Solubility of metal oxide nanomaterials: cautionary notes on sample preparation.

M-L Avramescu¹, M Chénier¹, H D Gardner² and P E Rasmussen¹,²*
¹Environmental Health Science and Research Bureau, Environmental and Radiation Health Sciences Directorate, HECSB, Health Canada, Ottawa, ON, Canada K1A0K9; ²Earth and Environmental Sciences Department, University of Ottawa, Ottawa, ON, Canada K1N 6N5; *corresponding author: pat.rasmussen@canada.ca

Abstract. Eight metal oxides were obtained to investigate the dissolution behaviour of engineered nanomaterials (ENMs) dispersed in biologically relevant media. Identities of the metal oxide compounds, and their crystal form and size were checked using powder X-ray diffraction (XRD). Methods for sonication of metal oxide nanoparticles were optimized to achieve stable stock dispersions, and methods for separation of dissolved metal ions from dispersed nanoparticles were evaluated. The results of the optimization experiments showed that each metal oxide ENM required a different combination of sonication time and power (% amplitude). Optimized values for delivered sonication energy (J/mL) ranged from 24 for CuO to 833 for γ-Al₂O₃. Centrifugation at 20000G was found to be more effective and less prone to artefacts than using commercially available ultrafiltration devices for separation of the dissolved metal fraction, under these experimental conditions. XRD results indicated that the composition of two metal oxide nanopowders (Mn₂O₃ and Fe₂O₃) did not meet the manufacturers’ claims, underscoring the importance of double-checking physical-chemical properties of commercial ENMs purchased for research.

1. Introduction
Metal oxide engineered nanomaterials (ENMs) are widely used as pigments, UV-absorption filters, fillers and semi-conductors. Solubility of ENMs is one of the most important physical-chemical properties used for hazard assessment [1-3], and international efforts are underway to develop standard methodologies for measuring solubility of ENMs dispersed in environmental matrices and biologically relevant fluids [4, 5]. As part of this effort, research was undertaken at Health Canada to develop protocols to determine solubility of eight metal oxide ENMs in cell culture medium: zinc oxide (ZnO), nickel (II) oxide (NiO), titanium dioxide (TiO₂), cerium oxide (CeO₂), copper (II) oxide (CuO), aluminum oxide (Al₂O₃), manganese oxide (MnO₂) and iron (III) oxide (Fe₂O₃). The formulation used in this study was Dulbecco’s Modified Eagle Medium (DMEM) containing 2% fetal bovine serum (FBS) as described by Decan et al (2015) [6].

For experiments investigating ENM dissolution behavior, the first step of sample preparation is to achieve a stable stock dispersion in water, which is subsequently diluted in the relevant aqueous biological medium. It has been previously established that dry powder ENMs do not retain their primary particle size when dispersed in aqueous media (not even in water) due to rapid agglomeration and aggregation [7-10]. Consequently, the goal of optimizing the sonication protocol is to achieve a uniform and reproducible dispersion with the smallest attainable size and minimal polydispersity [8, 9, 11]. The final preparation step in an ENM dissolution test is to separate the solid ENM particles that remain in the dispersion from the dissolved metal fraction that has been released into solution. A
variety of separation methods have been employed for this purpose, including centrifugation at various high speeds and ultrafiltration using filter devices [12-16]. Using cell culture media for dissolution testing presents a particular challenge, in that released metal ions tend to bind to components of the media (e.g., proteins) and may be retained by filters during separation [12, 17].

This paper focuses on aspects of the protocol development where particular caution was needed: confirmation of the identity of purchased ENMs; optimization of sonication methods to achieve stable stock dispersions in water; and avoidance of analytical artefacts when separating dissolved metal ions from solid ENM particles dispersed in cell culture medium.

2. Materials and methods

Suppliers of high quality metal oxide ENMs were identified by internet search, and dry nanopowder products were selected based on particle size (≤ 60 nm using TEM) and the availability of other characterization information (e.g. specific surface area, purity). A Rigaku Ultima IV X-ray diffractometer (XRD Bragg-Brentano geometry) was used to confirm the identity of the compounds present in the purchased nanopowders.

To prepare stock dispersions, the delivered sonication energy (J/ml) was optimized separately for each metal oxide ENM, to achieve the material-specific critical delivered sonication energy (DSE) as described by Taurozzi et al. [10] and Cohen et al. [8]. The metal oxide ENMs were dispersed in deionized water (MilliQ 18.2 MΩ) at a concentration of 1 g/L and sonicated using a Branson Ultrasonics Sonifier™ SFX550 equipped with a ½ in horn (with extension) and removable flat tip. The sonication system was calibrated using the calorimetric method as described by Taurozzi et al [18]. The 100 mL beaker containing the ENM water dispersion was placed in an ice-water bath and the horn extension immersed to the half-way point (2.5cm) between the surface and the bottom of the nanoparticle suspension. Optimization of the delivered sonication energy (J/mL) was performed by first varying the power (% amplitude) while keeping sonication time constant, and then varying the sonication time while keeping power constant. The stability of the optimized stock dispersion was evaluated using a DLS (Zetasizer Nano ZSP, Malvern Panalytical) to determine hydrodynamic diameter (Dh), polydispersity index (PDI) and zeta potential (ZP), at time zero and after 24 h. The pH was measured using a Mettler Toledo Seven Compact pH/Ion S220 pH meter.

For separation of dissolved metal ions from dispersed nanoparticles two different separation methods were assessed using CuO dispersions: centrifugation at 20000G (3 x 30 min) using an Allegra 64R centrifuge (Beckman Coulter) and centrifugal ultrafiltration, using commercially available centrifugal filter devices. Two centrifugal filter devices were included in the evaluation: Vivaspin 6 (3KDa PES) with 10000G for 20 min (as specified by the manufacturers), and Amicon Ultra 15 (3KDa Ultracel regenerated cellulose) with 4500G centrifugation for 30 min. In the case of the Amicon device it was necessary to decrease the speed from 5000G (recommended) to 4500G to avoid tube breakage and loss of samples; otherwise manufacturers’ specifications were followed. Also increased centrifugation time did not result in increased filtrate volume indicating that the filter blocked. Both the centrifugation and ultrafiltration techniques were tested using CuO dispersions in DMEM with 2% FBS (DMEM+FBS) and in deionized water (DIW), both at room temperature. Copper standard solution (10000 mg/L) was used to prepare Cu spikes for spike recovery tests in DMEM+FBS and DIW. The dissolved Cu concentration was determined using inductively-coupled optical emission spectroscopy (5100 Synchronous Vertical Dual View (SVDV) ICP-OES, Agilent Technologies). Blank correction was not necessary due to the relatively low background concentration of Cu in DMEM+FBS (0.0037-0.0039 mg/L in the blank compared to 6-8 mg/L in the sample).

3. Results and discussion

3.1. The need to confirm identity of purchased metal oxide nanopowder products.

Out of the eight metal oxide nanopowders selected, powder XRD analyses confirmed that six were acceptable for the purposes of the project. However, two metal oxide nanopowders did not meet the
manufacturers’ claims: Mn$_2$O$_3$ and Fe$_2$O$_3$. Mn$_2$O$_3$ was purchased from two suppliers but neither contained Mn$_2$O$_3$ according to XRD analysis. One nanopowder sold as Mn$_2$O$_3$ (Figure 1) consisted mainly of alpha MnO$_2$ (avg crystal size 21 nm) and hausmannite (Mn$_3$O$_4$; avg crystal size 46 nm), with a small fraction of an unidentified compound (avg crystal size 55 nm). As shown in Figure 1, if Mn$_2$O$_3$ had been present in this sample, the four characteristic peaks would have occurred at 2θ = 32.95° (intensity =100), 2θ = 55.19° (intensity =28), 2θ = 23.13° (intensity =16), and 2θ = 38.23° (intensity =14). The second nanopowder sold as Mn$_2$O$_3$ contained two MnO$_2$ compounds: akhtenskite (83 wt%; avg crystal size 20 nm) and ramsdellite (17 wt%; avg crystal size 32 nm). Knowledge of the identity of compounds being studied is important not only for accurate reporting, but also for entering the correct refractive index when making DLS measurements (e.g., refractive index for MnO = 2.16; MnO$_2$ = 2.4; Mn$_3$O$_4$ = 2.46).

![X-ray diffractogram of nanopowder incorrectly identified as Mn$_2$O$_3$ by the supplier.](image1.png)

*asterisks indicate locations of characteristics peaks of missing compound Mn$_2$O$_3$ (from ICCD 2016 Powder Diffraction File # 00-041-1442).

Results for one Fe$_2$O$_3$ nanopowder indicated the presence of two different compounds: gamma Fe$_2$O$_3$ (88 wt%) and synthetic hematite (12 wt%). As this mixed product was likely to yield inconsistent solubility results, a different supplier was selected whose Fe$_2$O$_3$ product was determined to contain > 98% synthetic hematite (avg crystal size 14 nm). These results underscore the importance of double-checking the physical and chemical characteristics of ENMs obtained for research purposes.

3.2. The need to optimize delivered sonication energy separately for each metal oxide nanopowder

The goal of optimizing the delivered sonication energy (DSE) was to “achieve a suspension with the smallest possible agglomerates that are minimally polydisperse and maximally stable over time” in the required media [8, 9]. The results of the optimization experiments showed that each metal oxide ENM required a different combination of sonication time and power (% amplitude) resulting in a variety of values for optimized DSE (J/mL) as shown in Table 1. The polydispersity index (PDI) and mean hydrodynamic diameter (Dh, nm) were measured after optimization. After sonication, less than 6% of particles occurred as agglomerates exceeding 1 µm Dh, for all studied ENMs. It was observed that some metal oxide ENMs (e.g. Al$_2$O$_3$, Fe$_2$O$_3$, CeO$_2$) tended to retain high polydispersity, even after
It was also observed that ZnO suspensions were harder to stabilize and consequently measure by DLS; the lower stability might be related to dissolution processes, evidenced by the decrease in Dh from 256±11 nm (0h) to 206±5 nm after 24h. Lower stability of the ZnO dispersion was also evidenced by zeta potential measurements (23.1±0.4; data not shown). Previous research also found that ZnO dispersions do not maintain their particle size [19] and electrostatic stability, attributed to possible formation of “unstable colloidal particles of Zn(OH)2(aq)” at pH between 7 and 12 [20].

Table 1. The delivered sonication energy (DSE, J/mL) necessary to obtain a stable stock dispersion in deionized water was optimized separately for each of the eight metal oxide ENMs. The results represent the mean and standard deviation (SD) for three replicate dispersions for each metal oxide, with each replicate measured in triplicate.\(^a\)

| ENM            | SSA (m\(^2\)/g) | DSE (J/mL) | PDI (SD) | Dh (SD) (nm) |
|----------------|-----------------|------------|----------|--------------|
| CuO (28 nm)   | 33              | 24         | 0.17 (0.02) | 186 (1.8)   |
| TiO\(_2\) (19/37 nm anatase/rutile) | 55.6 | 555 | 0.16 (0.01) | 157 (3.2) |
| ZnO (35-45 nm) | ~65             | 269        | 0.34 (0.01) | 256 (11.3) |
| γ-Al\(_2\)O\(_3\) (<50 nm) | >40        | 833        | 0.28 (0.06) | 295 (13.4) |
| NiO (15-35 nm) | 50-100          | 319        | 0.37 (0.02) | 165 (6.1)  |
| CeO\(_2\) (10-30 nm) | 30-50 | 319 | 0.36 (0.03) | 222 (5.9) |
| MnO\(_2\) (40-60 nm)\(^b\) | ~13.5 | 96 | 0.20 (0.01) | 146 (3.6) |
| α-Fe\(_2\)O\(_3\) (30 nm) | 20-60 | 278 | 0.37 (0.04) | 165 (7.6) |

\(^a\)PDI=polydispersity index and Dh=mean hydrodynamic diameter (nm) were both measured using DLS; SSA=specific surface area (m\(^2\)/g) provided by manufacturer. \(^b\)sold as Mn\(_2\)O\(_3\) by the manufacturer.

It was noted that the optimized DSE values in Table 1 are equipment dependent: even a small change (such as replacing the flat tip of the sonicator horn) may influence the outcome of optimization. In addition, lot-to-lot variability may influence the dispersion stability, as was observed in the case of nano-CuO (Aldrich catalogue # 544868). Results from the optimization exercise (Table 2) indicated that three different lots of the same nano-CuO product (same catalogue #; all described as “<50 nm”) required three different DSE values. Further investigation revealed that each lot was characterized by a different particle size (Table 2), as indicated on the certificate of analysis provided for each lot on the manufacturer’s website. These findings, that the sonication protocol needs to be optimized for each ENM and each set of equipment, indicate that optimized DSE values reported for metal oxide ENMs (such as Table 1) should be regarded only as a starting point.

Table 2. Variation of required delivered sonication energy (DSE) necessary to disperse CuO nanopowder (<50nm) in deionized water, for different lots of the same product catalogue number (Aldrich # 544868). The results represent the mean and standard deviation (SD) for three replicate dispersions for each metal oxide, with each replicate measured in triplicate (except for CuO(3)).

| ENM   | Average Particle Size | SSA (m\(^2\)/g) | Lot           | DSE (J/mL) | PDI | Dh (SD) (nm) |
|-------|-----------------------|-----------------|---------------|------------|-----|--------------|
| CuO(1) | <50nm                 | 25 - 40         | MKBH9047V     | 384        | 0.32 (0.02) | 365 (93)     |
| CuO(2) | 28 nm                 | 33              | MKAA0633      | 24         | 0.16 (0.02) | 186 (2)      |
| CuO(3) | 40nm                  | 25 - 40         | MKBT8894V     | 72         | 0.34 | 289          |
3.3. Evaluation of methods for separating dissolved metal fraction from dispersed nanoparticles.

Figure 2 summarizes the comparison of methods for separating dissolved Cu from CuO nanoparticles in DMEM+FBS dispersions using centrifugation alone versus the Amicon filter device (Figure 2a) or the Vivaspin filter device (Figure 2b). These results show that the concentration of dissolved Cu obtained using either filtration device was significantly lower (p<0.001) than that obtained using centrifugation, indicating the need for caution when selecting separation methods. Dissolved Cu recovery in DMEM+FBS after Amicon filtration (3.4 ± 0.31 mg/L) was only about 42% of the recovery after 60 min centrifugation (8.35 ± 0.32 mg/L; Figure 2a). Dissolved Cu recovery in DMEM+FBS Vivaspin filtrate (2.66 ± 0.05 mg/L Cu) was only about 45% of that recovered using 60 min centrifugation (5.87 ± 0.06 mg/L Cu; Figure 2b). Similarly poor recoveries were observed using DIW dispersions (not shown in Figure 2), and it was also noted that less filtrate was collected with DMEM+FBS (3 mL) than with DIW (4.5-5 mL) using the Vivaspin device.

The results in Figure 2a indicate no significant difference (p>0.05) in dissolved Cu concentration after 30, 60 or 90 min centrifugation, indicating that there was no advantage in repeating the centrifugation step. Figure 2b confirms these results, showing no difference in dissolved Cu concentration in the DMEM+FBS centrifugate after 30 min (5.98 ± 0.07 mg/L Cu) compared to 60 min (5.87 ± 0.06 mg/L Cu). This observation contrasts with results using DIW as the dispersion medium (data not shown): that is, a 30 min centrifugation was insufficient for a DIW dispersion as indicated by a significantly higher Cu concentration (5.79 ± 0.81 mg/L) compared to that obtained after 60 min centrifugation (3.01 ± 0.003 mg/L) and 90 min centrifugation (2.98 ± 0.02 mg/L).

Figure 2. Effectiveness of separating dissolved Cu from nano-CuO dispersion (200 mg/L) in cell culture medium (DMEM+FBS) using (a) Amicon filtration compared to centrifugation and (b) Vivaspin filtration compared to centrifugation. Error bars represent the standard deviation (SD) of the mean of three replicate dispersions. (Variations from 6-8 mg/L dissolved CuO in centrifugate, as shown for different batches of cell culture medium prepared at different times, are within uncertainty of solubility measurements).

Figure 3 shows DLS characterization of non-ENM components of the above DMEM+FBS centrifugates and filtrates (containing dissolved Cu as in Figure 2). These measurements indicated that Dh (nm) values in the Amicon filtrate were about half those obtained from centrifugation alone (Figure 3a). Similar results were obtained for the Vivaspin filtrate (Figure 3b).
Figure 3. Hydrodynamic diameter (Dh, nm) measurements of DMEM+FBS centrifugate containing dissolved Cu after separation (a) using Amicon filtration compared to centrifugation alone, and (b) using Vivaspin filtration compared to centrifugation alone. Error bars represent the standard deviation (SD) of the mean of three replicate dispersions.

The cause for incomplete dissolved Cu recovery observed with filter devices (Figures 2 and 3) may be related to the tendency of the filter membrane to retain larger colloidal components of the medium (e.g. Cu ions bound to proteins), as reported in previous studies [12]. This tendency was confirmed using DLS measurements of blank DMEM+FBS (Figure 4). Particle size measurements of blank medium that was not subjected to any separation method or was simply centrifuged (Dh = 13-16 nm; Figure 4) were similar to those of the centrifugation filtrate containing dissolved Cu (Dh = 13-16 nm; Figure 3). In contrast, Vivaspin filtration significantly affected the average particle size of the biomolecules observed in the blank DMEM+FBS (Figure 4): average Dh measurements of the Vivaspin blank filtrates (2-3 nm) were only a fraction of Dh measurements following centrifugation alone (13-15 nm).

Figure 4. Hydrodynamic diameter (Dh, nm) measurements of blank DMEM+FBS after no separation, centrifugation alone, and Vivaspin filtration. Error bars represent the standard deviation (SD) of the mean of three replicate dispersions.

Several lines of evidence from the above experiments indicated that the Amicon/Vivaspin ultrafiltration systems introduced an analytical artefact causing some dissolved Cu to be retained on the filter membrane. The results shown in Figure 2 indicated that about 55-58% of the dissolved Cu was retained by the filtration devices, confirmed using a combination of DLS and ICP-OES measurements. Filtration even affected the average particle size of the biomolecules observed in the blank cell culture medium (Figure 4).
Further research would assist in identifying the precise mechanism(s) causing retention on the ultrafiltration membrane, which may be related to complexation of dissolved Cu by specific media components [12] and/or adsorption of Cu ions by the membrane itself [17]. With the aim of investigating potential artefacts associated with ultrafiltration, a spike recovery experiment was conducted using 10-100 µg/L solutions of Cu in DIW, comparing the two ultrafiltration devices (Amicon and Vivaspin) with separation by centrifugation alone. Spike solutions of soluble Cu (n=3) were prepared in DIW and incubated for 2h (37°C, 100 rpm shaking); after 2h an aliquot from each replicate was subjected to Amicon and Vivaspin centrifugal separations, and the rest of sample was centrifuged at 20000G (2x30 min). A third aliquot was used without any separation, for reference. The results (not shown) indicated that both filter devices retained dissolved Cu to varying degrees, resulting in lower Cu recoveries than with centrifugation alone. For the 10 µg/L Cu spike, recoveries were in the following order: 14% Amicon < 29% Vivaspin < 95% centrifugation at 20000G (2x30 min). For the 100 µg/L Cu spike, recoveries were: 26% Amicon < 74% Vivaspin < 103% centrifugation at 20000G (2x30 min). As these results suggested that spike recoveries may be concentration dependent, it is possible that ultrafiltration may be useful at higher concentrations than used in the present study. At the concentration ranges used in these separation experiments, centrifugation at 20000G (60 min) was the most effective method for separating dissolved Cu from both cell culture medium and DIW. Although no advantage was observed using longer centrifugation (90 min vs 60 min) at 20000G for Cu in the present study, it was noted that longer centrifugation times did prove to be effective for separation of dissolved forms of certain other metal oxide ENMs (e.g. TiO₂, Al₂O₃).

4. Conclusions
This study confirmed that the critical DSE is ENM-specific, with each of the eight studied metal oxide ENMs requiring individual optimization of the sonication method. Even different lots of the same product catalogue number demonstrated differences in optimized DSE values (e.g. ranging from 24 to 384 J/min for different lots of CuO nanopowder). Thus, analysts would be advised to optimize the sonication settings for each new ENM, using the recommended value only as a starting point.

For the metal oxide concentrations used in the present study, it was concluded that centrifugation at 20000G (60 or 90 min) was more effective, and less prone to artefacts, than using commercially available filtration devices for separation of dissolved metal ions from dispersed nanoparticles. While commercially available filtration devices have many applications and are easy to use, analysts would be advised to check for adsorption of metal ions of interest on the ultrafiltration membrane.

The importance of double-checking the identity and purity of purchased ENMs was underscored by the finding that two out of the eight metal oxide ENMs obtained for this project did not meet the manufacturers’ claims.

5. References
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