A common European approach to the regulatory testing of nanomaterials

The ENPRA dispersion protocol for NANoREG

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ENPRA Risk Assessment of Engineered NanoParticles

Version 1.0

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1 Introduction

This protocol describes the ENPRA stock dispersion protocol developed for *in vitro* and *in vivo* testing of powders and liquid dispersions of pigments and nanomaterials (NM). The protocol also contains specific recommendations for handling specific nanopowders and dispersions with NM in relation to complying with this protocol. The protocol is a revision of the original ENPRA dispersion by Jacobsen et al. (2010) to specifically address the NANoREG Technical Guidance dosument.

2 Definitions

Material vial: The vial with content as distributed in the vials to the partners

MilliQ-filtered (Sterile-filtered) de-ionized water: De-ionized water, which has been sterile-filtered through filter with a 0.45μm pore size or smaller. Use the highest quality of sterile-filtered de-ionized water available in your laboratory.

Dispersion media: MilliQ-filtered sterile-filtered water (or higher purity) added 2 vol.% serum

Stock suspensions: Particles dispersed in dispersion media at 2.56 mg/ml.

Incubation media: Stock suspensions diluted in cell media and used for in vitro exposure.

Instillation fluid: Stock suspensions used as is or further diluted with dispersion medium and used for in vivo instillation.

3 Materials and media

3.1 List of ingredients and supplies

Purified and sterile-filtered water
Serum (sterile)
Ethanol (96 vol%) for hydrophobic NM
1 flask for 2% v/v serum-water dispersion medium
Vials: 20ml Scint-Burk glass pp-lock+Alu-foil (WHEA986581; Wheaton Industries Inc.) for weighing out NM and probe-sonication
Steel and glass spatula
Pipette and pipette tips
Weighing boat/weighing paper
Electrostatic neutralizer
Weigh
Control or reference weights
Probe sonicator (13 mm tip is recommended)
Ice (for ice-water)

3.2 Water quality

It is recommended to use the purest available water. The protocol has been used using both Nanopure Diamond UV and Millipore MilliQ-filtered water (Figures 1 and 2). No differences were observed in the dispersion characteristics 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 similar 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. Blank controls should always be used.

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.

3.2.1 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 (γ-irradiated Barnstead D3750 Hollow fibre filter). The water quality is listed as:

Resistivity: ≤ 18.2 MΩ-cm at 25ºC
Pyrogens: < 0.001 EU/ml
Total Organic Carbon: < 3.0 ppb
Other: nuclease-free (RNase andDNase).

Figure 1. Example of Thermo Nanopure Diamond® UV-water system (Thermo Scientific).

3.2.2 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 25ºC
Pyrogens: <0.02 EU/ml
TOC: 5-10 ppb
Silicates: <0.1 ppb
Heavy metals ≤ 0.1 ppb
Microorganisms: ≤1 cfu/ml

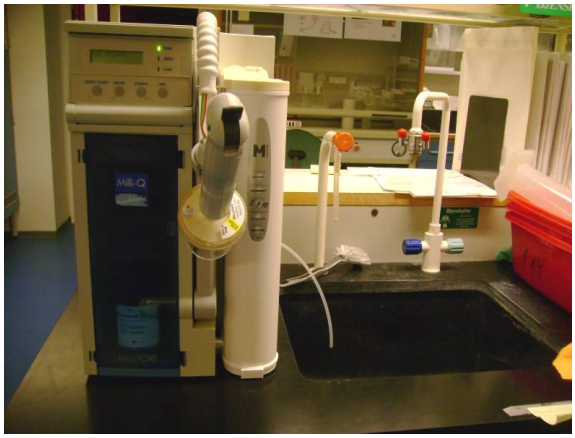

Figure 2. Example of a Millipore water filtration system (Millipore).

3.3 Sera

The following sera were selected at the time of the SOP development. Please ensure that similar sera are used and checked for quality.

- Bovine serum (Sigma B9433; batch 098K0372). Sigma has ca. 60 in stock (March - 2010).
- Mouse serum (Sigma M5905; Batch 059K6022). Sigma has above 80 in stock (March - 2010) and more than 300 in USA.

B9433 is sterile-filtered, cell culture tested. Sigma has performed numerous tests. E.g. it was tested to contain less than 5 Endotoxin Units/ml. We suppose this is below detection limit. Based on this, we do not see any problems with using same product (B9433) but other batch numbers should this (should this batch be unavailable).

M5905 is not sterile filtered or tested. It contains 0.01% Thimerosal. Sigma has not able to inform us of the tests performed on this product. I.e. this product should be tested for endotoxins.

Sera have not been selected for other species.

When preparing standard dispersion media; use the highest standard of de-ionized and filtered water (≤ 0.45 µm) available. The MilliQ-filtered water is added 2 vol.% serum. Water with serum can be stored frozen at -20 °C for long term storage.

4 Nanomaterials

The ENPRA protocol has been applied to a number of different NM. It was originally developed and validated for the TiO_2, ZnO, Ag, and MWCNT NM listed in Table 1.

Table 1. Nanomaterials for which the ENPRA protocol was developed

Material	NM code	Label name	label second line	CAS-Number	Source
TiO2	NM-101	Titanium Dioxide, thermal photocat 7	rutile-anatase, thermal, 7 nm	13463-67-7	Hombikat UV100
TiO2	NRCWE-001	Small	rutile, XRD size 10 nm	13463-67-7	NanoAmor
TiO2	NRCWE-002	Small, positively charged small	rutile, XRD size 10 nm		Modified NRCWE-001
TiO2	NRCWE-003	Small, negatively charged	rutile, XRD size 10 nm		Modified NRCWE-001
TiO2	NRCWE-004	Large	rutile, XRD size 94 nm	13463-67-7	NaBond
ZnO uncoated	NM-110	Zinc Oxide	uncoated 100 nm	1314-13-2, *EINECS* 215-222-5	BASF Z-Cote
ZnO coated	NM-111	Zinc Oxide	coated triethoxyca-prylylsilane 130	1314-13-2, 2943-75-1 *EINECS* 215-222-5, 220-941-2	BASF Z-Cote HP1
Silver	NM-300	Silver		7440-22-4	RAS GmbH
MWCNT	NM-400	Multi-walled carbon nanotubes	entangled, diameter 30 nm, length 5 µm	7782-42-5, EINECS 231-955-3	Nanocyl
MWCNT	NM-402	Multi-walled carbon nanotubes	entangled, diameter 30 nm, length 20 µm	7782-42-5, EINECS 231-955-3	Arkema Graphistrength C100

5 Handling of the test materials

All vials should be stored desiccated at room temperature in dark. The materials have been distributed in vials under argon. Be especially careful when using silver particles in dispersions, such as NM300K and NM302K. Experience from working with NM300K has shown that this material is very sensitive. Therefore, please check your vials for sediments before opening and follow the specific protocol designed for handling of the silver NM. We recommend that NM-300K and NM302K should only be opened only once. If you need to reuse vials with these two NM, we recommend that you work with the vials under argon (see section 5.3). The other materials should to be relatively stable under air, but handling under argon is strongly recommended if re-use is required.

5.1 Handling liquid dispersions (example for NM-300K)

The vial with NM300K contains 0.5 g silver to 5 ml de-ionised water (75%) with stabilizing agent 7% ammonium nitrate and 8% emulsifiers (4% each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene(20) Sorbitan mono-Laurat (Tween 20). The silver concentration is roughly 10% w/w (100mg/ml), which means that each vial contains 500 mg of silver (See data sheets for exact concentrations). The material is very viscous, and weighing is therefore recommended for dilution.

For dilution use at least 25 ml dispersion media. - E.g. 1 ml NM-300K to 38.0625 ml dispersion media will give a concentration of 2.56 mg/ml.

WORK FAST, once the material vial is open! Keep the vial upright during handling to minimize escape of the argon.

1) Weigh the vial for the stock suspension with cap
2) Remove cap from stock suspension vial
3) Remove cap from material vial
4) Pipette calculated volume from material vial to mixing vial. (At least material enough for 25 ml dispersion)
5) Close the material vial. If you choose to use the silver again, seal it under argon.
6) Close the mixing vial
7) Weigh the mixing vial and calculate mass difference
8) Add calculated amount of dispersion medium (2% serum water v/v) and stir it gently by hand.
9) Pipette 6 ml from mixing vial to stock suspension vial.
10) Close the stock suspension vial.
11) Proceed to probe-sonication (section 6).

5.2 Handling of powders

Powder materials should be stored in darkness, dry (desiccated if possible), and at room temperature. BE FAST, once the material vial is open! Keep the vial upright during handling to minimize escape of the argon. Use clean glass ware and spatulas.

Weigh app. 15.36 mg particles corresponding to 6 ml dispersion media. However, for hydrophobic NM (e.g., NM111[ZnO]), wetting with 0.5 vol.% EtOH is essential before addition of dispersion medium and sonicating to achieve a good suspension in the dispersion media.

1) Weigh the vial for the stock suspension with cap
2) Remove cap from suspension vial
3) Remove cap from material vial
4) Transfer material to stock suspension vial (Use enough NM for 6 ml dispersion)
5) Close the material vial
6) Close the stock suspension vial
7) Weigh the stock suspension vial and calculate mass difference
8) Add calculated amount of dispersion media
9) Proceed to probe-sonication (section 6).

5.3 Sealing vials under argon

In order to avoid or at least limit the potential nanomaterial release to the laboratory surroundings to a minimum, we suggest the following procedure when re-sealing the vials under argon. The material vial and cap is placed in a box a little higher than the vial itself. See Figure 1. The box is placed in a fume hood. Open for the argon inlet and slowly fill the box. After the appropriate amount of argon has been delivered and the atmospheric air has been replaced seal the vial with the cap. Keep the argon on until the vial is fully sealed.

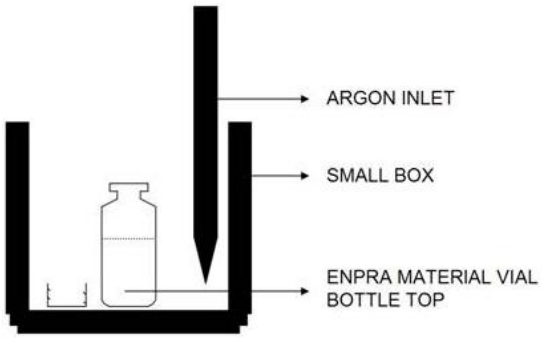

Figure 1. Illustration of a possible setup for re-sealing NM vials in an argon bath.

6 Dispersion by probe-sonication

Remember for the sonication vial, it is requested to use the specific 10 ml Schott Duran glass beakers D=2.6 cm listed under materials to enable comparability.

Important: The **suggested sonifier** is a 400 W, 20 kHz Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037). The horn can be seen at www.sonifier.com/pdf/DISRUPTO.PDF. The sonicator can be seen at http://www.sonifier.com/s450_digital.asp.). **Other sonifiers** may be used. **All sonifiers must calibrated** using the ***NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing***.

The samples are continuously cooled in an ice-water bath to prevent heating of the sampling during the sonication procedure. See Figure 2. Add pre-cooled MilliQ to the insulated box (e.g., styrofoam) with ice in order to ensure a more direct cooling of the sample.

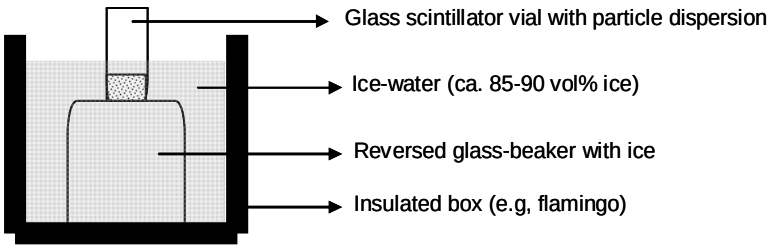

Figure 2. Illustration showing a principle set-up for placement of sonication vial in ice-water bath. Different configurations are possible. NB Flamingo is styrofoam!

1) Fill a 250 ml glass beaker with ice and place it upside-down in a insulation box (e.g., styrofoam)

2) Add ca. 85-90 vol% ice into the insulation box
3) Add ca. 10-15 vol% cold (e.g.,. refrigerated) 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. One may fix the vial using a clip or burette holder to ensure that the vial is not moving during sonication.
5) Insert the sonication probe as close as possible one third into the dispersion. Never immerse it less than the upper quarter and never lower than half-way into the dispersion (find the correct height using a vial with 2% serum-water alone).
6) Start sonication and run it for 16 min at the settings identified of your specific probe-sonifier using the *NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing*, while controlling that the sonication probe does not touch the walls of the scintillation vial.
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.

Procedure CNT/TiO_2/AgNP Prepare a stock suspension of 2.56 mg particles/ml dispersion media. Sonicate 1x16 min without pause. Make sure that the sample is continuously cooled by ice/water as depicted in Figure 2.

Procedure for hydrophobic NM (e.g., NM-111): Tilt the vial so the material is gathered in a small area. Wet the particles with 0.5% vol/vol EtOH (96%). Let the EtOH move back and forth over the particles for 1 min. Prepare a stock suspension of 2.56 mg by adding 99.5% standard dispersion media. Addition of EtOH is required to disperse the hydrophobic NM. We suggest to use the EtOH wetting for NM that needs direct comparison as could be the case for e.g., NM110[ZnO] and NM-111[ZnO]. Sonicate 1x16 min without pause. Make sure that the sample is continuously cooled by ice/water as depicted in Figure 2.

Important: The sonicator horn must not touch the sides and bottom of the glass when ON. Make sure that *the horn is immersed as close as possible to the upper one third in the medium and not above the upper quarter*. Do not start the sonicator before the probe has penetrated the liquid. Use approximately 6 ml of dispersion medium and never less than 4 ml.

7 Quality control

After sonication, the dispersions must be checked ***using dynamic light-scattering (DLS) or alternative techniques*** following the requests in the NANoREG Technical Guidance Document. For the first assessment, it is suggested to use the standard viscosity of water. It should be noted, however, that the viscosities of some dispersion may differ significantly from that of water (and even for some materials also from time to time). Viscosity measurements are usually time-consuming and will have to be made in parallel or by subsequent correction of the DLS data.

Samples for electron microscopy (TEM or SEM) or Atomic Force Microscopy must be prepared at the same time as DLS measurements are done. Check for protocols on CIRCA as specified in the NANoREG Technical Guidance Document.

8 Stability of the stock suspension

The stock dispersions are meta-stable due to the relatively high particle concentrations. For the materials listed in Table 1, suspensions remain stable for 30 to 60 minutes depending on the NM. To ensure homogeneity, one should always gently shake or Vortex the stock dispersion at low to intermediate speed for 10 sec before use. It has been shown that this, re-establishes the original dispersion characteristics for NM in Table 1. It is assumed that this observation can be extrapolated to other NM.

Alternatively ultrasound bath treatment may be used, but the result may vary with type of equipment.

9 Application of the stock dispersion

The stock dispersion may now be used for *in vitro* and *in vivo* exposure studies. Please follow the NANoREG Technical Guidance Document regarding the documentation and qualification requirements for the stock dispersion medium and exposure media.

9.1 In vivo studies

Procedure: The stock suspension will be used as is (Highest concentration; 128 ug/50 µL) or diluted with dispersion medium (MilliQ with 2% mouse serum) to the lower concentrations (1-64 µg/mL).

It should be noted that the dispersion characteristics of these diluted dispersions have not been documented yet. Better dispersion and stabilities is generally expected for stock dispersion diluted in pure de-ionized sterile-filtered water.

9.2 In vitro studies

The NANoREG Management Committee did not approve the use of the ENPRA dispersion protocol for in vitro studies. If interested for NANoREG studies, please consult your Work-package leader and the Management Committee.

Procedure: The stock suspension 2.56 mg/ml (particles in MilliQ w. 2% serum of choice) should be diluted at least 10 times with full normal cell media. Highest tested concentration will thus be 256 ug/ml. Add MilliQ containing 2% serum to cell media to determine if the dilution has any effect on your cells/assay.

10 References

Jacobsen NR, Pojano G, Wallin H and Jensen KA (2010). Nanomaterial dispersion protocol for toxicological studies in ENPRA. Internal ENPRA report. March 2010. National Research Centre for the Working Environment. 8 pp.

Changes to protocol

No changes from vs. 1.0 has been made