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Issues surrounding the testing of nanoparticles for ecotoxicological studies

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Abstract

There is a genuine worry about how engineered nanoparticles affect the environment and this had resulted in a two-part study to be presented here. The first part of the study investigates some of the issues surrounding the development of dispersion and characterisation of nanoparticle suspension, critical in order to carry out appropriate ecotoxicological testing to understand the toxicological relevance of nanoparticles in the environment. Cerium oxide (CeO₂) nanoparticles were dispersed in de-ionised water and subsequently characterised using Dynamic Light Scattering and Scanning Electron Microscope. Results show that reliable data relies on the need to control the dispersion step and to understand limitations associated with current tools. The second part of the study investigates the fate of nanoparticles when dispersed in three different ecotox media (seawater compared with media of fish and daphnia), in an attempt to identify initial concerns exist. A visual sedimentation experiment was carried out, which showed that nanoparticles (within a two day period) are relatively unstable in these ecotox media (relative if dispersion was carried out in DI water). Although most particles aggregated into larger clusters, SEM images showed the presence of nanosize clusters (of less than 800 nm) still present in these media. It is the presence of these nanosize particles that will be of utmost concern, if the hypothesis that relates particle size and toxicological activity holds true.

Keywords: nanoparticles, aquatic ecotoxicity, characterisation, dispersion

1 Introduction

Over the past few years, research concerning nanoparticle toxicity has attracted public concern [1]. Particularly, in assessing their toxicological
significance, several studies [2] [3] indicate that nanoparticle toxicity is governed by their small size and high surface area, which subsequently will produce greater chemical reactivity. In an attempt to address this public concern, the OECD (Organization for Economic Co-operation and Development) has recently launched a sponsorship program on nanoparticle safety assessment that requires global co-operation. This has resulted in the United Kingdom to launch the PROPeCt LINK project, which aims to fulfil the UK’s contribution for the testing of zinc oxide (ZnO) and cerium oxide (CeO2) nanoparticles.

Central to toxicological investigation of nanoparticle is the need to link toxicological activity with physicochemical properties [4]. In other words, what are the physical/chemical properties of the nanoparticles that is most responsible for toxicological activity? This type of research has been conducted in the past but the conclusions drawn from such studies are often contradictory in nature, suggesting the need to successfully develop and establish standardised protocols, to be agreed amongst the research community. For example, toxicity of carbon nanotubes has been one of the most pressing questions in nanotechnology [5]. Recently, Donaldson and co-workers [6] have shown experimentally that nanotubes show similar toxic responses to asbestos fibres. However, findings from Koyama and co-workers [7] have reported that the extent of toxicity of carbon nanotubes was low if compared to asbestos.

The purpose of the present study is to fulfil two objectives. The first objective concerns issues surrounding development of protocols. This research explores some of the issues associated with dispersing and subsequently characterising CeO2 in DI water; DI water was used as past results showed good stability when nanoparticles are dispersed in such media as reflected by their corresponding high zeta-potential values [8] [9]. Results showing effect of using different de-agglomeration tools to disperse the nanoparticles will be presented. In the development of characterisation protocols, the importance of understanding the limitations of the technique (particularly “limit of quantification”) for the intended use will be evaluated. For example, as nanotoxicological investigations often require the need to conduct analysis at extremely low particle concentrations, ~ nanogram per litre (ng/L) or less [10], the effects on data acquired (from various techniques: Dynamic Light Scattering (DLS) for particle size and zeta-potential measurement, and scanning electron microscope (SEM)) upon dilution of nanoparticle concentration will be shown. A technology roadmap will be presented to show the limitations associated with such tools.

The second objective of this study relates to the fate of the nanoparticles in ecotox media and to identify any initial concerns. According to hypothesis, it is aspects of particle size characteristics that dominate the toxic profile of nanoparticles [11]. At high salt concentration, the ecotox media in this study is expected to result in particle instability upon dispersion, to result in the formation of large aggregates/agglomerates that would eventually sediment out [12] and thus rendering less toxic. The central question here is: Will all of the nanoparticles sediment out? To assess this, dispersions of CeO2 and ZnO will be made in three different ecotox media (seawater, media of fish and daphnia) and results from a visual sedimentation type experiment will be presented; results
will be compared relative to dispersions in DI water. SEM analysis will then be carried out on the dispersions after the two-day period, to see if nanosize particles still exist.

2 Experimental

2.1 Materials and sample preparation

Z-Cote Zinc Oxide (ZnO with a reported primary particle diameter size of 100 nm) and Nanograin (CeO₂, with a reported average particle size of ~ 50 – 70 nm) was supplied from BASF SE and Umicore Belgium, respectively. Nanoparticles were dispersed using the protocol below, in one of four possible aqueous liquid media: de-ionised (DI) water and three ecotox media (seawater and media of fish and daphnia). DI water from Millipore, MilliQ system was used to prepare all of aqueous solutions/suspensions. Ecotox media was prepared as follows, according to the recipes provided by University of Exeter: a) Seawater - 25 g per L of Tropic Marin Sea Salt (Tropical and Marine Limited), was prepared, pH ~7.5. b) Daphnia freshwater media. Salts (196 mg CaCl₂·2H₂O, 82 mg MgSO₄·7H₂O, 65 mg NaHCO₃, 0.002 mg Na₂SeO₃ (as obtained from appropriate dilutions from 2mg/ml concentration stock) were dissolved in 1 L volume of DI water. Upon continued stirring, DI water was further added such that final pH ~ 7.5 and conductivity is between ~ 360 – 480 µS/cm. End volume ~ 1 – 1.5 L. c) Fish freshwater media. This was prepared in three separate steps. First, salts (11.76 g CaCl₂·2H₂O, 4.93 g MgSO₄·7H₂O, 2.59 g NaHCO₃, 0.23 g KCl) were dissolved separately in DI water (1L volume) to make four separate stock solutions. Second, 25 mL of each salt stock solution was aliquoted into a clean bottle and diluted in DI water (made up to 1 L volume). Third, 200 ml of the stock solution from Step 2 was aliquoted and further diluted with DI water (made up to 1L volume). For long-term storage, these ecotox solutions were autoclaved and stored in the fridge until needed.

2.2 Nanoparticle dispersion in aqueous liquid media

The appropriate amount of nanoparticle powder was weighed in a small pre-cleaned vial using an analytical mass balance. To disperse, a few drops of the appropriate liquid media was added to the vial and was mixed into a thick paste using a spatula. ~ 15 mL of liquid media was then added to the paste and the whole mixture stirred gently using the spatula. De-agglomeration step was then carried out and is very much dependent of which de-agglomeration tools was employed. If an ultrasonic probe (130 Watt Ultrasonic Processors, from Cole Palmer) was used, the ultrasonic probe tip (6 mm titanium tip) was inserted halfway down the 15 ml volume of dispersed nanoparticles and sonicated with 90 % amplitude for 20 s (unless state otherwise); temperature measurements were made using a standard laboratory digital thermometer (Fisher Scientific) before
and after the sonication step. After sonication, nanoparticle suspension was further diluted using the appropriate liquid media, in order to make up to 1 L total volume (unless stated otherwise); a glass rod was used to gently mix the final dispersion, to ensure homogeneity. If other de-agglomeration tools were used instead (PowerGen Fisherbrand 500 homogeniser or Kinematica Overhead stirrer PX-SR 90 D), then the initial 15 ml dispersion mixture was diluted straightaway into the appropriate liquid media, to make up a total 1 L in volume. The homogeniser or overhead stirrer was lowered to the dispersion and ran at a constant speed for 1 minute (to create maximum vortex action without spillage in a 1 L beaker).

For the purpose of investigating “limit of quantification”, CeO$_2$ was dispersed in DI water using ultrasonic probe. A stock solution of 500 mg/L was prepared and appropriate dilutions with DI water were made from this stock. For the purpose of “visual sedimentation” tests, eight separate nanoparticle suspensions (500 mg/L) were prepared (ZnO and CeO$_2$ dispersed separately in DI water and three ecotox media) in media bottles. Images of the bottles, showing the state of the dispersion in the bottles, were taken (using Compact Sony Cyber-shot DSC – T900) at various intervals during a period of two days; in between capturing images, bottles were stored in the dark.

2.3 DLS (particle Size and zeta-potential) analysis

The instrument employed for particle size analysis and zeta-potential measurements was a Zetasizer Nano ZS (Malvern Instruments, UK) with 633 nm red laser. The same instrument is also able to make zeta-potential measurements, by using a laser Doppler electrophoresis set up. Detailed protocols for DLS particle size and zeta-potential measurements has been reported elsewhere [8].

2.4 Scanning electron microscopy (SEM) analysis

Scanning electron microscope images were collected using a Carl Zeiss Supra 40 electron microscope, in which the optimal spatial resolution of the microscope is a few nanometres. Images were acquired with an accelerating voltage of 15 kV, working distance of ~ 3 mm, tilting angle 0°, and recorded with an in-lens secondary electron detector. For analysis of the “as received” nanoparticle powder, a small scoop of the nanoparticle powder was sprinkled over an SEM carbon adhesive discs (Agar Scientific); one side of the carbon disc was placed securely on a metal stub, whilst the other side was exposed to the nanoparticle powder. Excess powder on top of the disc was removed by gently tapping the stub on its side until an even (light) coating of powder on the surface was apparent. Detailed protocols associated with sample preparation (of depositing nanoparticles dispersed in liquid media on to poly-L-lysine slides) suitable for SEM analysis has been reported elsewhere [8].
3 Results and discussion

3.1 Nanoparticle dispersion

Figure 1a shows the SEM images of “as received powders” for ZnO and CeO$_2$ and results show that polydispersity for both particle size and shape is high. SEM images also show evidence of extensive aggregation and agglomeration (fusion of particles) that exists in both nanoparticles, which is particularly evident in CeO$_2$.

![SEM images of as received powders for ZnO and CeO$_2$.](image)

Figure 1: Nanoparticle Dispersion in Aqueous Media: a) a schematic of the dispersion step from the “as received” powders (SEM images shown; scale bars 200nm for ZnO and 100 nm for CeO$_2$) b) Particle size distribution of CeO$_2$ in DI water (50 mg/L) and the effects of using different de-agglomeration tools (with “exposure time” of 1 minute).

Figure 1a shows a schematic illustrating the basic steps of the dispersion protocol, as detailed in the Method section. Overall, this involved two essentials steps: a) the wetting of the nanoparticle powder into a paste, so as to substitute solid air-interfaces with solid liquid interfaces, as recommended by ISO 14887: 2000 [13] b) de-agglomeration of nanoparticles using an appropriate tool, so as to introduce sufficient shear energy such that aggregates/agglomerates are
broken down using an appropriate de-agglomeration tool, ideally to individual primary particles [14]. Figure 1b shows the particle size distribution (by intensity as reported by DLS) of CeO$_2$ (50 mg/L) in DI water, when dispersed using an ultrasonic probe, with exposure time of 1 minute. Results show a particle size distribution between 68 - 615 nm in size. The plot also shows the effect of altering the dispersion protocol step, when either an overhead stirrer or homogeniser was employed instead of ultrasonic probe. Results show a much broader particle distribution, with particle sizes as big as 1 micron; the much bigger size particles found in the dispersions using these tools can only be explained by the much lower shear energy provided (to result in insufficient de-agglomeration/de-aggregation) if compared to the ultrasonic probe. Undoubtedly, the stability of the final stability after the dispersion will be governed by the inherent properties of the liquid media and their interactions with liquid media [15]. Overall, due to its effectiveness in de-agglomerating, the ultrasonic probe is the tool of choice for the dispersion protocol.

Another variable that can potentially affect the particle size distribution is the length of time that the dispersion is exposed to i.e. the “exposure time”. Figure 2a. shows the effect of changing the “exposure time” to the mean particle size. As expected, increasing the de-agglomeration time from 5s to 50 s resulted in a reduction of particle size; the longer the exposure time the more de-agglomeration the smaller the particle size. However, increasing the exposure time beyond 50 s does not seem to result in further breaking of nanoparticles; it is hypothesised that shear energy provided by the ultrasonic probe was sufficient to de-aggregate but cannot sufficiently break nanoparticles that have fused together (i.e. agglomerates). A side effect of ultrasonication is the increase in temperature of the dispersion, which is expected due to the high shear energy that it provides [16]. Figure 2b shows the change in temperature that occurred as result of dispersion at various “exposure time” and relationship between the two variables is shown to be linear. Ideally, temperature change in the dispersion should be minimised and so, a 20 s exposure time was chosen for our dispersion protocol as this gave ~ 5 C temperature increase (with corresponding particle size of 215 nm as shown in the DLS and the corresponding SEM image in Figure 2a). Results so far have shown the importance in having well controlled protocols for dispersions, as changes in the protocols can potentially affect the particle size distribution of the resultant dispersion.
20 s exposure using ultrasonic probe gave average particle size of 215nm.

Figure 2: Effect of varying “exposure time” (using an ultrasonic probe) on the DLS mean particle size of CeO₂ in DI water (50 mg/L) on: a) mean particle size (inset: corresponding SEM image, when 20 s exposure time was used; scale bar shown reads 100 nm) and b) corresponding temperature change measured in the dispersion, after exposing the dispersion with the ultrasonic probe.

3.2 Limitations of characterisation tools

Figure 3a and 3b shows the effect of reducing CeO₂ nanoparticle concentration in DI water (from 500 mg/L to 0.001 mg/L) on mean particle size and zeta-potential, respectively. Both plots show that values measured are similar within a certain concentration range. However, under “extreme dilution” conditions, data value shifts significantly upon yielding what is thought to be erroneous results. The erroneous data defines the limit of quantification for particle size and zeta-potential to be 0.1 and 50 mg/L, respectively. The results at extreme dilution is not surprising and explanations have been previously attributed: the inherent homodyne configuration of the optics b) a combination of: increase in signal contribution due to extraneous particles and inherent sensitivity of the detector, which defines the limit of quantification for particle size and zeta-potential, respectively [17]. Figure 3c shows a series of SEM images of CeO₂ (dispersed in DI water and subsequently adsorbed on the surface of poly-l-lysine substrates), upon changing nanoparticle concentration within the dispersion. It is apparent that the particle size distribution changes dramatically when nanoparticle
concentration in the dispersion is diluted from 500 mg/L to 10 mg/L. A much-reduced number of nanoparticles and a tendency for smaller particles adhering to the surface were observed under the “extreme dilution” conditions.

Figure 3. CeO$_2$ dispersed in DI water and the effect of varying nanoparticle concentration on: a) DLS mean particle size b) zeta-potential c) SEM of nanoparticles adsorbed on poly-L-lysine slides. Scale bars SEM: for 500 mg/L (reads 2 μm and 200 nm, for low and high magnification, respectively) and for 10 mg/L (reads 1 μm for both).

Unlike DLS instrument, the limit of quantification will be governed by the adsorption kinetics of the nanoparticles on to the substrates during the sample preparation step. At low nanoparticle concentrations, it is the diffusion rate of the particle that will dominate and this in turn explains why the smaller particles are preferentially adsorbed [12]. Overall, identifying the limit of quantification for an individual analytical procedure is important, so as to understand when data becomes scientifically unreliable. This is particularly of importance to nanoeccotoxicological investigations as researchers in this field are often
interested in making measurements under extreme dilute conditions, in the order of less than few ng/L [10].

Figure 4 aims to identify where common laboratory techniques like DLS and SEM sit on the technology roadmap. Three important criteria have been identified as being essential: sensitivity (x-axis), selectivity (y-axis) and representativeness (z-axis). Ideally, an instrument should have a high degree of sensitivity (to single particle level), high selectivity (to measure in the presence of potentially interfering substances in the ecotox media [10] and high representativeness (such that the data is a representation of the entire population rather than a subset; this will subsequently contribute towards the accuracy and repeatability of the measurements); the ideal tool sits on vertex C. According to the technology road map, the DLS and SEM sit on vertex A and B, respectively. The DLS, belongs to a category of “population based methods”; they may not have the desired combination of high sensitivity and selectivity but data are highly representative of the entire population. SEM on the other hand, belongs to a category of “single particle based methods”, which are highly sensitive and selective but data obtained lacks “representativeness”. Future research activities should therefore employ and subsequently validate a technique that belongs on vertex C on this roadmap.

Figure 4. Technology roadmap: tools/techniques for nanoparticle characterisation in complex environmental media assessed against three identified criteria of: sensitivity (x-axis), selectivity (y-axis) and “representativeness” (z-axis).

3.3 Visual sedimentation

Figure 5 shows a typical result from the visual sedimentation experiment of when nanoparticles (either ZnO or CeO2) are dispersed in one of the ecotox media (500 mg/L). After two days, the dispersion inside the bottle shows
complete sedimentation, leaving a clear solution above the sediment. Interestingly, if nanoparticles were dispersed in DI water instead, a different result is apparent in that a cloudy suspension is still observed on Day 2. This suggests that particles are more stable in DI water and subsequently a much slower sedimentation rate observed. In the presence of salts in the ecotox media, is shown to sufficiently cause particle instability within these media and resulted in particle aggregation/agglomeration [18]. The addition of salts in the ecotox liquid formulation employed in this study was sufficient to cause particle aggregation, resulting in a much faster sedimentation rate. If such sedimentation events occur in ecotoxicological relevant media, then this raises the question as to whether we should be concerned. On day 3, sample inside the bottles were analysed with an SEM; most particles present in the dispersion was shown to constitute largely of micron size particles, as expected. However, upon careful examination, some smaller clusters of both nanoparticles (particle diameter size of less than ~ 800 nm) were present in all four media, as shown in Figure 5; particle diameter size was estimated by assuming spherical shape cluster. From the SEM images, this is the case for ZnO disperse in DI water, seawater and daphnia liquid media; for the case of CeO2, this is true upon dispersion in DI water and fish media. If the hypothesis that relates particle size with toxicity holds true, then it is the presence of such nanosize clusters that should be of utmost concern to the aquatic environment.

Figure 5. Visual sedimentation experiment of nanoparticles (typical of either ZnO or CeO2 dispersed in an ecotox media (seawater or daphnia or fish media), during a two-day period. SEM images show the presence
of nanosize clusters of nanoparticles present in the bottles for both ZnO and CeO₂ (obtained on third day); scale bar on all SEM reads 200 nm.

4 Conclusion

While most nanoparticles were shown to have aggregate out of solution when immersed in ecotoxicological media, some nanosized clusters were evident. If this was to occur in a real environmental setting, then there is potential for aquatic organisms to ingest such small particles. Through time, these small particles can accumulate and it is the accumulation of dose that can subsequently formulate a problem. In terms of protocol developments in ecotoxicological investigations, there is a need to:

a) have well controlled, agreed protocols on dispersion and characterisation
b) to identify, develop and validate suitable tools/technology that can offer a combination of high sensitivity, selectivity and “representativeness”

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