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A metabolomic study on the responses of *daphnia magna* exposed to silver nitrate and coated silver nanoparticles

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ABSTRACT

We examined the short-term toxicity of AgNPs and $AgNO_3$ to *Daphnia magna* at sublethal levels using ^{1}H NMR-based metabolomics. Two sizes of polyvinylpyrrolidone-coated AgNPs (10 and 40 nm) were synthesized and characterized and their Ag^+ release was studied using centrifugal ultrafiltration and inductively coupled plasma mass spectrometry. Multivariate statistical analysis of the ^{1}H NMR spectra showed significant changes in the *D. magna* metabolic profiles following 48 h exposure to both AgNP particle sizes and Ag^+ exposure. Most of the metabolic biomarkers for AgNP exposure, including 3-hydroxybutyrate, arginine, lysine and phosphocholine, were identical to those of the Ag^+-exposed groups, suggesting that the dominant effects of both AgNPs were due to released Ag^+. The observed metabolic changes implied that the released Ag^+ induced disturbance in energy metabolism and oxidative stress, a proposed mechanism of AgNP toxicity. Elevated levels of lactate in all AgNP-treated but not in Ag^+-treated groups provided evidence for Ag-NP enhanced anaerobic metabolism. These findings show that ^{1}H NMR-based metabolomics provides a sensitive measure of *D. magna* response to AgNPs and that further targeted assays are needed to elucidate mechanisms of action of nanoparticle-induced toxicity.

1. Introduction

With the increased application of nanosized silver in consumer products, predominately as bactericide, an increasing quantity of silver nanoparticles (AgNPs) is finding its way into wastewater and hence to rivers and streams. In addition to being bactericidal, AgNPs are toxic to other taxa. To date, most of the available data on adverse effects of AgNPs involve freshwater species. The documented effects include developmental deformities in zebrafish (Massarsky et al., 2013), altered stress-related gene expressions in Japanese medaka (Chae et al., 2009), and respiratory stress in Eurasian perch (Bilberg et al., 2010).

Despite many recent publications on toxicological effects of AgNPs, it remains unclear whether observed toxicity is specifically related to nanoparticles or is due to the effects of dissolved forms of Ag released from AgNPs. Laban et al. (2010) stated that the mortality of *Pimephales promelas* embryos after 96 h exposure to AgNPs was not attributed solely to the released Ag ions but rather to the AgNPs themselves. Li et al. (2010) also showed that the active component in the toxicity testing of AgNPs to *Daphnia magna* was the reduced form of silver and not the presence of excess dissolved Ag^+. It was also concluded that Ag had an enhanced acute toxicity to freshwater cladocerans, *Ceriodaphnia dubia*, if present as AgNPs compared to when present as the ionic form (Griffitt, et al., 2008). Other authors have published evidence that the toxicity of AgNPs can entirely be explained from co-occurring Ag^+ (Meyer et al., 2010; Kim et al., 2011). Using genetic analysis, Meyer et al. (2010) found evidence that the toxicity of polyvinylpyrrolidone (PVP) coated AgNPs to *Caenorhabditis elegans* was mediated by the release of ionic silver. Kim et al. (2011) also

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concluded that AgNP suspensions were not acutely toxic to *D. magna* when excluding Ag^+ with synthesized sorbents. Thus, there is an ongoing debate over whether the observed toxicity is due to the release of Ag^+ alone, or if the nanoparticles themselves exert a direct toxic effect.

For evaluation of the potential toxicity of nanoparticles, fast and high throughput methods are needed. The omics technologies are particularly well suited to evaluate toxicity in both *in vitro* and *in vivo* systems. Metabolomics can rapidly screen for biomarkers related to predefined pathways or processes in biofluids and tissues (Lin et al., 2006; Viant et al., 2003). Specifically, little is known about the mechanisms of action of nanoparticles which generally are difficult to measure by conventional methods. Thus, metabolomics can provide possible mechanistic insight into nanotoxicity. Metabolomics has been used to study the toxic effects of a wide variety of environmental contaminants to aquatic organisms (Bundy et al., 2009; Liu et al., 2011) and was recently applied to identify mechanisms of toxicity of AgNPs in rats (Hadrup et al., 2012) and titanium dioxide nanomaterial in earthworms (Whitfield Aslund et al., 2012). Yet, despite the cladoceran *D. magna* being one of the most widely utilized aquatic test species, few metabolomics studies have been reported, especially for nanomaterials. This is a knowledge gap that this research aims to redress.

D. magna is a freshwater invertebrate with many attributes that make it an ideal sentinel organism and it plays important ecological roles in freshwater habitats. It is among the most sensitive organisms used in ecotoxicology and a standard test organism for the standardized protocols of the U.S. Environmental Protection Agency (EPA), Organization for Economic Cooperation and Development (OECD), and International Standardization Organization (ISO) (Baun et al., 2008; OECD Guidelines test no. 202, 2004).

In this study, the metabolomics approach was applied to evaluate the potential toxicological effects of PVP-coated AgNPs at sublethal concentrations (2 μg/L and 10 μg Ag/L), using *D. magna* as the test organism. An $AgNO_3$ solution was used to compare the toxic effects of Ag^+ to those of the AgNPs. Using global analysis of metabolomics for the discovery of biomarkers, we sought to examine whether AgNP toxicity could be attributed to nanoparticles or to dissolved silver released from the AgNPs. The extent to which AgNP sizes modifies toxicity to *D. magna* was determined by using two types of size-controlled AgNPs.

2. Materials and methods

2.1. Preparation and characterization of metal salt solutions and nanoparticle suspensions

Silver nitrate ($AgNO_3$; ACS reagent, 99.0%; Sigma-Aldrich) was used as a source of Ag^+ for toxicity testing. Two sizes of PVP coated AgNPs (10 and 40 nm) were synthesized by reducing an $AgNO_3$ solution, using sodium hypophosphite and stabilizing with PVP. Details of particle synthesis, purification and storage are described in detail by Liu et al. (2009) and are summarized in Supplementary information. The exact Ag concentrations of NPs solutions were measured using inductively coupled plasma-mass spectrometry (ICP-MS; Agilent 7500i, Agilent Technologies Co. Ltd, USA) after digestion with 65% HNO_3.

The morphology and size of the obtained AgNPs were determined by transmission electron microscopy (TEM) (H-7500, Hitachi, Japan) at 80 kV accelerated voltage on a Philips EM420 at 120 kV and scanning electron microscopy (SEM) (S-4800, Hitachi, Japan) coupled with an energy dispersive X-ray spectroscopy (EDS) (Oxford, UK) at 15 kV. TEM samples were prepared by placing a drop of fresh AgNP suspension in water on a copper grid with a continuous carbon film coating, followed by solvent evaporation overnight at room temperature. The size distribution of the nanoparticles was estimated using Image-Pro plus software (Media Cybernetics). At least 300 particles were counted from a multipicture in each case. The UV–vis spectra at 300–700 nm were obtained using a Shimadzu UV-1700 PharmaSpec. AgNPs were characterized in de-ionized water and exposure medium under experimental conditions for size and Zeta potential by Dynamic Light Scattering (DLS) using a Zeta Sizer (Nano ZS, Malvern Instruments). Three successive measurements within an interval of two minutes were performed on two samples.

The release of soluble Ag at low (2 μg Ag/L) and high (10 μg Ag/L) AgNP concentrations in reconstituted water (RW, see below) was determined at the end of the exposures by ultracentrifugation through a 3 kDa membrane (pore size around 1 nm, Millipore, USA) as described by Navarro et al. (2008).

2.2. Animals and exposure study

D. magna were kindly provided by Dr. Qiaoguo Tan (Xiamen University, P.R. China). The RW water used had a pH of 7.2, hardness of 73 mg/L as $CaCO_3$,containing 24 mg/L Ca, 3.1 mg/L Mg, 2.1 mg/L of K and 26.3 mg/L of Na. It was prepared from the following analytical reagent-grade chemicals: Sodium bicarbonate, calcium sulfate, magnesium sulfate, potassium nitrate (Sigma-Aldrich, Shanghai, China) added to purified Milli-Qt water (Milli-Q, Millipore, Bedford, MA, USA, 18.2 MΩ cm). To avoid an uncontrolled loss of silver ions from RW caused by precipitation as AgCl, we used a specially designed chloride free RW in this study. At least 10 mL of water was allocated to each individual *D. magna* and refreshed every 2 days. The freshwater green alga, *Chlamydomonas reinhardtii*, was offered as food daily at a dose of $0.5–1.0 \times 10^6$ cells per individual depending on the age of the animals. The routine culture and all experiments were conducted under a light/dark cycle of 14 h:10 h and a temperature of 24 °C.

2.3. Acute toxicity tests

The acute (48 h) toxicity tests were conducted following methods described by Zhao and Wang (2012), see Supplementary information for more details. To compare the effects of AgNPs and the released Ag^+ ions on *D. magna*, two different experiments were performed. In the first experiment, the impact of NP size and concentration on AgNP toxicity was investigated. A series of preliminary experiments were conducted to determine the range of chemical concentrations that caused mortality of *D. magna*. Based on the determined concentration ranges, a low concentration of 2 μg/L and a high concentration of 10 μg/L were selected for the AgNP exposures. One hundred 10-day old individuals were placed in 1000 mL of RW containing a low or a high AgNP concentration for 48 h and media were refreshed every 24 h to maintain the NPs in suspension. Five replicates were used for each treatment. The animals were not fed during the test. To compare the difference in toxicity between AgNPs and Ag^+, an $AgNO_3$ standard solution (1 g Ag/L) was used as a stock to prepare different Ag^+ concentrations in the second experiment. $AgNO_3$ concentrations were chosen based on the concentration of soluble Ag released from AgNPs after 48 h under the experimental conditions. Four replicates per treatment were used in the experiment with approximately one hundred 10-day old *D. magna* in each replicate sample.

2.4. Metabolite extraction

For the metabolomics analyses, polar metabolites in each *D. magna* sample were extracted by the modified extraction protocol described previously (Wu et al., 2008). Briefly, each *D. magna*