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Sulfidation of Silver Nanoparticles: Natural Antidote to Their Toxicity

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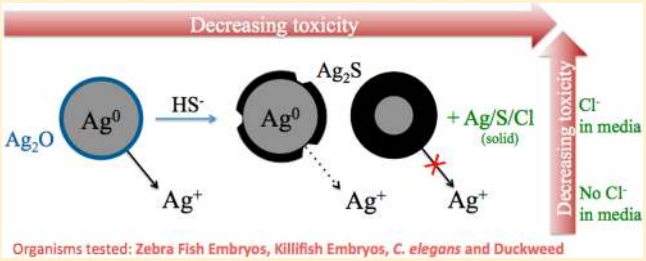
Supporting Information

ABSTRACT: Nanomaterials are highly dynamic in biological and environmental media. A critical need for advancing environmental health and safety research for nanomaterials is to identify physical and chemical transformations that affect the nanomaterial properties and their toxicity. Silver nanoparticles, one of the most toxic and well-studied nanomaterials, readily react with sulfide to form Ag(0)/Ag_2S core−shell particles. Here, we show that sulfidation decreased silver nanoparticle toxicity to four diverse types of aquatic and terrestrial eukaryotic organisms (*Danio rerio* (zebrafish), *Fundulus heteroclitus* (killifish), *Caenorhabditis elegans* (nematode worm), and the aquatic plant *Lemna minuta* (least duckweed)). Toxicity reduction, which was dramatic in killifish and duckweed even for low extents of sulfidation (about 2 mol % S), is primarily associated with a decrease in Ag^+ concentration after sulfidation due to the lower solubility of Ag_2S relative to elemental Ag (Ag^0). These results suggest that even partial sulfidation of AgNP will decrease the toxicity of AgNPs relative to their pristine counterparts. We also show that, for a given organism, the presence of chloride in the exposure media strongly affects the toxicity results by affecting Ag speciation. These results highlight the need to consider environmental transformations of NPs in assessing their toxicity to accurately portray their potential environmental risks.



INTRODUCTION

While a great deal of studies have examined the risks of pristine nanomaterials, increasing emphasis is being placed on studying the "aged" forms of the materials that are expected to dominate in the environment[1,2] This is because physical, chemical, and biological transformations of many nanomaterials (e.g., dissolution to release toxic metal ions,[3,4] oxidation and reduction,[5,6] adsorption of biomacromolecules[7] or natural organic matter[8,9]) can affect the properties and toxicity of the materials.[10] For example, carbon nanomaterials can be partially oxidized *in vivo*[11] or by exposure to sunlight.[12] Carbon

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nanotubes that have been partially oxidized by peroxidases induce much less pulmonary inflammation than pristine nanotubes.[13] Despite this growing body of knowledge about nanomaterial transformations in environmental and biological media, a great majority of toxicity testing still utilizes as-manufactured or pristine nanomaterials.[3,14,15]

Similar trends are observed for assessment of silver nanoparticle (AgNP) toxicity. Most recent studies[16−31] have used pristine AgNPs, which organisms are not likely to encounter. Pristine particles are used in order to carefully assess the effect of intrinsic particle properties on toxicity (e.g., particle size,[32] shape,[21] and the nature of the surfactant[30]). However, toxicity risk will depend in part on the speciation of the nanomaterial that the organism encounters. Less has been done to assess the effect of environmental constituents on particle properties and how these alterations impact particle behavior and toxicity.

AgNPs have repeatedly been found to readily transform to Ag_2S or AgCl, depending on the environment.[33−40] Elemental silver in the AgNPs is oxidized to Ag^+, which then reacts with inorganic sulfide to form Ag_2S NPs or core−shell Ag:Ag_2S particles. Depending on the Cl/Ag ratio, Ag^+ may also react with chloride to form solid or aqueous Ag−Cl species.[34,35,38,41] Thus, there is a range of dissolved silver species (e.g., AgCl(aq), $AgCl_2^-$, $AgCl_3^{2-}$) that may result depending on the composition of the water.[38,42] Metal sulfidized nanoparticles in particular have been found in wastewater treatment biosolids and in samples taken from pilot studies using simulated treatment.[40,43,44] This suggests that AgNPs entering the environment via sewage sludge or WWTP effluent will be partly or fully sulfidized.[39] In another study, AgNPs added to a freshwater mesocosm simulating an emergent wetland were shown to be mostly sulfidized after 18 months of aging in the sediment.[37] Several studies have demonstrated that toxicity of AgNPs toward microorganisms including nitrifying bacteria[45,46] and *Escherichia coli*[47] is reduced by sulfidation. While the effect of ligands on dissolved Ag toxicity has been reviewed,[42] the ability of sulfidation to decrease the toxicity of pristine AgNPs to organisms other than bacteria has yet to be evaluated, especially as a function of the degree of sulfidation. The latter is important because the rate and extent of sulfidation of AgNPs released to the environment is not yet well characterized. Media effects on AgNP dissolution and aggregation are also not yet well tested.

In this study, pristine AgNPs and AgNPs that have been partially sulfidized to varying degrees were synthesized and characterized with regard to size and electrophoretic mobility; their toxicity to four multicellular organisms, with each organism in a low and high ionic strength medium; and their solubility and aggregation state in the different media used in the toxicity assays. Our main objective was to test the hypothesis that partial sulfidation of AgNPs reduces their toxicity (i.e., mortality or growth suppression) to a diverse range of organisms (i.e., vertebrates, invertebrates, plants). Our second objective was to test the hypothesis that the composition of the exposure media (especially Cl^- concentration) could strongly affect the observed toxicity of AgNPs, as has been documented for dissolved Ag species.[42] We found that partial sulfidation of AgNPs significantly reduced toxicity for all organisms studied and that even a low amount of sulfidation (~mol 2%) was typically sufficient to greatly reduce toxicity. Chloride in the exposure media decreased toxicity for 3 organisms. This latter finding raises the possibility that discrepancies in reported toxicity values are not solely due to organismal differences but also due to differences in media chemistry[24,48] that are typically poorly characterized. These results highlight the importance of considering both the environmental transformation of nanoparticles and chemical interactions with media when assessing their toxicity, in order to accurately portray their potential toxicity risks.

MATERIAL AND METHODS

Particle Synthesis and Characterization. A batch of polyvinylpyrrolidone (PVP)-coated AgNPs with an average size of 37 nm and a standard deviation of 5.8 nm (Figure 1) were synthesized and characterized along with partly sulfidized PVP-coated AgNPs (molar S/Ag ratio = 0.019, 0.073, and 0.432), then distributed for immediate use. In brief, the AgNPs were sulfidized using a 10^{-3} M sodium sulfide (Na_2S) solution in a 0.01 M $NaNO_3$ electrolyte. After 24 h, the final solutions were centrifuged and washed with DI water. Details of the synthesis and complete characterization of these particles are given in Levard et al.[35] Briefly, the relative abundances of Ag^0/Ag_2S were determined by X-ray absorption spectroscopy. The Ag_2S fraction represents 3, 10, and 64 mol % of total silver for the S/Ag molar ratios of 0.019, 0.073, and 0.432, respectively, which are consistent with the range of sulfidation extent that was observed for AgNPs aged in more realistic environmental scenarios.[37,39,43,49,50] The pristine and the sulfidized AgNPs were negatively charged at pH 7.5 (−15 mV in 10 mM $NaNO_3$).[35] Before exposure to the different organisms, the particles were maintained in aqueous suspension to avoid the irreversible aggregation that typically occurs upon drying. Prior to characterization or toxicity testing, particle suspensions were stored for as short a time as possible (less than a month) in a sealed bottle in the dark.

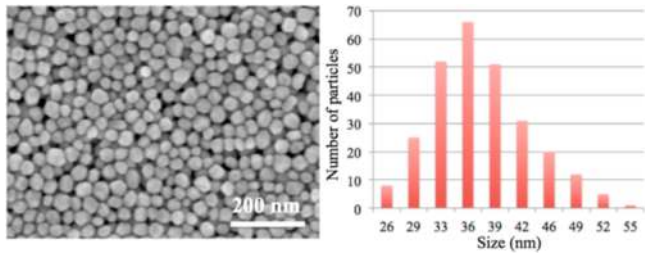

Figure 1. Nonsulfidized silver nanoparticles analyzed by scanning electron microscopy (SEM). Image analysis revealed that particles had an average size of 37.3 nm with a standard deviation of 5.8 nm. The size distribution determined from counting 260 particles on 2 separate SEM images.

The hydrodynamic diameter and aggregation rates of the particles were determined in each type of exposure medium by dynamic light scattering (ALV/CGS-3, Langen Germany). For all aggregation experiments, a 50 mg/L AgNP stock suspension was added to DI water and diluted to a concentration of 25 mg/L using double strength media to provide the same solution conditions as in the exposure study. The dynamic light scattering measurements were started immediately after dilution. Initial hydrodynamic diameters ranged from 40 nm to 360 nm depending on the composition of the medium (Media compositions are listed in Table 1 and Supporting Information (SI) Table 1). Aggregation rates were approximated from the initial slopes of hydrodynamic diameter vs time measurements.

Table 1. Organisms and Properties of the Medium Types Used in This Study[a]

medium type	organism	chloride ion [mM]	ionic strength [mM]	pH	cond. [mS]	hydrodynamic diam. (nm) pristine AgNP	hydrodynamic diam. (nm) Ag_2S NP (S/Ag = 0.019)
DI water	zebrafish embryo, killifish embryo					80	140
EPA medium	*C. elegans*	0.1	3.6	7.7	3.6	80	140
Hutner's medium	duckweed	0	27.0	5.0	1.9	40	500
Hutner's + 1.75‰ ASW	duckweed	32.4	66.0	5.1	2.4	60	400
K^+ medium	*C. elegans*	89	104	5.7	83.0	300	300
embryo medium	zebrafish embryo	17.5	23.5	7.3	23.5	60	140
10 ‰ IO[45]	killifish embryo	176	224.3	8.1	10.0	340	300

[a]Complete information on media ion compositions is available in the Supporting Information (Supplementary Table 1).

To determine the effect of sulfidation and the media composition on the concentration of soluble silver species, the concentration of soluble Ag species was measured for the pristine AgNPs and for those sulfidized at an S/Ag molar ratio of 0.019 (the lowest used) for one AgNP concentration. Given the limited amount of the more highly sulfidized AgNPs, only the least sulfidized particles could be used to assess the effect of sulfidation and medium composition on dissolved Ag concentration. Either pristine AgNPs or sulfidized AgNPs (S/Ag molar ratio of 0.019) were added to media to a concentration between 144 to 221 ppm. These concentrations, which were similar to or higher than the doses used in the toxicity testing, were needed to measure the dissolved Ag released from the samples in each medium given the detection limits of the analytical method (discussed below). The particles were separated from the supernatant after 48 and 120 h by filtering using Amicon Ultra 3 kDa MWCO centrifuge tubes. Supernatant silver ion concentrations were measured using inductively coupled plasma–atomic emission spectrometry (ICP-AES; TJA IRIS Advantage/1000 Radial ICAP Spectrometer; detection limit, 10 ppb) after acid digestion with concentrated HNO_3. Duplicates of each sample were measured, with ion concentrations normalized by initial particle concentrations.

Organisms and Media. Four model organisms were selected for this study: two species of fish, *Danio rerio* (zebrafish) and *Fundulus heteroclitus* (killifish); the nematode worm *Caenorhabditis elegans*; and the aquatic plant *Lemna minuta* (least duckweed). These organisms were selected to provide a diverse set of organisms and uptake pathways for AgNPs and Ag ions. Six common exposure media and DI water were used. All media were prepared using high purity chemicals and deionized water, with the exception of the salt blend, Instant Ocean (IO, Foster & Smith, Rhinelander, WI, U.S.A.), which was diluted to 10 parts per 1000 (10 ‰ on a mass per mass basis) with deionized water. Table 1 lists the organisms and six exposure media types used, and compares their chloride concentration, ionic strength, pH, and conductivity. The exact composition of 10 ‰ IO was estimated from an elemental composition analysis performed by ICP-AES.[51] Artificial seawater (ASW) was prepared according to Kester et al. (1967).[52]

Toxicology. In all assays, PVP-only controls were analyzed along with the AgNPs. The PVP concentration ranged between 20 and 200 mg/L, depending on the organism and particle concentrations used. These PVP concentrations are much higher than the concentration of PVP that would result if all of the PVP adsorbed to the particle surfaces were to desorb in the experiment. Additionally, adsorption of PVP to the particles is essentially irreversible over the time scales used in these studies,[53] so these controls are quite conservative with respect to PVP toxicity. These controls consistently showed no adverse effect.

Zebrafish Embryos (*Danio rerio*). Adult Tropical 5D zebrafish were housed and reared at Oregon State University Sinnhuber Aquatic Research Laboratory. Adult zebrafish were group-spawned and embryos were collected and staged according to Kimmel et al.[54] For each sample, working stocks of AgNPs (both unsulfidized and sulfidized) were mixed to 100 μg/mL (ppm) by diluting the AgNPs in reverse osmosis (RO) water or embryo medium. Samples were then placed into a water bath sonicator for 3 min. To increase bioavailability, the embryonic chorion was removed at four hours post fertilization (hpf).[55] Embryos were rested for 30 min prior to exposures in individual wells of a 96-well plate with 100 μL of prepared AgNPs suspension. Exposure plates were sealed and wrapped with aluminum foil to prevent evaporation and minimize light exposure. Embryos were exposed to five concentrations of silver nanoparticles with the highest concentration at 50 ppm and the lowest concentration at 0.08 ppm. An equivalent amount of DI water or embryo medium without AgNPs was added as a negative control (n = 16, two replicates). Trimethyltin chloride (5 μM) was used as a positive control to confirm the embryonic responsiveness. The static nanoparticle exposure continued until 120 hpf. At 120 hpf, embryos were assessed for mortality.[55]

Killifish Embryos (*Fundulus heteroclitus*). Adult killifish were collected from King's Creek, VA, U.S.A. (37° 18′16.2″N, 76° 24′58.9″W). Killifish were kept in 30 or 40 L tanks in a flow-through system. Water was maintained at 25 °C and 15‰ IO (made from diluted Instant Ocean, Foster & Smith, Rhinelander, WI, U.S.A.) on a 14:10 h light/dark cycle. Fish were fed pelleted food *ad libidum* (Aquamax Fingerling Starter 300, PMI Nutritional International, LLC, Brentwood, MO, U.S.A.). Embryos were obtained for experiments by manual spawning and fertilization. At least 1 h following fertilization, embryos were rinsed in 0.3% hydrogen peroxide followed by 3 washes in 20‰ ASW.

Embryos were screened for normal development at the 4–8 cell stage and immediately dosed in 0.2 mL dosing solution/embryo in 96-well plates with n = 24 for each concentration.[56] 1000 ppm stock AgNPs suspensions were sonicated for 15 s in a bath sonicator. Embryos were exposed to four concentrations ranging from 1 to 200 ppm Ag for each of the four AgNPs solutions in both DI water and in 10 ‰ ASW. Experiments

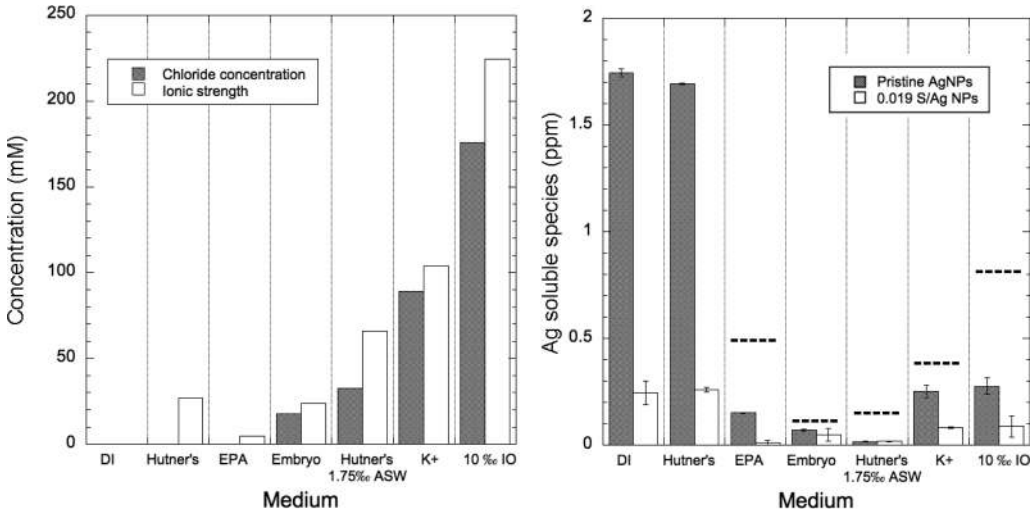

Figure 2. Effects of sulfidation and media composition on the concentration of dissolved Ag species. Chloride concentration and ionic strength are represented on the left. The dissolution of initial (black bars) and 0.019 S/Ag particles (white bars) was measured in six media types and DI water over 120 h (right). The total concentration of Ag was between 144 and 221 ppm for both particle types. The dashed lines above the bars correspond to the thermodynamically expected concentration of dissolved Ag species calculated using Medusa based on the chemical composition of each media (except for DI water and Hutner's medium, which had no Cl^- and thus no AgCl).[61] The same trends were observed after 48 h in all media types (SI Figure 2).

were screened for mortality, which is defined as cessation of heartbeat at 24 and 48 h post dosing.

***C. elegans* (*Caenorhabditis elegans*).** The nematode *Caenorhabditis elegans* was tested for acute lethality and growth inhibitory effects of the pristine AgNPs and sulfidized AgNPs in high- and low-ionic strength *C. elegans* liquid media (K^+ medium and EPA water, respectively). One lethality and three growth assays were performed. Exposure concentrations were based on previous AgNP toxicity studies.[23,30] Age-synchronized L1 larval stage *C. elegans* of the wild-type N2 (Bristol) strain were obtained by overnight hatch of purified egg preparations, as previously described.[23] To test lethality, L1 cultures were exposed in 96-well plates, without food, to pristine AgNPs and sulfidized AgNPs. After 24 h, nematodes were probed for movement with a worm pick for 15 s, and scored as alive (responsive) or dead (unresponsive).

For growth assays, age-synchronized L1 larvae were obtained, as described above. Stock solutions were sonicated for 15 s in a bath sonicator and then diluted to target exposure concentrations in EPA and K^+ medium, with the volume of stock solution balanced by the same volume of double ionic strength medium to preserve medium composition. Nematodes were fed UVC-inactivated *uvrA* (DNA damage repair-deficient) bacteria[23] daily during the exposure, as described previously.[30] Nematode size (EXT, extinction coefficient) was measured using a COPAS Biosort at 72 h after the exposure began, as previously described.[30] Control nematodes progressed from the L1 to the young adult stage during this time.

Duckweed (*Lemna minuta*). Samples of the floating aquatic plant *Lemna minuta* were collected from the coastal plain of North Carolina and grown in pure-culture using half-strength Hutner's medium[57] under cool fluorescent lights at 100 μEinsteins m^{-2} s^{-1}. For experimental treatments, *L. minuta* were grown in 10 mL of either Hutner's medium (a lower ionic strength medium) or a modified Hutner's medium made in 1.75‰ ASW.[52] Experiments were conducted in 6 cm × 1.5 cm Petri dishes (Genesee Scientific, San Diego, USA). Plants were exposed to 0.3, 1, 3, 10, 30, and 100 mg Ag L^{-1} for each particle type for 7 days. Each treatment consisted of four replicates, each starting with 5 fronds of *L. minuta*, where one frond is one individual leaf. Growth suppression was measured by dry weight relative to controls.

Statistical Analysis of Toxicity Assay Results. The mortality data from the zebrafish and killifish (48 h postdosing) assays were fit to a set of dichotomous models (logistic, probit, log−logistic, log-probit, Weibull) using the EPA software BMDS (Benchmark Dose Software). Since the model selection criterion AIC (Akaike information criterion) did not differ significantly across model types, a probit model was considered appropriate for both organisms. For *C. elegans*, the extinction (EXT) values obtained from the Biosort (72 h postdosing) were fit to linear and exponential models, which are more appropriate for assays with a continuous end point. An exponential model (Model 4 in BMDS, with an assumed log-normal distribution of the response) was selected because the dose−response curves fit using this model achieved significantly lower AIC values than alternatives. For duckweed, dry weight measurements were fit to a linear logistic regression model using the software JMP. This model accounts for the subtoxic stimulus of growth (i.e., hormesis) observed at low concentrations of AgNPs.

For zebrafish and killifish, LC_{50} values were estimated as 50% extra risk over background. For *C. elegans* and duckweed, EC_{50} values were estimated as 50% relative deviation in the mean response from the background mean. One-sided 95% confidence limits were estimated from each fit distribution. Global goodness of fit statistics and dose−response curves are provided as Supporting Information (Supplementary Table 2, Supplementary Figure 1) along with the estimated EC_{50} and LC_{50} values. To ensure the quality of reported estimates, EC_{50} values and LC_{50} values exceeding the range of concentrations tested for each organisms were simply reported as being in excess of this range.

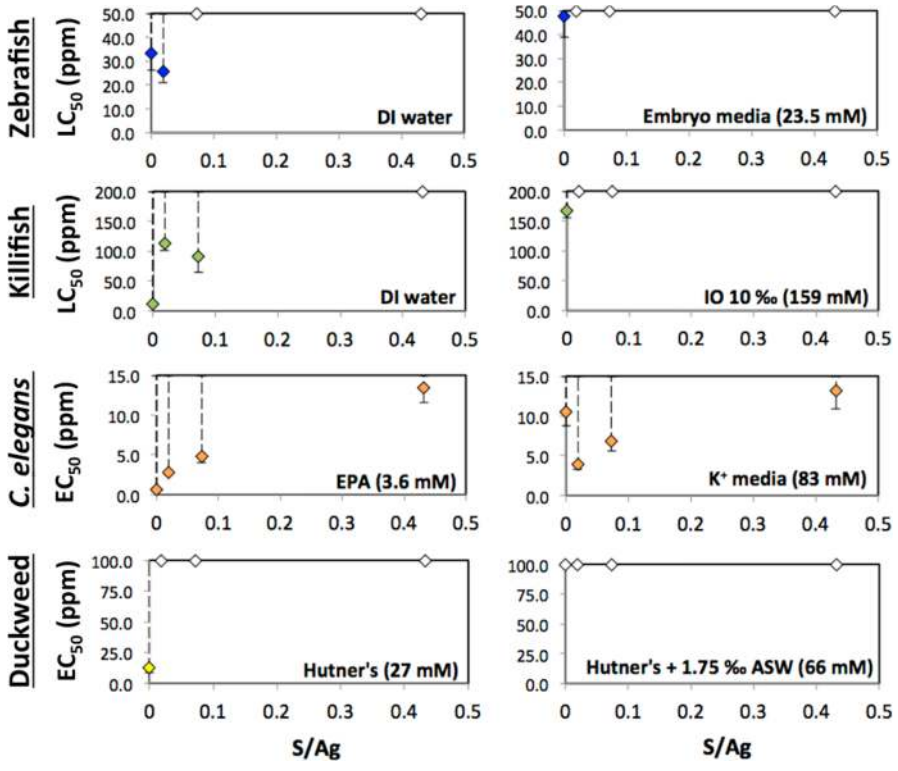

Figure 3. LC_{50} and EC_{50} responses (in ppm) of zebrafish embryos, killifish embryos, *C. elegans*, and duckweed to the presence of silver nanoparticles. Error bars denote a one-sided 95% confidence interval. One-sided intervals calculated by BMDS reflect the fact that potential toxicity (lower bound) is generally of greater concern than potential nontoxicity (effective upper bound of infinity). Open diamonds indicate that the predicted EC_{50} or LC_{50} exceeded the tested range of AgNP concentrations (error bars not shown). As sulfidation of the silver particles increases along the *x*-axis (from 0 to 0.5, where 0.5 represents a 100% Ag_2S AgNP), all organisms are overall less sensitive to the particles. For three of the four organisms (results for *C. elegans* are ambiguous), exposures at relatively higher ionic strengths (right column) show relatively higher toxicity than the lower ionic strength media (left column).

RESULTS AND DISCUSSION

Impacts of Media on Particle Stability (Dissolution and Aggregation). Two key parameters that can potentially affect AgNP toxicity are dissolution and aggregation. Free Ag^+ ion, which has previously been shown to be a strong determinant of toxicity for a number of species exposed to various forms of soluble silver and bulk $Ag_2S_{(s)}$,[42] appears to be the main cause of toxicity for AgNPs in this study (described later in the paper). In the literature, multiple modes of toxicity resulting from exposure to AgNPs have been reported. Some evidence points to a nanoparticle-specific effect,[24,48] whereas other evidence suggests that toxicity derives primarily from the release of Ag^+ ions.[4,24] Nanoparticle-specific effects may result from exogenous or endogenous production of ROS (e.g., superoxide or hydroxyl radicals), which induces oxidative stress,[32,48,58,59] or they may result from locally elevated dissolved Ag concentrations in the vicinity of the AgNPs.[47] However, the observed toxicity may also correlate with the total concentration of soluble Ag species in the bulk solution (i.e., there may be no particle effect). Dissolved Ag species can react with sulfur- and phosphorus-bearing moieties in cellular components (e.g., membrane-bound proteins), leading to the observed toxicity.[42,60] Specific interactions between the AgNPs, released Ag^+, and components of the media can affect toxicity. Therefore, the concentration of soluble Ag species was measured in each exposure medium that had been amended with AgNPs or the sulfidized AgNPs (0.019 S/Ag ratio; Figure 2 (right) and SI Figure 2). In each medium, the partly sulfidized materials (0.019 S/Ag molar ratio) released less dissolved silver compared to the pristine AgNPs over 48 and 120 h. The Ag_2S layer formed on the AgNPs' surfaces, representing only about 3 mol % of the total silver added, decreases the rate of oxidation and Ag^+ release by an order of magnitude as shown in a previous study.[35]

Additionally, significant differences in the dissolved silver species concentration were observed for each particle type between media with no chloride (e.g., DI water and Hutner's medium) and all the other media that contain chloride. Chloride is known to strongly react with Ag^+ ions, potentially forming quite insoluble AgCl(s) ($K_{sp} = 1.77 \times 10^{-10}$)[34] or dissolved silver chloride species ($AgCl_{aq}$, $AgCl_2^-$, $AgCl_3^{2-}$, and $AgCl_4^{3-}$). Speciation will depend on the Cl/Ag ratio in solution.[38] The measured concentrations of dissolved silver species, including Ag^+ and the dissolved Ag−Cl complexes, are consistent with those calculated assuming that the Ag^+ ion in solution is in equilibrium with the chloride ion present in the media (horizontal dashed lines in Figure 2). Therefore, the decrease in the soluble silver species concentration in media containing chloride (EPA, embryo, Hutner's plus 1.75‰ ASW, K+, and 10‰ IO) compared with those without chloride (DI water and Hutner's medium) can be attributed to the formation of $AgCl_{(s)}$ precipitates and soluble Ag−Cl complexes.[62] This result shows the importance of considering particle-media interaction in solubility assays. Considering that dissolved silver species will likely play a role in the observed toxicity, these results suggest that the toxicity for a given organism will depend

on the nature of the media used. This is consistent with results for Ag ion toxicity across a range of organisms tested.[42]

The composition of the media not only affects AgNP dissolution but also aggregation. This in turn can affect the route of exposure and biouptake.[63] The hydrodynamic diameter of pristine PVP-coated AgNPs and sulfidized PVP-coated AgNPs was measured in each exposure medium using dynamic light scattering. Measurements performed on the pristine AgNPs indicate that they had very low rates of aggregation for all media except the K^+ medium and the IO medium (SI Figure 3). This finding is consistent with the fact that the K^+ and IO media have chloride concentrations above the reported critical coagulation concentration (112 mM) for PVP-coated AgNPs,[64] whereas the other media do not. In contrast, the sulfidized AgNPs are rapidly aggregated when added to the exposure medium (Table 1and SI Figure 3), and the degree of aggregation is dependent on the medium in which they are dispersed. Measured hydrodynamic diameters ranged from 60 to 250 nm. AgNP stability strongly depends on the composition of the media, which can potentially play an important role on the toxicity.

Effect of sulfidation on toxicity. To examine the extent to which the degree of sulfidation changes AgNP toxicity, four types of organisms (zebrafish embryos, killifish embryos, *C. elegans*, and duckweed) were exposed to pristine AgNPs and AgNPs that had been sulfidized to three different levels. Overall, increasing the degree of sulfidation of the AgNPs reduced their toxicity to all organisms tested (Figure 3).

There were species-specific differences in the magnitude of the effect of sulfidation. For example, when moving from pristine to 0.019 S/Ag (~3% of the silver is sulfidized) particles, sulfidation increased the EC_{50} and LC_{50} of the AgNPs for duckweed and killifish embryos, respectively, by up to an order of magnitude. Comparing the same particle types for *C. elegans* and zebrafish, the trend in EC_{50} and LC_{50} with increasing sulfidation is less clear. For zebrafish in DI water and *C. elegans* in both media, low levels of sulfidation (S/Ag = 0.019) had less impact on toxicity than for duckweed and killifish embryos. The nonmonotonic behavior observed for zebrafish in DI water and *C. elegans* in K^+ media may be a result of experimental error. In particular, large variance in the *C. elegans* response (EXT values) resulted in particularly poor model fits for this organism (see SI Table 2). Regardless, higher levels of sulfidation (S/Ag > 0.019) always led to lower toxicity. Differences between organisms are likely due to the different sensitivities of the organisms to Ag, and potentially to differences in the exposure route that the assay exploits.

Effect of Media Components on Toxicity. The toxicity of AgNPs was also impacted by the composition of the media used in the assay. For killifish, zebrafish, and duckweed, pristine AgNP toxicity was lower in the higher ionic strength media than it was in the lower ionic strength media (Figure 3). In fact, at the higher ionic strength, there was no observed toxicity for exposure of killifish, zebrafish, or duckweed to either pristine or sulfidized AgNPs at exposure doses (e.g., >50 mg/kg), which are higher than the concentrations expected in surface water (ng/L) and loadings to sediments (μg/kg/y).[65] Observed differences in toxicity can probably be attributed to the presence of chloride in the higher ionic strength media, which was shown to lower the amount of dissolved silver in the system (Figure 2). Aggregation may also partly explain those differences.

The *C. elegans* growth is less sensitive to the choice of exposure media than the end points for the other tested organisms (Figure 3). This contrasts with a previous report showing that the effect of AgNPs on growth was greater in the lower ionic strength EPA media.[30] The variance in the extinction coefficient data may explain this discrepancy. It is worth noting that both the EPA medium and the K^+ medium contain Cl^- and yielded similar measured and calculated concentrations of soluble silver species (Figure 2). This is an indirect indication that dissolved silver species are controlling the toxicity in this assay. However, in lethality tests, *C. elegans* L1 larvae were less susceptible to the lethal effects of the AgNPs in the higher ionic strength medium (K^+ medium) compared to the lower ionic strength medium (EPA medium, Figure 4).

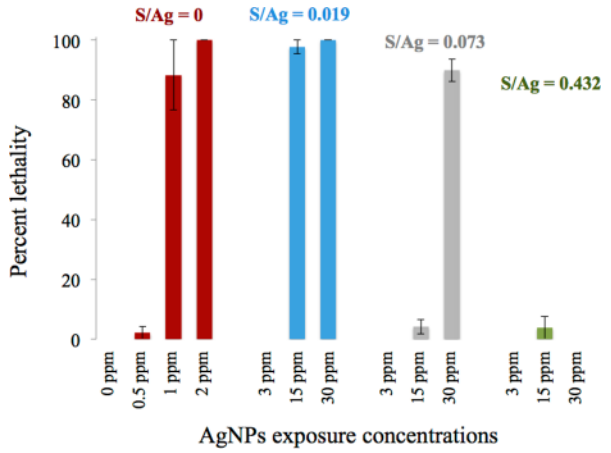

Figure 4. Influence of sulfidation on the mortality caused by AgNPs for *C. elegans* in EPA water (low ionic strength and low Cl^-). Mortality is defined as the number of unresponsive organisms over the total number of organisms. Error bars represent the standard error of the mean (four replicates). At high ionic strength, there was no mortality for pristine AgNPs or any of the sulfidized AgNPs (data not shown).

This agrees with results previously reported for AgNPs ranging in size from 5 to 75 nm in diameter (measured by TEM) and with various coatings including gum arabic, polyvinylpyrrolidone (PVP), and citrate.[30]

Studies have shown that the aggregation of AgNPs can decrease the rate of release of Ag^+ from the particles.[66] In this study, both aggregation and the presence of chloride ion can decrease the amount of Ag^+ and other soluble Ag species and therefore toxicity. While is it difficult to separate these effects in this study, there was little correlation between the size of the aggregates (Table 1) and the observed toxicity. This suggests that the reduction in Ag^+ due to $AgCl_{(s)}$ formation had the dominant effect on toxicity. This is most evident in the comparison of toxicity assay results for the pristine AgNPs in the low ionic strength and high ionic strength media. The data in Figure 3 indicate that there was a reduction in toxicity of the pristine AgNPs in the embryo medium compared to DI water for the zebrafish assay even though there was no aggregation of the AgNPs in either medium (Table 1 and SI Figure 3). Thus, the reduction in zebrafish toxicity can be attributed solely to the decrease in the concentration of dissolved silver. Similarly, in the case of the duckweed, there was no aggregation of the particles in Hutner's medium or Hutner's plus 1.75‰ ASW; however, there was a decrease in the observed toxicity and a decrease in dissolved Ag, presumably due to formation of

$AgCl_{(s)}$. A second line of evidence suggesting the negligible effect of AgNP aggregation on toxicity is seen in the *C. elegans* data. Even though there was significant aggregation in the K^+ medium compared to the EPA medium, there was relatively little difference in the measured LC_{50} values for the pristine AgNPs in these different media. Additionally, both the measured and thermodynamically expected dissolved silver concentration in solution is similar for both media. This suggests again the higher impact of dissolved silver species on toxicity compared to aggregation. A similar comparison cannot easily be made with the Ag^0/Ag_2S NPs, since these particles rapidly aggregated in the media and we cannot distinguish between the effects of sulfidation and aggregation on Ag^+ released from the particles. For the particles with the lowest degree of sulfidation, toxicity in the high ionic strength media was less than in the lower ionic strength media for each organism. However, there was no apparent trend between aggregation state and toxicity (Table 1 and SI Figure 3). This suggests that chloride in the media is controlling the dissolved silver concentration in addition to the effects of sulfidation.

Environmental Implications. This study demonstrates that even a low degree of sulfidation of the AgNPs dramatically decreased their toxicity to killifish and duckweed. Overall, partial sulfidation to a level greater than 0.073 S/Ag mol ratio decreased AgNPs toxicity for all organisms in a wide range of exposure media. This decrease in toxicity is related to decreases in silver ions released from the AgNPs because of the formation of a nearly insoluble Ag_2S layer ($K_{sp} = 10^{-51}$) on the surface of the AgNPs. Additionally, the presence of chloride in exposure media decreases the toxicity due (most probably) to the precipitation of $AgCl_{(s)}$. The decrease in AgNP toxicity due to the precipitation of $AgCl_{(s)}$ or the precipitation of Ag_2S on the particle surface was recently shown for *E. coli* in two separate studies.[38,47] These findings for AgNPs are consistent with previous work determining Ag toxicity toward a variety of organisms using (primarily) $AgNO_3$.[42] AgNP aggregation state did not affect the toxicity results, likely because the effects of sulfide and chloride on free silver ion concentration masked any possible effects of aggregation state.

Many engineered nanomaterials aside from Ag are made using soft metal cations (e.g., Zn, Cu, Cd). These metals all have a high affinity for inorganic sulfide and therefore will also readily sulfidize to form metal sulfide solids with relatively low aqueous solubilities.[67] An initial release of unsulfidized nanomaterials made from these metal cations would likely have a deleterious effect in natural systems, but the long-term effects would be lessened through sulfidation of the nanoparticles. Based on this work, efforts should focus on determining the types, rates, and extents of transformation of nanoparticles in the environment. Testing protocols for assessing the toxicity of NPs made from soft metal cations that react with inorganic sulfide should consider use of sulfidized (transformed) nanoparticles made from metal cations known to form metal sulfides, since this transformation may affect toxicity. Assessments should also be conducted in media representative of the environment under consideration (e.g., brackish waters vs freshwater), since the compositions of different media can impact the measured LC_{50} and EC_{50} through interaction with the nanoparticles or by determining the concentration of toxic metal cations.

Finally, it is important to note that these transformed products are not necessarily the stable end products. Rather, their stability depends on local solution conditions (e.g., pH, redox). Although the sulfidized form of AgNPs is highly stable under reducing conditions, it is possible that they may be readily oxidized if the environmental conditions became oxic (e.g., dispersed into the water column). Thus, the long-term fate of metal sulfides must be determined. This is especially true if the sulfidized products are small in size, as has been shown for the sulfidation of ZnO and CuO NPs,[67,68] because the reactivity and rates of oxidation may be higher than for larger sized metal-sulfides.

■ ASSOCIATED CONTENT

Supporting Information

Chemical composition of the different media used, the results of the statistical analysis used to calculate EC_{50} and LC_{50} values, the effects of sulfidation and media on the available dissolved silver species after 48 h, and the aggregation of initial silver particles and S/Ag (0.019) nanoparticles in the different media. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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