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Impacts of Select Organic Ligands on the Colloidal Stability, Dissolution Dynamics, and Toxicity of Silver Nanoparticles

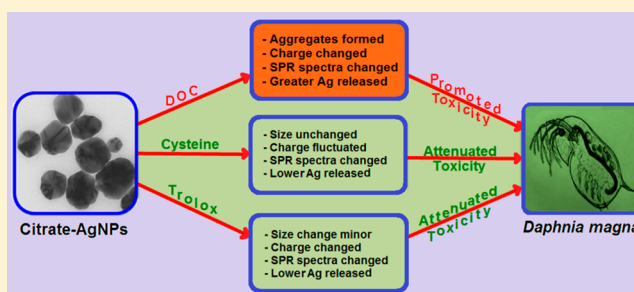
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S Supporting Information

ABSTRACT: Key understanding of potential transformations that may occur on silver nanoparticle (AgNP) surface upon interaction with naturally ubiquitous organic ligands (e.g., –SH (thiol), humic acid, or –COO (carboxylate)) is limited. Herein we investigated how dissolved organic carbon (DOC), –SH (in cysteine, a well-known Ag⁺ chelating agent), and –COO (in trolox, a well-known antioxidant) could alter the colloidal stability, dissolution rate, and toxicity of citrate-functionalized AgNPs (citrate–AgNPs) against a keystone crustacean *Daphnia magna*. Cysteine, DOC, or trolox amendment of citrate–AgNPs differentially modified particle size, surface properties (charge, plasmonic spectra), and ion release dynamics, thereby attenuating (with cysteine or trolox) or promoting (with DOC) AgNP toxicity. Except with DOC amendment, the combined toxicity of AgNPs and released Ag under cysteine or trolox amendment was lower than of AgNO₃ alone. The results of this study show that citrate–AgNP toxicity can be associated with oxidative stress, ion release, and the organism biology. Our evidence suggests that specific organic ligands available in the receiving waters can differentially surface modify AgNPs and alter their environmental persistence (changing dissolution dynamics) and subsequently the toxicity; hence, we caveat to generalize that surface modified nanoparticles upon environmental release may not be toxic to receptor organisms.



INTRODUCTION

When risks pertaining engineered nanoparticles (ENP) have remained unclear, nanotechnology continues to grow with evolution of the novel ENPs and the associated nanoenabled products in the commercial market.^{1,2} Silver nanoparticle (AgNP) is among the most widely used ENPs in both the consumer and medical applications for its prominent catalytic, antimicrobial, and plasmonic properties^{2,3} and has therefore generated prominent interest for understanding its underlying toxicity mechanism.^{1,4–8} Identifying AgNP toxicity mechanism(s) would inform the risk managers to find ways of mitigating the risks (if any).

The concept that ENPs are surface modified upon entering the natural environment has emerged as a generally accepted premise in environmental nanoscience and toxicology.^{1,5,9–11} An apparent problem with this tenet, however, is that different types of NPs may not show similar surface modifications following their release into aquatic or terrestrial environments where various organic and inorganic ligands exist.^{5,9–13} Therefore, a need to evaluate the potential changes that might occur on the ENP surface, and how such changes would modulate ENP toxicity, may arise on a case-by-case basis.^{5,14}

Natural organic ligands that are ubiquitous in aquatic systems include reduced –SH (a functional group in cysteine), –COOH (a functional group in organic acids, including

amino acids and vitamins), humic and fulvic substances (with variable aliphatic and aromatic groups including R–OH, –COOH, –OH, –OCH₃, –NH₂, etc.), and carbohydrates, among others, which are the products of metabolic and decomposition processes emanating of both the aquatic and terrestrial systems.¹³ As environmental release of AgNPs from the nanoenabled products is inevitable,¹⁵ key information about the potential transformations that might occur once model AgNPs are released into aquatic environments^{5,9–11} and how such modifications would change the toxicity is limited to a few studies;^{1,16,17} therefore, more research is warranted to explain if and how AgNPs are surface modified and to elucidate underlying toxicity mechanisms. Previous studies have reported mixed results: some show particle-specific toxicity,^{7,16,18,19} others predominantly show free Ag⁺ toxicity,^{6,20} while the reports revealing combined effects of particles and released ions to be lower than of free Ag⁺ ions alone are rare.¹ Assay-dependent toxicity^{21,22} or species-specific sensitivity to NPs is emerging as a viable explanation – one that implicates differences in species biology (Gram positive/Gram negative

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bacteria, feeding behavior in higher organisms, predator–prey interactions, etc.) – to ENP toxicity.^{1,7,12,23–25}

In this study, we investigate the potential modulating effects of L-cysteine, dissolved organic carbon (DOC), and trolox on the colloidal stability, ion release rate, and aquatic toxicity of a widely studied model citrate-coated AgNPs (citrate–AgNPs) against the keystone crustacean *Daphnia magna*. We measure changes in hydrodynamic diameter (HDD), zeta (ζ) potential, and localized surface Plasmon resonance to assess citrate–AgNP colloidal stability in the presence of three types of organic ligands/compounds. Rate of Ag ion release in the presence of each ligand type is determined to understand whether the released Ag ion has any effect on citrate–AgNP toxicity under the conditions analyzed. Our strategic utilization of organic ligands, i.e., cysteine and humic acid, including a well-known antioxidant (trolox) offered complementary ways to investigate the toxicity of the model citrate–AgNPs and enabled us to associate the changes in colloidal stability and ion release rate with the observed toxicity in *D. magna*. As a free Ag⁺ source, AgNO₃ toxicity is assessed concomitantly in the presence of each of the three organic ligands, thus allowing us to differentiate between the toxicity of AgNPs versus that of ionic Ag⁺.

2. MATERIALS AND METHODS

Nanoparticle Synthesis, Purification, and Characterization. Citrate-coated silver nanoparticles (citrate–AgNPs) were synthesized following a previously reported method.^{3,10} Briefly, 1 mM AgNO₃ and 10 mM sodium citrate dihydrate solutions were mixed together in a volume ratio of 2:1, respectively, and the mixture was heated at 70 °C in a water bath for 4 h which led to the formation of citrate–AgNPs. Following synthesis, citrate–AgNPs were purified using KrosFlo Research Ili Tangential flow filtration (TFF) system equipped with 10 kD hollow fiber membranes (P/N: X31S-300-02P, surface area = 145 cm²; Spectrum Laboratories, CA, USA). Details on purification were described previously (SI Figure S2; Table S1).^{3,10} To determine silver concentration (as total Ag) in the nanosuspension, Flame-Atomic absorption spectroscopy (AAS, Varian 220Z/220FS) was used, prior to which the purified sample was digested using conc. HNO₃ (trace metal grade) following the standard method 3050B.²⁶

The hydrodynamic diameter (HDD, volume-weighted) and zeta (ζ) potential of the purified citrate–AgNP suspension were recorded using the dynamic light scattering (DLS) method (PSS NICOMP Particle Sizing Systems, CA). The measurement accuracy of DLS unit was assessed using Duke 500 (491 nm) NIST 3490A standard (PSS Nicomp, FL, USA) prior to recording the HDD and ζ potential measurements. The localized surface Plasmon resonance (SPR) spectra were obtained using the UV/vis spectrophotometer (Shimadzu PharmaSpec UV-1700). Carbon-coated copper Formvar grid (Ted Pella, Cat. # 01813-F) was used as a support onto which an aliquot of sample was dropped and air-dried before a Transmission electron microscope (Philips EM 420; operated at 120 kV in the bright-field mode) was used to visualize the morphology of citrate–AgNPs; the images captured were used to estimate particle size distribution (PSD) and circularity using an ImageJ 1.44.^{1,3}

Toxicity Bioassay and Exposure Conditions. *Daphnia magna* 48 h acute toxicity test was conducted following the standard USEPA guidelines.²⁷ This static, nonrenewable test followed a randomized block design. Ten less than 24 h old *D.*

magna neonates were introduced randomly into each 50 mL test beaker under variable test conditions (detailed in the following section). Each block of treatments or controls were randomized for their spatial placement during the test period. A range-finding test preceded the definitive tests. Tests were performed in a minimum of triplicates (total 30 daphnids for each concentration) in moderately hard water (MHW)²⁶ used as a test matrix. Animals were unfed during the test period but were fed during the culture and maintenance, per the guideline.²⁷ A 16:8 h photoperiod was maintained using 2 feet long wide-spectrum fluorescent tubes. At the end of 48 h, total dead daphnids in each test chamber were counted to assess mortality under various treatment conditions as mentioned below.

To assess for potential influence (attenuate or enhance) of cysteine (CYS), humic acid (DOC, dissolved organic carbon), or trolox (TRX) on the toxicity of citrate–AgNPs against *Daphnia magna*, tests were performed using several freshly prepared concentrations of each organic ligand: 0, 2, 5, 10, 15, 20, and 25 mg DOC/L;²⁸ 0, 0.01, 0.05, 0.25, 0.5, 1, 2, 5, 10, and 20 mg CYS/L;²⁹ and 0, 0.5, 1, 10, 25, 50, 100, and 200 mg TRX/L.³⁰ Concurrently, AgNO₃ was used to test for free Ag⁺ ion-specific toxicity under the same treatment conditions as stated above for citrate–AgNPs. Cu²⁺ (as CuSO₄) that was used as a positive control revealed that *D. magna* neonates were appropriately sensitive to be used as a bioassay for evaluating citrate–AgNPs and free Ag⁺ toxicity. MHW (with no added AgNP) was used as a negative control.

Preparation of Test Solutions for Exposure Study.

MHW was used to prepare DOC by stirring a known quantity of Leonardite humic acid (International Humic Substances Society; Cat # 1S104H-5) overnight and was vacuum filtered using a 0.45 μ m pore size Millipore filter. The concentration of DOC thus prepared was determined by measuring UV–vis absorbance at 280 nm (SI Figure S3), and verified following the method SM 5310C coupled with persulfate-UV oxidation procedure.¹ Before use, we stored the solution in the dark at 4 °C. The chemical characteristics of this humic acid have been published in detail by Thorn et al.³¹

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Cat# 218940250) and L-cysteine (C₃H₇NO₂S; Cat# 173601000) were procured from Fisher Scientific, USA. Both trolox and cysteine solutions were prepared in MHW by stirring (with magnetic stirrer) overnight at 1000 rpm and were used as-prepared with pH adjusted to 7.0; concentrations reported for multiple dilutions are nominal values for TRX and CYS only. Unlike CYS and DOC, the purpose of using TRX was to indirectly assess if oxidative stress was the cause of AgNP toxicity as was previously reported.^{32–34} Because ~85% mortality of daphnids occurred at 1 mg/L citrate–AgNPs or at 7.5 μ g/L Ag⁺ (used as AgNO₃), these concentrations were further considered for the detail toxicity experiments with an aim to observe measurable mitigating effects in the presence of organic ligands as indicated by the scarce literature.^{4,7,16} Each organic ligand in solution was added to citrate–AgNP suspension or AgNO₃ solution and stirred (using magnetic stirrer) for 5 min at 350 rpm, to gently allow some interactions to occur between each ligand and AgNP/Ag⁺, and pH was maintained at 7.0 before the daphnids were exposed to the treatments or controls in the test chambers. To obtain a reliable/reproducible measurement for assessing AgNP stability in the evaluated test conditions, citrate–AgNP concentration was necessary to be doubled (2 mg Ag/L) for the DLS and

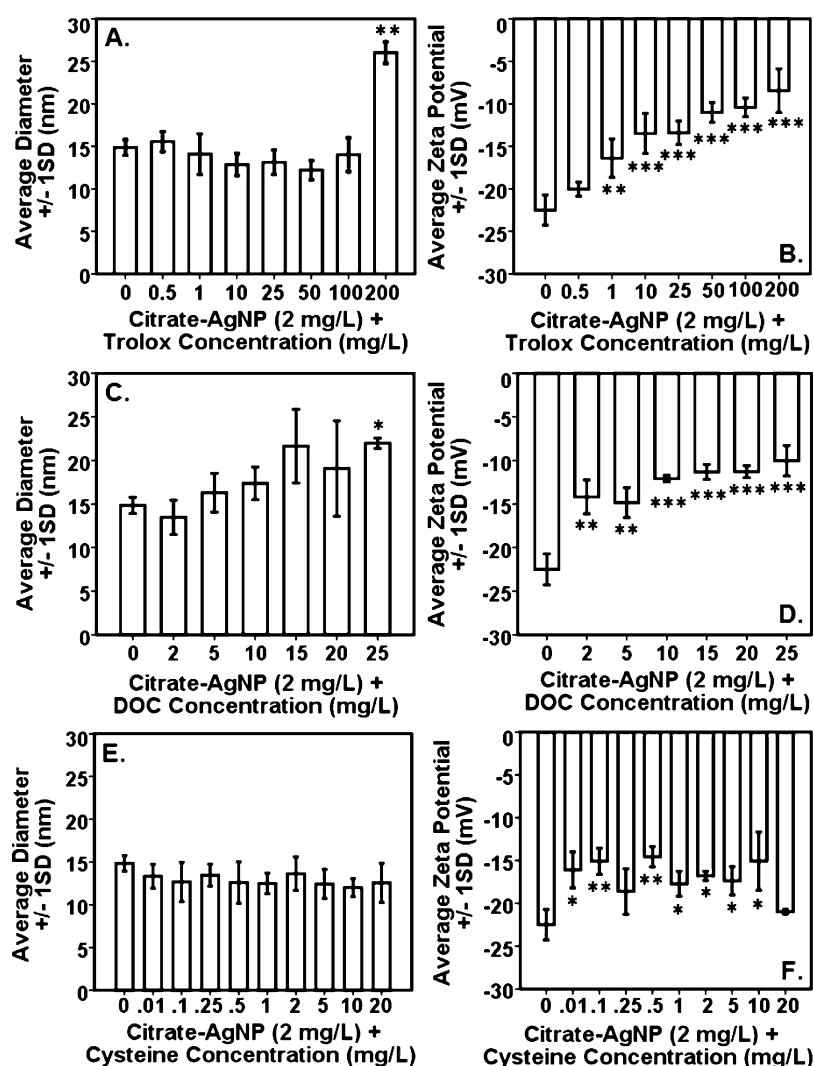


Figure 1. Effects of multiple organic compounds (trolox, DOC, or cysteine) on the (A, C, E) hydrodynamic diameter (HDD) and (B, D, F) surface charge (as zeta potential) of citrate-AgNPs. To obtain the reliable DLS measurements for HDD and ζ potential, 2 mg/L citrate-AgNP were used with each ligand amendment. DOC, dissolved organic carbon. '*' denotes $p \leq 0.01$; '**' denotes $p \leq 0.001$; and '***' denotes $p \leq 0.0001$ as compared to the baseline of 0 mg DOC/L, pH 7, and 280 mg hardness/L (as CaCO_3); significant difference was analyzed by ANOVA followed by Dunnett t (posthoc) test.

UV-vis methods. Ion release and toxicity experiments were also concomitantly conducted for each organic ligand alone as a vehicle control.

Ion Release Rate under Variable Water Chemistry Conditions. Duplicate samples were run with each ligand to assess for Ag dissociation rate from the citrate-AgNP suspension. Twenty-five mL of each sample type was incubated in a plastic centrifuge tube placed on the same shelf where toxicity assay was performed and a 16:8 h photoperiod was maintained at 20 ± 2 °C for 48 h. pH of the suspensions was maintained at 7.0 during the experiment. Each sample was ultracentrifuged (Thermo Scientific Sorvall WX Ultra Series Centrifuge SN# N13 V-427288-NV) at 45,000 rpm (205,835 g at the bottom, 146,347 g in the middle, and 86,858 g at the topmost part) in a polycarbonate bottle (Thermo Scientific Cat. # 314348) for an hour, following which 3 mL of supernatant was pipetted; the samples were digested using the same volume of ultrapure HNO_3 and the total Ag concentration was determined using the Graphite furnace-AAS. Data are reported as an average of the duplicate runs.¹

Statistical Analysis. Linear regression analysis was used to estimate EC_{50} (i.e., effective concentrations that kill 50% of the test population) values and are reported as an average of three replicates. One-way ANOVA (analysis of variance) was used to test for significant difference between the sample EC_{50} s and the controls, followed by the Dunnett t test for multiple comparisons. Significant difference was established at the $p \leq 0.05$ using IBM SPSS ver. 20.³⁵

3. RESULTS AND DISCUSSION

Characteristics of Citrate-AgNPs. The characteristics of citrate-AgNPs are previously documented in our publications (see SI Figures S1,S2; Tables S1,S2).^{3,23} Briefly, citrate-AgNPs showed 14.8 ± 0.9 nm as HDD and 56.5 ± 19.2 nm as TEM diameter, with ζ potential of -22.5 ± 1.8 mV (SI Figure S1, Table S2). The noted difference in particle diameter as revealed by different characterization tools (TEM vs DLS) can be ascribed to the fundamental differences in the procedures used for particle sizing and data presentation.^{1,3,10} Comparable ζ potentials before and after cleaning (SI Table S2) and the

particles as revealed by the TEM imagery strongly suggest a stable colloidal dispersion and that the TFF did not change citrate–AgNP properties.³ Observed localized SPR peak was as characterized by Mie theory (SI Figure S1C).³⁶ The citrate–AgNPs showed circularity of 0.88 and were of high purity as previously reported.^{1,3,16}

Modulation of AgNP Stability by Trolox, DOC, and Cysteine. The measures of HDD, ζ potential, and SPR spectra have routinely offered information on characterizing the colloidal stability of nanoparticles in the aqueous media.^{37,38} Using the DLS and UV–vis methods, the data collected for HDD and ζ potential are then compared with the measurable modifications in SPR spectra to assess for potential changes in particle stability in the presence of each of the three organic ligands in MHW. On average, the DLS measurements revealed a slight decrease in HDD with increasing TRX concentrations, which can be attributed to increasing ionic strength upon TRX addition leading to electrical double layer to contract as the TRX molecules compete with counterions in the suspension.^{1,10,38} However, at 200 mg TRX/L the HDD abruptly increased by 1.75-fold (from ~ 15 nm to ~ 26 nm). The steep decline in ζ potential that occurred with increasing TRX concentrations suggests potential agglomeration of citrate–AgNPs at greater TRX concentrations. At 200 mg TRX/L, average zeta potential decreased by more than 3-fold compared to citrate–AgNPs alone (2 mg Ag/L, with no added TRX; Figure 1B) – the result corroborated our observed increase in HDD (Figure 1A). Increasing TRX concentrations reduced absorbance and blue-shifted the localized SPR peak. Our observation of the complete masking of the localized SPR absorbance at ≥ 100 mg TRX/L, with an emergence of new peak centered around λ of 350 nm (Figure 2A), was a novel finding. This suggests an alteration in characteristics at the AgNP/dielectric interface,³⁹ which can be attributed to potential coordination at an oxygen site of the trolox molecule with the cations (Ca^{2+} , Mg^{2+} , Na^+ , or K^+ readily available in moderately hard water used as the test medium; denoted by M^+ in SI Figure S4B) interacting with available COO^- on AgNP surface; the process likely influenced by the planar benzene ring and the carboxylate substituent of the 6-membered, non-aromatic ring of the trolox molecule (SI Figure S4B).

Interparticle bridging of AgNPs at higher DOC concentrations (≥ 15 mg DOC/L)³⁸ apparently resulted in larger particles, which corresponded to a steep decline in ζ potential (Figure 1C,D). This is consistent with the change in localized SPR absorbance and broadening, with no (blue/red) shift, of the SPR peak with increasing DOC concentrations (Figure 2B). High affinity of thiolate bonding (of CYS) with metallic ions (Ca^{2+} , Mg^{2+} , Na^+ , or K^+ readily available in moderately hard water used as the test medium; denoted by M^+ in SI Figure S4A) interacting with carboxylate coating of AgNPs (SI Figure S4A) may explain our observation of the slight blue-shift (shifted to the left) of the localized SPR peaks, which can thus be attributed to surface modification of citrate–AgNPs with increasing CYS concentrations.^{29,41} CYS could significantly mask citrate–AgNP SPR absorbance in a concentration-dependent manner (Figure 2C). Furthermore, relatively lower SPR absorbance of citrate–AgNPs at higher CYS concentrations is suggestive of adequate CYS-coating of AgNP surface as reported by Liu et al.⁴¹ (SI Figure S4A). Despite consistent ζ potential fluctuations, no significant change in HDD under a wide range of CYS concentrations, the result consistent with a

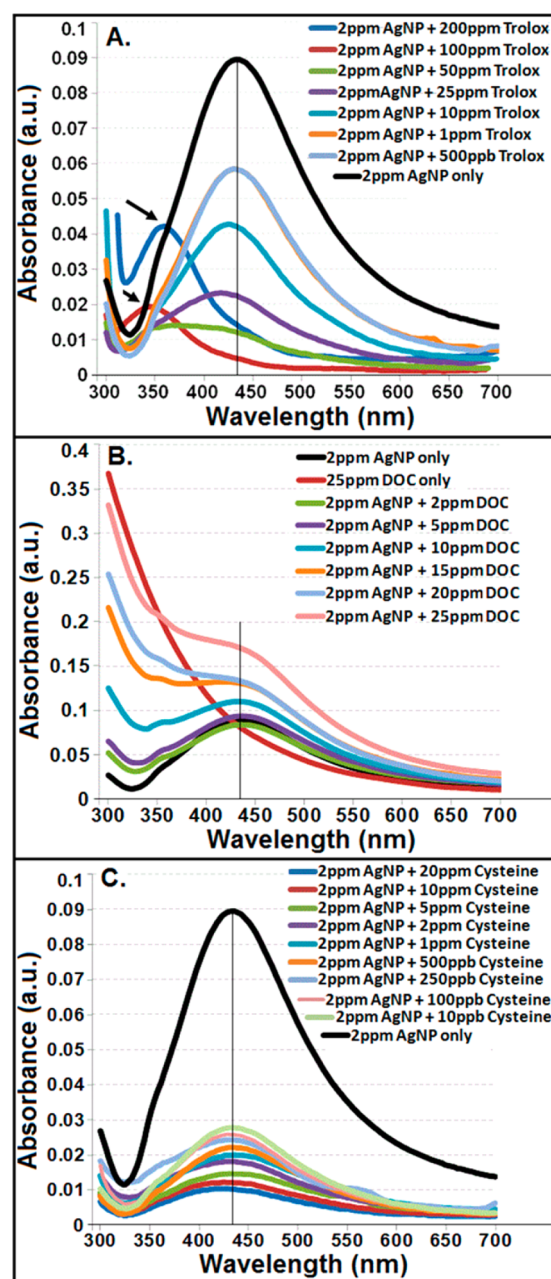


Figure 2. Changes in the localized surface Plasmon resonance (SPR) spectra of citrate–AgNPs upon amendment with variable concentrations of (A) trolox, (B) dissolved organic carbon (DOC), or (C) L-cysteine.

previous report by Gondikas et al.,²⁹ suggests that citrate–AgNPs were fairly stable in the test matrix (Figure 1E,F).

Modulation of Ion Release Rate by Trolox, DOC, and Cysteine. Incubation of citrate–AgNPs with different organic ligands for 48 h under conditions similar to the *D. magna* toxicity bioassay revealed remarkable differences in the rates of Ag dissociation from citrate–AgNPs (Figure 3). The dissolved Ag release rate (%) steeply declined as a function of TRX concentrations. However, at 0.5 mg TRX/L the Ag release rate was enhanced (4.65%), suggesting that at this low concentration TRX was unable to lower oxidative environment, instead appeared stimulatory for oxidative dissolution to occur. At 10–200 mg TRX/L, the Ag release rate was lower (1.38–0.95%) than in pure citrate–AgNP suspension without added

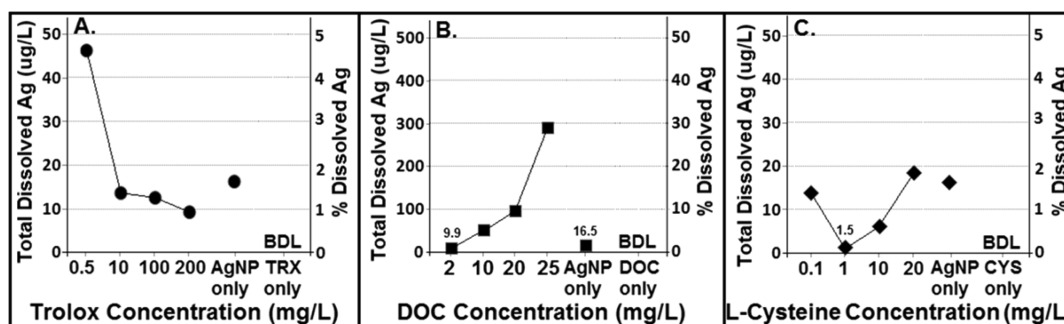


Figure 3. Silver dissolution (measured as total Ag) from citrate-AgNPs under variable ligand amendments: (A) trolox (TRX), (B) dissolved organic carbon (DOC), or (C) cysteine (CYS). BDL, below detection limit of GF-AAS. Numerals above the dots in scatter plots are the concentrations of Ag ions in $\mu\text{g/L}$.

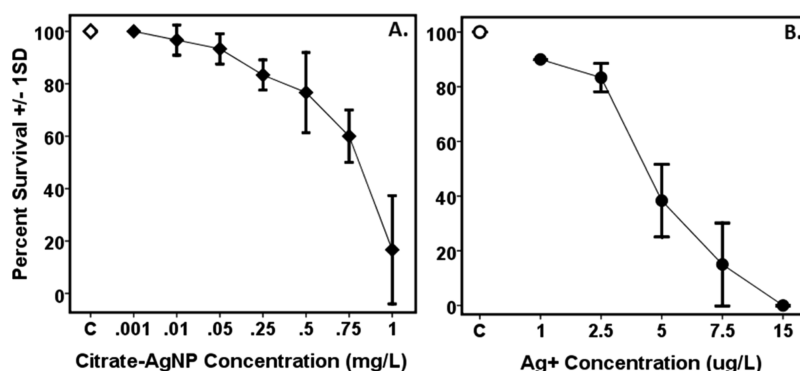


Figure 4. Concentration-dependent effects on survival of *Daphnia magna* neonates in moderately hard water (without organic ligands) upon exposure to (A) citrate-AgNPs or (B) dissolved Ag^+ ions (as AgNO_3). Error bars represent ± 1 standard deviation (SD) of at least triplicate test runs.

TRX (1.65% Ag released of initial concentration 1 mg AgNP/L). This suggests that higher TRX concentrations (10–200 mg TRX/L) could, in fact, inhibit Ag release, verifying the former role as an antioxidant, i.e., TRX scavenges the reactive oxygen species present or otherwise formed in the test environment, thus providing a less conducive condition for oxidative dissolution of Ag to occur from citrate-AgNP surfaces.

As previously reported,¹ the Ag release rate steeply increased as a function of DOC concentrations. At 25 mg DOC/L (Figure 3B), the Ag release rate was 29.5-fold higher than at 2 mg DOC/L, and this was 17.7-fold higher than without added DOC. In a 4 h experimental period, previously we found only 3.6% released Ag from the same citrate-AgNP suspension with 20 mg DOC/L and with an order of magnitude higher initial citrate-AgNP concentration.¹ Nearly 30% Ag that dissociated in this study from citrate-AgNPs can be attributed to the disparate test conditions between the two studies (4 h dissolution test conducted in dark in the previous study¹ vs 48 h test with 16 h photoperiod in this study). Hence, these data suggest that Ag dissociation into ions is dependent upon test duration and treatment conditions (favored by light).

The lower overall Ag release rate (0.15–1.41%) upon CYS treatment (0.1–10 mg CYS/L) is indicative of less favorable environment for citrate-AgNPs to dissociate into Ag ions (Figure 3C). Thiolate bonding of CYS with the cations M^+ (see SI Figure S4A; M^+ represents Ca^{2+} , Mg^{2+} , Na^+ , or K^+ readily available in moderately hard water used as the test medium) interacting with citrate-coating of AgNPs might have rendered minimal oxidation of citrate-AgNPs in the presence of $-\text{SH}$ ligand (Figure 3C), thereby resulting in lower Ag release.^{40,41}

Because no measurable amount of Ag was detected in the highest concentration of different organic compounds used in this study, this justifies that the source of released Ag under the evaluated conditions was the citrate-AgNPs, not the TRX, DOC (Leonardite humic acid), or CYS itself.

Modulation of AgNP Toxicity by Trolox, DOC, and Cysteine. A concentration-dependent mortality of *D. magna* neonates was observed for both the citrate-AgNPs and free Ag^+ (as added AgNO_3 ; Figure 4A,B). Comparison of EC_{50} s revealed citrate-AgNPs ($\text{EC}_{50} = 798 \pm 156 \mu\text{g Ag/L}$) 185 times less toxic than free Ag^+ ($\text{EC}_{50} = 4.3 \pm 0.4 \mu\text{g Ag/L}$).

Figure 5 provides insights into how different organic ligands modulate aquatic toxicity of citrate-AgNPs and Ag^+ in *D. magna*. In this study, *D. magna* survival was enhanced with increasing TRX concentrations; at ≥ 100 mg TRX/L daphnid survival reached 100%, while at 0.5 mg TRX/L no protection was conferred upon exposure to 1 mg/L citrate-AgNPs (Figure 5A). On the other hand, TRX was unable to protect daphnids against free Ag^+ (as added AgNO_3 ; 7.5 $\mu\text{g Ag/L}$) even at as high as 800 mg TRX/L (Figure 5B). Surface interactions of TRX with citrate-AgNPs, as suggested by complete masking and blue-shifted localized SPR peak at 100 or 200 mg TRX/L, coupled with low Ag ion release at higher TRX concentrations can be directly associated with 100% daphnid survival.

Known for its cell-permeability owing to its hydrophilicity, trolox is a derivative of Vitamin E with inherently antioxidant and membrane-protective characteristics,⁴² which are justified by its routine use as a standard/positive control in many antioxidant assays,⁴³ including in the studies evaluating aging and neuronal death under oxidative stress.⁴⁴ Adequate

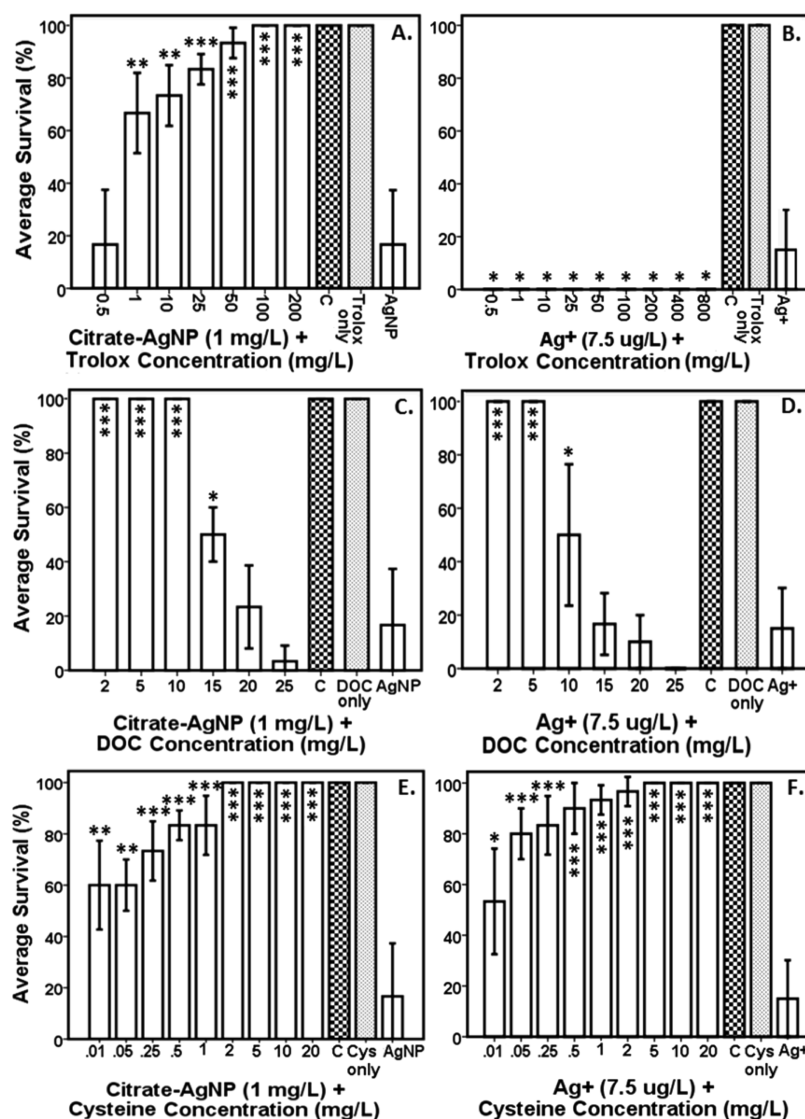


Figure 5. Influence of variable organic ligands (trolox, DOC, or cysteine) on the toxicity of (A, C, E) citrate-AgNPs, or (B, D, F) dissolved Ag⁺ ions (as AgNO₃) measured as change in *Daphnia magna* survival. Error bars represent ±1 standard deviation (SD) of at least triplicate test runs. '*' denotes $p \leq 0.01$; '**' denotes $p \leq 0.001$; and '***' denotes $p \leq 0.0001$; significant difference was analyzed by ANOVA followed by Dunnett t (posthoc) test for multiple comparisons. DOC, dissolved organic carbon; C, moderately hard water (MHW) control; AgNP, 1 mg AgNP/L in MHW; Ag⁺, 7.5 μg Ag⁺/L in MHW; trolox only, only trolox was used in MHW at both its highest and lowest concentrations evaluated; DOC only, only DOC was used in MHW at both its highest and lowest concentrations evaluated; Cys only, only cysteine was used in MHW at both its highest and lowest concentrations evaluated. A fixed concentration of citrate-AgNPs (1 mg/L as total Ag) or Ag⁺ (7.5 μg /L as total Ag) was used to assess the effects of a wide range of trolox, DOC, or cysteine on the survival of *D. magna* neonates.

protection conferred at higher concentrations against citrate-AgNPs might be associated with trolox's potent antioxidant (as it quenches reactive oxygen radicals) and membrane-protective properties, thus providing an indirect evidence of oxidative stress due to the presence of citrate-AgNPs in the polar medium.^{28,45} It has been reported that citrate-AgNPs could lead to the generation of strong oxidant such as H₂O₂ in the aqueous media, whereas H₂O₂ was not detected with ionic Ag⁺ (used as AgClO₄; 2 mg Ag/L) in solution.²⁸ Contrasting toxicity results obtained here for citrate-AgNPs and free Ag⁺ strongly suggest distinct mechanism underlying the toxicity of zerovalent Ag⁰ versus the monovalent Ag⁺, with oxidative stress apparently being a cause of citrate-AgNP toxicity^{32–34} versus the direct ionic effects (disrupting ionic balance across the membrane) of free Ag⁺,^{46,47} and this indicates that oxidative

stress was not the mechanism responsible for free Ag⁺ toxicity.²⁹

D. magna survival was not compromised at lower DOC concentrations for citrate-AgNPs (up to 10 mg DOC/L) or for free Ag⁺ (up to 5 mg DOC/L) (Figure 5C,D). Interestingly, however, daphnids mortality increased significantly at higher DOC concentrations; i.e., at ≥ 15 mg DOC/L with citrate-AgNPs or at ≥ 10 mg DOC/L with free Ag⁺ (Figure 5C,D), which appeared to be counterintuitive though. Humic acid, which binds to NPs and/or ions can also eliminate some reactive oxygen radicals present or formed in aqueous suspension,^{48,49} was expected to quench available oxygen radicals during the experiment and also reduce bioavailability of citrate-AgNPs and dissolved Ag⁺ ions present in the test suspension, hence minimal toxicity was expected. Since this was not the case, alternately, it seems plausible that larger

aggregates of the DOC-citrate–AgNPs/DOC-citrate–AgNPs–Ag⁺ complex that were apparently formed under higher DOC concentrations (Figure 1C) might have led daphnids to filter-feed on the available aggregates, therefore exposing them to a higher internal dose of Ag then when lower amounts of DOC were present in the nanosuspension and resulting in higher mortality. As with DOC, antioxidative property as that of TRX and Ag ion chelating property as that of CYS cannot be expected. As Ag ion release from citrate–AgNPs upon DOC treatment also increased remarkably (97.6–292.0 $\mu\text{g Ag/L}$ with 20–25 mg DOC/L amendments) among the ligands evaluated in this study, it is plausible that oxidative stress coupled with released ionic Ag, the latter likely influenced by daphnids filter-feeding of the aggregates resulting in higher internal Ag dose, might have contributed to higher toxicity of citrate–AgNPs (Figure 5C). Because protective effects also declined with increasing DOC concentrations under free Ag⁺ (as added AgNO₃) treatment, it can be inferred that daphnids feeding behavior might have led to greater exposure to DOC–Ag⁺ complexes and thus higher toxicity observed (Figure 5D).

Amendment with –SH ligand (as cysteine) conferred protection to daphnids upon exposure to citrate–AgNPs or free Ag⁺ (Figure 5E,F). At 2 mg CYS/L or higher, daphnid survival reached 100% with citrate–AgNPs or free Ag⁺ treatment, suggesting that the applied CYS concentration was optimal to confer adequate protection against Ag (AgNP and Ag⁺) stress. As CYS is known for its higher affinity to complex with free Ag⁺,^{40,41} it is expected to scavenge free Ag⁺ that might be present or otherwise formed within the citrate–AgNP suspension during the test period. Our results imply that CYS could chelate the monovalent Ag⁺ as present in AgNO₃ solution and therefore protected daphnids from free Ag⁺ effects. The toxicity results observed for citrate–AgNPs might explain enough CYS-coating of AgNP surface, as verified by lower absorbance (Figure 2C; and previously reported by Liu et al.⁴¹), and scavenging of dissolved Ag⁺ emanating from citrate–AgNP surface by CYS molecules.

Toxicity evaluation using various organic ligands (without citrate–AgNPs or free Ag⁺) alone used in this study at both the lowest and highest concentrations showed no toxic effects on daphnid survival (Figure 5), confirming that the daphnids mortality that occurred under the tested conditions were not due to the effects of ligands added to citrate–AgNPs suspension or free Ag⁺ (as added AgNO₃) solution. The amount of dissolved Ag released from citrate–AgNPs is greater than the calculated EC₅₀ value for Ag⁺ (as AgNO₃). Because citrate–AgNPs were less toxic even with a greater amount of dissolved Ag release in the test suspensions, it suggests that the released Ag may not have been in the highly toxic monovalent form (Ag⁺); instead it may likely have been in the less harmful Ag species (e.g., AgCl) or other Ag-complexes, given the chemical makeup of the organic compounds coupled with the ionic constituents inherent to the MHW (NaHCO₃ = 96 mg/L, CaSO₄·2H₂O = 60 mg/L, MgSO₄ = 60 mg/L, KCl = 4 mg/L; USEPA²⁷) used as the test medium. Future studies may elucidate what different Ag species/complexes may have formed under these different organic ligands' treatments in similar test media (e.g., MHW). Alternately, the results suggest that the combined effects of AgNPs and released Ag ions were lower than that of free Ag⁺ (as added AgNO₃), except under higher DOC concentrations tested that showed higher combined toxicity.

The MHW used as a test matrix in *D. magna* bioassay is considered suitable for TRX solubility owing to the presence of its two functional groups, –COOH and –OH, with dissociation constants (pK) 3.89 and 11.92, respectively, per molecule of TRX, thus offering higher solubility in aqueous media at circumneutral pH.^{50,51} In this study, TRX amendment of citrate–AgNP suspension (i) modified AgNP surface properties (verified by changes in ζ potential and localized SPR spectra), (ii) inhibited Ag dissociation, possibly due to quenching of reactive oxygen present or formed during the experiment which otherwise would have promoted oxidative dissolution of Ag,²⁸ and (iii) consequently rescued daphnids from the toxicity of the widely used model AgNPs. DOC amendment (i) altered particle size (HDD) and surface properties (revealed by changes in ζ potential and localized SPR spectra), (ii) which enabled formation of larger DOC-citrate–AgNPs/DOC-citrate–AgNPs–Ag⁺ aggregates (revealed by HDD), (iii) followed by filter-feeding of the aggregates by daphnids potentially leading to higher internal Ag dose, and (iv) consequently, higher toxicity resulting in daphnids mortality. This finding suggests the role of species biology (i.e., filter-feeding behavior of *D. magna*) as an important factor to be considered when investigating nanoparticle toxicity in aquatic systems. We tested this hypothesis by evaluating the effects of comparable citrate–AgNPs (different batches of citrate–AgNPs but with similar physicochemical properties) amended with the chemically same DOC at analogous concentrations and using *Escherichia coli* as the test organism (data published in Pokhrel et al. in press).¹ Unlike in *D. magna* for which we observed increased toxicity of citrate–AgNPs with increasing DOC concentrations in this study, toxicity was rather attenuated in our previous study with *E. coli*,¹ supporting the hypothesis that variability in species sensitivity can alter the toxicity due to distinct biology of the organism employed in toxicity testing.^{1,52} Thiol amendment of citrate–AgNP suspension (i) modified the surface properties (revealed by slight blue-shift and broadening of localized SPR peaks, and lower UV–vis absorbance), (ii) inhibited Ag release, (iii) scavenged released Ag ions, and (iv) promoted daphnid survival, perhaps due to a decreased amount of bioavailable Ag as a result of CYS-citrate–AgNP complexation and quenching of free Ag⁺ by the available CYS molecules in the suspension. In summary, based on the specific organic ligands employed in this study the results suggest that citrate–AgNP toxicity can be best associated with oxidative stress, ion release, and the organism biology; while the direct physical contact as one of the probable mechanisms of citrate–AgNP toxicity, as was previously demonstrated,^{7,16} cannot be ruled out as we did not assess it explicitly in this study.

Environmental Implications. The tenet that AgNPs can be environmentally transformed is generally well-received and has served as *a priori* concept, *albeit* it has been less well explored and understood.^{1,5,9–11} In this study, we systematically evaluated how different organic ligands including –SH (in cysteine), –COOH (in trolox), and DOC (Leonardite humic acid with high aromaticity, $f_a = 0.58$, as determined by Thorn et al.³¹) could influence colloidal stability, Ag release dynamics, and aquatic toxicity of citrate-functionalized AgNPs. Our results show that ligands that are ubiquitous in the environment (such as –SH, –COOH, and humic acid) could surface modify citrate–AgNPs, alter their colloidal persistence and rate of dissolution, and subsequently could attenuate or enhance nanotoxicity; the latter likely dependent on oxidative stress,

amount of Ag ion released, and the species biology⁵² (as revealed by comparing results for *E. coli*¹ versus *D. magna*). Except for DOC amendment, the combined toxic effects of AgNPs and released Ag upon cysteine or trolox amendment was lower than that of AgNO₃ alone under the evaluated conditions; the findings are unique to the literature.^{34,53} These data caveat to extending toxicity results obtained for one species to another as the obvious differences in the organism biology^{1,6,7} may result in significantly different toxicity of zerovalent Ag⁰ or monovalent Ag⁺ in the aquatic systems, and that surface modified nanoparticles upon environmental release may no longer retain its inherent toxic potency cannot be generalized. Whether a low level (ng/L) of naturally occurring cysteine in sediment porewater and surface water⁵⁴ would confer protection against naturally modified engineered AgNPs at lower (ng/L) doses (should they be toxic) as predicted for aquatic systems⁵⁵ could be an important thesis for future research. 'How would various humic substances (e.g., Suwanee River humic/fulvic acids, Leonardite himic acid, soil humic/fulvic acids, peat humic acid, other bulk natural organic matters) alone or in combination transform (size, aggregation/sedimentation, coating density, surface charge/reactivity) AgNPs and consequently the toxicity in aquatic receptors?' is a question not only heuristically interesting but one that also carries significant ecological importance. Results from such studies may offer meaningful and novel insights into the nanochemistry potentially occurring in natural aquatic systems enriched with various types of organic ligands.

■ ASSOCIATED CONTENT

■ Supporting Information

TEM/UV-vis characterization of AgNPs; citrate-AgNP purification using TFF method; DOC calibration curve using absorbance; Molecular interaction of L-cysteine and trolox with AgNP; Analysis of water quality parameters; and Quality Control. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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associated institutions. Any mention of the trade names does not imply their endorsements or recommendation for use.

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