

The effects of silver nanoparticles on fathead minnow (*Pimephales promelas*) embryos

Geoff Laban · Loring F. Nies · Ronald F. Turco ·
John W. Bickham · Maria S. Sepúlveda

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Abstract Nanoparticles are being used in many commercial applications. We describe the toxicity of two commercial silver (Ag) nanoparticle (NP) products, NanoAmor and Sigma on *Pimephales promelas* embryos. Embryos were exposed to varying concentrations of either sonicated or stirred NP solutions for 96 h. LC₅₀ values for NanoAmor and Sigma Ag NPs were 9.4 and 10.6 mg/L for stirred and 1.25 and 1.36 mg/L for sonicated NPs, respectively. Uptake of Ag NPs into the embryos was observed after 24 h using Transmission Electron Microscopy and Ag NPs induced a concentration-dependent increase in larval abnormalities, mostly edema. Dissolved Ag released from Ag NPs was measured using Inductively Coupled-Mass Spectrometry and the effects tested were found to be three times less toxic when compared to Ag nitrate (AgNO₃). The percentage of dissolved Ag released was inversely proportional to the concentration of Ag NPs with the lowest (0.625 mg/L) and highest (20 mg/L) concentrations tested releasing 3.7 and 0.45% dissolved Ag,

respectively and percent release was similar regardless if concentrations were stirred or sonicated. Thus increased toxicity after sonication cannot be solely explained by dissolved Ag. We conclude that both dissolved and particulate forms of Ag elicited toxicity to fish embryos.

Keywords Ecotoxicology · Fish early life stages · Nanoparticles · Silver

Introduction

Nanomaterials are part of a commercial revolution that has resulted in an explosion of hundreds of novel products due to their diverse physico-chemical properties, enabling their usage in a wide range of innovative applications (Salata 2004; Gwinn and Vallyathan 2006). Nanotechnology is generally defined as the controlled modification of particles or materials resulting in at least one dimension between 1 and 100 nm (Roco 2005). However, toxicological studies have included particles and aggregates that are as large as several hundred nanometers (Handy and Shaw 2007). Nevertheless, the driving force behind this new technology is manufacturing control that results in increased surface area, and altered conductivity or surface chemistry of the nanoparticles (NPs) that impart unique physical and chemical properties to NPs (Masciangioli and Zhang 2003; Hoet et al. 2004).

Commercialization of nanotechnology is progressing at a much faster rate than the understanding of its potential environmental impact and effects (Maynard et al. 2004; Guzman et al. 2006; Nowack and Bucheli 2007). In fact, the market for products using NPs is expected to be worth US \$1 trillion by 2015 (Roco 2003, 2005). Presently, there are over 600 commercialized products on the market made

G. Laban · J. W. Bickham · M. S. Sepúlveda (✉)
Department of Forestry & Natural Resources, Purdue University,
195 Marsteller St., West Lafayette, IN 47907, USA
e-mail: mssepulv@purdue.edu

L. F. Nies · M. S. Sepúlveda
School of Civil Engineering, Purdue University, 550 Stadium
Mall Dr., West Lafayette, IN 47907, USA

R. F. Turco
Department of Agronomy, Purdue University, 915 W. State St.,
West Lafayette, IN 47907, USA

G. Laban · J. W. Bickham
Center for the Environment, Purdue University, 503
Northwestern Av., West Lafayette, IN 47907, USA

from engineered nanomaterials and silver (Ag) NPs are contained in almost one quarter of these products according to the Woodrow Wilson International Center for Scholars database (<http://www.nanotechproject.org>).

Silver NPs are known for their high antimicrobial properties (Sondi and Salopek-Sondi 2004; Baker et al. 2005; Kim et al. 2007; Lok et al. 2007) and are used in products ranging from surgical instruments, contraceptive devices, bandages, water purificants, paints, laundry detergents, socks, and underwear (Cheng et al. 2004; Lansdown 2006; Cohen et al. 2007; Lee et al. 2007a, b; Chen and Schluesener 2008). While there have been studies exploring the toxicity of metal NPs (Hillyer and Albrecht 2001; Takenaka et al. 2001; Schins 2002; Hostynek 2003; Service 2003; Huang et al. 2008), including Ag (Ji et al. 2007; Kim et al. 2008), currently there is insufficient toxicity and exposure data necessary to fill the knowledge gap for the “source-pathway-receptor-impact” framework (Owen and Handy 2007) necessary for proper risk assessment of Ag NPs (Karn et al. 2003; Dreher 2004).

There is a need for a sensitive but cost effective high-throughput methodology for determining the toxicological effects of NPs in complex biological ecosystems. Of particular concern is the wide commercial use of Ag NPs and the number of products soon to be available as potential sources of Ag NPs to aquatic biosystems. As an example, there is evidence that both ionic Ag and Ag NPs contained in clothing fibers (e.g., socks) could be leached to the environment when washed (Benn and Westerhoff 2008). Presently, there is no data for Ag NP concentrations in the environment but it is estimated that by 2010, as much as 15% of the Ag emitted into European waters will come from biocidal plastics and textiles containing Ag NPs (Blaser et al. 2008). The concept of Ag either leaching or being released into water systems is of particular concern considering the many years of research showing that ionic Ag is highly toxic to various freshwater aquatic species with LC_{50} s ranging from 0.01 to 70 $\mu\text{g/L}$ depending on the species (Nebeker et al. 1983; LeBlanc et al. 1984; Luoma et al. 1995; Hogstrand and Wood 1998; Zhu et al. 2006; Dethloff et al. 2007; Naddy et al. 2007a, b). In addition, Ag ions are known to inhibit Na^+ and Cl^- transport in gills of fish (Morgan et al. 1997). Currently, there is limited knowledge about the possible adverse effects that Ag nanotechnologies can exert to aquatic organisms but there could be a potential for increased exposure to both ionic Ag and Ag NPs because of the rapid development of commercialized nano-products (Luoma 2008).

To evaluate the ecotoxicological effects of metal based NPs, we examined the effects of two commercially available Ag nanoproducts. First we compared the effects of Ag NPs purchased from NanoAmor (35 nm in size) and Sigma (≤ 100 nm in size) on the mortality and phenotypic effects

exerted on exposed fathead minnow (*Pimephales promelas*) embryos. Since Ag NPs are known to release ionic Ag, we measured the amount of total dissolved Ag released by the commercial Ag NPs named above and compared the effects with silver nitrate (AgNO_3) which was used both as a positive control and a proxy for dissolved Ag. We hypothesized that exposure to smaller NanoAmor (35 nm) Ag NPs would result in increased toxicity to the developing embryos compared to larger Sigma (≤ 100 nm) Ag NPs and that sonication would enhance toxicity. We also investigated the uptake of Ag NPs by embryos. We hope that the in vivo protocol described here will be one that could be used to develop a rapid, sensitive method for high-throughput analyses of NP toxicity using a vertebrate organism. In addition, testing the effects of commercial products that are currently used and can possibly end up in aquatic waters would greatly assist in future risk assessments studies.

Materials and methods

Particle characterization

Two types of commercially available Ag NPs were utilized in these studies: commercially described as 35 nm in size (NanoAmor, Houston, TX) and ≤ 100 nm in size (Sigma-Aldrich, St. Louis, MO) both 99.99% purity. Stock solutions (1,000 mg/L) of the two groups of NPs were prepared in ultrapure MilliporeTM water and then sonicated at 15 kHz for 1.5 h in a water bath sonicator (Branson Ultrasonics, 2510 Ultra sonic cleaner, Kimco Distributing Corp, Mentor, OH). Particle sizes were confirmed by placing a drop of the homogenous suspension in a Transmission Electron Microscopy (TEM) copper grid with a lacy carbon film and viewed using a Titan 80–300 KeV Field-Emission Environmental TEM from FEI Corporation. For each NP, a series of digital images were taken before exposure and at the end of the exposure period (96 h) and later analyzed (by measuring a total of 50 particles per image) using Sigma Scan 5.0 (HALLoGRAM Publishing, Aurora, CO). TEM images were also taken from sonicated and stirred treatments to compare the sizes of agglomeration before and after 96 h.

Toxicity testing of Ag NPs and dissolved Ag

A reproductive colony of fathead minnows was established at the Baker Aquatic Research Laboratory, Purdue University (West Lafayette, IN) from breeding adult fathead minnows (aged 6–12 m) obtained from the US Environmental Protection Agency (USEPA, Duluth, MN and

Cincinnati, OH). Breeding pairs (1:1 sex ratio) were maintained in 9.5 L aerated, flow-through tanks (well water) held at $25 \pm 1^\circ\text{C}$ and a photoperiod of 16 h light:8 h dark. Adult fish were fed frozen brine shrimp (Brine Shrimp Direct, Ogden, UT) daily to satiation. Each tank was provided with a piece of 3" diameter PVC cut in half as breeding substrate which was checked daily for eggs. Newly fertilized (<24 h old) eggs were collected from each PVC substrate for the toxicity studies. Acute (96 h) static nonrenewal tests were conducted in accordance to standard methods. Ten eggs were placed in 50 mL glass beakers and set in an environmental chamber at 25°C with a 16:8h light:dark cycle. Each beaker containing the fish tank water was spiked with stock solutions of Ag NPs (either NanoAmor or Sigma) in order to achieve target nominal concentrations of 0.625, 1.25, 2.5, 5, 7.5, 10, 15, 20, 25 mg/L with a final volume of 30 mL. A negative control (no Ag NPs) was used in all experiments and all conditions were tested in triplicate. In order to compare the acute toxicity of Ag NPs to that of dissolved Ag released, fathead minnow embryos were exposed to a range of dissolved Ag concentrations so as to cover the range released from all doses of Ag NPs tested which ranged from 0 to 100 $\mu\text{g/L}$. The LC_{50} for AgNO_3 on fathead minnow embryos was determined (used as a proxy for ionic Ag toxicity) and tested at similar concentrations of dissolved Ag released from Ag NPs. To avoid settling of particles especially at higher doses, all treatment solutions were sonicated for an additional 5 min prior to addition of the eggs. Since this additional sonication appeared to significantly decrease the settling of particles, we tested the effects of Ag NPs without sonication (stirred only) or with sonication. Test solutions from stirred and sonicated treatments were collected for TEM analysis as described above. Samples of test beakers were checked daily for the presence of any dead embryos and dissolved oxygen concentrations (DO) were maintained >7.5 mg/L (by bubbling using a glass Pasteur pipette attached to a portable air pump). Stirring of the eggs "mimics" the movement of fins by adult males and increases embryo survival. Water quality was measured for the concentrations of total (Mg, Ca, Na, K, Fe, Cu, and Zn) which can affect NP chemistry and the mean \pm SD ($n = 30$) of the various elements were: 16 ± 3.9 , 39.1 ± 9.4 , 3.76 ± 7.4 , 9.64 ± 2.3 , $0.07 \pm .01$, 0.009 ± 0.001 , and 0.05 ± 0.04 mg/L, respectively. The pH was measured daily and ranged between 8.3 and 8.5. Water hardness (mg/L CaCO_3) was 240 and 215 mg/L on days 1 and 4, respectively.

Quantification of dissolved silver

The concentration of dissolved Ag released (defined as ionic Ag released from Ag NPs and dissolved Ag NPs) was

measured from the Sigma Ag NPs. An aliquot of Sigma Ag NPs from each exposure group (with no eggs) was calculated by taking 12 mL from each treatment and centrifuging it at 15,000 rpm for 1 h. A total of 10 mL was then filtered through a 0.02 μm Anopore membrane disc (Whatman International Ltd, Maidstone England). A volume of 8 mL of this supernatant was placed in 15 mL falcon tubes and 2 mL of 2% nitric acid added to enhance dissolution. Concentrations of dissolved Ag in these samples were quantified by a Perkin Elmer Sciex ICP Mass Spectrometer ELAN DRC-e (Perkin Elmer, Waltham, MA) using AgNO_3 as a standard with concentrations ranging from 0.5 to 500 $\mu\text{g/L}$ to make a seven point standard curve.

Mortality and abnormalities

After the 96-h exposure toxicity test, the number of dead embryos as well the frequency and type (if any) of abnormalities were assessed from digital images obtained using an Olympus Optical DP70 digital camera attached to an Olympus dissecting scope.

Uptake of Ag NPs

To investigate uptake of Ag NPs by fathead minnow embryos, eggs were collected 24 h after exposure to 5 mg/L (stirred) for both sizes of Ag NPs. Embryos were biologically fixed using microwave enhanced processing with a 2% glutaraldehyde solution in a 0.1 M cacodylate buffer, then stained with 0.5% OsO_4 cacodylate buffer followed by dehydration of the cells using a series of 10, 30, 50, 70, 90, and 100% ethanol and propylene oxide solutions. Infiltration and embedding of cells were performed in an Epson generic resin and cells were left to polymerize in an oven at 60°C for 48 h. Then, 2 μm ultrathin sections were collected and images with a FEI/Phillips CM-100/1000TM TEM (FEI Company, Hillsboro, OR) using accelerated voltage of 80 KV and varied magnification were taken to find NPs in the embryo with similar shape, size and density as compared to images taken of just Ag NPs stock solutions.

Data analysis

Lethal concentrations ($\text{LC}_{50\text{s}}$) for each NP were determined using the USEPA Toxicity Data Analysis Software Version 1.5 (USEPA 1994). All other statistical analyses were performed using SAS 9.1. Statistical differences were compared to controls using ANOVA (PROC GLM) followed by Dunnett's multiple comparison test. Differences in the frequency distribution of developmental abnormalities were compared across treatments using Chi Square (PROC FREQ). Unless otherwise noted,

experimental data is plotted as means \pm SD (n = average of 10 embryos per condition). Differences were considered statistically significant at $p \leq 0.05$ unless otherwise noted.

Results

Particle characterization

The TEM images revealed that the actual particle sizes obtained from the Ag NPs experimental group NanoAmor were in fact highly variable. Indeed, the size distribution ranged from 29 to 100 nm with the majority of particles falling within the 31–50 nm range (Fig. 1a). Sigma Ag NPs described as ≤ 100 nm also ranged greatly in size (from 21 to 280 nm) with over half of the particles measured falling within the 21–60 nm size range (Fig. 1b). Sonication resulted in a decrease in the formation of aggregates compared to solutions that were only stirred, regardless of

NP size (Fig. 1c, d for NanoAmor and Fig. 1e, f for Sigma).

Toxicity tests

Although Ag NPs were toxic to *P. promelas* embryos after the 96-h static test, there was no correlation between particle size and toxicity as exposure to NanoAmor and Sigma Ag NPs resulted in similar mortality rates (LC_{50} s of 9.4 and 10.6 mg/L, respectively, Fig. 2a, b). However, a short sonication period (5 min) resulted in a significant increase in mortality compared to exposure to particles that were just stirred. Indeed, LC_{50} s decreased from 9.4 to 1.25 mg/L and from 10.6 to 1.36 mg/L for the NanoAmor and Sigma Ag NPs, respectively, when test solutions were sonicated (Fig. 2a, b). The estimated LC_{50} for $AgNO_3$ was 15 μ g/L (Fig. 2c) and the lowest estimated concentration of dissolved Ag released for any NP group was 18.43 μ g/L for day 4 sonicated 0.625 mg/L Sigma Ag NP (Fig. 3b). The toxicity of $AgNO_3$ at the concentrations estimated to equal

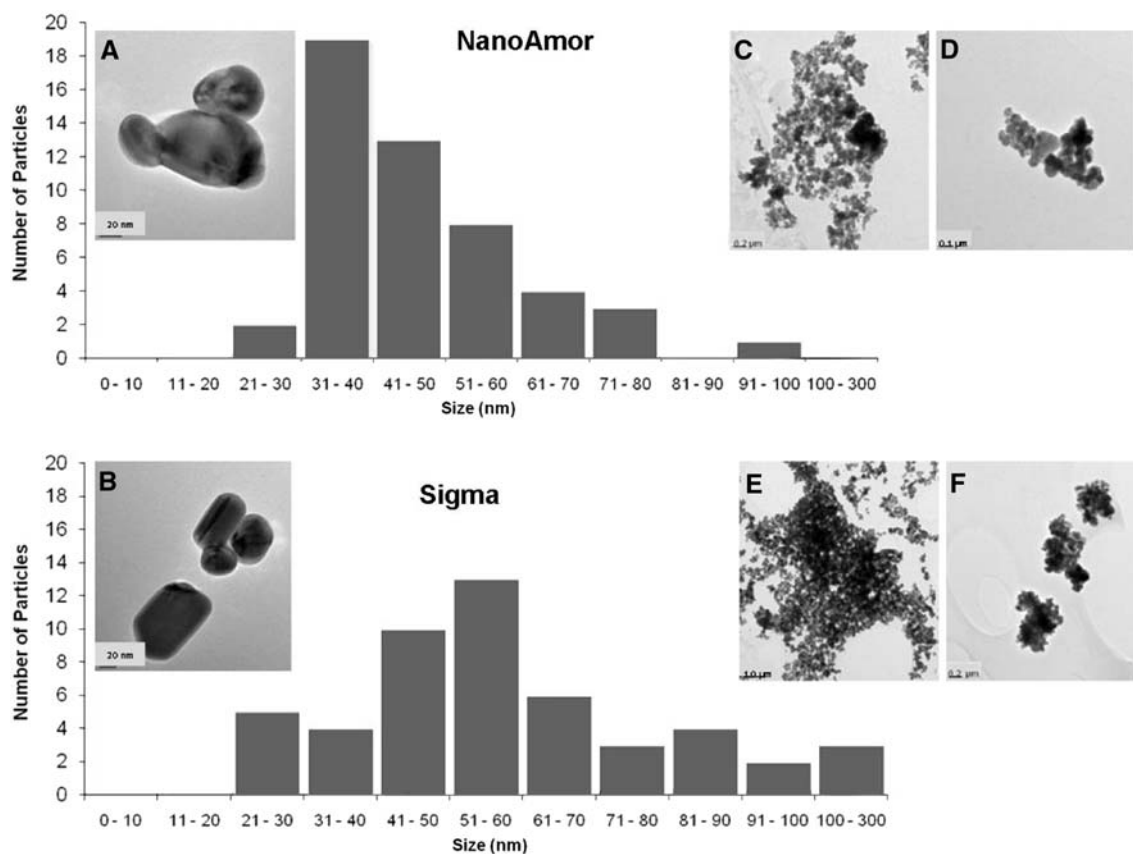


Fig. 1 Characterization of silver nanoparticle (Ag NP) size using transmission electron microscopy (TEM) for **a** NanoAmor (35 nm) and **b** Sigma (≤ 100 nm) Ag NPs. For each size, a total of 50 Ag NPs were measured from digital images using Sigma Scan. The image

shown for each type of Ag NP was typical of the size variation observed. Samples of test solutions were also photographed 96 h after either being stirred or sonicated (**c**, **d** for NanoAmor and **e**, **f** for Sigma). Note that these photographs were taken at a μ m scale

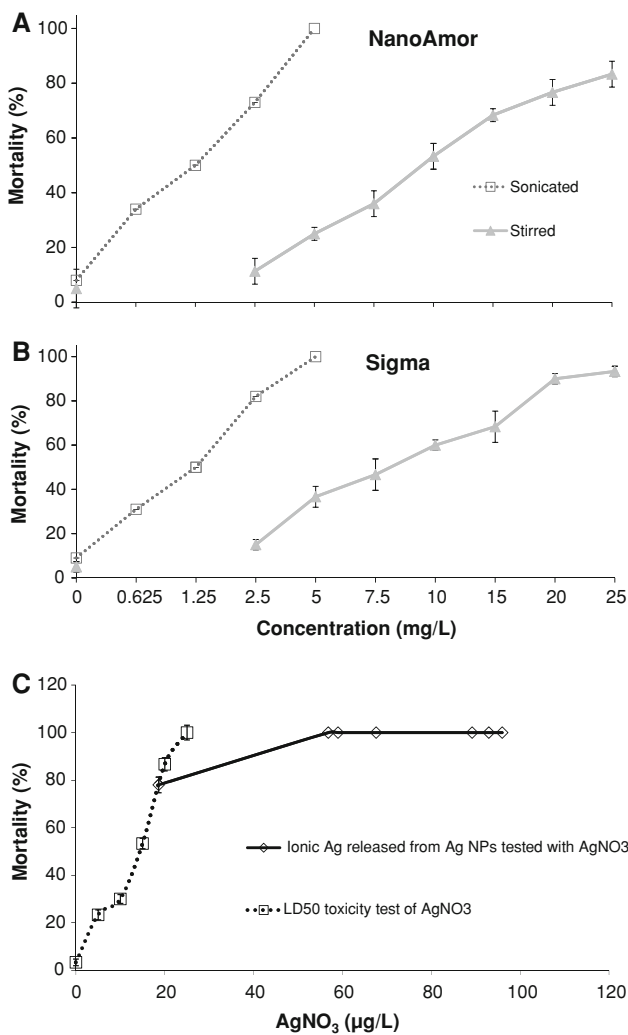


Fig. 2 Effects of **a** NanoAmor and **b** Sigma silver nanoparticles (Ag NPs) on fathead minnow embryo mortality (means \pm SD). Shown also are the effects of sonication compared to stirring on increasing the toxicity of Ag NPs. Note that the two lowest concentrations of Ag NPs (0.625 and 1.25 mg/L) were not tested in the stirred solutions. **c** Effects of silver nitrate (AgNO₃) on fathead minnow embryo mortality. Shown are means \pm SD of concentrations tested that bracketed the concentrations of dissolved silver released from the different concentrations of Sigma Ag NPs solutions tested (μ g/L)

the levels of dissolved Ag released from the Ag NP exposure groups was very high (Fig. 2c).

Quantification of dissolved silver released

The concentration of dissolved Ag released from Ag NPs was estimated using ICP-MS (Fig. 3). Results indicate that similar amounts of dissolved Ag were released from the Sigma Ag NPs tested regardless if solutions were stirred or sonicated ($p > 0.05$), but with concentrations increasing significantly with increasing concentrations (all

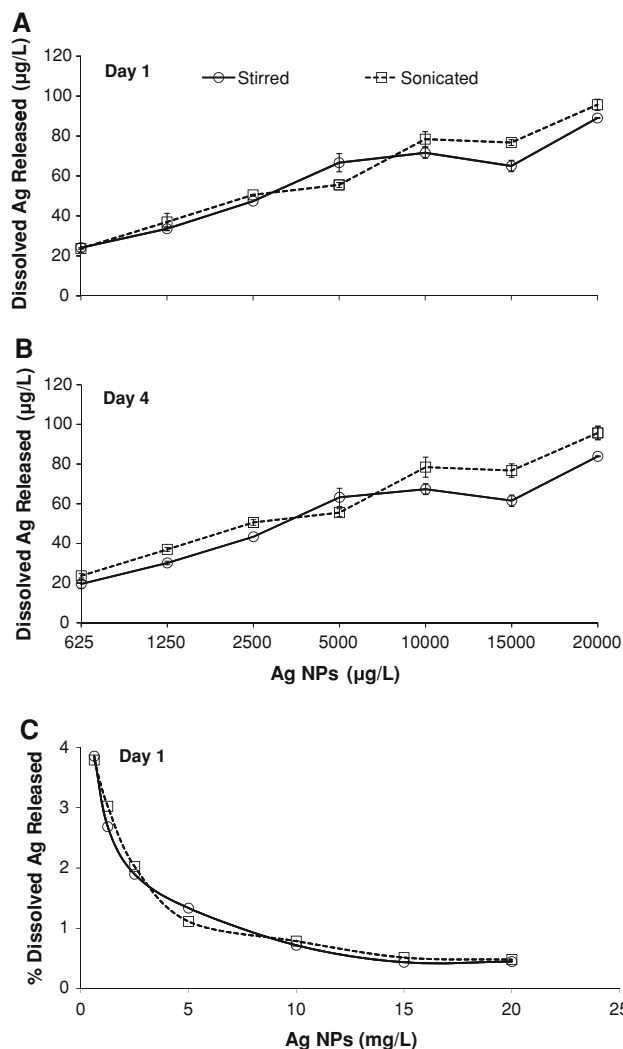


Fig. 3 Means \pm SD of dissolved silver released by seven different concentrations of silver nanoparticles (Ag NPs, only the Sigma ≤ 100 nm) ranging from 625 to 20,000 μ g/L after either being stirred or sonicated for 5 min. Concentrations of dissolved silver were measured using inductively coupled-mass spectrometry at **a** initiation (day 1) and **b** termination (day 4) of the exposure. $N = 3$ per concentration point. **c** Shows the percentage of dissolved silver released by Ag NPs on day 1

comparisons $p > 0.05$). Percent Ag released from Ag NPs exposures showed an inverse relationship with increasing concentration with 0.625 and 20 mg/L releasing 3.7 and 0.47% dissolved Ag, respectively (Fig. 3c).

Characterization of abnormalities

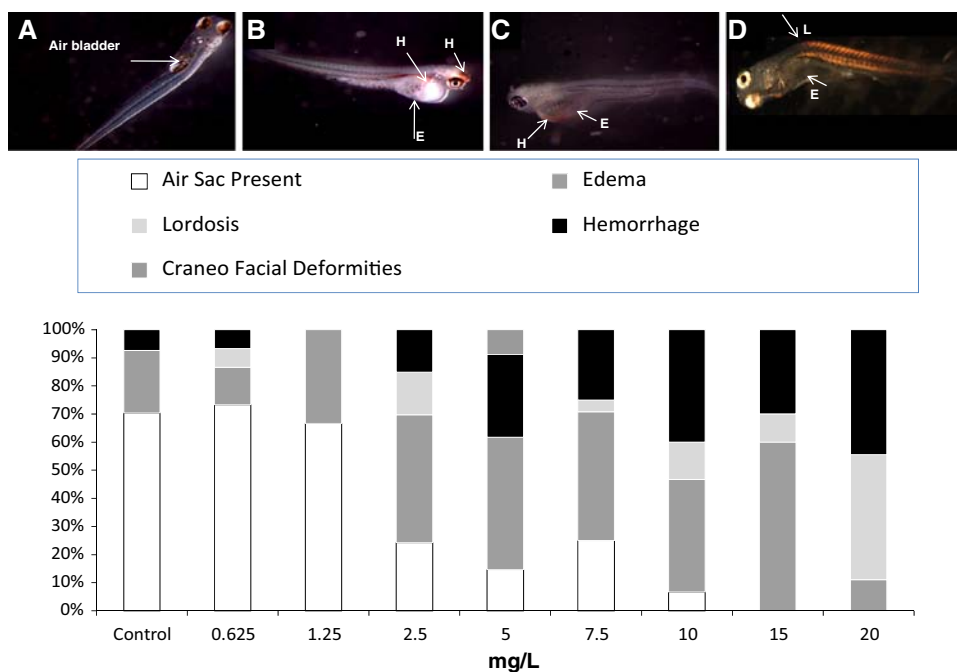
Exposure of fathead minnow embryos to Ag NPs induced a variety of developmental abnormalities in hatching larvae. Since there were no differences in the types and frequencies of these abnormalities between the two size Ag NPs tested, data were pooled for statistical comparisons to the

control group. Abnormalities included absence of noticeable air sac ($p < 0.0001$, $X^2 = 78$); development of moderate ($p < 0.0001$, $X^2 = 43$) and severe ($p < 0.0001$, $X^2 = 52$) pericardial and yolk sac edema; hemorrhages to the head and pericardial area ($p < 0.0001$, $X^2 = 33$); and lordosis or bending upwards of the vertebral column ($p = 0.0002$, $X^2 = 30$). Slight edema and craniofacial abnormalities (small heads) were also observed, but did not differ across treatments. Representative photographs showing these abnormalities are presented in Fig. 4a–d. The bar graph in Fig. 4 summarizes the types and frequency of abnormalities observed for all treatments.

Uptake of silver NPs

No detectable Ag NPs were observed in the control embryos (Fig. 5a–c). In contrast, Ag NPs were easily detectable in embryos exposed to both NanoAmor and Sigma particles (Fig. 5d–i). It is interesting to note that these photographs were taken only 24 h after exposure began. The photographs show NPs first attaching in large quantities to the surface of the egg (chorion; Fig. 5d) and later embedded throughout *P. promelas* embryos in “clumps” (Fig. 5e–i). Electron diffraction verified that the particles observed were metallic and not artifacts, but because of the size and concentration of the particles the ring patterns produced were not strong enough to confirm they were Ag. However, by comparing the size and shape of the particles observed in the embryos to that of the Ag NPs stock solution we were able to confirm that the particles were Ag NPs.

Fig. 4 Captured images of fathead minnow larvae exposed to silver nanoparticles (Ag NPs) (a control; b and c exposed to 10 mg/L of NanoAmor and Sigma Ag NPs, respectively; and d exposed to 2.5 mg/L of NanoAmor Ag NPs). Note the absence of air bladder in treated larvae. Bar graph summarizes the types and frequency of deformities per treatment group with $N = 30$ observed compared to controls (no Ag NP added)



Discussion

Particle characterization

An objective of the present study was to evaluate the effects of two different commercial NPs with differing particle sizes on the toxicity to *P. promelas* embryos. Previous studies have shown that smaller-sized NPs are positively correlated to increased toxicity (Hussain et al. 2005; Pan et al. 2007). In the present study however, there were no detectable differences in the toxicity of the two batches of Ag NPs tested. This was likely due to the unexpected variation in actual particle sizes obtained as compared to the manufacturer’s labeled product. TEM images showed that the NanoAmor Ag NPs described as 35 nm were in actuality larger, with most of them measuring ≥ 41 nm. Similarly, Sigma Ag NPs described as 100 nm ranged in size from 21 to over 300 nm. More importantly, the similarity in actual particle size distribution (Fig. 1) between the two commercial Ag NPs used in this study did not allow for detection of size-dependent differences in toxicity since both treatments contained particles of overlapping sizes. Finally, agglomerations were observed for both NanoAmor and Sigma NPs during the course of the experiment, which could have masked any effects due to particle size alone.

Toxicity tests

An important observation was the ~ 10 -fold increase in toxicity in treatment solutions that were sonicated for an

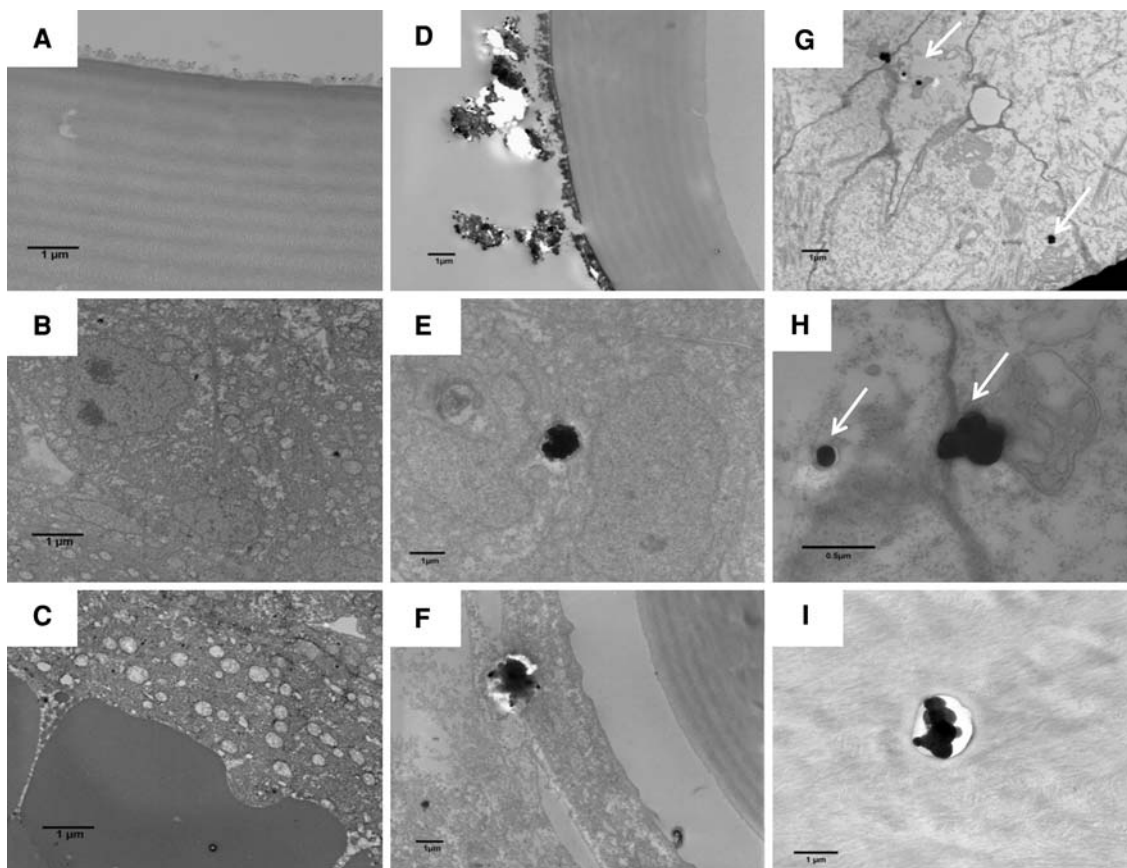


Fig. 5 Transmission Electron Microscopes (TEM) photographs showing: **a** outer surface of embryo of control (no silver added) $\times 3,900$; **b** and **c** controls showing the inside of the embryo, $\times 3,900$; **d** many silver nanoparticles (Ag NPs) aggregated to the outer membrane, $\times 11,500$; **e** and **f** aggregates of Ag NPs engulfed by a sac

inside the embryo, $\times 11,500$; **g–i** both single and agglomerated Ag NPs inside embryo, $\times 21,000$. Note, (**d–i**) images of fathead minnow embryos were exposed to 2.5 mg/L for 24 h. Arrows point to Ag NPs taken-up by the embryo

additional 5 min compared to those that were just stirred. Higher amounts of aggregation were observed in the stirred treatment solutions especially at the higher concentrations (Fig. 1c, e) compared to the sonicated ones (Fig. 1d, f) which would cause agglomerated NPs to move out of solution much faster with a subsequent reduction in actual concentration in the water column. Previous studies have shown that particle size distribution results from the kinetic competition between coagulation of colloids and sedimentation (Gustafsson et al. 2001) and this could have been altered by sonication which is known to reduce agglomeration (Murdock et al. 2008). Agglomeration can nullify the properties associated with nanosized particles by reducing its effective surface area (Greulich et al. 2009). Therefore, the increase in toxicity after sonication could be attributed to the creation of a more stable colloidal suspension during exposure as a higher concentration of Ag NPs would be left in the water column when compared to stirred solutions. An increased number of single NPs and the presence of smaller aggregates staying in solution

longer presented a larger effective surface area for interaction with embryos in the sonicated treatments. This could lead to increased physical damage to the egg chorion (Fig. 5d). One known mechanism of Ag NP toxicity is through cell membrane damage leading to increase in permeability and altered transport capabilities (Sondi and Salopek-Sondi 2004). As discussed in more detail below, this enhanced toxicity is not explained by a higher concentration of dissolved Ag since stirred and sonicated solutions released similar concentrations of this heavy metal (Fig. 3).

Toxicity effects of dissolved silver released

To test whether the concentration of dissolved Ag released from Ag NPs were causing the toxic effects observed, we quantified the amount of dissolved Ag released into the test solutions from these NPs and tested these concentrations in a way similar to those described for NPs. Since there were no observed differences in particle size and mortality

between NanoAmor and Sigma Ag NPs tested, it was assumed that they released similar amounts of dissolved Ag, thus we only measured this release from the Sigma Ag NPs test solutions. The highest concentration of dissolved Ag was detected on day 1 for both stirred and sonicated Ag NPs, with concentrations showing a non-significant decline during the next 3 days of the experiment (Fig. 3b). This decline is likely due to the formation of insoluble Ag complexes with various ligands like chlorides, sulphides and thiosulphates that are known to be in the water (Glover and Wood 2005; Naddy et al. 2007b; Schnizler et al. 2007), or re-association with the NPs (Morones et al. 2005). A low percentage of dissolution was observed (Fabrega et al. 2009) but the lower concentrations of Ag NPs released a higher percentage of dissolved Ag (Fig. 3c). This was due to the large amount of aggregation observed at the higher concentrations (Fig. 1c, d) which reduced surface area and hence reduced the percentage of dissolved Ag being released at higher mass concentrations.

When embryos were exposed to concentrations of AgNO₃ that bracketed the levels of dissolved Ag released from the various concentrations of Ag NPs, a significantly enhanced mortality was observed (Fig. 2c). The LC₅₀ for AgNO₃ was 15 µg/L, which is below the range of dissolved Ag concentrations released from all the Ag NPs test solutions tested (18–95 µg/L, Fig. 3a). If toxicity was purely due to the dissolved Ag released by the Ag NPs, one would expect ~100% mortality at all the treatment groups. However, this is not what was observed, suggesting that the toxicity produced by AgNO₃ is more potent than that produced by dissolved Ag released by Ag NPs. Similarly to what we observed, previously published research has shown that AgNO₃ is highly toxic in biological systems with a median LC₅₀ ranging between 5 and 60 µg/L (Lemke 1981; Nebeker et al. 1983; LeBlanc et al. 1984; Bury et al. 1999). It is well known that AgNO₃ dissociates completely (with a log_K value = -0.3; Morel and Hering 1993; Wood et al. 1996) releasing a large portion of free Ag ions very rapidly. Such a rapid release of high levels of free Ag ions could explain the enhanced toxicity of AgNO₃ compared to the slower rate of release of ionic Ag by Ag NPs which results in very little existing as free Ag ions. Conversely, increased pH, hardness and various ligands found in aquatic systems will probably result in lower concentrations of free Ag ions (Hogstrand and Wood 1998) as was the case in our test system considering our pH was 8.4 and hardness was 240 mg/L. Thus, approximately 40 µg/L of dissolved Ag released at LC₅₀ (1.34 mg/L) for Ag NPs is not as toxic to fathead minnow embryos as 40 µg/L AgNO₃ and is almost three times less toxic than the LC₅₀ of AgNO₃ (15 µg/L), since very little of it will exist as free Ag ions. Our findings are further supported by

previous studies using fathead minnows that have shown that complexed forms of Ag (chlorides and thiosulphates) are less toxic than AgNO₃ (Terharr et al. 1972; LeBlanc et al. 1984). In addition, Ag NPs are known to have a different toxic effect on micro-organisms than AgNO₃ (Morones et al. 2005) which further reinforces the idea that the toxicity caused by dissolved Ag released from NPs is likely different from Ag ions released from AgNO₃.

Nonetheless, the concentration of dissolved Ag released from the Ag NPs was concentration-dependent and could have contributed to some of the toxicity observed as a small portion could exist as free Ag ions. However, similar concentrations of dissolved Ag were released from both stirred and sonicated treatments (Fig. 3a) and mortality was significantly increased after sonication (Fig. 2a). Thus, the significant difference in mortality observed between stirred and sonicated treatment groups is likely not due solely to the concentration of dissolved Ag released (Fig. 3a) since they both released similar amounts. We conclude that the increase in toxicity observed in this study is due to a combination of the particulate Ag NPs and the dissolved Ag concentration released. The particulate Ag NPs may have a physical effect damaging the embryo membrane enhancing the toxic mode of action of dissolved Ag which may have a portion existing in the very potent free Ag ion form, especially in sonicated samples. This finding is further supported by recent studies showing that both ionic Ag and particulate Ag influences toxicity in algae, *Chlamydomonas reinhardtii* (Navarro et al. 2008) and that both particulate and ionic Ag NPs cause toxicity in zebrafish (*Danio rerio*) gills with the Ag NPs causing greater toxicity (Griffitt et al. 2008). These findings lay the groundwork for exciting questions to be asked and research to be done in kinetics, physical chemistry, and mechanisms of toxicity of Ag NPs.

Characterization of abnormalities

As the concentration of Ag NPs increased, air sacs were not visible in exposed fry. It is unknown as to whether this was due to the inability of fry to swim up to the water surface and fill their air sacs with air and/or to an abnormal development of the air sac. Edema (pericardial and yolk sac) was a very commonly observed abnormality in all the treatment groups and are similar to the phenotypic effects exerted by Ag NPs in zebrafish embryos (Lee et al. 2007a, b; Asharani et al. 2008). Hemorrhaging of the head and pericardial area also showed a dose response as the higher concentrations showed a higher prevalence of this deformity. The various abnormalities observed are of concern as most of these affected fry would likely not survive.

Uptake of silver NPs

There have been concerns about the ease at which NPs can be taken up and translocated within cells (Suzuki et al. 2007) and in vitro studies have shown the uptake of NPs in mammalian cell lines (Chang et al. 2007; Yu et al. 2008). Although Kashiwada (2006) reported uptake of NPs using a fish model, most of the ecological studies conducted so far have shown uptake of NPs in either unicellular organisms (bacteria) or invertebrate (*Daphnia*) species (Brayner et al. 2006; Zhu et al. 2006; Roberts et al. 2007). Size has been shown to be a factor in the uptake of NPs by cells (Limbach et al. 2005; Chithrani et al. 2006; Chithrani and Chan 2007) as NPs show a greater capacity to be taken up and cause damage at both the cellular and tissue levels compared to micro-particles (Ferin et al. 1992; Brooking et al. 2001; Rejman et al. 2004). However, the overlapping in particles sizes in the two samples used in this study prevented an analysis of the effects of particle size on uptake and toxicity. Single Ag NPs have been shown to enter zebrafish chorion pores via diffusion using a fluorescent probe (Lee et al. 2007a, b). Nonetheless, TEM examination of exposed embryos in this study revealed the uptake of NPs for both NanoAmor and Sigma particles after only 24 h. The actual mechanism(s) behind this uptake were not examined, but can be the result of particles falling out of solution onto the surface of eggs (chorion), and then either diffusing across membrane pores and/or being actively taken up by endocytosis. The latter can be seen in Fig. 5g–i, as evidenced by the presence of vacuoles engulfing Ag NPs. Furthermore, NPs were not only taken up as single particles into fathead minnow embryos but also as agglomerates. The increased rate of developmental abnormalities and mortality observed after exposure to Ag NPs suggest that this uptake can result in significant toxicity and deformities to fathead minnow embryos and is supported by work showing that Ag NPs crossing the chorion pores resulted in deformities and toxicity in zebrafish embryos (Lee et al. 2007a, b; Asharani et al. 2008).

Fathead minnow toxicity model

This study demonstrates that fathead minnow embryos can be used as a high-throughput, highly efficient, cost-effective, and sensitive platform for investigating the toxicity of Ag NPs with results obtained within a few days. Most biological systems used to investigate toxicity of NPs are limited by either the range of tests possible or by the model used, with limited characterization of genotoxic or metabolic effects. Previous studies with invertebrates, bacteria, and in vitro cellular platforms lacked the complexity of vertebrate organisms (Braydich-Stolle et al. 2005; Hussain et al. 2005; Kashiwada 2006). The zebrafish model already

has been used successfully to test the toxicity and uptake of AgNPs (Lee et al. 2007a, b; Asharani et al. 2008; Griffitt et al. 2008). However, the fathead minnow test system used in the present study offers several advantages especially for investigating the environmental effects of nano-materials. First, it is a well-characterized environmental model and the species is native to the US. Second, the availability of extensive genetic information on this fish lends to its efficient use in detailed molecular characterization of subcellular effects using available microarrays (Ankley et al. 2001; Larkin et al. 2007; Kane et al. 2008) or other quantitative genetic molecular techniques. Finally, it is the preferred environmental model of the USEPA for use in toxicity studies and thus our knowledge of the biology of this animal will continue to increase through time.

Conclusions

Commercial development of nanotechnology without a more detailed understanding of the fate and toxic action of NPs to environmental receptors presents a potential biohazard and is not prudent. Here, we propose an efficient and cost-effective method for determining the toxicity of Ag NPs in aquatic organisms. An important finding from the present study was the increased embryo toxicity after NP solutions were briefly sonicated as opposed to just stirred. Presently, we do not know what concentrations are environmentally relevant because of a lack of field data but this work highlights the importance of reporting detailed information on solution preparation, as well as the difficulty of creating a homogenous and stable NP solution for toxicity testing. Tests reproducing environmental conditions (stirred) compared to more controlled laboratory settings (sonicated) must be done to allow better extrapolation of the results for risk assessment. Our results also indicate that the observed increase in toxicity was due to both Ag NPs and the dissolved Ag released from these NPs but further studies are needed that investigate the ionic and particulate effects associated with metal NPs in dynamic aquatic systems. Differing environmental conditions can alter the toxicity of Ag NPs by either increasing or decreasing particle aggregation which could increase or reduce the amount of ionic Ag released. In addition, Ag NPs and the dissolved silver released from them were less toxic than AgNO₃. This fish embryo model could be easily adapted to test the toxicity of various types of NPs proposed or already in industrial/medical use and will be potentially directly transferrable to field studies. Although LC₅₀ data is useful and provides a good baseline for toxicity tests, further molecular studies can be more focused using the results from the phenotypic effects on the fathead minnow model. This will allow for the investigation of the

mechanisms that lead to the phenotypic deformities characterized in this paper. Developing a sensitive, high-throughput procedure for toxicity screening such as this one, is a necessary first step for screening and testing a broad spectrum of NPs and the fathead minnow model is ideal for achieving efficient results.

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