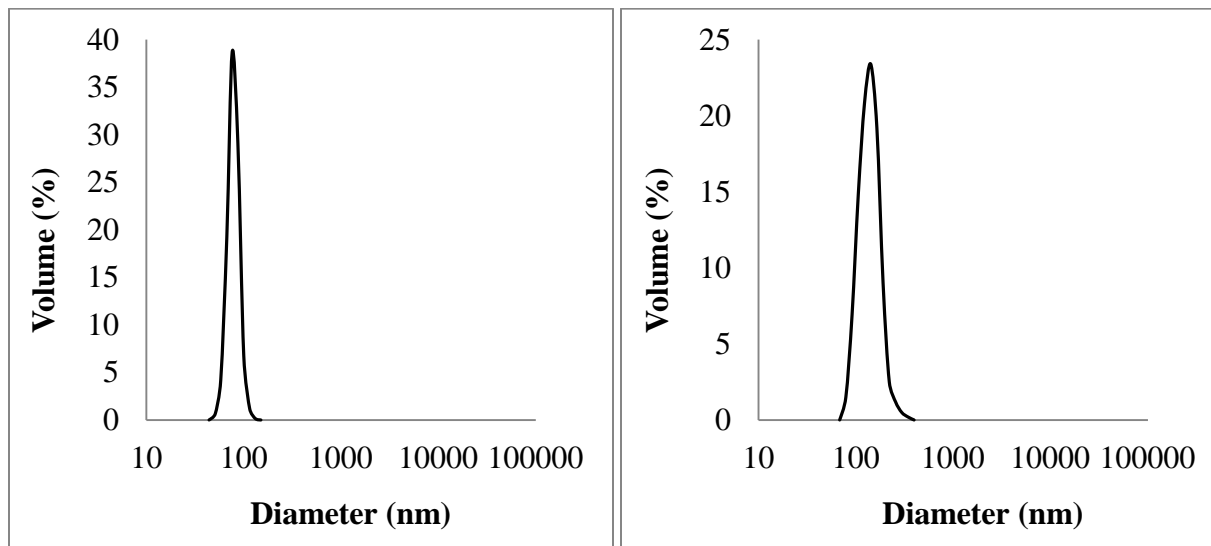

Figure A1. LDS (left) and DLS (right) volume-based PSD profiles of HA modified SRM 1898 dispersions in the OECD synthetic hard water prepared following the procedure described in this protocol. The x-axis is shown on a log scale.

Table A1. LDS summary for freshly prepared triplicate samples in OECD hard water.

Replicate #	Mean size	D10	D90	pH
1	75.1	60.8	89.4	7.0
2	73.6	60.3	86.5	7.0
3	77.9	61.7	95.8	7.0
SD	2.2	0.7	4.8	
95% CI	5.3	1.8	11.8	
Average	75.6	60.9	90.6	
Range up	81	63	102	
Range down	70	59	79	

Coverage factor k = 2.48 for a 95 % confidence interval.

Table A2. DLS summary for freshly prepared triplicate samples in OECD hard water.

Replicate #	Mean size	D10	D90
1	122.6	74.3	154.0
2	123.3	81.4	151.5
3	125.1	76.6	158.0
SD	1.3	3.6	3.3
95% CI	3.3	9.0	8.2
Mean	123.7	77.4	154.5
Range up	127	86	163
Range down	120	68	146

Coverage factor k = 2.48 for a 95 % confidence interval.

The triplicate LDS results are presented in stacked format in Figure A2 for each test time following the initial preparation in the OECD hard water. Note the appearance of a micrometer scale component (agglomerates) after 7 d. The data clearly show that the suspensions are stable toward significant agglomeration for more than the typical 24 h to 96 h acute toxicity assay range. Even after 9 d, the nanoscale component is predominant while the agglomeration appears to proceed slowly, with little apparent difference between 7 d and 9 d.
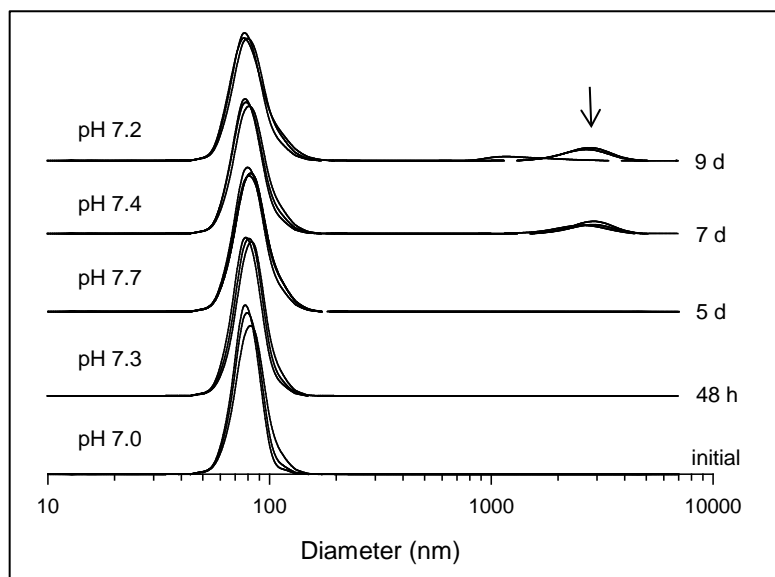

Figure A2. LDS volume-based PSD profiles of HA modified SRM 1898 suspensions in OECD synthetic hard water prepared in triplicate following the procedure described in this protocol. The x-axis is presented on a log scale. Time after sample preparation is indicated on the right side. The mean pH of the suspensions is indicated on the left side. The appearance of a micrometer scale component (indicated by arrow) is apparent after 7 days, suggesting the onset of significant agglomeration.

A.3. Tests using EPA synthetic hard freshwater

The prescribed protocol was also tested using EPA standard synthetic hard water with a nominal hardness of 170 mg/L as $CaCO_3$. Directions for preparing the EPA water can be found in Ref. 17 (using the composition identified as "Hard" in Table 7 of that document).The PSD was determined up to 5 d after initial preparation using independently prepared triplicate test suspensions. The LDS PSD profiles are presented in stacked format in Figure A3. Since this was a secondary test medium, only three time points were analyzed, but the results clearly show that the protocol yields a monomodal nanoscale suspension that is stable for at least 24 h and possibly up to 5 d. After 5 d the PSDs show the growth of microscale agglomerates, though the nanoscale component still predominates the volume or mass distribution of particles.
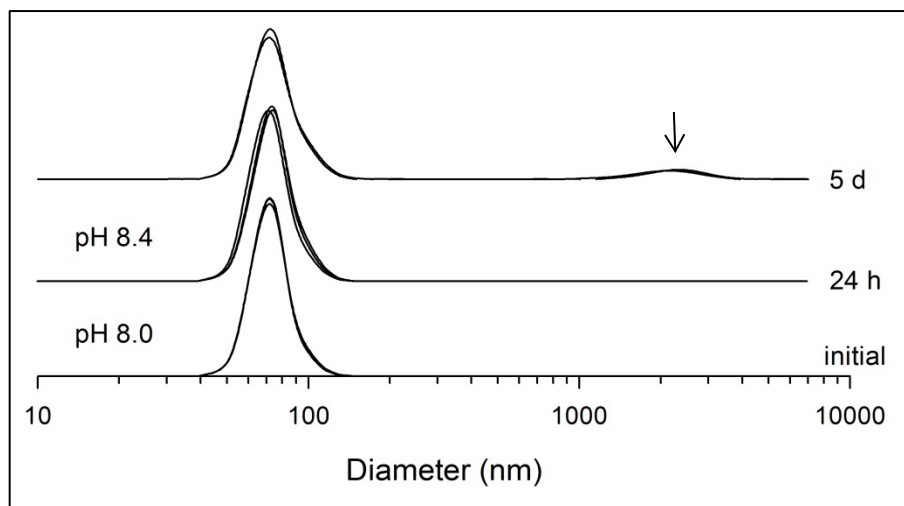

Figure A3. LDS volume-based PSD profiles of HA modified SRM 1898 suspensions in EPA hard water prepared in triplicate following the protocol. The x-axis is presented on a log scale. Time after sample preparation is indicated on the right side. The mean pH of the suspensions is indicated on the left side. The appearance of a micrometer scale component (indicated by arrow) is apparent after 5 days, suggesting the onset of significant agglomeration.

Table A3. LDS summary for freshly prepared triplicate samples in EPA hard water.

Replicate #	Mean size	D10	D90
1	76.6	61.6	96.1
2	76.7	61.5	97.0
3	76.6	61.6	95.9
SD	0.1	0.1	0.6
95% CI	0.1	0.1	1.5
Mean	76.6	61.6	96.3
Range up	76.7	61.6	97.0
Range down	76.6	61.5	95.9

Coverage factor k = 2.48 for a 95 % confidence interval.