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Synthesis of isotopically modified ZnO nanoparticles and their potential as nanotoxicity tracers

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ZnO nanoparticles with distinct isotopic composition can be tailor synthesized to be used as tracers of environmental fate and uptake by organisms.

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ABSTRACT

Understanding the behavior of engineered nanoparticles in the environment and within organisms is perhaps the biggest obstacle to the safe development of nanotechnologies. Reliable tracing is a particular issue for nanoparticles such as ZnO, because Zn is an essential element and a common pollutant thus present at elevated background concentrations. We synthesized isotopically enriched (89.6%) with a rare isotope of Zn (^{67}Zn) ZnO nanoparticles and measured the uptake of ^{67}Zn by *L. stagnalis* exposed to diatoms amended with the particles. Stable isotope technique is sufficiently sensitive to determine the uptake of Zn at an exposure equivalent to lower concentration range (<15 μg g^{-1}). Without a tracer, detection of newly accumulated Zn is significant at Zn exposure concentration only above 5000 μg g^{-1} which represents some of the most contaminated Zn conditions. Only by using a tracer we can study Zn uptake at a range of environmentally realistic exposure conditions.

1. Introduction

It is increasingly recognized that the environmental implications of nanotechnologies should be understood better before further technological development takes place. Quantities that reach the environment, the fate, bioavailability and toxicity will determine the environmental implications of specific nanomaterials. In addition to potential novel forms of toxicity unique to nanosized particles, metal-bearing nanoparticles also cause concern because of the potential toxicity of their dissolved components.

Zinc oxide (ZnO) is an important material for nanotechnology and increasingly employed in commercial products, notably in sunscreens (Nohynek et al., 2007) but also in the photocatalytic degradation of organic contaminants for applications in water remediation (Comparelli et al., 2004), bioimaging (Kachynski et al., 2008) and as gas sensors for detecting pollutants, toxic gases, alcohol, and food freshness (Tang et al., 2006; Ristoscu et al., 2007). A number of nano-ZnO consumer products exist (see for example the Project on Emerging Nanotechnologies' Nanomaterial Inventory: http://www.nanotechproject.org/inventories/consumer), and since reference to particle size is not required in product labels, perhaps many more are purchased by consumers unknowingly. A wide range of applications can potentially lead to a large-scale manufacturing of nanoparticulate ZnO for both industrial and domestic use and likely accidental or end of life-cycle environmental releases.

Recent research has been directed towards the potential toxicity of ZnO as well as other metal oxide nanoparticles and nanomaterials in general (Limbach et al., 2005; Oberdörster et al., 2005; Adams et al., 2006; Bruner et al., 2006; Nel et al., 2006; Franklin et al., 2007; Kasemets et al., 2009). Although a rich body of literature is now available, we are still surprisingly uncertain as to whether engineered nanoparticles are inherently toxic or not. This is to a large extent due to inconsistent results in the literature. For example, CeO_2 nanoparticles have been described as promising neuro-protectants (Das et al., 2007) but in other studies reported as toxic to bacteria and mammalian cells (e.g. Bruner et al., 2006). Jang and Kim (2007) showed toxicity of TiO_2 to be dependant on a crystalline phase with anatase indicated as more cytotoxic then rutile while no such effects was observed by Simon-Deckers et al. (2009).

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Research on the environmental impact of engineered nanoparticles is still hampered by a lack of reliable tools to detect, visualize and quantitatively trace particle movement and transfer in complex environmental and biological systems. Labeling of particles to make them distinctive and thus easily detectable is one way to resolve this problem. Various approaches have been developed including radioactive with gamma or beta emitters labeling (Oughton et al., 2008; Petersen et al., 2008; Hong et al., 2009) and the use of various types of dyes for fluorescence microscopy imaging (Kirchner et al., 2005). Each of the above labeling strategies requires post-synthesis manipulation of the particles to introduce the label and modification of the surface properties of the particles in the case of fluorescent dyes. The use of gamma-emitting radioisotope tracers is limited to specific elements and to licensed laboratories (given the handling and disposal hazards associated with radioactivity). Such issues mean that only a limited number of relevant studies have been undertaken and that radiolabelled particles could never be used as tracers in real scenarios.

Work with metal ions shows that the drawbacks inherent to using radioactive or surface modified tracers can be avoided by using stable isotope tracers (Croteau et al., 2004), which allow quantifying uptake and loss rates for most metals (since most elements have 2 or more stable isotopes), and discriminating background from added amount of tracer. The latter is particularly important while working with elements such as Zn present in high abundance in natural environment.

Zinc is a ubiquitous element in soils and sediments with concentrations ranging from few to a few hundred $\mu g\ g^{-1}$ (Adriano, 2001). Large quantities of Zn are also released to the environment due to industrial activities (e.g. application of sewage sludge on soils or atmospheric emissions) which adds to the natural background concentration (McGrath, 1997). Although potentially toxic at high doses (Luoma and Rainbow, 2008), Zn is an essential element to organisms, i.e. it is required for the functioning of the metabolism. White and Rainbow (1985) estimated that 35 $\mu g\ g^{-1}$ of Zn is essential to fulfill the enzyme requirements of marine molluscs and crustaceans. Background Zn concentrations in biological tissues are thus high compared to non-essential elements such as cadmium or silver. In animal tissues, zinc concentrations can range from 40 to several thousand $\mu g\ g^{-1}$ (Phillips and Rainbow, 1988). The ubiquitous presence of Zn in the environment limits the ability to study its bioaccumulation dynamics upon short time exposures to environmentally realistic concentrations (Luoma and Rainbow, 2005). However, these limitations can be overcome by using stable isotope labelled nanoparticles.

Gulson and Wong (2006) suggested that stable isotopes tracing may prove valuable for monitoring nanoparticles in the field and addressing emerging research questions but neither has yet been demonstrated. Beside the costs, there are several technical challenges in creating stable isotope labelled nanoparticles and tracing their fate in biota including: 1) availability of suitable isotopically enriched precursor for the synthesis of the material 2) quantitative measurement of the newly accumulated metal, distinguishing that from background concentrations, particularly while working at low exposure concentrations with essential metals like Zn. 3) detection of the tracer in the animal tissue following short time exposures at environmentally realistic concentrations.

In this work we demonstrate, for the first time, that it is possible to synthesize isotopically modified ZnO nanoparticles that satisfy all these conditions and are therefore suitable as biological/environmental nanotoxicity tracers. We further show that bioaccumulation experiments using Zn (dissolved or as ZnO nanoparticles) require a tracer such as an enriched stable isotope of Zn (Croteau et al., 2004) because the high background Zn concentrations limit the ability to detect bioaccumulation over short time exposures at environmentally realistic concentrations.

1.1. Nanotoxicity standard

Although a wide range of synthesis protocols for ZnO nanoparticles are available in the literature, many are not suitable for a nanotoxicity standard. This is because many protocols focus on the synthesis for specific industrial applications but are lacking considerations for the fundamental principles of nanotoxicity standard which are (see also Valsami-Jones et al., 2008): 1) Non-toxic starting materials; in numerous protocols toxic chemicals such as amines, tri-octylphosphineoxide (TOPO), surfactants (e.g. hexadecyltrimethylammonium bromide HTAB or cetyl trimethylammonium bromide CTAB) are used 2) Aqueous synthesis; particles synthesized in media such as alcohols are unstable when re-suspended in biological/environmental (i.e. aqueous) media 3) Tuneable size range protocols; in order to demonstrate size related toxicity the protocols need to be flexible enough to generate a suitable range of sizes for testing.

Creation of stable isotope labelled particles requires some additional considerations to be satisfied. Specifically, the starting materials for synthesis must be available in an isotopically labelled form. For example, in the case of nano-ZnO, a commonly used precursor for its synthesis is Zn acetate. Isotopically enriched Zn is however available mainly as metal or metal oxide. Custom synthesis in another chemical form is prohibitively expensive for routine use. Such cost can be a major obstacle to the wide use of the materials as standards, so in this work we attempted to find alternative low cost approaches. It should be noted that isotopically labeled ZnO is available for purchase at a reasonable cost. However, this is supplied as a micron sized bulk material which may potentially be transformed to nanoparticles by milling, but this produces polydisperse sample and is limited in the size range it can generate.

The present work had the following objectives: optimization of the ZnO synthesis method based on the criteria described above; adaptation of the optimum synthesis method to isotopically modified materials and finally, testing of the labelled materials for their efficiency as tracers in biological experiments.

2. Materials and methods

2.1. Synthesis of isotopically enriched ZnO nanoparticles (^{67}ZnO-NP)

ZnO nanoparticles were first synthesized from non-isotopically modified Zn metal powder (AnalaR, BDH Chemicals, CAS 7740-66-6) to optimize the synthesis conditions (reaction temperature and time, precursor concentration). The optimized protocol was then followed while working with isotopically enriched metal powder (enrichment level of ^{67}Zn 89.6%, purchased from Isoflex, Moscow, Russia).

Briefly, the metal powder (500 mg) was heated with acetic acid (100%, 50 ml, AnalaR, BDH Laboratory Supplies) at 80 °C for 3 days, which yielded a Zn acetate precursor (ZAP, approximately 1 g). After 48 h of drying at 50 °C, ZAP was transformed into ZnO nanoparticles via either thermal decomposition or forced hydrolysis in diethylene glycol (DEG, ReagentPlus 99%, Sigma Aldrich) using a protocol modified from Feldmann and Jungk (2001).

2.1.1. Thermal decomposition

The Zn acetate precursor was decomposed in air in porcelain crucibles placed in a pre-heated Griffin electric furnace. Following decomposition, the samples were cooled to room temperature in a dessicator. We tested both the effect of changing the temperature (150–800 °C) and time (5 min – 56 h) on the transformation of the precursor into the final product (ZnO) and on the particle size of the final product. Isotopically modified particles were prepared by thermal decomposition (in air) at 300 °C for 15 min. Such prepared sample was washed 3 times with deionised water (DIW) and dried in the oven at 50 °C for 24 h. There were large losses of material via thermal decomposition synthesis route with reaction yield of about 25 % only. From approximately 200 mg of the precursor we obtained approximately 25 mg of the final product ZnO via this route.

2.1.2. Hydrolysis

The Zn acetate precursor was first dissolved in DEG at 80 °C and then hydrolyzed (in oil bath) for 1 h at 170 °C following addition of DIW (Millipore, <18 MΩ cm) to provide a molar ratio of water to Zn of 10:1. The following concentrations of the precursor were tested: 500 mg, 300 mg and 100 mg in 50 ml of DEG. Isotopically modified particles were prepared using 100 mg of the precursor hydrolyzed with 100 μL of DIW for 1 h in DEG at 170 °C.

2.1.3. Particle characterization

X-ray diffraction XRD, Enraf-Nonius diffractometer coupled to INEL CPS 120 position-sensitive detector with Cu Kα radiation was used for phase identification with STOE software. High temperature XRD stage Enraf-Nonius FR590 coupled with INEL curved, position-sensitive detector (PSD) within a static beam-sample geometry, fitted with a GeniX system with Xenocs FOX2D CU 10_30P mirror to generate ultra-high brightness Cu Kα was used to study the decomposition of zinc acetate precursor into ZnO. A small portion of the precursor (few mg) was heated on a Pt holder from 50 to 800 °C using 50 °C increment steps with 60 s temperature ramp time and 30 s holding time at selected test temperatures (100, 200, 300 °C etc). The shape and size of the particles were analyzed using Scanning Electron Microscopy (SEM) (Phillips XL30) and Transmission Electron Microscopy (TEM) (Hitachi 7100) operating at an accelerating voltage of 5 kV and 100 kV respectively. ImageJ software was used to measure particle size from SEM images (particles counted manually). Particle size in solution (DIW, 25 °C) was analyzed using Dynamic Light Scattering (DLS) on Malvern Zetasizer Nano instrument (Malvern Instruments Ltd, UK) equipped with He–Ne 633 nm laser. Colloids were prepared for DLS measurement by diluting 100 μL in 10 ml of DIW. Powders were suspended in DIW (1 mg in 10 ml) with the aid of ultrasonicating probe UP100H (100 W, 30 kHz, 30 s). Surface charge of the samples was measured in DIW at 25 °C, pH = 7.4, using the zeta potential module of the Malvern instrument.

2.1.4. Experimental organisms

Freshwater snails (*Lymnaea stagnalis*) were reared in the laboratory in moderately hard water (MOD, hardness of 80–100 mg of $CaCO_3$ L^{-1}; pH 7.0; U.S. EPA, 2002). Three days prior to each experiment, snails of a restricted size range (mean soft tissue dry weight of 6.8 ± 0.6 mg 95% CI, $n = 112$) were transferred to a 10 L glass aquarium filled with MOD water. Food was withheld during this period.

2.1.5. Dietborne exposures to enriched ^{67}Zn

The benthic diatom *Nitzschia palea* was grown axenically for several generations in an S-diatom medium (Irving et al., 2003). Diatoms were harvested onto a 1.2 μm Isopore membrane filter (Millipore) and rinsed with synthetic softwater (SO, hardness of 40–48 mg of $CaCO_3$ L^{-1}; pH 7.0, U.S. EPA, 2002)

To achieve dietborne Zn concentration up to 1000 μg g^{-1}, we used the protocol described by Croteau and Luoma (2009) which involves labeling algae with enriched stable metal isotopes. Dietborne Zn concentrations achieved using this procedure are, however, limited by the algae tolerance to the acute dissolved metal exposure. Briefly, algae were re-suspended into a 20 mL acid-washed glass scintillation vial filled with SO water spiked with different concentrations (10–5000 μg L^{-1}) of a commercially purchased standard, isotopically enriched in ^{67}Zn (94%, Trace Sciences International, Canada) for 24 h. After exposure, labelled-diatoms were harvested onto a 1.2-μm Isopore™ membrane filter (Millipore) and rinsed with SO water. Small sections of the filters (less than 10% of the filter area, which is approximately 17 cm^2) holding the labeled diatoms were sampled and dried for 24 h at 40 °C prior to metal analysis. The remaining filters coated with diatoms labeled with enriched ^{67}Zn were offered as food to *L. stagnalis*.

To achieve dietborne Zn concentrations higher than 1000 μg g^{-1}, we used the protocol described by Croteau et al. (in press) which involves amending diatom mats with isotopically modified nanoparticles. The use of metal enriched nanoparticles allows achieving concentrations much higher than if algae were exposed to dissolved metal (see above).

Briefly, serially diluted suspensions of isotopically modified ZnO nanoparticles made by forced hydrolysis (average size 110 nm ± 11 $n = 100$, surface charge of 24 ± 5 mV) were poured onto algal mats and filtered through under low vacuum (<10 mm Hg) to deposit particles. Small sections of the filters (i.e. less then 10% of the total filter area) holding the diatoms amended with ZnO nanoparticles were sampled and dried for 24 h at 40 °C prior to metal analysis. The remaining filters coated with diatoms mixed with isotopically enriched ^{67}ZnO NPs were offered as food to *L. stagnalis*.

2.1.6. Dietborne uptake experiments

For each treatment, 8–10 acclimated snails were transferred to a feeding chamber made from a 150 ml polypropylene vial that had two 4-cm diameter holes (opposite edges) covered with a 100 μm nylon mesh. Each feeding chamber was acid-washed prior to being partially submerged in 20 L glass tank filled with MOD water as described in Croteau et al. (in press). For each treatment, a filter holding the ^{67}Zn labeled diatoms was laid flat on the bottom of the feeding chamber. Snails were exposed to the labeled food for 3–4 h, which is shorter than food gut residence time (Croteau et al., 2007), thereby minimizing the confounding influences of efflux and isotopes recycling (Luoma and Fisher, 1997). After feeding on the labeled food, snails were removed, rinsed with DIW and allowed to depurate for 48 h in 20 L glass tanks filled with MOD water. Unlabelled lettuce was provided ad libitum during depuration. After depuration the snails were frozen.

2.2. Sample preparation, analysis and calculation of accumulated ^{67}Zn concentrations

Partially thawed *L. stagnalis* were dissected to remove soft tissue, placed individually on a piece of acid-washed Teflon sheeting and allowed to dry at 50 °C for 3 days. Dried snails and diatoms were weighed and digested at room temperature in Teflon vials with concentrated nitric acid (Baker Ultrex II grade, 100 μL mg dry wt $sample^{-1}$) for 5–7 days. Hydrogen peroxide (Baker Ultrex II grade, 40 μL mg dry wt $sample^{-1}$) was added prior to final dilution with DIW. Similar weight samples of the certified reference material NIST-2976 (mussel tissue from National Institute of Standards and Technology) were submitted to the same digestion procedure during each analytical run.

Water and digested samples were analyzed for the naturally occurring stable isotopes of Zn (^{64}Zn, ^{66}Zn, ^{67}Zn, ^{68}Zn and ^{70}Zn) by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) as described in Croteau et al. (2004). To account for instrument drift and change in sensitivity, internal standardization was performed by addition of germanium (^{74}Ge) to all samples and standards, but the calibration blanks. We also re-analyzed one of our standards after every 10 samples. Deviations from standard value were in general less than 5% for the analyzed isotopes.

We used an isotope tracing technique that allows tracking newly accumulated tracers independently from background levels (Croteau et al., 2004). Briefly, the relative abundance of ^{67}Zn tracer (i.e. p^{67}) is determined using the signal intensities of each isotope in the calibration standards i.e.,

$$p^{67} = \text{Intensity}\left(\frac{^{67}Zn}{^{64}Zn + ^{66}Zn + ^{67}Zn + ^{68}Zn + ^{70}Zn}\right)_{\text{standard}} \quad (1)$$

Concentrations of tracer in the experimental organisms ($[^{67}Zn]_{\hat{e}}$) are calculated as the product of p^{67} and the total metal concentrations inferred by the ICP-MS software from tracer intensity ($[T^{67}Zn]$), i.e.,

$$[^{67}Zn]_{\hat{e}} = p^{67} \times \left[T^{67}Zn\right] \quad (2)$$

The original load of tracer ($[^{67}Zn]^{0}_{\hat{e}}$) that occurred in each sample in the absence of a spike is calculated as the product of p^{67} and the total metal concentrations inferred from the intensity of the most abundant isotope that is minimally affected by isobaric and polyatomic interferences (Croteau et al., 2007) (e.g. ^{66}Zn), i.e.,

$$[^{67}Zn]^{0}_{\hat{e}} = p^{67} \times \left[T^{66}Zn\right] \quad (3)$$

The net tracer uptake ($\Delta[^{67}Zn]_{\hat{e}}$) is derived from the total experimental metal concen tration ($[^{67}Zn]_{\hat{e}}$, equation (2)) minus the pre-existing concentration of tracer ($[^{67}Zn]^{0}_{\hat{e}}$, equation (3)).

$$\Delta[^{67}Zn]_{\hat{e}} = p^{67} \times \left(\left[T^{67}Zn\right] - \left[T^{66}Zn\right]\right) \quad (4)$$

3. Results and discussion

3.1. ZnO synthesis optimization with non-isotopically modified material

Starting with a Zn metal powder, a Zn acetate "in house" precursor (ZAP) was synthesized by reacting the powder with concentrated acetic acid. Two different routes were then used to transform the precursor into ZnO nanoparticles: 1) thermal decomposition and 2) forced hydrolysis (wet synthesis route). The thermal decomposition of various zinc salts provides a simple route for the synthesis of nanosized ZnO (Yang et al., 2004; Maensiri et al., 2006; Lin and Li, 2009). Zinc acetate is often chosen as a precursor due to its relatively low decomposition temperature (<300 °C). Based on the thermal properties of zinc acetate (Lin and Li, 2009) temperatures varying from 150 to 800 °C were selected for decomposition of our in house synthesized precursor.

High temperature XRD stage was used to study the transformation and changes in crystallinity and particle size of the precursor as a function of temperature (Fig. 1A). Zinc oxide peaks were observed to emerge on the pattern as the temperature reached 250–300 °C indicating the onset of the precursor transformation. As the temperature increased ZnO peaks became

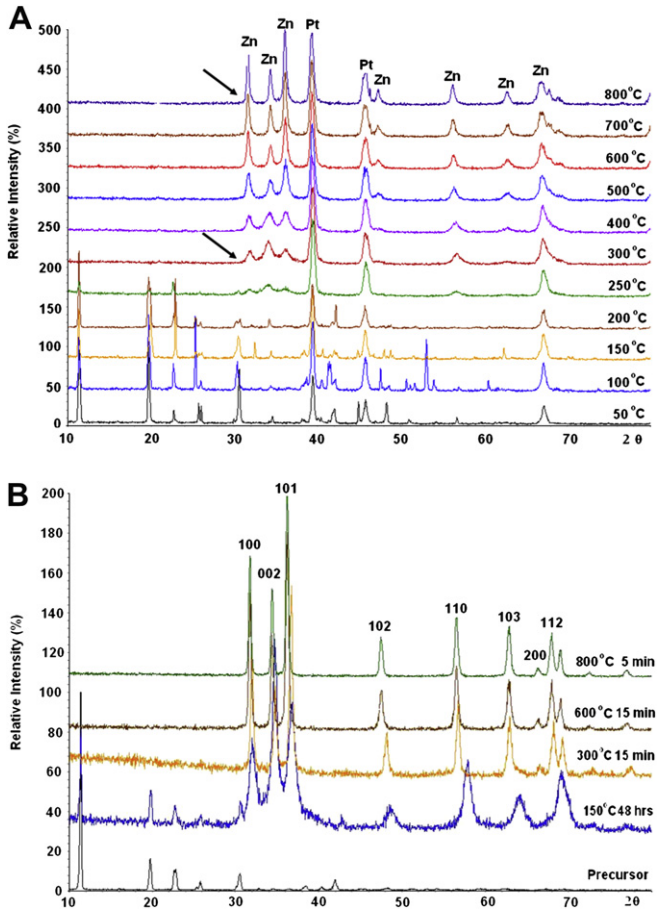

Fig. 1. XRD patterns showing transformation of in house synthesized Zn acetate precursor into ZnO after thermal decomposition at a range of temperatures. A. Sample heated from 50 to 800 °C on a platinum holder of high temperature stage XRD. ZnO and Pt peaks marked. ZnO peaks emerge on the pattern as the temperature reached 250–300 °C and increase in sharpness as the temperature increases indicating crystallite size increasing. B. Sample heated from 150 to 800 °C in porcelain crucibles in the electric furnace. The transformation time is inversely related to the temperature; ZnO formation at 150 °C was observed after hours of heating while it took only minutes for complete transformation at 800 °C.

sharper indicating an increase in crystallinity as well as in crystallite size of such obtained ZnO.

To decide on the optimum temperature for thermal decomposition, particle size distribution in samples heated at selected temperatures was measured using DLS. At temperatures 150 and 800 °C, DLS indicated the formation of large sedimenting particles, for which it was not possible to obtain stable readings. The sample heated at 600 °C showed a bimodal distribution with peaks at 130 nm and 720 nm whilst sample heated at 300 °C appeared to be optimum, showing a monomodal particle size distribution with a peak at 190 nm.

Based on the XRD data (Fig. 1A and B) and on the particle size measurements (SEM and DLS) of the powders decomposed at varying temperatures (Fig. 2A), 300 °C was selected as an optimal decomposition temperature to obtain pure ZnO with the smallest and most uniform particle size distribution (200–300 nm average agglomerates size from DLS).

Although thermal decomposition is a simple route to produce ZnO, the particles agglomerate before dispersing in any media and are also relatively polydisperse in both size and shape (Fig. 2A). Wet synthesis routes on the other hand lead usually to monodispersed samples and offer better possibilities of tuning the size and/or shape within one synthesis protocol. Based on the literature survey and laboratory trials of several published protocols, forced hydrolysis in DEG was chosen as a simple protocol, and repeatedly providing pure phase monodispersed ZnO colloidal suspensions (Feldmann, 2003; Bryner et al., 2006). By experimenting with the in house synthesized Zn acetate precursor we tuned the size of ZnO obtained via this method in the range from 150 nm to 550 nm (Fig. 2B) by changing the precursor concentration (from 100 to 500 mg in 50 ml). The smallest particles were obtained at the lowest precursor concentration. The TEM images of individual particles (in particular for the smallest size obtained) indicated possible formation of the particles by coalescence of much smaller, sub-10 nm, crystals.

3.2. Synthesis of isotopically modified ZnO nanoparticles

Once the synthesis conditions were optimized, we repeated the above experiments with isotopically modified Zn metal as a starting material and two sets of isotopically modified ZnO particles were generated: 1) in a powder form following thermal decomposition of the precursor at 300 °C and for 15 min 2) in a colloidal suspension form following forced hydrolysis in DEG (100 mg of precursor in 50 ml DEG). The characterization data for both samples are shown in Fig. 3A (SEM and TEM and 3B (XRD and DLS).

The XRD patterns (see Fig. 3B) confirmed the presence of pure phase ZnO in both powder and colloid sample. In the latter, a large scattering peak was observed at approximately $2\theta = 22.4$ which can be attributed to the polymer in which the particles were suspended as: 1) the same peak was observed on the pattern from the polymer sample only and 2) the intensity of this peak was observed to decrease as the colloid sample was pre-concentrated by washing and thus polymer was removed.

Sample 1 (powder) was agglomerated with individual particles varying in size from 20 nm to 70 nm (TEM) and also of variable shape (i.e. spherical, trigonal, rods). The average size of agglomerates, as indicated by DLS measurements, was 245 nm in this sample. Sample 2 (colloid) was monodisperse in size and shape with highly symmetrical particles of average size of 125 nm as from DLS measurements and 110 ± 11 nm ($n = 100$) from SEM. Surface charge was measured for both samples and was negative (-17.7 ± 0.64 mV) for sample 1 (powder) and positive (23.6 ± 5.17 mV) for sample 2 (colloid). The difference in surface charge between the powder and colloid is likely to be due to changes in the surface properties of the powder (surface speciation) as a result of the process of drying and dehydration.

The stability of the colloidal suspension (sample 2) was followed for 4 months by taking DLS measurements over time. There was no indication of a significant particle growth or agglomeration over time but the polydispersity index increased from 0.054 to 0.174. Changes over time are an important consideration because environmentally realistic eco-toxicological tests often necessitate exposures for long periods (e.g. weeks) and/or storage of stocks before use.

The isotopically labeled ZnO particles described in this study were slightly larger then the nominal definition of a nanoparticle (e.g. NNI 2010, http://www.nano.gov/html/facts/faqs.html). However, there is evidence in the literature that particles above this nanosize regime (i.e. above 100 nm) may also present some potential hazard as can also migrate into the blood circulation and thus be transported to other organs in the body (e.g. Rothen-Rutishauser et al., 2006; Furuyama et al., 2009).

3.3. Tracing the biological fate of isotopically modified ZnO

We conducted experiments to determine whether ^{67}Zn enriched nanoparticles could be detected in animal tissues after

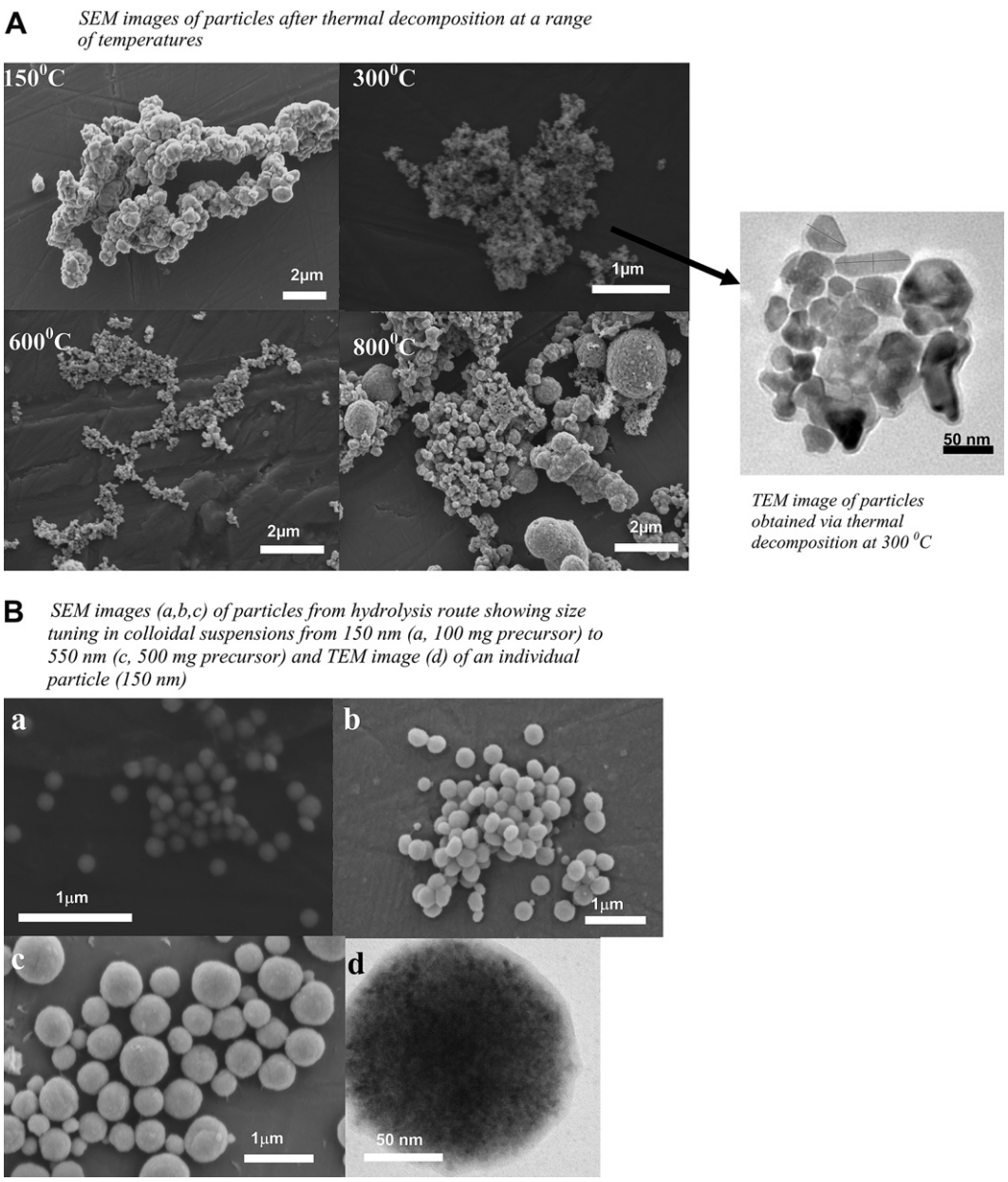

Fig. 2. Size and shape of particles synthesized from non-isotopically modified precursor via thermal decomposition (A) and hydrolysis (B).

short dietary exposures. To do this we fed freshwater snails (*L. stagnalis)* diatoms mixed with different amounts of the synthesized ^{67}ZnO nanoparticles (made by forced hydrolysis) and then fed them unlabelled food to clear their gut. The ^{67}Zn retained after gut clearance (48 h as defined by Croteau et al., 2007) was used to define assimilation. We also fed snails diatoms labelled with ^{67}Zn to show how a tracer can improve experimental sensitivity, thereby overcoming the limitations imposed by the high natural concentrations of total Zn in animals and the environment. Fig. 4 shows that Zn concentration as low as 1 μg g^{-1} can be detected in freshwater snails exposed to about 15 μg g^{-1} of dietborne Zn when an enriched stable isotope of Zn is used as a tracer (^{67}Zn). In contrast, exposures 333-times higher are required to detect Zn uptake if no tracer is used. That is, detection is significant at Zn exposure concentration only above about 5000 μg g^{-1} if no tracer is used. Thus, the enriched stable isotope technique is sufficiently sensitive to determine the uptake rate of Zn in *L. stagnalis* at an exposure equivalent to the lowest concentrations of Zn that might be expected in environmental media. Without the tracer, uptake rates can only be determined at concentrations equivalent to some of the most contaminated Zn conditions (or much longer exposure times), reducing the feasibility of studying a range of environmentally realistic exposure conditions. A companion paper (Croteau et al., in press) presents detailed experiments addressing the questions whether Zn in ZnO nanoparticles is bioavailable and toxic using the isotopically modified ZnO nanoparticles described here.

Additional experimental work is required to specifically characterize Zn partitioning within the tissues since Zn from the ZnO might have solubilized in the gut of the snail. The precise identification of accumulated metal species in biological tissues (i.e. speciation) is, however, difficult to determine due to their ease of

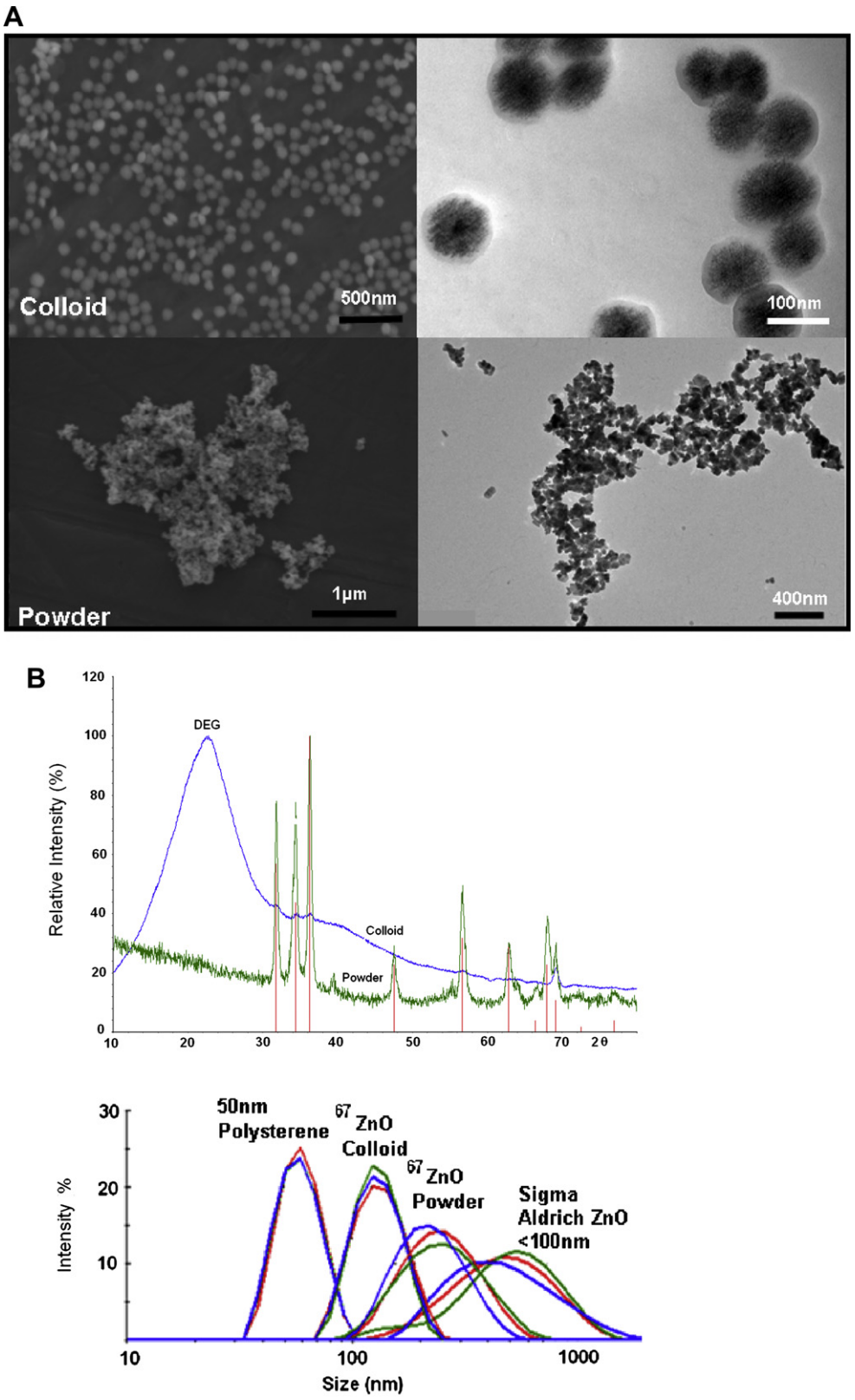

Fig. 3. A) SEM and TEM images of ^{67}ZnO samples obtained by hydrolysis of the synthesized isotopically modified precursor (colloid) and its thermal decomposition product (powder) and B) XRD patterns of the same two samples and DLS size distribution data of the samples (in DIW) and also showing a 50 nm polysterene particle size standard (Duke Scientific) and a commercial sample of ZnO nanoparticles (Sigma Aldrich) for comparison.

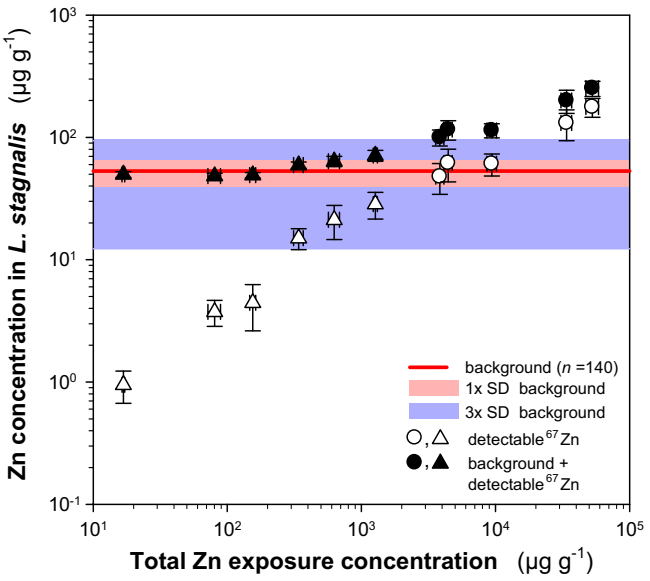

Fig. 4. Zinc concentrations in snails exposed for 3–4 h to dietborne ^{67}Zn. The red line across the exposure concentrations displays the mean background Zn concentrations (total Zn) measured in 140 snails. The shaded areas represent the error surrounding the background Zn concentration; 1× (pink) and 3× (blue) the SD of the mean. The open symbols represent the detectable ^{67}Zn (newly accumulated Zn) after each exposure i.e. diatoms labelled with ^{67}Zn (triangles) and diatoms mixed with ^{67}ZnO nanoparticles (circles). Each concentration was derived from the total measured ^{67}Zn concentration minus background. The closed symbols represent the sum of the detectable Zn and the corresponding background concentrations i.e. diatoms labelled with ^{67}Zn (triangles) and diatoms mixed with ^{67}ZnO nanoparticles (circles). (For the interpretation of the reference to color in this figure legend the reader is referred to the web version of this article.)

transformation under various extraction and analytical procedures. Sub-cellular fractionation procedures combined with synchrotron-based X-ray absorption spectroscopy show promises to study the sub-cellular disposition of metals (Andre et al., 2009) and by extension a metal-bearing nanoparticle and their potential toxicity, and this will be a future direction for our work.

4. Conclusions

This study showed, for the first time, the synthesis of isotopically unique nanoparticles and the advantage of using these in tracing uptake of essential elements such as Zn. The stable isotope tracing approach has applications not only in studying bio-uptake but also in tracing the fate and transport of labelled nanomaterials in soils or sediments. This technique can be extended for tracing the fate of nanoparticles comprised of other elements present in high abundance in the natural environment such as iron or copper. We are currently working on isotopic labeling of CuO nanoparticles.

This work forms the basis for our ongoing efforts to create a collection of suitable reference nanomaterials with distinct properties, including where appropriate isotopic composition, which can be easily traced in both laboratory and fieldwork experiments and thus become standards for nanotoxicology.

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