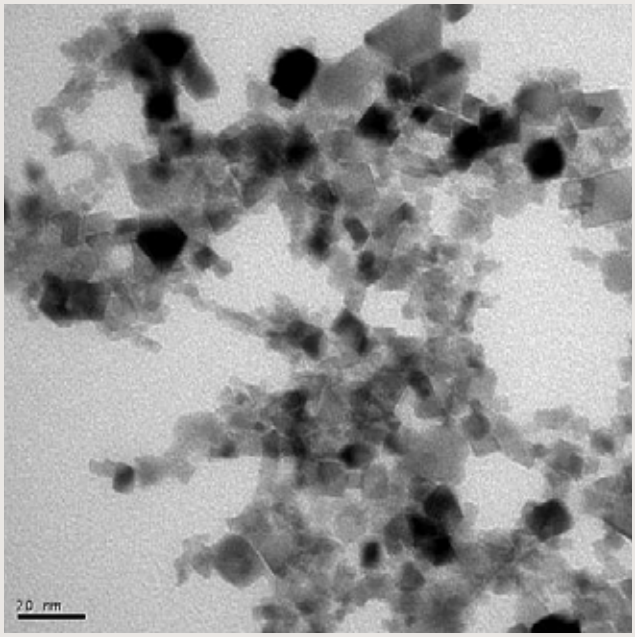
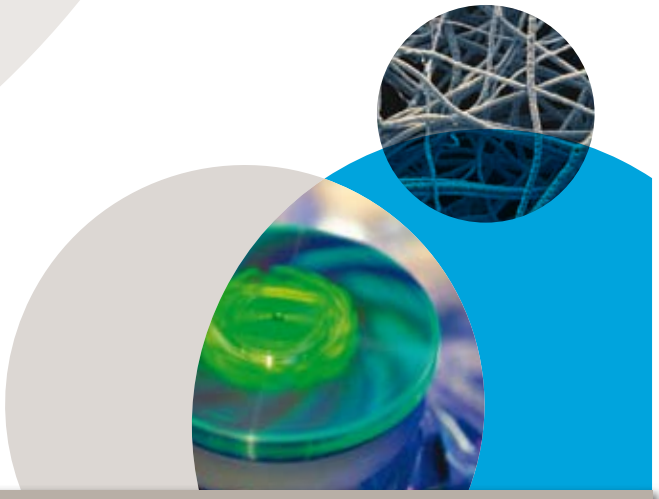
PROSPECT
Global Nanomaterials Safety

Protocol for Nanoparticle Dispersion





18 May 2010

IN SUPPORT OF PROSPECT:

Ecotoxicology Test Protocols for Representative Nanomaterials in Support of the OECD Sponsorship Programme

Table of Contents

Cover photo: UMICORE NanoGrain® cerium oxide nano particles (Image courtesy of UMICORE S.A./N.V.)

FOREWORD/PREFACE

PROSPEcT [1] is the UK's contribution to the OECD Sponsorship Programme [2] to examine the environmental safety of nanomaterials in accordance with the agreed OECD WPMN *'Guidance Manual for Sponsors of the OECD Sponsorship Programme for the Testing of Manufacture Nanomaterials'*. [1] It will provide crucial data to the OECD work, by addressing gaps in the current level of knowledge on the physico-chemical and ecotoxicological properties of these materials, followed by fundamental scientific research leading to establishing scientific test methodologies to study those endpoints that may not be assessed through standard tests used for bulk chemicals.

PROSPECT is a public-private-partnership dedicated to supporting the safe and responsible exploitation of nanomaterials, and developing a better understanding of their impact on humans, and the environment. PROSPECT has been created and is lead by the Nanotechnology Industries Association (NIA). More Information on PROSPEcT is available at the following websites: PROSPECT: http://www.nanotechia-prospect.org/home/home and NIA: http://www.nanotechia.org.

This document is to be submitted as the protocol for nanoparticles dispersion. It is part of the third deliverable of the PROSPEcT Project.

Any mention of trade names or commercial products in this document does not constitute endorsement or recommendation for use.

[1] **PROSPEcT: Ec**otoxicology **T**est **P**rotocols for **R**epresentative Nanomaterials in Support of the **O**ECD **S**ponsorship **P**rogramme'

[2] OECD's Working Party on Manufactured Nanomaterials (WPMN) launched a Sponsorship Programme in November 2007. The programme involves OECD member countries, as well as some non-member economies and other stakeholders to pool expertise and to fund the safety testing of specific Manufactured Nanomaterials (MNs). In launching this Sponsorship Programme, the WPMN agreed on a priority list of 14 MNs for testing (based on materials which are in, or close to, commerce). They also agreed a list of endpoints for which they should be tested. Much valuable information on the safety of MNs can be derived by testing a representative set for human health and environmental safety.

1.0 Introduction

This dispersion protocol is developed to take into consideration our findings from the relevant literature, and recently published report entitled 'Evaluation and Assignment of Nanoparticle Dispersion/Characterisation Methodologies, to be developed under PROSPEcT'. A copy of this report is available for download from the following website, http://www.nanotechia-prospect.org/publications.

The protocol presented here is designed for laboratories equipped with a limited range of equipment, for the nanoparticles ZnO and CeO_2. The broad principles are applicable to a wide range of nanoparticles but individual cases may require specific additional, individual steps or other modifications of the procedure.

There is a video to accompany this protocol and it is recommended that the user view the video prior to using the protocol. A copy of this video can be viewed from the following website, http://www.nanotechia-prospect.org/publications.

2.0 Safety

The user is recommended to perform this work in a well-ventilated area such as a fume cupboard. Please ensure to conduct a full risk assessment i.e. COSHH and RISK assessment forms, for both normal operating practices as well as for possible spillage or other accidents. Ensure that the operator has appropriate personal protective equipment to include goggles, anti-static gloves and a lab coat.

3.0 Dispersion Considerations

Prior to the dispersion step, researchers must:-

a) Consider the research criteria for dispersion

Important criteria to consider:

1. if surfactants additives or naturally occurring dissolved organic constituents are to be added
2. if to impose some restrictions as to particle size to be analysed e.g. if large aggregates should be removed
3. the choice of dose parameter (surface area, mass or number)

In the accordance to the meeting held on April 30, 2009 between NPL and Exeter University, one criterion that was agreed upon is that no surfactants or other additives are to be added into the dispersion. The rationale behind this is to prevent any toxicological response effects that may arise from the action of additives in the dispersion; it may also be the case that additives can potentially render the nanoparticle non-toxic. Another criteria identified, is that the protocols are to be developed (where appropriate) from principles and guidelines found from international standards organizations, such as ISO. The recipe for dispersion presented here aims to reflect on the agreed criteria for dispersion.

b) Understand the history of the powders, particularly how the powders have been sub-sampled

It is important to ensure that the correct sampling practices have been carried out and for methods of sampling to be sufficiently validated. For example, if a spinning riffler is to be used then a mass validation method, as detailed in ISO 14488, should be carried out before using other methods e.g. BET, laser diffraction, etc.

c) Store the sub-sampled powders in Vial 1 (as detailed below) under controlled storage conditions

The sub-sampled powders to be used in the PROSPEcT project will be distributed by the European Commission Joint Research Centre (JRC). The nanoparticle powder has been sub-sampled into pre-cleaned vials, through the use of a spinning riffler and is packed under Argon gas to protect the sample from the atmosphere. In the remainder of the document, these vials will be referred to as "Vial 1". In accordance to the JRC sample handling protocol, Vial 1 should be stored (upright) at room temperature and in the dark; temperature and humidity should be monitored and recorded periodically. Note that the nanoparticles may be hygroscopic in nature and therefore it is important to avoid any kind of water uptake by the sample. Once Vial 1 has been opened, the content should be used and any remaining material should be discarded.

d) Weigh the desired amount from Vial 1 into Vial 2 (as detailed below)

Prior to opening Vial 1, the identification number (as indicated on the label) should be recorded for future reference. "Vial 2" in this step is a pre-cleaned glass container (~20 ml, 28 x 60 mm). The dimensions of Vial 2 container was chosen to allow:-

a) sufficient space for an ultrasonic probe to be introduced into the container
b) fast/ effective sonication.

Weighing the appropriate amount of nanoparticles should be done under inert atmosphere and hence, it is ideal to house an analytical balance inside a glove box. However, prior to its use, the correct functioning of the balance must be calibrated against a set of commercially available calibrations weights. With the aid of a clean spatula, ~ 15 mg of nanoparticle powder should be transferred from Vial 1 to Vial 2 and the appropriate final mass differences calculated to correspond to the total mass of the nanoparticle powder in Vial 2. It is important to minimise any material loss through handling.

4.0 Dispersion Method

The method below is written for the preparation of Nanograin cerium oxide (CeO_2) dispersion in DI water (concentration of 15 mg/L) but can be easily adopted for ZnO (at some other concentration) and aqueous based liquid media. This method is not particularly suited for the dispersion of the coated zinc oxide nanoparticles and the researcher is advised to use the method described under Section 4.3.

4.1 Materials

1 large glass beaker (1 L)
Volumetric (glass) flask (1 L)
DI water (resistivity of ~ 18 Ohm)

Ultrasonic probe[3] (Cole-Parmer ® 130-Watt Ultrasonic Processors (50/60 Hz, VAC 220); product number EW-04714-51); the probe is a 6 mm (1/4") titanium and is tuned to resonate at 20 kHz, ±50 Hz)
Mini Lab Jack
Stainless steel spatula
Disposable pipette (preferably standard glass Pasteur pipette, 150 mm length)
Vial 2 (as detailed above) containing Nanograin CeO2 (~15 mg)
Vial 3 (pre-cleaned, with no specific dimensions) to contain a suitable volume of DI water (or ecotox media), such that you will end up with 1 mg/ml nanoparticle concentration in Vial 2

4.2 Method

Step 1: Add a few drops of DI water (or liquid media) taken from Vial 3, using a glass pipette to the nanoparticle powder in Vial 2, in order to create a thick paste. Do this whilst mixing using a pre-cleaned spatula and apply sufficient energy to remove visible aggregates in the paste. The purpose of this wetting step is to sufficiently substitute solid-air interface with solid-liquid interface, as recommended by guidelines in BS ISO 14887 (2000) ["Sample Preparation – dispersing procedures for powders in liquids].

Step 2: Add the rest of DI water from Vial 3 into Vial 2 (containing the paste of nanoparticle powder) and gently mix using a clean spatula.

Step 3: Place Vial 2 on to a lab jack and insert the ultrasonic probe tip half way down the small vial. De-agglomerate using an ultrasonic probe for 20 s (at 90 % amplitude; this should give a temperature rise of ~5 °C in the dispersion). The operator should determine the acceptable temperature rise during sonication in the given time period. If longer sonication time is required then the operator must provide a better control of the temperature inside the vial. One option is to immerse Vial 2 in an ice bath during the sonication. During sonication, ensure that the tip is not touching the sides of the glass vial. In addition, do not place your hands near the de-agglomerating unit whilst it is operating.

Step 4: Once completed, transfer the nanoparticle suspension to the desired total volume (to make the "stock") and mix gently with a glass rod. Flush the small vial with further DI Water (or liquid media) and add this to the rest of the suspension. This "washing" step is important to ensure that all of the nanoparticles are transferred from the small vial to the larger beaker, such that dosage measurement (by mass) can be interpreted accurately. Gently stir with a glass rod. For greater accuracy, make up to the desired volume using appropriate volumetric flask/ pipette.

Step 5: The dispersion is now ready for analysis. For the nanoparticle analysis, this will involve the sample splitting of "the stock". From guidelines found in ISO 14488: 2007 "[Particulate materials sampling and sample splitting for the determination of particulate properties"] sample splitting using a pipette is recommended as this method (relative to sample splitting using multiple capillary tubes) is simple to do and less prone to contamination. Prior to taking an aliquot out of the stock, agitate the stock dispersion; this

[3] Although exposure of the nanoparticles to a high intensity ultrasonic probe appears to be more effective than other de-agglomeration tools, its limitations have not been fully investigated. For example, probe tip disintegration/ erosion through time can potentially contaminate samples. Probes can also have highly variable performance, particularly at the lower end of the market. In addition, the high amount shear provided by the ultrasonic probe can alter nanoparticle architecture and also increase the temperature of the dispersion.

can be achieved by gently mixing using a clean glass rod to ensure homogeneity of the sample.

4.3 Dispersion protocol for coated zinc oxide (Z-Cote HP1) nanoparticles

The Z-Cote HP1 nanoparticles contain a coating of hydrophobic silane, which renders it difficult to disperse using the above method. In order to prepare a dispersion of the HP1 particles, the following method is recommended. [2] An alternative method to disperse coated zinc oxide particles has also been published by Wiench et al. [3]

Method

Weigh approximately 10 to 15 mg of particles corresponding to approximately 4 to 6 ml of dispersion media. Wetting with 0.5 vol% of ethanol (EtOH) is essential before sonicating to achieve a good suspension in the dispersion media.

1. Weigh the vial for the stock suspension with the cap
2. Remove cap from suspension vial
3. Remove cap from material vial
4. Transfer material to stock suspension vial (at least sufficient material for 25 ml suspension)
5. Close the material vial
6. Close the stock suspension vial
7. Weigh the stock suspension vial and calculate the mass difference
8. Add calculated amount of dispersion media

Use the highest standard of de-ionised water available and filtration through a 0.45 µm filter or smaller. Add 2 vol% serum to obtain the dispersion medium. The dispersion medium can be frozen at -20 °C for long term storage.

The recommended sonifier is a Branson Sonifier S-450d (Branson Ultrasonics Corp., Danbury, CT, USA) equipped with a standard 13 mm disruptor horn (Model number: 101-147-037). Further details of the can be obtained from http://www.sonifier.com/pdf/DISRUPTO.PDF. More details of the sonicator can be obtained from http://www.sonifier.com/s450_digital.asp. We recommend the use of 10 ml Schott Duran glass beakers, D=2.6 cm. The sample is continuously cooled in ice during the heat build up caused by the sonication procedure, see Figure 1. Add pre-cooled MilliQ to the insulated box with ice in order to ensure a more direct cooling of the sample.

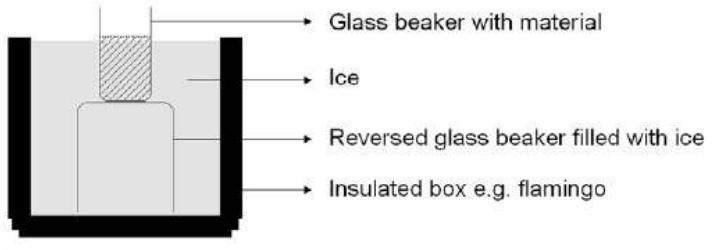


Figure 1 Illustration of the arrangement of the vial containing stock dispersion during the sonication procedure. [2]

Tilt the vial so the nanoparticles are gathered in a small area. Wet the particles with 0.5% vol/vol EtOH (96%) for 1 minute. Prepare a stock suspension of 2.56 mg by adding 99.5% standard dispersion media. Addition of EtOH is required to disperse the hydrophobic coating particles. It is suggested that both coated and uncoated zinc oxide particles be prepared this way for comparability. Sonicate continuously for 16 minutes. Make sure that the sample is continuously cooled by ice/water.

Ensure that the sonicator horn does not touch the bottom of the glass when it is switched on. Make sure that the horn is located in the top half of the liquid but below the liquid surface. Do not start the sonicator before the probe has penetrated the liquid. Use approximately 4 to 6 ml of MilliQ.

For in vivo: The stock suspension will be used as is or diluted with dispersion medium (MilliQ with 2% mouse serum) to the lower concentrations (1-64 µg/ml).

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

The dispersion should be stable within the hour but it is recommended that it is used immediately.

5.0 Dispersion Stability Testing

In accordance to BS ISO 14488, successful (liquid) sample splitting can only be conducted if a homogeneous dispersion has been achieved – otherwise this will result in a much higher sampling error. Prior to sample splitting, the operator should check that dispersion is sufficiently stable during the time that is required to perform sample splitting and subsequent reliable characterisation of the nanoparticle dispersion using a predetermined tool. For example, if the nanoparticle dispersion is to be characterised by Dynamic Light Scattering (DLS), then suitable aliquot should be pipetted out from stock and the mean particle size acquired. Six replicates should be acquired to ensure that the sample is sufficiently stable within a reasonable amount of time.

If there is evidence of aggregation/sedimentation in the sample, then the dispersion is not stable enough to allow subsampling to be carried out without incurring sub-sampling error. In addition to errors incurred from sub-sampling steps, stability testing of the dispersion is important for nanoparticle characterisation. For example, one of the pre-requisites for reliable and accurate DLS measurement is to have a sample that is stable with no signs of sedimentation, as DLS is applicable only to particles that remain fully suspended undergoing Brownian diffusional motion, throughout the measurement.

6.0 Dispersion Characterisation Tools

Whatever the choice of characterisation tools chosen, operators must be aware of the limitations posed by the various techniques. It is beyond the scope of this dispersion protocol to give detailed description of limitations of various techniques and so it is left for the operator to ensure that the technique chosen is suitable for a given nanoparticle dispersion under analysis. For example, in the case of DLS, this tool is not suitable to resolve a broad particle size distribution, as potentially larger particles can mask the signal of the

smaller nanoparticles. In order to resolve multi-modal particle distribution, techniques that have a separation mechanism element integrated in analytical tool will be more suitable e.g. CPS disc centrifuge.

References

[1] OECD WPMN *'Guidance Manual for Sponsors of the OECD Sponsorship Programme for the Testing of Manufacture Nanomaterials'*, ENV/JM/MONO(2009)20, 9 July 2009. Available at: http://www.olis.oecd.org/olis/2009doc.nsf/linkto/env-jm-mono%282009%2920.

[2] Jacobsen, NR. et al., '*Nanomaterial dispersion protocol for toxicological studies in ENPRA*', March 2010.

[3] Wiench, K. et al., *Acute and chronic effects of nano- and non-nano-scale TiO_2 and ZnO particles on mobility and reproduction of the freshwater invertebrate Daphnia magna*, Chemosphere 76 (2009) 1356–1365.