A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***SOP for Dispersion***
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Owner and co-owner(s)	Romain Grall (CEA) Sylvie Chevillard (CEA)
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1 General description

This SOP was developed for preparation of general batch dispersions for in vitro and in vivo toxicity testing in NANoREG. The method aims to produce a highly dispersed state of any MN by ethanol prewetting to handle hydrophobic materials followed by dispersion in sterile-filtered 0.05% w/v BSA-water at a fixed concentration of 2.56 mg/ml using probe sonication. The protocol may not produce the smallest particle size in the dispersion as possible, but is a generically applicable procedure that ensures reasonable dispersion of the test materials selected for NanoReg. Moreover, the protocol is developed with the aim to use or characterize the dispersion immediately after its production. The current protocol can ensure stable dispersion for 0.5 to 1 hour before considering redispersing due to onset of agglomeration and/or sedimentation. However, the possible sedimented particles can easily be re-dispersed by vortexing the dispersion for 10 seconds. Long-term storage of MN in liquids should also be avoided in general due to risk of partial alteration of the original MN or its coatings and other hydrochemical reactions.

2 Materials and Methods

2.1 Preparation of stock dispersion

A 2.56 mg/ml stock dispersion is prepared by prewetting powder in 0.5 vol% ethanol (96% purity) followed by dispersion in 0.05 wt% BSA-water during 16 minutes of probe sonication. For harmonization of dispersion energy and stabilities it is recommended to produce a 6-8 ml dispersion in the 20 ml tall glass scintillation vials. It takes 15.36 mg to produce a 6 ml 2.56 mg/ml stock dispersion (see below for further details).

2.1.1 Materials

- Pure and sterile-filtered water
- Bovine Serum Albumin (sterile)
- Ethanol (96 vol%)
- 1 flask for batch dissolution of BSA
- 1 flask for sterile-filtered 1% w/v stock BSA-water solution
- 1 flask for 0.05% w/v BSA-dispersion medium
- Sterile filter (0.2 m)
- Vials: 20ml Scint-Burk glass pp-lock+Alu-foil (WHEA986581; Wheaton Industries Inc.) for weighing out
- MN and sonication
- Steel and glass spatula
- Pipette and pipette tips
- Weighing boat/weighing paper
- Electrostatic neutralizer
- Weigh Control or reference weights
- Probe sonicator Ice (Ice-water)

Water

It is recommended to use the purest available water. The protocol has been tested using both Nanopure Diamond UV and Millipore MilliQ-filtered water. No differences were observed in the

quality of the batch dispersions. However, if chemical analyses of especially Fe and Zn are to be conducted, it may be recommended to use Nanopure or controlled water because water from some Millipore systems has been found to be contaminated with these elements at promille levels even after mounted after de-ionization units.

Quality control of the water may be done before use. Especially in case that analysis and experiments may be influenced by trace-elements at low concentrations.

For general sampling and validation, we suggest collection of water in acid cleaned chemically stable bottles suitable for elemental analysis. Control the water quality (e.g., particles by DLS, elemental concentration by Atomic Absorption Spectrometry (AAS) or Inductive Coupled Mass Spectrometry (ICP-MS), CFU and endotoxin by growth in petri dishes or specific analysis) before use. If the water sample passes the quality test, the water is evaluated as pure and can be used for the experiment.

a. Nanopure Diamond UV water:

The Thermo Nanopure Diamond® UV-water system (Thermo Scientific) water purification system is made by Thermo Scientific and designed with 4 stages of de-ionization combined with UV light- treatment and final particle filtration Filter: 0.2 µm filter (-irradiated Barnstead D3750 Hollow fibre filter). The water quality is listed as:

Resistivity: ≤ 18.2 MΩ-cm at 25°C

Pyrogens: < 0.001 EU/ml

Total Organic Carbon: < 3.0 ppb

Other: nuclease-free (RNase and DNase).

b. MilliQ-water:

Most Millipore water purification systems in toxicology laboratories are designed to filter water that has already been de-ionized. It contains an internal filtration unit and a final 0.22 µm MilliPark Gammagold (Millipore) filter for particle filtration. The quality of the Millipore water is listed as:

Resistivity: 18.2 MΩ-cm at 25°C

Pyrogens: <0.02 EU/ml

TOC: 5-10 ppb Silicates: <0.1 ppb

Heavy metals ≤ 0.1 ppb

Microorganisms: ≤1 cfu/ml

Serum Albumin

Several types of albumin may be selected to fit specific toxicological in vitro and in vivo tests systems (e.g., calf, mouse and rat). It is important that the selected albumin has passed tests for purity and sterility. This protocol was developed using Bovine Serum Albumin (BSA). The specifications are:

- Bovine Serum Albumin (Fraction V), Sigma (catalogue number: A-9418)
- Cell culture tested
- Analysis: 16 wt% Nitrogen and 1.9 wt% H2O
- Number of amino acid residues: 607
- Molecular weight: 66,399 Da

- Isoelectric point in water at 25 °C: pH = 4.7
- Physical dimensions: 140 x 40 x 40 Å (prolate ellipsoid where a = b < c) (Wright and Thompson, 1975)

2.1.2 Production of sterile-filtered BSA water

The production of the 0.05% w/v BSA-water (the dispersion medium) is done in two steps: 1) Preparing a sterile-filtered 1% w/v BSA stock solution and 2) Dilution to reach a 0.05% w/v BSA dispersion medium.

a. Procedure for making a 1% w/v BSA water stock solution:

1) Add from pipette 50 ml water Nanopure (or MilliQ) to a 100 ml mixing flask (e.g., re-useable acid-washed blue-cap flasks or similar).
2) Weigh out 1 g BSA (powder) in a weighing boat and pour it into the flask with 50 ml water, rinse the weighing boat into the mixing bottle with Nanopure (or MilliQ) water to retrieve as much BSA as possible into the mixing flask.
3) Fill Nanopure water (or MilliQ) into the mixing flask up until 100 ml to reach a 1 % w/v BSA-water solution.
4) Gently stir or swirl the BSA-solution for a few minutes (be careful to avoid foam by not using agitated stirring) and leave the mixing flask in the refrigerator over-night for complete dissolution of the BSA.
5) Sterile filter the solution into a new flask through a 0.2 µm sterile disposable filter ware with collection flasks after complete dissolution of BSA in the mixing flask. A subsequent step of sterile filtration of the volume to be used for each toxicity test is recommended to ensure no bacterial contamination in the tests. Sterile filtration causes about 28% loss of BSA resulting in a true BSA concentration of 0.036% w/v in the stock dispersion as determined by a Pierce BCA protein Assay Kit for microplate reading.

b. Procedure for making the "0.05% w/v BSA-water solution" for nanomaterial dispersion:

The 0.05% w/v BSA solution to be used for test item preparation is achieved by simple dilution of the sterile-filtered 1% w/v BSA batch solution. Remember that the 0.05% w/v BSA solution in reality contains ca. 0.036% BSA w/v due to loss in the sterile-filtration.

Example: 2 ml 1 % w/v BSA is diluted with 38 ml Nanopure water (or MilliQ) (Dilution factor = 20x) to reach a batch solution of 0.05 w/v%.

2.1.3 Weighing nanomaterial powder

Weighing should be done in a ventilated weighing box, a glove box or a fume hood designated for sensitive weighing with an accuracy of at least 0.1 mg or better.

Materials

- Microweigh with accuracy of 0.1 mg or better
- Reference or control weights with masses within the scale the weighing project
- Wet and dry wipes for cleaning
- Weighing boat
- Steel and glass spatula's
- Vials: 20ml Scint-Burk glass pp-lock+Alu-foil (WHEA986581; Wheaton Induistries Inc.)[a]
- Vials with nanomaterials

- Tray for storage of vials
- Argon source[b]

[a] Can be re-used after acid washing or regular washing followed rinsing in ethanol. Add new pp-lock+Alu-foil lids.

[b] Maybe needed for handling NM-series materials distributed from JRC. See below.

Preparation of weighing area

1) Turn on the weighing box, glove box, fume-hood 15-30 minutes before use.
2) Ensure wearing appropriate work dress (two- or three layer gloves*, lab-coat, laboratory shoes) and that personal respiratory protection equipment is easily accessible in case of accident.
3) Ensure all material to be used for weighing and storage is present (nanomaterials, bottles/vials for weighing material in, cleaning tissue (both wet and dry wipes) before commencing the work.
4) Calibrate the weigh with traceable reference weight and log the data. Check that accuracy is within acceptance.

* It is recommended to use two- or three layers of gloves for dermal protection. 1) Inner glove in textile 2) and or inner glove using long powder free nitril or latex rubber glove, 3) powder free nitril glove.

Weighing out the nanomaterial

5) a. Open a clean empty vial for preparation of the stock dispersion and place it on the weigh. b. Tara the weigh.
6) Carefully open the vial without shaking it (NM-materials are packed in argon atmosphere and a special strategy may required for weighing and potential re-use for NM-samples – see below).
7) Remove the electrostatic charge on the vial using a neutralizer (e.g., ionization blower) and carefully weigh out the required mass with a spatula in steel or glass.
8) Close the lid on both vials
9) After completion of weighing materials, clean the weigh and work area for potential spills using wet and dry wipes.
10) Waste is packed in a suitable waste bag and discarded according to local or institutional directions.
11) Ventilate the work-area (ventilated weighing station, fume hood, glove box etc.) for 15 minutes

Calculations for preparing the 2.56 mg/ml stock dispersion

For preparing a 2.56 mg/ml stock dispersion in a 6 ml EtOH and BSA-water, each vial must contain at least 15.36 mg nanomaterial. For harmonization of the dispersion energy it is recommended to stay as close as possible to 6 ml EtOH and BSA-water.

Calculation of the correct volume is done simply according to equation 1:

1: $V = m / c$

m = mass of nanomaterial (mg)

c = concentration (normally 2.56 mg/ml)
V = volume of dispersion medium (ml)

6 ml EtOH + BSA-water is required to disperse 15.36 mg powder. 0.5 vol% EtOH (96% or higher) is used for pre-wetting: 6 ml x 0.5/100 = 0.03 ml (30 99.5 vol% sterile-filtered BSA-water (0.05% w/v) is used as dispersion medium: 6 – 0.03 = 5.97 ml BSA-water

2.1.4 Particle dispersion

Particles are pre-wetted using ethanol (EtOH). This procedure was previously introduced to enable dispersion of hydrophobic materials in water-based systems. In this protocol, we have introduced EtOH pre-wetting for all materials to harmonize the treatment for all powder materials.

Pre-wetting procedure (volumes for 15.36 mg powder)

1) Carefully open the glass scintillation vial with pre-weighed powder (ideally 15.36 mg).
2) Tilt the scintillation vial ca. 45° and add 30 µl EtOH drop-by-drop onto the particles in the vial by pipette.
3) Screw on the lid and gently mix the EtOH and powder by simultaneous gently tapping the vial on the table-top while rotating the tilted 45° vial from side to side between your fingers for approximately one minute.
4) Add 970 µl 0.05 % BSA water by pipette while slowly rotating and swirling the 45° tilted scintillation glass. Be careful to avoid foaming of BSA. The last ml BSA-water or so is added along the top of the inner wall of the vial to collect the powder in the fluid at the vial bottom.
5) Add the remaining 5 ml 0.05 % BSA water by pipette along the sidewalls of the scintillation vial to wash down any particles that may be stuck to the sidewalls.
6) Place the vial on ice for at least 5 minutes while the sonicator and ice-water is prepared.

2.1.5 Probe Sonication

The protocol was developed using a 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037).

The particle dispersions are continuously cooled in an ice-water bath to minimize heat development during sonication.

Sonication procedure

1) Fill a 250 ml glass beaker with ice and place it upside-down in an insulation box (flamingo/styrofoam)
2) Add ca. 85-90 vol% ice into the insulation box
3) Add ca. 10-15 vol% cold water into the insulation box
4) Carefully place the glass scintillation vial with powder on top of the upside-down glass-beaker and pack the ice-water around the vial to keep the dispersion cooled.
5) Insert the sonication probe (between the upper quarter and upper half of the BSA-water volume in the scintillation beaker (e.g., find the correct height using a vial with BSA-water alone).

6) Start sonication and run it for 16 min at 400 W and 10% amplitude while controlling that the sonication probe does not touch the walls of the scintillation vial. Use of different sonication conditions (power and amplitude) may require different sonication times as discussed below.
7) Remove the scintillation vial and add the lid.
8) Clean the sonication probe by sonication for 5 minutes (similar sonication settings) with the probe fully immersed in a 50:50 water-EtOH (>96%) mixture followed by rinsing in EtOH using a dispenser and a collection bottle underneath. The probe is allowed to air-dry in the fume-hood. Other in-house cleaning methods may also apply.

Selection of sonication time

Analysis of the energy consumption of different sonicators in the project showed that the Branson S- 450D probe sonicator used for development of this dispersion protocol delivered 3,136 MJ/m3 during the 16 min sonication at 10% amplitude. It was found that a similar probe at a different laboratory delivered only 2,096 MJ/m3 within the same time-frame resulting. Different efficiencies of different sonicators may result in slightly different average particle sizes when using the same sonication time as shown below in tests of NM-105 (Figure 3.5). However, the average particle size decreases systematically as a power function of sonication time using any sonication instrument and the resulting particle sizes appears comparable at the same delivered energy.