Distinct transcriptomic responses of *Caenorhabditis elegans* to pristine and sulfidized silver nanoparticles

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TITLE RUNNING HEAD: Toxicogenomic responses to sulfidized Ag nanoparticles

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Capsule

The toxicity of fully sulfidized Ag nanoparticles is not due to release of Ag ions.

Abstract

Manufactured nanoparticles (MNP) rapidly undergo aging processes once released from products. Silver sulfide (Ag_2S) is the major transformation product formed during the wastewater treatment process for Ag-MNP. We examined toxicogenomic responses of pristine Ag-MNP, sulfidized Ag-MNP (sAg-MNP), and AgNO_3 to a model soil organism, *Caenorhabditis elegans*. Transcriptomic profiling of nematodes which were exposed at the EC_{30} for reproduction for AgNO_3 , Ag-MNP, and sAg-MNP resulted in 571 differentially expressed genes. We independently verified expression of 4 genes (*numr-1*, *rol-8*, *col-158*, and *gri-20*) using qRT-PCR. Only 11% of differentially expressed genes were common among the three treatments. Gene ontology enrichment analysis also revealed that Ag-MNP and sAg-MNP had distinct toxicity mechanisms and did not share any of the biological processes. The processes most affected by Ag-MNP relate to metabolism, while those processes most affected by sAg-MNP relate to molting and the cuticle, and the most impacted processes for AgNO_3 exposed nematodes was stress related. Additionally, as observed from qRT-PCR and mutant experiments, the responses to sAg-MNP were distinct from AgNO_3 while some of the effects of pristine MNPs were similar to AgNO_3 , suggesting that effects from Ag-MNP is partially due to dissolved silver ions.

Keywords: antimicrobial, nanomaterial, toxicogenomics, soil, wastewater

Introduction

Silver nanoparticles (Ag-MNP) are the third highest manufactured nanoparticle (MNP) produced by volume, with current annual production exceeding 300 tons globally (Hendren et al., 2011). Ag-MNP are used in textiles, plastics, and medical devices principally for their anti-microbial properties (Osório et al., 2012; Prabhu and Poulouse, 2012), although they are also used in electronics, such as in conductive inks (Perelaer et al., 2006). Previous studies demonstrate that Ag-MNPs can be released from composite materials and enter wastewater streams, where the majority of Ag-MNP partition to the sludge (Benn and Westerhoff, 2008),(Kaegi et al., 2013). Nearly 75% of all households in the United States are serviced by wastewater treatment plants (WWTP), with the majority of the sludge generated being land applied as biosolids to agricultural fields (EPA, 1995). Repeated applications of Ag-MNP laden biosolids constitute the primary route by which Ag-MNPs are expected to be introduced to terrestrial ecosystems (Blaser et al., 2008; Hendren et al., 2013; Whitley et al., 2013). Once released to the environment, many metal and metal oxide MNPs have been shown to undergo biogeochemical transformations (Baalousha et al., 2010; Chun-Nam et al., 2007; Henglein, 1998; Liu et al., 2005). Both laboratory incubation, pilot, and full scale WWTP experiments demonstrate that Ag-MNPs are subject to rapid transformation from Ag(0) to Ag₂S (Kaegi et al., 2011; Levard et al., 2012; Lombi et al., 2013; Ma et al., 2014).

Notwithstanding the likely sulfidation of Ag MNPs, the vast majority of ecotoxicological studies involving Ag-MNPs, have focused on the effects of pristine Ag-MNPs and not sulfidized forms. Several studies have previously shown that pristine Ag-MNPs can cause increased mortality, reduced body length, and compromised reproductive performance in the model soil nematode *Caenorhabditis elegans* (Choi et al., 2014; Ellegaard-Jensen et al., 2012; Kim et al., 2012; Meyer et al., 2010; Roh et al., 2009; Tyne et al., 2013; Yang et al., 2014b). We previously

demonstrated that sulfidation of Ag-MNPs greatly reduced their adverse effects on *C. elegans* mortality, growth, and reproduction (Starnes et al., 2015). Utilizing synchrotron-based X-ray fluorescence (XRF) microscopy we demonstrated that the uptake of Ag was lower for *C. elegans* exposed to sulfidized Ag-MNP compared to pristine Ag-MNP. This demonstrated that the observed toxicity from sAg-MNP was not likely due to uptake and accumulation of Ag. Further, we quantified particle dissolution and its contribution to toxicity in that study which was very low for the sAg-MNP treatment (Starnes et al., 2015). It is possible that the toxicity of sAg-MNP could be explained by cuticle damage since internal tissue Ag concentrations were so low. It has also demonstrated that sulfidation significantly reduced the toxicity (mortality and growth) of Ag-MNP to *C. elegans* (Levard et al., 2013; Starnes et al., 2015). Decreases in toxicity of sulfidized Ag-MNPs compared to pristine Ag-MNPs have also been demonstrated for zebrafish (*Danio rerio*), duckweed (*Lemna minuta*), killfish (*Fundulus heteroclitus*), and *Escherichia coli* (Levard et al., 2013; Reinsch et al., 2012).

Whether the driving force behind Ag-MNP toxicity in ecological receptors is due solely to effects of released Ag⁺ ions to particle specific effects, or a combination of the two is currently debated. For example, Xiu et al. and Yang et al. independently reported that toxicity, in *E. coli* and *C. elegans* respectively, was driven by ions released from Ag-MNP (Xiu et al., 2012; Yang et al., 2012), while others have found that the toxicity of Ag-MNP exceed the equivalent metal salt concentrations in nitrifying bacteria cultures (Choi and Hu, 2008; Meyer et al., 2010; Yin et al., 2011). It has also been reported that toxicity can be driven by a combination of ions and intact nanoparticles (Yang, 2014). Our previous work demonstrates that the importance for toxicity of dissolved ion relative to particle specific effects are dependent on the exposure concentration (Starnes et al., 2015). At low exposure concentrations, effects are dominated by

release of Ag⁺ ions, while at higher exposure concentrations particle specific effects are more prominent (Starnes et al., 2015).

A powerful emerging tool for investigating potential environmental and ecotoxicological impacts of MNP is the model organism *C. elegans*, an ubiquitous soil dwelling nematode is primarily associated with decaying plant material (Ellegaard-Jensen et al., 2012; Kim et al., 2012; Maupas, 1901; Meyer et al., 2010; Roh et al., 2010; Roh et al., 2009; Tsyusko et al., 2012; Williams and Dusenbery, 1990). The attractiveness of *C. elegans* to test MNP toxicity is attributable to the fully mapped genome, invariable cell fate map, short generation time, availability of mutants covering much of the genome, low maintenance cost, and amenability to RNA interference methods (Choi and Hu, 2008; Handy et al., 2012; Kim et al., 2012; Leung et al., 2008; Ma et al., 2009; Roh et al., 2010; Roh et al., 2009; Williams and Dusenbery, 1990; Zhang et al., 2011). Genome wide screening of *C. elegans* have been already used to investigate the effects of MNPs (Ag-, Au-, CeO₂-, and TiO₂) (Cui et al., 2006; Menzel et al., 2009; Roh et al., 2010; Roh et al., 2009; Tsyusko et al., 2012; Viñuela et al., 2010). However, to our knowledge, this is the first study to examine transcriptomic responses to aged sulfidized Ag-MNPs by a model organism. It is anticipated that transcriptomic responses will elucidate mechanisms of sulfidized Ag-MNP toxicity that we previously reported were not explained by the release of Ag⁺ ions (Starnes et al., 2015).

The objective of this study was to compare the transcriptomic responses of *C. elegans* exposed to pristine and sulfidized Ag-MNP and AgNO₃. We hypothesized that that the mode of toxicity is different between pristine and aged MNPs and that the responses of *C. elegans* to MNPs would differ from those in response to AgNO₃ exposure. To confirm the role of select genes in the mechanisms of toxicity, we also employed experiments with mutant strains and

RNA interference for selected genes that demonstrated opposite responses to one or more treatments.

Materials and Methods

Silver nanoparticle synthesis and characterization

Polyvinylpyrrolidone (PVP) coated (pristine) Ag-MNPs were synthesized and then fully sulfidized (completely converted to Ag₂S). Synthesis and sulfidation as well as characterization of the pristine Ag-MNPs and sulfidized Ag-MNPs (sAg-MNPs) are described in our previous work (Starnes et al., 2015). The particle synthesis methods are also described in the supplemental information (SI). Polyvinylpyrrolidone is one of the most common coatings used for dispersal of Ag-MNPs (Wang et al., 2014). As reported previously, the Ag-MNPs and fully sulfidized Ag-MNP had a zeta potentials in the exposure medium of -6.1 mV and -28.1 mV, respectively, as determined by phase analysis light scattering. The Z-average hydrodynamic diameters for Ag-MNP and sAg-MNP suspended in the exposure medium (described below) as determined by dynamic light scattering was 79.6 ± 0.5 nm and 88.5 ± 0.5 nm, respectively. Complete sulfidation of the Ag-MNPs was verified by powder X-ray diffraction as described in Starnes et al., (2015). Ag concentrations of all stock solutions used to prepare the exposures were determined using inductively coupled plasma mass spectrometry (ICP-MS) after digestion of the particles in aqua regia. We also quantified the dissolution of the of both Ag-MNP and sAg-MNP over the exposure period of 24 h without presence of bacteria and *C. elegans* using ultracentrifugation and ICP-MS in our previous work and found dissolution rates of 5.8% and 0.43% respectively (Starnes et al, 2015) Unfortunately, dissolution is not readily measured in the presence of biomass. Any Ag ions bound to bacterial cells or nematodes would be removed by ultrafiltration or ultracentrifugation and not accounted for in the dissolution measurement. To

measure loss of Ag to the exposure container walls, we incubated asset of three replicates of exposure solutions for 24 h and then removed the solutions and analyzed them. Concentrations were compared to solutions acid digested directly in the tubes without removal.

Nematode exposure and microarrays

Wild type N2 Bristol strain of *C. elegans* were obtained from the *Caenorhabditis* Genetics Center (CGC). *Caenorhabditis elegans* were age-synchronized using NaClO/NaOH solution to isolate eggs. The eggs were placed on K-agar plates with bacterial lawns until needed. All exposures were carried out in moderately hard reconstituted water (4 mg L⁻¹ KCl, 60 mg L⁻¹ MgSO₄, 60 mg L⁻¹ CaSO₄, mg L⁻¹, 96 mg L⁻¹ NaHCO₃) (EPA, 2015).

Approximately 5000 L1 stage nematodes per replicate were exposed to AgNO₃ (10 µg Ag L⁻¹), Ag-MNP (350 µg Ag L⁻¹), and sAg-MNP (1500 µg Ag L⁻¹) for 48 hours. The selected concentrations were the EC₃₀ values for reproduction based on our previous work (Starnes et al., 2015). The equitoxic concentrations, corresponding to the EC₃₀ for reproduction for each substance, were chosen so that modes of toxicity could be differentiated where the severity and nature of the effects was similar rather than examining transcriptomic responses at similar Ag concentrations, which would have resulted in a very different nature and severity of effects. The exposures were conducted in the presence of bacterial food, *E. coli* (OP50 strain), at the rate of 10 µL stock solution per mL of exposure solution (OD₆₀₀ = 1 for stock solution). To ensure a consistent exposure over the course of 48 hours exposure solutions were replaced after 24 h with fresh ones and fresh bacterial food was added. Exposures were carried out in 15 mL polypropylene centrifuge tubes containing 4 mL of exposure solution. The tubes were arranged horizontally in the incubator and three replicates per treatment were prepared. After exposure the nematodes were twice washed for 30 minutes with bacteria free moderately hard

reconstituted water to remove most of the bacteria from the nematodes. Bacterial RNA contamination is not a concern since the techniques for measuring mRNA concentrations are sequence specific to *C. elegans*. RNA was then extracted from each replicate using Trizol with subsequent purification with Qiagen RNAeasy Kit (Qiagen, Chatsworth, CA). Cells were lysed using multiple freeze-thaw cycles (-80 and 37°C) as previously described (Tsyusko et al., 2012). All samples were checked for RNA degradation using a Nanodrop ND-2000 spectrophotometer and Agilent 2100 Bioanalyzer to examine the quality and quantity of the RNA samples. All RNA samples had RNA integrity numbers of 9 and above and were sent to the Microarray Core Facility at the University of Kentucky to be processed using Affymetrix *C. elegans* whole genome microarrays. Total of 1 µg of RNA was used to prepare the biotin-labeled cRNA probes, which were hybridized to the microarray chips for 16, washed in fluidics station, and stained following standard GeneChip expression analysis technical manual. The chips were scanned using Affymetrix GCS 3000 7G scanner. Raw data files were normalized using the robust multi-array average (RMA) algorithm followed by quantile normalization implemented in Partek Genomics Suite 6.4 (Partek, Inc., St. Louis, MO)(Irizarry et al., 2003a). Data were screened for differences of each treatment (Ag-MNP, sAg-MNP, and AgNO₃) from control using one-way ANOVA with contrasts. The false discovery rate (FDR) threshold was set at 0.2 and the fold change was required to be greater than ± 1.5 , and a P-value ≤ 0.05 . The FDR of 0.2 was selected to balance the protection against false positives while minimizing the rate of false negatives. Using Partek, genes that were significantly different from control in at least one treatment were then analyzed using agglomerative hierarchical cluster analysis (HCA). HCA is a 2-Pass clustering method; the first pass is a K-means clustering and in the second pass the K-means clusters are joined by agglomerative clustering (Partek, 2009). Lists of genes that were

significantly different than control for each treatment were generated in Partek and exported to Integrated Pathway Analysis (IPA, Ingenuity Systems, www.ingenuity.com) to investigate the possible involvement of relevant pathways. These lists were also uploaded to the Database for Annotation, Visualization and Integrated Discovery v 6.7 (DAVID) utilizing functional clustering tools to identify significant changes in enrichments annotations between treatments (Huang et al., 2008). In DAVID functional annotation tool gene ontology terms for biological process (BP), molecular function (MF), cellular compartment (CC), and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway were selected. To avoid redundancy in the same gene sets being represented in multiple functional clusters the DAVID options of “GOTERM_BP_ALL”, “GOTERM_MF_ALL”, and “GOTERM_CC_ALL” were selected. All microarray data have been submitted to the National Center for Biotechnology Information (NCBI) (Accession number GSE70509).

qRT-PCR: Validation of Microarray Data.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses were performed on samples in triplicates (both biological and technical) from independent experiment. Glucose-6 phosphate isomerase I (*gpi-1*) was selected as the reference gene, which had no significant changes across treatments in either microarray or qRT-PCR analysis (Fig. S1). Four differentially expressed genes with one of the highest expression levels and that had opposite responses to one or two treatments were selected for qRT-PCR confirmation. Among them were collagen (*col-158*), ground-like (*grl-20*), roller (*rol-8*), and nuclear localized metal responsive (*numr-1*) genes. 300 ng of RNA were converted to cDNA using a High-Capacity RNA-to-cDNA kit™ (Applied Biosystems) and RNA was cleaned with DNase I for 15 minutes prior to

cDNA conversion (Qiagen, Chatsworth, CA). qRT-PCR reactions were carried out in 10 μ L volumes using TaqMan fast advanced master mix, TaqMan gene expression arrays for each gene (Table S1), and diluted 1:19 cDNA. All gene probes (except for *numr-1*) spanned introns and StepOne Plus system (Applied Biosystems) was used for all amplifications with a program of 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C. All treatments for each gene were run in triplicates. Negative controls and minus reverse transcription (–RT) negative controls were run for every gene/sample to check for DNA contamination.

Toxicity testing using mutant strains and RNA interference.

Mutant strains for *rol-8* and *numr-1* were obtained from the CGC. Mortality testing for mutant strains were carried out using age synchronized L3 stage nematodes exposed for 24 hours at 25°C in the dark to 3 concentrations of AgNO₃ (6.1, 7.5, 9.1 μ g Ag L⁻¹), Ag-MNP (52, 75, 85 μ g Ag L⁻¹), and sAg-MNP (525, 2000, 3500 μ g Ag L⁻¹). Two sample student t-tests were used to test for significant changes in mortality from N2 wild type at each treatment and concentration.

Testing of selected differentially expressed genes (*col-158* and *grl-20*) using RNAi knock-down was employed by feeding N2 wild-type nematodes *E. coli* strains that expressed the required dsRNA for 2 generations prior to age synchronization for mortality testing(Williams and Dusenbery, 1988). *Escherichia coli* strains were obtained from Source Bioscience (<http://www.sourcebioscience.com/>) and standard RNAi protocols were followed (Fraser et al., 2000). Mortality screens for RNAi experiments were carried out under the same conditions described above. To confirm that RNAi was successful, reduction in expression levels of *col-158* and *grl-20* were verified using qRT-PCR.

Results and Discussion

The purpose of this study was to compare the transcriptomic effects of a pristine Ag-MNP, sulfidized sAg-MNP and AgNO₃ at similar effect concentrations (EC₃₀ for reproduction) on *C. elegans*. Each of the three treatments caused a distinct response with only 20 differentially expressed genes shared among them. However, out of these shared genes, 11 responded in opposite directions with 5 ground-like genes (*grl*) and 6 collagen genes (*col*) being upregulated in the sAg-MNP and down-regulated in the AgNO₃ and Ag-MNP treatments (Fig. S2). These differential responses for the shared genes further confirm that the transcriptomic responses are unique to each treatment. A hierarchical cluster analysis based on 571 differentially expressed genes reveals that replicates cluster within their respective treatments, indicating that replicates within a treatment have gene expression profiles with greater similarity to each other than to replicates from a different treatment (Fig. 1). All treatments are grouped in two major clusters, with AgNO₃ forming one cluster and the control and both MNPs the second. Within the second cluster, Ag-MNP showed the greatest dissimilarity to both controls and sAg-MNP.

Figure 1. Hierarchical clustering of significantly expressed genes from *Caenorhabditis elegans* exposed for 48 hours to Ag^+ , pristine silver manufactured nanoparticles (Ag-MNP), and sulfidized silver manufactured nanoparticles (sAg-MNP) in moderately hard reconstituted water.

Within the set of the 571 differentially expressed genes, only 3.5% were significantly expressed in all three treatments. Over 85% of these genes were unique to one of the three treatments. The greatest overlap in significantly expressed genes occurred between AgNO_3 and Ag-MNPs and accounted for approximately 10% of the total differentially expressed genes while the overlap between Ag-MNPs and sAg-MNPs accounted for approximately 5% of the genes (Fig. 2). These low percentages of shared genes indicate that genomic responses for the sAg-MNPs are different from the pristine Ag-MNPs. Our previous work showed more than 10 times as much dissolved Ag was released from the pristine Ag-MNP than from the aged sAg-MNP over the course of the exposure (Starnes et al., 2015). The amount of dissolved Ag released

from Ag MNP in the microarray experiment was likely affected by the presence of nematodes and bacteria during exposures. However, our transcriptomic results still indicate that the observed toxicity was not solely caused by the release of Ag ions. Had the toxicity of the Ag-MNPs been due solely to effects of Ag ions, the replicates of hierarchical clustering would have been randomly distributed and the list of overlapping genes between each MNPs and AgNO₃ would have been much larger (Figs. 1 and 2). The percent of Ag lost to the container walls is estimated to be 0 ± 3.5 , 33.4 ± 1.2 and 30.2 ± 2.7 in the AgNO₃, Ag-MNP and sAg-MNP treatments, respectively. It is unclear how this affects exposure since the nematodes dwell on the bottom of the container. It also has no implications for the interpretation of the results since the transcriptomic profiles are compared at equitoxic concentrations rather than equal mass concentrations. The toxicity of the sAg-MNP particles could not be due to the release of S since the concentration of S in the particles was only 1.6 μ M compared to the S concentration in the exposure medium of around 850 μ M. Similarly our previous studies showed that the toxicity of the AgMNPs was not due to free PVP polymer in solution (Starnes et al., 2015). Finally, the toxicity is unlikely to be due to an indirect effect on the *E. coli* OP50 since previous studies have shown that Ag MNP toxicity occurs in *E. coli* at concentrations approximately 50-100 times greater than our exposure concentrations (Sondi and Salopek-Sondi, 2004). We also did not observe a decrease in cell density at our exposure concentrations.

Figure 2. Venn diagram of the significantly up/down regulated genes with a fold change greater than ± 1.5 , FDR of 0.2, and p-value ≤ 0.05 . *Caenorhabditis elegans* exposed for 48 hours to AgNO₃, pristine Ag manufactured nanoparticles (Ag-MNP), and sulfidized silver manufactured nanoparticles (sAg-MNP) in moderately hard reconstituted water.

There are few toxicogenomic studies examining the effects of MNPs such as Ag-, Au-, and TiO₂-MNP to *C. elegans* in the literature (Rocheleau et al.; Roh et al., 2009; Tsyusko et al., 2012). These studies have shown that MNPs can alter the gene expression profiles of *C. elegans* in pathways associated with stress response, reproduction, and lysosomal activity. There are limited data to indicate whether the toxicity of soluble NPs, such as Ag and ZnO, is primarily due to the ions or the intact particles. Taken together our data and the data from other *C. elegans* studies suggest that the toxicity of Ag-MNPs is a result of both ionic and intact nanoparticles (Ellegaard-Jensen et al., 2012; Kim et al., 2012; Meyer et al., 2010; Starnes et al., 2015).

qRT-PCR confirmation

The expressions of four genes selected from the shared and unique gene expression pools were confirmed independently with qRT-PCR (Fig. S3). Among these four genes, there are three related to cuticle function and two of these (*col-158*, *grl-20*) are from the shared pool that were significantly up-regulated only in response to aged sAg-MNP, but down-regulated to Ag-MNP and AgNO₃. The third gene, roller (*rol-8*), is from the unique genes that responded only to sAg-MNPs and this was also up-regulated. The fourth gene, nuclear metal responsive gene (*numr-1*), is shared between Ag-MNP and AgNO₃ and was up-regulated in both exposures, but showed no effect in sAg-MNP exposure. *Numr-1* is a nuclear localized metal response gene and therefore should be closely related due to toxicity from metal ion exposure (Tvermoes et al., 2010).

Biological Pathways and Gene Ontology

No pathways were identified in any treatment using Integrated Pathway Analysis. However, four KEGG pathways in total were identified using DAVID for all treatments (1 for each of the MNPs and two for AgNO₃). These pathways (Table 1) showed no commonality between any of the treatments further confirming uniqueness of the responses. None of these pathways were found by Roh et al., where *C. elegans* was exposed to uncoated pristine Ag-MNP, but this was not unexpected since the current study utilized PVP coated Ag-MNP, a different testing medium, and exposure times that were nearly three times longer than used by Roh (Roh et al., 2009).

Table 1. List of pathways that were identified by DAVID using lists of significantly altered genes from N2 wild type nematodes when exposed to Ag⁺, pristine silver manufactured nanoparticles (Ag-MNP), and sulfidized silver manufactured nanoparticles (sAg-MNP) at the LC30 for 48 hours.

KEGG ID	Pathway	Genes in Pathway	Fold Enrichment	P-Value	Exposure
cel00982	Drug Metabolism	5	1.8	0.0026	AgNO ₃
cel00680	Methane Metabolism	3	1.1	0.0083	AgNO ₃
cel04142	Lysosome	5	7.5	0.0025	Ag-MNP
cel04020	Calcium Signaling Pathway	3	1.5	0.0057	sAg-MNP

For pristine Ag-MNP, 5 genes were identified in the lysosomal pathway suggesting that lysosomes are involved in processing the particles. Results from previous studies of MNP in both *in-vivo* as well as *in-vitro* models have implicated that lysosomes are involved in the detoxification of several types of MNP (Au, Ag, and Fe), the acidic nature of the lysosome may promote the dissolution of the MNP (Levy et al., 2011; McCarthy et al., 2013; Shukla et al., 2005; Yang et al., 2014a). Yang et al. demonstrated that using lysosomal function mutants greatly enhanced the toxicity of Ag-MNP, though different genes and Ag-MNP were utilized in the study, there is a clear indication that lysosomes are integral to the toxic response in *C. elegans* (Yang, 2014). Three of the genes in the lysosome pathway were hypothetical proteins and two were cysteine protease related genes (*cpr-1* and *cpr-2*) that encode lysosomal proteins. Both *cpr-1* and *cpr-2* were significantly down-regulated in Ag-MNP exposures. One of the cysteine protease related genes, *cpr-1*, has been linked to the proper protein degradation within lysosomes while the other, *cpr-2*, has been linked to immune response of *C. elegans* to *Staphylococcus aureus* infection (Britton et al., 1998; JebaMercy et al., 2011; Levard et al., 2011). Additionally, *cpr-2* is a orthologue of the human cathepsin b gene (*ctsb*) which is a lysosomal cysteine protease and has a role in the progression of chronic inflammatory diseases

such as rheumatoid arthritis and neurodegenerative diseases such as Alzheimer's (Hook et al., 2014; Tong et al., 2014).

In the sAg-MNP exposure, the calcium signaling pathway was identified and the three genes associated with it were all found to be significantly down-regulated such as (egg laying deficient (*egl-30*), and two uncoordinated (*unc-43*, *unc-68*) genes. All three of these genes have been linked to egg laying, additionally *unc-43* and *unc-68* are also involved in locomotion, cell fate determination, and synaptic regulation (EPA, 2015; Huang et al., 2008; Irizarry et al., 2003b; Williams and Dusenbery, 1988). This finding provide support for our previous results which showed that reproduction was significantly reduced when the nematodes were exposed to sAg-MNP (Adachi and Kagawa, 2003; Reiner et al., 1999; Reiner et al., 1995; Starnes et al., 2015; Wang and Wadsworth, 2002). Interestingly, sAg-MNP induced a calcium signaling pathway (cel04020) that was also identified by Tsyusko et al., when exposing *C. elegans* to Au nanoparticles (Tsyusko et al., 2012). However, the genes associated with this pathway in this study were different from those in the Au-NP study.

Two pathways were identified in the AgNO₃ exposure. The first, drug metabolism pathway, contained two significantly down-regulated genes (hypothetical dehydrogenase protein and flavin containing monooxygenase *fmo-2*) and three genes that were significantly upregulated (glutathione S-transferase *gst-10*, *gst-30*, and UDP-glucuronosyl transferase *ugt-50*). One of the significantly down-regulated genes, *fmo-2*, has already been shown upregulated in AgNO₃ and Ag-MNP toxicity (Eom et al., 2013). However, Eom et al. used a shorter exposure time with a higher dose of AgNO₃ than the current study (Eom et al., 2013). The three upregulated genes in the pathway are all phase II xenobiotic detoxification enzymes (Hasegawa and Miwa, 2010; Hasegawa et al., 2010; Nair and Choi, 2011). The second pathway, methane metabolism, had

three catalase (*ctl*) genes associated with the pathway that were significantly upregulated. These genes, *ctl-1*, 2, and 3 are three catalase genes found in *C. elegans*, and knockout mutants for *ctl-2* have been previously shown to cause increases in mortality when exposed to Ag-MNP (Ahn et al., 2014). In addition, *ctl-1* and 2 are upregulated by *daf-16* which is a key part of the stress and aging response associated with the insulin signaling pathway (Murphy, 2013).

For gene ontologies, when investigating the gene list for each treatment a total of 18 Biological Processes (BP), six Molecular Function (MF), and no Cellular Compartment (CC) were identified (Table 2). There was little commonality among the treatments. Ag-MNP gene list returned GO terms related to metabolic processing of enzymes (GO: 0006732) and cofactors (GO: 0018988), while sAg-MNP returned regulation of growth (GO: 0045927), molting cycle (GO: 0018988) and post-embryonic development (GO: 00009791) terms. The tenth GO term for aging identified for Ag-MNPs (GO: 0007568) was shared with AgNO₃ and was the only BP GO term that was shared among the treatments. AgNO₃ had nine out of ten GO terms relating to stress terms such as heat (GO: 0009408), unfolded protein (GO: 0006986), reactive oxygen species (GO: 0000302), and abiotic stimulus (GO: 0009628) .

Table 2. List of Biological Process (BP) and Molecular Function (MF) Gene Ontology terms that were identified by DAVID using lists of significantly altered genes from N2 wild type nematodes when exposed to AgNO₃, pristine Ag manufactured nanoparticles (Ag-MNP), and sulfidized silver nanoparticles (sAg-MNP) at the EC30 for 48 hours.

Go Term BP	Category	Genes in Category	P-Value	Fold Enrichment	Exposure
GO:34976	Response to ER stress	5	8.60E-06	4.03	AgNO ₃
GO:0033554	Cellular Response to Stress	10	2.50E-05	4.03	AgNO ₃
GO:0006986	Response to unfolded protein	5	2.80E-05	4.03	AgNO ₃
GO:0009408	Response to Heat	5	6.90E-04	4.03	AgNO ₃
GO:0010033	Response to Organic Substances	5	3.80E-03	4.03	AgNO ₃
GO:0009628	Response to abiotic stimulus	6	9.00E-03	4.03	AgNO ₃

GO:0007568	Aging	12	8.40E-04	3.17	AgNO ₃
GO:0042542	Response to Hydrogen Peroxide	4	1.60E-04	1.88	AgNO ₃
GO:0000302	Response to ROS	4	3.40E-04	1.88	AgNO ₃
GO:0007568	Aging	8	8.10E-04	3.09	Ag-MNP
GO:0006084	Acetyl-CoA metabolic process	3	9.30E-04	1.69	Ag-MNP
GO:0006732	Coenzyme metabolic process	4	1.80E-02	1.69	Ag-MNP
GO:0051186	Cofactor metabolic process	4	5.00E-02	1.69	Ag-MNP
GO:0018988	Molting cycle, protein-based cuticle	10	5.00E-04	3.29	sAg-MNP
GO:0045927	Positive Regulation of growth	28	1.20E-02	1.47	sAg-MNP
GO:0006030	Chitin metabolic process	3	2.80E-02	1.38	sAg-MNP
GO:0006022	aminoglycan metabolic process	3	3.62E-02	1.38	sAg-MNP
GO:00009791	Post-Embryonic Development	25	3.40E-02	1.27	sAg-MNP
GO Term MF	Category	Genes in Category	P-Value	Fold Enrichment	Exposure
GO:0008233	peptidase activity	15	1.50E-02	1.83	AgNO ₃
GO:0017171	Serine hydrolase activity	6	1.10E-02	1.73	AgNO ₃
GO:0008238	exopeptidase activity	4	3.60E-02	1.73	AgNO ₃
GO:0004096	Catalase Activity	3	3.00E-03	1.12	AgNO ₃
GO:0008061	Chitin binding	3	1.50E-02	1.4	sAg-MNP
GO:0001871	Pattern Binding	3	2.30E-02	1.4	sAg-MNP

When the MF clustering was run using DAVID only AgNO₃ and sAg-MNP had significant clusters indicated and neither lists had any common terms. GO terms of chitin binding (GO: 0008061) and pattern binding (GO: 0001871) were identified for sAg-MNP. The AgNO₃ gene list contained GO terms such as peptidase activity (GO: 0008233), serine hydrolase activity (GO: 0017171), and catalase activity (GO: 0004096).

All of the MF GO for Ag-MNPs are enzymatic terms whereas the MF GO terms for sAg-MNP related to structural cuticle components of the nematode. This is in agreement with our previous study and supports our hypothesis that the cuticle is the primary target for sAg-MNPs (Starnes et al., 2015). In that study we determined the distribution of Ag in animals exposed to equitoxic concentrations (LC30 over 4hr) of AgNO₃, Ag-MNP and sAg-MNP using synchrotron-based X-

ray fluorescence microscopy. The nematodes exposed to sAg-MNP had non-detectable internal Ag concentrations. Animals exposed to pristine Ag-MNP or AgNO₃ had readily detectable internal Ag concentrations with those in the Ag-MNP treatment being less than the AgNO₃ treatment. We hypothesized that the toxicity of sAg-MNP could be explained by cuticle damage and these gene expression profiles support this hypothesis, since many genes involved in cuticle structure were differentially expressed in the sAg-MNP treatment.

Toxicity using Mutant Strains and RNA interference

The four genes used for the qRT-PCR confirmation involved in cuticle function (*rol-8*, *col-158*, *grl-20*) and metal ion response (*numr-1*) were further used for the functional testing with *C. elegans* mutant strains or using RNAi. If there is an increase or decrease in toxicity in the mutant or RNAi knockdowns versus wild type nematodes after exposures to one or several treatments that indicates that these genes are possibly involved in toxicity or detoxification mechanisms. When mortality testing was conducted on the *rol-8* mutant strain (Fig. 3a) there were significant increases in toxicity as compared to wild type nematodes for AgNO₃ at 7.5 and 9.1 µg Ag L⁻¹, Ag-MNP only at 52 µg L⁻¹, and all levels of sAg-MNP. *rol-8* is associated with deformations in the cuticle of the nematode which causes them to move using a lefthand barrel roll motion (Higgins and Hirsh, 1977). Based on the results of the microarray and qRT-PCR experiments, the significant increase in *rol-8* expression was only observed for the Ag-MNP and sAg-MNP. However, it was surprising to see AgNO₃ causing an increase in toxicity in *rol-8* mutant. This result may on one hand suggest that the response of this gene is due to released ions. However, since both microarray analysis and the independent qRT-PCR experiments did not show up-regulation of *rol-8*, the increased mortality in *rol-8* mutants could be due to the mutant strain being more susceptible to any stressor and or toxicant due to decreased integrity of the cuticle.

In *numr-1* mutants, significant increases in mortality as compared to wild type nematodes were found at all treatment levels in both AgNO₃ and Ag-MNP, but no such increase was observed in sAg-MNP at any level (Fig. 3B). *Numr-1* has been shown to be responsive to AgNO₃ and its response to pristine Ag-MNP and AgNO₃ is indicative that some of the effects are also due to dissolved ions (Tvermoes et al., 2010). Our previous study showed that the dissolved Ag released from sAg-MNP (0.43%) over 24 hr is more than 13 times lower than from pristine Ag-MNP (5.8%) (Starnes et al., 2015), which would explain why we did not observe up-regulation in *numr-1* expression in response to sAg-MNP. This suggests that *numr-1* is involved in toxicity mechanism due to released ions from only pristine but not sAg-MNP.

RNAi knockdown was used for two genes, *col-158* and *grl-20*. The *col* genes are linked to the structure and function of the cuticle of the nematodes, the *grl* genes are in the ground-like group which is the hedgehog related superfamily, whose function, though not yet fully understood, has been linked to cuticle construction (Burglin; Levy and Kramer, 1993; Merris et al., 2007). The *grl-20* and another collagen gene *col-36*, which is also up-regulated in response to sAg-MNP, have been previously suggested to co-regulate each other suggesting similar function. The successful knockdown of both genes was confirmed using qRT-PCR (Fig. S4). No significant changes in the mortality as compared to wild type nematodes in any treatment or level were observed for these genes (Fig. SI 5a and b). The lack of the effect in these genes could be due to the differences in length of exposure between the mortality experiments and the reproduction experiments. The *col-158* and *grl-20* could be involved at time points in the growth cycle outside of the 24 hour mortality experiments. Additionally there are at least 130 cuticle *col* genes and at least 33 *grl* genes that could be compensating for the knockdown of these individual genes (Burglin, 2006; Page, 2007). In addition to these two *col* and *grl* genes, there were 9 more

col or *gri* genes in the shared pool that were also up-regulated only in response to sAg-MNP. It is possible that multiple genes must be knocked down to observe an increase in toxicity.

Figure 3. Mortality of L3 *Caenorhabditis elegans* after 24 hours without feeding exposure to Ag⁺, pristine silver manufactured nanoparticles (Ag-MNP), and sulfidized silver manufactured nanoparticles (sAg-MNP) in moderately hard reconstituted water. Data are presented as mean percent mortality with error bars indicating standard error of the mean. An asterisk (*) indicates significantly different than N2 at $\alpha = 0.05$ within each treatment and concentration.

Conclusions

This study demonstrates that exposure of *C. elegans* to Ag-MNP and sAg-MNP at EC₃₀-reproduction results in different transcriptomic profiles. Based on these transcriptomic results combined with our previous results relating dissolution, toxicity and Ag bioaccumulation, we conclude that the toxicity of Ag-MNP and sAg-MNP is due to different mechanisms. While the toxicity of pristine Ag-MNP is partly explained by dissolution and release of Ag ions, a proportion of the toxicity is due to particle specific effects. The relative contribution of ionic versus particle specific effects was previously shown to be concentration dependent, however, both mechanisms involve uptake and bioaccumulation of Ag. In contrast, the toxicity of sAg-

MNP is largely independent of free ion release and Ag bioaccumulation. The results presented here support the hypothesis that cuticle damage is the primary mechanism of toxicity of sAg-MNP to *C. elegans*. While the decreased solubility of sAg-MNP relative to Ag-MNP is correlated with decreased toxicity, release of free ions is not the primary mechanism of reproductive toxicity of fully sulfidized sAg-MNP.

Supporting Information

Additional text with methods for Ag-MNP synthesis and characterization, four figures, and one table as described in the text. This material is available free of charge via the internet at the Journal of Environmental Science and Technology.

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