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Assessing the exposure to nanosilver and silver nitrate on fathead minnow gill gene expression and mucus production

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ABSTRACT

Silver exposure is toxic to fish due to disturbances of normal gill function. A proposed toxicity mechanism of silver nanoparticles (AgNP) is derived from the release of silver ions, similar to silver nitrate ($AgNO_3$). However, some datasets support the fact that AgNP can have unique toxic effects that are mediated at the gill. To determine if differences between $AgNO_3$ and AgNP toxicities exist, fathead minnows were exposed to 20 nm PVP- or citrate-coated silver nanoparticles (50.3 μg/L PVP-AgNP; 56.0 μg/L citrate-AgNP) or 3.81 μg/L $AgNO_3$ for 96 h. These concentrations were applied to approximate the dissolved fraction of Ag in the AgNP suspensions. Mucus production in the water was measured. While mucus production was initially significantly increased in the first 4 h of exposure in all silver treatments compared to control, a decrease in mucus production was observed following 24–96 h of exposure. To determine which genes/pathways are driving this shift in mucus production, gills were dissected and microarray analysis was performed. Hierarchical clustering of differentially expressed genes revealed that all samples distinctly clustered by treatment. There were 109 differentially expressed genes shared among all Ag treatments compared to controls. However, there were 185, 423, and 615 differentially expressed genes unique to $AgNO_3$, PVP-AgNP, and citrate-AgNP, relative to control. While functional analysis indicated several common enriched pathways, such as aryl hydrocarbon receptor signaling, this analysis also indicated some unique pathways between nanosilver and $AgNO_3$. Our results show that $AgNO_3$, PVP-AgNP, and citrate-AgNP exposure affected mucus production in fish gills and also lead to common and unique transcriptional changes.

1. Introduction

Engineered nanomaterials are being increasingly manufactured worldwide. Of those, silver nanoparticles (AgNP) are of particular interest due to the antimicrobial properties of silver. Nanosilver production has been estimated at 2.8–20 tons per year in the United States alone (Hendren et al., 2011). As a result, AgNP are appearing in an increasing number of commercial products such as textiles, athletic equipment, medical devices, keyboards, baby bottles, stuffed animals, and food containers (Seltenrich, 2013). These products can release both silver ions and AgNP into the environment creating potential toxicity to aquatic organisms (Fabrega et al., 2011). This is of substantial concern, especially given the high toxicity of silver to aquatic organisms, second only to mercury among metals (Seltenrich, 2013). Despite many recent studies suggesting that AgNP have the potential to cause toxicity in humans and wildlife (Christen et al., 2013; Fabrega et al., 2011; Garcia-Reyero et al., 2014; Hadrup et al., 2012; McCarthy et al., 2013; Powers et al., 2011a, 2010; Suliman et al., 2013; Xu et al., 2013), there is still significant uncertainty in both the scientific and regulatory communities regarding the specific biological impacts of such substances. Furthermore, while the potential adverse effects of nanoparticles (NP) in the environment at the population or ecosystem levels have been minimally explored, recent studies suggest that the impact could be more significant than previously suspected (Colman et al., 2013; Levard et al., 2012; Oberdörster et al., 2005; Pokhrel and Dubey, 2012; Unrine et al., 2012), stress-

Abbreviations: AgNP, silver nanoparticles; DEG, differentially expressed genes; FHM, fathead minnows; NP, nanoparticles; PVP, polyvinylpyrrolidone.

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ing the need to understand the adverse effects of both ionic silver and NP in the environment. However, it also must be understood that the bioavailability of silver can be altered by transformations, photooxidation and interactions with environmental ligands (Kennedy et al., 2014). The reactivity of NP depends on complex physico–chemical properties such as size, agglomeration state, capping agent, dissolution kinetics, polydispersity, zeta potential, specific surface area or coating material. While toxicity due to AgNP is generally thought to be due to the dissolution of the silver from NP (Groh et al., 2014; Kennedy et al., 2010; Newton et al., 2013; van Aerle et al., 2013), several studies suggest that it cannot be explained exclusively by the effects of ionic silver (Garcia-Reyero et al., 2014,b; Powers et al., 2011a,b; Shaw and Handy, 2011). Differences may also relate to the extent to which ions are released, organism type and acute versus chronic exposure duration (Yang et al., 2012). Recent studies suggest that AgNP could be converted to more stable silver sulfides in certain oxygen-free environments such as wastewater plants. This conversion would significantly reduce the particles' ability to release silver ions and kill bacteria (Kaegi et al., 2013). Other studies also show the size or coating-dependent toxicity of NP (Ahn et al., 2014; Ivask et al., 2014; Shang et al., 2014), or even how the exposure medium, such as content of chloride ions (Groh et al., 2014), or dissolved organic carbon (Kennedy et al., 2012) can influence NP toxicity. This information stresses the need to understand the potential specific toxicity of the NP either due to their shape, coating, or to their ability to release silver ions.

The anatomy of fish gills reflects their primary function as gas-exchange, osmoregulatory, and excretory organ (Di Giulio and Hinton, 2008). The physiological and anatomical features of fish gills that promote efficient respiration also contribute to the uptake of xenobiotics and other compounds directly from water (Di Giulio and Hinton, 2008). Fish then use mucus hypersecretion as a response to the stress (Shephard, 1994) provoked by toxicants and irritants like metals (McDonald and Wood, 1992), carbon nanotubes (Smith et al., 2007), TiO_2 NP (Federici et al., 2007), $AgNO_3$ and AgNP (Bilberg et al., 2012; Bilberg et al., 2010; Bilberg et al., 2012, 2010; Hawkins et al., 2014b). Mucus hypersecretion protects not only by trapping and sloughing chemicals, but also by bringing innate immune proteins to pathogens (Mallatt, 1985). Nevertheless, concerns about the potential ability of mucus to eventually enhance toxicity have also been raised. If the mucus layer becomes more rigid and/or if chemical stressors such as NP are concentrated at the gill surface (Lichtenfels et al., 1996), toxicity could be increased. Alternatively, altered mucus homeostasis could adversely affect ionic regulation and a gas exchange.

Here we exposed fathead minnows (*Pimephales promelas*, FHM) to polyvinylpyrrolidone (PVP)-coated AgNP, citrate-coated AgNP, or silver nitrate ($AgNO_3$) in order to examine whether observed effects were due to AgNP, dissolved silver, or a combination of the two and to compare the effects of the two different coatings. We examined the mucus production after initial chemical insult (4 and 28 h) and transcriptional changes in gill after 96 h of exposure using microarrays. Microarrays have the potential to give information about mechanism of action for specific classes of chemicals and provide what is known as compound signature. The parallel analysis of multiple biochemical pathways at the mRNA level can provide a systems-wide understanding of toxicity that can be correlated to phenotypic changes (reviewed in (Denslow et al., 2007)).

2. Material and methods

2.1. Fish source, care and handling

Male fathead minnows (*P. promelas*) at eight months old were obtained from Aquatic Bio Systems (Fort Collins, CO, USA) and cultured according to University of Mississippi IACUC approved conditions. The fish were allowed to acclimate in glass chambers containing 1.5 L of moderately hard water (MHW) prepared according to U.S. EPA guidelines (Technology, 2002) for four days prior to the exposure. During acclimation, water was changed daily and fish were fed daily with Tetramin flakes.

Table 1
Linear regression of mucus production for 1–4 h post dosing. Slopes within the same column with different letters are statistically different ($n = 5$).

Treatment	Day 0		Day 1	
	Slope	R^2	Slope	R^2
Control	0.1093 ± 0.0053^a	0.948	0.1623 ± 0.0101^a	0.918
3.8 $AgNO_3$	0.3403 ± 0.0157^b	0.953	0.0127 ± 0.0029^b	0.454
50 PVP	0.3369 ± 0.0163^b	0.949	0.0402 ± 0.0042^c	0.796
56 Citrate	0.3664 ± 0.0104^b	0.982	0.0081 ± 0.0008^b	0.835

2.2. Silver

PVP-AgNP and citrate-AgNP were obtained from Nano Composix (San Diego, CA, USA) at a concentration of 1 mg/ml and a nominal size of 20 nm. Stock preparation and particle characterization have been previously described by (Hawkins et al., 2014a). Briefly, primary particle size was determined by transmission electron microscopy and image analysis of particle diameter and hydrodynamic diameter was determined by dynamic light scattering (DLS) and field flow fractionation (FFF). Silver nitrate was obtained from Sigma–Aldrich (St. Louis, MO, USA) prepared in distilled deionized water and diluted to a working stock with a nominal concentration of 10 μg/ml.

2.3. Exposure

Fathead minnows were exposed to control, PVP-AgNP and citrate-AgNP at 50.3 or 56.0 μg/L, respectively, or silver nitrate ($AgNO_3$) at 3.81 μg/L for 96 h (measured concentrations; $n = 5$ jars/treatment; 3 fish/jar; 1.5 L water/jar). The selected doses were targeted to provide a similar dissolved Ag concentration in the PVP-AgNP and citrate-AgNP treatments, when compared to the concentration of $AgNO_3$. Exposure water was changed and redosed daily. The fish were fed once at 48 h, 30 min before water change. Water quality was 289 ± 2 μS/cm, 231 ± 1 ppm TDS, 26.4 ± 0.8 °C, 151 ± 1 ppm salinity, and pH 8.09 ± 0.3. At 96 h, the fish were euthanized with buffered MS-222. Body weight and length were recorded. Gills were removed, preserved in RNA later and stored at −80 °C until analysis.

2.4. Mucus water concentrations

The estimation of mucus water concentration was done by using the phenol sulfuric acid assay protocol established by (DuBois et al., 1956) and modified for this application by (Parrish and Kroen, 1988). Glucose was used as a standard curve to represent the mucus carbohydrate content. Details on this assay have been reported by (Hawkins et al., 2014b). Briefly, water samples ($n = 5$ tanks/treatment/day) were collected on day 0 (at 0–4 h) and day 1 (immediately following water change and redosing; at 24–28 h) of the exposure.

2.5. Water sampling and ICP-MS analysis

Water samples were taken immediately following dosing to confirm Ag concentration ($n = 5$/treatment/collection). Details on water sampling for total and dissolved Ag and ICP-MS analysis have been described in (Hawkins et al., 2014a).