The concentration-dependent aggregation of Ag NPs induced by cystine

Afshinnia K, Gibson I, Merrifield R and Baalousha M*

Center for Environmental Nanoscience and Risk, Department of Environmental Health Sciences, Arnold School of Public Health, University South Carolina, Columbia, South Carolina 29208

Corresponding author: * mbaalous@mailbox.sc.edu.

Abstract

Cystine is widely used in cell culture media. Cysteine, the reduced form of cystine, is widely used to scavenge dissolved Ag in eco-toxicological studies to differentiate dissolved vs. nanoparticle uptake and toxicity. However, little is known about the impact of cysteine and cystine on the aggregation behavior of Ag NPs, in particular as a function of Ag NP concentration. Herein, we investigate how cystine (0-300 μM) affects the stability of citrate-, polyvinylpyrrolidone-, and polyethylene glycol- coated silver nanoparticles (cit-Ag NPs, PVP-Ag NPs and PEG-Ag NPs, respectively) with and without Suwannee River fulvic acid (SRFA) as a function of Ag NPs concentration using UV-vis spectroscopy at environmentally and ecotoxicologically relevant Ag NP concentrations (ca. 125-1000 $\mu\text{g L}^{-1}$). The results demonstrate, for the first time, the concentration-dependent aggregation of cit-Ag NPs in the presence of cystine with a shift in the critical coagulation concentration (CCC) to lower cystine concentrations at lower cit-Ag NP concentrations. At the highest cit-Ag NP concentration (1000 $\mu\text{g L}^{-1}$), reaction limited aggregation was only observed and no CCC was measured. SRFA slowed the aggregation of cit-Ag NPs by cystine and aggregation occurred in reaction limited aggregation (RLA) regime only. No CCC value was measured in the presence of SRFA. Cystine replaces citrate, PVP and PEG coatings, resulting in aggregation of both electrostatically and sterically stabilized Ag NPs. These findings are important in understanding the factors determining the behavior of Ag NPs in cell culture media. Also due to the similarity between cystine and cysteine, these results are important in understanding the uptake and toxicity of Ag NPs vs. Ag ions, and suggest that the reduction of the toxicity of Ag NPs in the presence of cysteine could be due to a combined effect of scavenging Ag^+ ions and Ag NP aggregation in the presence of cysteine.

Introduction

The rapid development of nanotechnology and concern regarding the potential environmental and human health implication of nanoparticles (NPs) has spurred extensive interest in understanding the behavior, fate and effects of NPs¹⁻³. Silver NPs (Ag NPs) are among the most widely used NPs in consumer products as antimicrobial agent⁴. Several studies have suggested that Ag NPs can be released from nano-enabled products and find their way to the environment, resulting in environmental and human exposure to Ag NPs^{5,6}.

Considerable research has been conducted to better understand the effect of Ag NPs on a wide range of organisms and cell lines⁷, with a particular attention given to differentiate the NP from dissolved metal effects due to the known solubility of Ag NPs⁸⁻¹¹. Several studies have reported mixed results; some show particle-specific toxicity^{8,12-14}, others predominantly relate the effect to free Ag⁺ ions^{15,16}, while other studies suggested combined effect of Ag NPs and free Ag⁺ ions^{17,18}. These controversies could be partially due to the lack of understanding of NP behavior in the ecotoxicological media⁷, in particular at eco-toxicologically relevant concentrations^{19,20}.

To differentiate Ag⁺ from Ag NPs effect, binding ligands (e.g. Cl⁻ and cysteine) are typically used to complex the released silver ions from Ag NPs^{8,18}, with little understanding of the impact of the ligands (e.g. cysteine) on the behavior of Ag NPs in the test medium. Several studies have reported reduced toxicity of Ag NP suspension in the presence of cysteine and attributed this reduction solely to the complexation of free Ag⁺ ions by cysteine^{8,18}, without considering the potential destabilization of Ag NPs in the presence of cysteine. Therefore, understanding the interaction between Ag NPs and cysteine is an important issue to underpin the assessment of dissolved silver and Ag NP uptake and toxicity in aquatic organisms. Additionally cystine, the oxidized form of cysteine, is widely used in cell culture media (<http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/media-expert/cysteine.html>). Cysteine and cystine are essential amino acids in cell culture media that must be supplied from an exogenous source.

On the other hand, the fate, transport and effects of Ag NPs in the environment depends on their interaction with naturally ubiquitous organic ligands such as thiols (cysteine), disulfide (cystine) and natural organic matter (NOM; fulvic and humic acids), which may chemically, or physically sorb onto NP surface and modify Ag NPs surface properties and colloidal stability^{21,22}. For example hydrosulfide groups in NOM can form strong covalent bonds with Ag NPs surface and replace the original surface coating such as polyvinylpyrrolidone (PVP)^{23,24}, and thus reduce PVP-Ag NPs stability by increasing ionic strength²⁵. Aggregation of NPs (e.g. Ag NPs and Au NPs) can lead to a reduction in NP toxicity^{26,27}.

Few studies have investigated the effect of cysteine on the stability of Ag NPs²² and their fate and transport in porous media²¹, typically at high Ag NP concentrations (ca. > 1 mg L⁻¹)^{7,21,22}, and observed slight aggregation of cit- and PVP- Ag NPs over a period of 48 hours²⁵. However, there are currently no studies on the impact of cystine on the behavior of Ag NPs, in particular at low NP concentrations.

The aim of this study is therefore to investigate the stability of citrate-, PVP- and PEG-coated Ag NPs following their interaction with cystine. In particular, we investigated the impact of cystine on cit-Ag NP aggregation kinetics at environmentally and eco-toxicologically relevant Ag NP concentrations (125-1000 µg L⁻¹), and the impact of SRFA on the interaction between cit-Ag NPs and cystine. We also investigated the aggregation of citrate, PVP and PEG-Ag NPs up to 24 hours post interaction with different concentrations of cystine. Cystine (disulfide form of cysteine) and Suwannee River fulvic acid (SRFA) were chosen to represent two distinct types of NOM. Cystine and cysteine represent a class of small molecular weight metal-complexing ligands with a thiol and disulfide groups and are commonly present in surface waters at low concentrations (nM to µM range)^{25,28}. Cysteine is also widely used in Ag NP toxicity experiments to differentiate the toxicity of NP vs. Ag⁺ ions. Cystine rather than cysteine was used in this study for the following reasons 1) the weaker affinity of the disulfide group to Ag⁺, thus minimizing the impact on Ag NP dissolution and enabling the use of UV-vis to study the aggregation behavior of Ag NPs, 2) cysteine is highly reactive and readily oxidizes to cystine under oxic conditions^{29,30}, and 3) Cystine is the predominant form of in plasma, extracellular body fluids and cell culture media³¹. SRFA represent a class of less-defined natural macromolecules, which usually stabilize NPs^{19,32}.

Materials and Methods

Chemicals

Silver nitrate (ACS grade, 99.9+%) and Sodium borahydrate (98%) were purchased from Alfa Aesar (Ward Hill, MA, USA). Trisodium citrate (Lab grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Cystine (L-cystine ≥98% TLC) was purchased from Sigma Aldrich (St Louis, MO, USA). SRFA was purchased from the International Humic Substances Society (St Paul, MN, USA). All chemicals were used without further purification.

Synthesis and characterization of silver nanoparticles

Citrate-coated silver NPs (cit-Ag NPs) were synthesized and characterized as described in previous publications^{33,34}. Briefly, cit-Ag NPs (cit-Ag NPs) were prepared by the reduction of

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4 silver nitrate in trisodium citrate. A 100 ml of 0.31 mM trisodium citrate, 100 ml of 0.25 mM silver
5 nitrate and 10 mM of sodium borohydride solutions were prepared in high purity water and kept
6 at 4 °C in the dark for 30 min. The silver nitrate and trisodium citrate solutions were mixed
7 together in a conical flask and vigorously stirred. Subsequently, 6 ml of the reducing agent,
8 sodium borohydride (NaBH₄), was added in one batch. After 10 min of stirring, the solution was
9 heated slowly to boiling and heated for a further 90 min, left overnight and cooled (4 °C, in the
10 dark). Ag NPs were then cleaned, to remove the excess reagents before use, by ultrafiltration
11 (Amicon, 1 kDa regenerated cellulose membrane, Millipore) using a diafiltration method to
12 prevent NP aggregation and drying. Ag NPs were re-dispersed in 0.31 mM trisodium citrate
13 solution to avoid further growth; this process was repeated at least three times. The
14 concentration of Ag NPs was measured by inductively coupled plasma-optical emission
15 spectroscopy and was about 10.0±0.3 mg L⁻¹.

16 PVP- and PEG- Ag NPs were prepared by ligand exchange approach using citrate-
17 coated Ag NPs as precursor as described elsewhere³⁵. Aliquots of cit-Ag NPs were converted
18 into PEG- and PVP- stabilized Ag NPs by adding 8 molecules of PVP, or 4 molecules of PEG
19 per nm² surface area of Cit-Ag NPs under vigorous stirring (e.g. 700 rpm) for at least 1 hour and
20 then cooled overnight at 4°C.

21 The size and electrophoretic mobility (EPM) was measured by dynamic light scattering
22 and laser Doppler electrophoresis, respectively using a Malvern Zetasizer NanoZS Instrument
23 (Malvern, USA). The Malvern Zeta potential transfer standard consisting of Latex dispersed in
24 pH 9.2 buffer and has an EPM value of -42 mV (DTS1235, Malvern Instrument Limited) was
25 used to verify the performance of the instrument and the zeta potential cell throughout the
26 experiments.

27 The optical absorbance spectrum of Ag NPs were collected by a Uv-vis spectrometer
28 (UV-2600 Shimadzu, Santa Clara, CA, USA) using a 100 mm path length cuvette, which
29 allowed analysis of Ag NPs at relatively low concentrations (125 to 1000 µg L⁻¹). Uv-vis spectra
30 of cit-Ag NPs suspensions were collected over wavelengths (λ) 200-900 nm.

31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 **Solution chemistry**

52 The aggregation behavior of cit-Ag NPs was investigated by Uv-vis spectroscopy in
53 suspensions containing variable concentration of Ag NPs (125 to 1000 µg L⁻¹; 1.16-9.27 µM Ag)
54 and cystine (0 to 72 mg L⁻¹; 0-300 µM), in the presence and absence of Suwannee River Fulvic
55 acid (SRFA, 1 mg L⁻¹). No buffer was used to avoid interferences from ions in the buffer
56 solution. For instance, phosphate anions (a component of the commonly used phosphate
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4 buffers) have been shown to induce the aggregation of hematite particles³⁶ and titanium dioxide
5 NPs³⁷. The pH was measured throughout the experiment and was in the range 5.5-5.7.

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7 A stock suspension of SRFA was prepared by dissolving 2 mg in 10 ml ultrahigh purity
8 water (UHPW), and the pH was adjusted by adding aliquots NaOH to pH 7.0. The suspension
9 was then filtered (450 nm) to remove any aggregated SRFA molecules. A stock suspension of
10 333 μM (80 mg L^{-1}) cystine was prepared by dissolving 8 mg of cystine in 100 ml of UHPW. The
11 cystine stock suspension was prepared daily before performing the aggregation experiments to
12 avoid losses of cystine by degradation²⁵.
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15 For all experiments performed in the absence of SRFA, an aliquot (125, 250, 500, and
16 1000 μL) of Ag NPs stock suspension was diluted in UHPW and mixed with different aliquots of
17 cystine to achieve a final volume of 10 mL, and thus NP concentrations of 125, 250, 500 and
18 1000 $\mu\text{g L}^{-1}$. For all experiments performed in the presence of SRFA, an aliquot of 2 and 4 ml of
19 Ag NPs stock suspension was mixed with 0.8 and 0.4 ml of SRFA stock suspension and topped
20 with UHPW to achieve a final volume of 8 mL, which resulted in a mixture of 20 and 10 mg L^{-1}
21 SRFA and concentrations of Ag NPs of 2.5 and 5.0 mg L^{-1} . The mixtures were then left for 24 h
22 to reach equilibrium before performing any aggregation kinetic experiments. Then 0.5 and 1 ml
23 of the mixtures were mixed with cystine and topped with UHPW to 10 mL to achieve a final
24 concentration of Ag NPs of 125 and 500 $\mu\text{g L}^{-1}$, SRFA of 1 mg L^{-1} , and cystine (0-266 μM).
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36 Aggregation kinetics

37 To quantitatively measure the aggregation kinetics of cit-Ag NPs by Uv-vis, the
38 concentration of individual cit-Ag NPs was monitored over time by performing a time scan of the
39 Uv-vis absorbance at $\lambda=394$ nm, corresponding to the specific plasmon resonance peak for the
40 individual Ag NPs (**Figure 1a**). The changes in the UV-vis absorbance $\lambda=394$ nm can be
41 attributed to NP aggregation or dissolution^{32,38}. Analysis of dissolved Ag concentration by ICP-
42 MS following centrifugal ultrafiltration at 3 kD over 24 hours, well beyond the time required to
43 determine NP aggregation kinetics, demonstrated that the differences in cit-Ag NP dissolution in
44 the presence or absence of cystine was not significant (**Figure S1**). Therefore, the changes in
45 the UV-vis absorbance $\lambda=394$ nm can be attributed to aggregation of primary Ag NPs. The
46 aggregation rate constant is proportional to rate of change of the Uv-vis at $\lambda=394$ nm ($da_{\lambda=394}/dt$,
47 a refers to Uv-vis absorption, Eq.1) during the early stage aggregation^{32,39}; that is slope of the
48 loss in the UV-vis absorbance at $\lambda=394$ within the first 30 seconds after mixing Ag NPs with
49 cystine, which was determined by fitting a linear correlation function.
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$$k = \frac{1}{oN} \frac{da_{\lambda=394}}{d_t} \quad (\text{Eq.1})$$

Where N is the initial particle concentration and o is the optical factor.

For colloidal systems, plots of the attachment efficiency ($\alpha=1/W$, determined according to Eq.2, where W is the stability ratio) against electrolyte concentration at a given experimental conditions can be used to characterize NP aggregation kinetics and suspension stability.

$$\alpha = \frac{1}{W} = \frac{k_{slow}}{k_{fast}} \quad (\text{Eq.2})$$

Where k_{slow} and k_{fast} represent the aggregation rate constant under reaction (RLA) and diffusion (DLA) limited aggregation regimes. The RLA regime occurs at counter ion concentrations below the critical coagulation concentration (CCC), whereas the DLA occurs at counter ion concentrations above the CCC. The CCC is frequently used to measure the stability of NP suspensions since it quantifies the minimum concentration of the ion/molecule that is required to completely destabilize the NP suspension⁴⁰. The attachment efficiencies under RLA and DLA regimes were fitted by linear functions and their intersections yield the respective CCC. The aggregation behavior of cit-Ag NPs was also monitored qualitatively by collecting the Uv-vis spectra of Ag NPs 10 minutes post interaction with different concentrations of cystine over wavelength range 300-900 nm.

The zeta potential of Ag NPs and Ag NPs mixed with cystine were measured by laser Doppler electrophoresis using the same Malvern instrument. The reported zeta potentials and standard deviations were determined from 10 replicates of 50-100 runs each. The performance of the zeta potential cells was verified by measuring the zeta potential of the Malvern Zeta potential transfer standard and the zeta potential cell was replaced when any deviation from the standard value was observed.

The aggregation behavior of cit-, PEG- and PVP-Ag NPs was also monitored qualitatively by collecting the Uv-vis spectra of Ag NPs over wavelength range 300-900 nm at 0, 6 and 24 hours post mixing with different concentrations of cystine.

Results and Discussion

Properties of the synthesized Ag NPs

According to the cumulant analysis, the $z\text{-}d_h$ and polydispersity index of cit-Ag NPs in stock suspension are 16.6 ± 0.2 nm, 0.2 ± 0.1 , respectively. Those of PEG-Ag NPs were 19.4 ± 0.3 , and 0.29 ± 0.003 and those of PVP-Ag NPs were 21.6 ± 0.3 nm and 0.27 ± 0.005 . The zeta

potential of cit-Ag NPs, PEG-Ag NPs and PVP-Ag NPs was -46.5 ± 4.5 mV, -28.5 ± 1.4 mV, and -23.6 ± 1.1 mV, respectively. These measurements suggest that cit-, PEG- and PVP-Ag NPs are all well dispersed. The Uv-vis spectra of the cit-Ag NPs, PEG-Ag NPs and PVP-Ag NPs in UHPW water shows a single peak centered at 394 nm, 394 nm, and 395 nm, respectively. Full characterization of the synthesized cit-Ag NPs are provided elsewhere³²⁻³⁴.

Cystine induced aggregation of cit-Ag NPs

The Uv-vis spectra of cit-Ag NPs, after 10 minutes of mixing with different concentrations of cystine (**Figure 1a-d**) show a broadening of the Uv-vis absorbance peak (centered on 394 nm) of as-synthesized cit-Ag NPs at low cystine concentrations (ca. 20 μ M). At higher concentrations of cystine (ca. 33-83 μ M), the Uv-vis spectra show the formation of a second peak centered on higher wavelengths in the visible and near infrared region (NIR) of the spectrum in the range of 500-650 nm. The increase in cystine concentration results in the decrease in the Uv-vis absorbance at 394 nm and the increase of the second peak absorbance at 550-560 nm as a consequence of NP interactions, which indicates the loss of primary cit-Ag NPs by aggregation as observed elsewhere³². Furthermore, at the same concentration of cystine (ca. 33 and 67 μ M, **Figure S2**), the second peak is shifted to higher wavelengths at lower NP concentrations compared to higher NP concentrations. The increased width of the second peak at lower NP concentrations is likely to be due to the formation of larger Ag NP aggregates³², which can be attributed to the higher decrease in Ag NP surface charge with the decrease in cit-Ag NPs concentration (**Figure 2a**) at the same concentration of cystine (discussed below). Under such conditions, more cystine molecules are available for interaction with the Ag surface atoms at lower Ag NP concentrations.

The UV absorbance at λ_{394} follows a first order decrease at low cystine concentrations and a more rapid, higher order decrease at high cystine concentrations (**Figure 2a-c**). At the same concentration of cystine (**Figure S4**), the relative loss in the UV-vis absorbance increase with the decrease in NP concentration, indicating higher removal of primary NPs by more aggregation at lower Ag NPs concentrations. The attachment efficiencies of cit-Ag NPs calculated from the loss of the Uv-vis at λ_{394} are plotted in **Figure 3a**, which generally shows RLA and DLA regimes and suggests that the aggregation kinetic of cit-Ag NPs in the presence of cystine are controlled by electrostatic interactions between the cystine coated AgNPs (see discussion below). The increased cystine concentration results in a decrease in the surface charge of the cit-Ag NPs (**Figure 2a**). In the RLA regime, the cit-Ag NPs are kinetically stabilized by an electrostatic barrier resulting from the double layer repulsion and the van der

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4 Waals attractions forces. In the DLA, the surface charge of the cit-Ag NPs is sufficiently reduced
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6 by the cystine and the energy barrier is no longer sufficient to kinetically stabilize the particles,
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8 and therefore, aggregation process is only driven by diffusion. It was not possible to calculate
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10 the attachment efficiency at cit-Ag NP concentration of $1000 \mu\text{g L}^{-1}$ Ag as the concentration of
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12 cystine, within its limit of solubility, was not sufficient to induce DLA and thus it was not possible
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14 to calculate the fast aggregation rate.

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16 At the same cystine concentration and under RLA regime (**Figure 3a**), the attachment
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18 efficiency decreases with the increase in Ag NP concentration. This behavior can be attributed
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20 to the higher abundance of cystine per surface atom of Ag NPs at lower NP concentrations,
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22 resulting in higher coverage of the NP with cystine and therefore higher reduction in Ag NP
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24 surface charge. The CCC as a function of Ag NP concentration increases with the increase in
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26 NP concentration (**Figure 3b**), which can be attributed again to the fact that cystine is more
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28 efficient in aggregating cit-Ag NPs at lower NP concentrations. This is the first time that cystine-
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30 induced concentration-dependent aggregation of cit-Ag NPs is demonstrated.

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Effect of cystine on cit-Ag NPs Surface charge

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32 Cit-Ag NPs are negatively charged (zeta potential of $-46.5 \pm 4.5 \text{ mV}$). **Figure 2a** shows
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34 the zeta potential of Ag NPs ($125\text{-}1000 \mu\text{g L}^{-1}$) as a function of cystine concentration, and shows
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36 that, for a fixed NP concentration, the magnitude of zeta potential decrease with the increase in
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38 cystine concentration. For a fixed cystine concentration, the magnitude of zeta potential
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40 decrease with the decrease in NP concentration. These observations are in agreement with the
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42 increased aggregation of Ag NPs at higher cystine concentrations and lower NP concentrations.

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44 High uncertainty was observed in the measured zeta potential at low NP concentrations
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46 as laser doppler electrophoresis requires high NP concentrations, in the range of several mg L^{-1}
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48 ⁴¹. Therefore, to further confirm the effect of cystine on the surface charge of cit-Ag NPs, a
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50 second set of experiments were performed at higher NP concentrations ($1 \text{ to } 3.5 \text{ mg L}^{-1}$) and at
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52 fixed cystine concentration ($225 \mu\text{M}$; **Figure 2b**). Again, the results demonstrate that the
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54 magnitude of zeta potential of cit-Ag NPs decreased to lower values with the decrease in NP
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56 concentration (**Figure 2b**). Thus, cystine is more efficient in reducing the surface charge of cit-
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58 Ag NPs at lower NP concentrations, which can be attributed to the higher cystine to surface Ag
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60 atom ratio at lower NP concentrations and thus the higher coverage of Ag NPs by cystine
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62 molecules.

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59 The cystine-induced aggregation and surface charge alteration of cit-Ag NPs can be
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61 explained by the chemical interaction of the surface Ag atoms with the disulfide group in the
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cystine molecule and the zwitterion-type (Zwitterion is a neutral molecule with a positive and a negative electrical charge, though multiple positive and negative charges can be present) surface charge behavior of cystine molecules. It has been demonstrated that similar to the thiol group in cysteine, disulfide groups in cystine can sorb on the surface of AgNPs^{42,43}. Additionally, Ag NPs have been shown to induce S-S cleavage in cystine at low concentrations, but can keep intact S-S bonds bond at a concentration threshold of about 200 μM ^{42,44,45}, which may explain the similarity in the effect of cystine and cysteine in inducing the aggregation of silver NPs.

After cystine adsorption on the Ag NPs, the cystine molecule has still two functional groups (carboxylic and amine) to form bonds between particles. The pK_a value of the carboxylic and amine groups are 1.96 and 8.18, respectively⁴⁶ and the isoelectric pH of cystine is 5.07. The pH of the Ag NPs-cystine mixture was about 5.5-5.7, thus cystine has a negative net charge at the experimental conditions. At this pH, the amine functional groups of cystine are protonated (positively charged, NH^{3+}), whereas the carboxylic groups are deprotonated (negatively charged, COO^-). Thus, the positively charged amino group in the cystine ($-\text{NH}^{3+}$) on the surface of one NP interacts with the negative charge on the surface of other Ag NP (carboxylic group, COO^-) through electrostatic attraction^{47,48}, and cause aggregation⁴⁶. Hence, the cystine molecule constitutes a bridge among Ag NPs, thus inducing Ag NPs aggregation.

The concentration-dependent aggregation of Ag NPs in the presence of cystine is due to the ratio between Ag and cystine, and thus the surface coverage of Ag NPs by cystine. At lower Ag NP concentration, more cystine molecules are available to react with the surface of Ag NPs, resulting in a higher surface coverage of Ag NPs by cystine, reduction of surface charge of Ag NPs, and increased aggregation of Ag NPs. This is further supported by the shift to higher wavelengths (approximately 10 nm) in the Plasmon resonance of Ag NPs at lower NP concentrations in the presence of cystine (**Figure 2c**), indicating increased interaction with cystine at low NP concentrations.

Aggregation impacted by fulvic acid

The UV-vis spectra of cit-Ag NPs 10 minutes post interaction with cystine in the presence of SRFA show little to no change in the spectra in term of peak broadening or absorbance loss at 394 nm (**Figure S5a and b**), suggesting that SRFA prevents (or reduce) cystine induced aggregation of cit-Ag NPs. The rate of the decay λ_{394} is generally slower and decays to higher absorbance in the presence of SRFA (~ 0.88 , **Figure S5a and b**) compared to the absence of SRFA (~ 0.6 , **Figure 2a and c**), presumably due to the reduced aggregation of cit-Ag NPs in the presence of SRFA. It was not possible to calculate the attachment efficiency in

the presence of SRFA because aggregation of cit-Ag NPs did not reach the fast aggregation regime (DLA) within the limit of cystine solubility in water (333 μM). Nonetheless, the rate loss of UV-vis₃₉₄ increases slightly with the increase in cystine concentration, but does not reach the fast aggregation rate (**Figure 4a**).

It is well known that SRFA forms a surface coating on cit-Ag NPs and increase the surface charge and provide steric stabilization^{19,49-51} to cit-Ag NPs. Therefore, the sorption of SRFA molecules on cit-Ag NPs increases electrosteric stabilization of cit-Ag NPs, but they do not completely inhibit surface charge screening and NP aggregation by electrolytes^{32,52}. It is worth noting that, in the presence of SRFA, the surface charge of cit-Ag NPs did not change with the increase in cystine concentrations (**Figure 4b**), which can be attributed to the lack of interaction between cystine and the surface of Ag NPs. Similarly to the case of without SRFA, another set of experiment was conducted to measure the zeta potential of Ag NPs 24 hours post interaction with cystine in the presence of SRFA at higher Ag NP concentrations (1.0-3.5 mg L⁻¹ Ag) to avoid any uncertainty in the measured zeta potential at low NP concentrations (**Figure 4c**). The results suggest that the magnitude of the zeta potential decreases with the decrease in NP concentration at constant cystine concentration (225 μM), indicating the increased efficiency of surface charge alteration at lower NP concentrations due to the increased probability of surface Ag atoms-cystine interactions. Nonetheless, the magnitude of the zeta potential in the presence of SRFA (ca. -28 to -44 mV) remains higher compared with that in the absence of SRFA (ca. -17 to -24 mV), indicating that SRFA form a surface coating on Ag NPs, enhance their surface charge and thus their stability in the presence of cystine. The Uv-vis of Ag NPs spectra 24 hours post interaction with cystine in the presence of SRFA shows no shift in the first peak position with the change in NP concentrations (**Figure 4d**) confirming that SRFA limits the interaction of cystine with surface of Ag NPs. Furthermore, Figure 4d shows the formation of a second peak at higher wavelengths. For the same concentration of Ag NPs, the absorbance of the second peak is lower in the presence of SRFA (Figure 4d) compared with that in the absence of SRFA (Figure 2c), again confirming the role of SRFA in reducing the cystine-induced aggregation of Ag NPs.

Aggregation impacted by NP surface coating

It is well known that surface coatings have a significant impact on NP stability and aggregation. Whereas citrate and PEG stabilize Ag NPs via electrostatic repulsion, PVP stabilizes Ag NPs via steric stabilization mechanism^{32,35}. Therefore, citrate and PEG coated Ag NPs are susceptible to aggregation by charge screening by electrolytes, whereas PVP coated

Ag NPs are generally stable at a wide range of ionic strengths and pHs^{32,35}. Here, we investigate the impact of cystine on the stability of PEG- and PVP-Ag NPs at ecotoxicologically relevant and near environmentally relevant Ag NP concentrations.

The UV-vis absorbance of Cit-, PEG- and PVP- coated Ag NPs (225 $\mu\text{g L}^{-1}$) immediately after addition of different concentrations of cystine (7-133 μ) are presented in **Figure 5a, d and f**, together with those after 6 hours (**Figure 5b, e and h**) and 24 hours (**Figure 5c, f and i**). All spectra show two peaks, the first centered on 394 to 402 nm, 393-400 nm and 393-412.5 nm immediately after addition of cystine for citrate-, PVP- and PEG coated Ag NPs, respectively. The position of the first peak shifts to higher wavelengths at higher concentrations of cystine indicating increased interaction between Ag NP surface atoms and cystine molecules. These observations confirm that cystine replaces the three types of surface coatings (citrate, PVP and PEG). The higher shift at higher cystine concentrations indicates increased interaction between Ag NP surface atoms and cystine molecules.

The first peak shifts to higher wavelength with time; ca. 394-422 nm, 394-415 nm, and 393-424 nm, for citrate-, PVP, and PEG coated Ag NPs six hours post interactions with cystine. The position of this peak remains constant beyond six hours up to 24 hours, indicating that cystine-Ag NP surface atom interaction reaches equilibrium within six hours.

The second peak is centered on 495 nm immediately after addition of cystine, shifts to higher wavelengths (ca. 530 nm) between 0 and 6 hours and remains constant afterwards. These results indicate that cystine induces the aggregation of citrate, PEG and PVP coated Ag NPs, and that under the selected experimental conditions, Ag NPs occur both as primary Ag NPs as well as aggregated Ag NPs. These findings suggest that cystine binds to the surface of Ag NPs and replaces PVP and PEG coating similar to citrate, resulting in modification of NP surface coating and charge and therefore their aggregation behavior, in good agreement with other studies²¹. The Plasmon resonance peaks was stable within 6 hours indicating that Ag NPs were stable at this time point and did not undergo any further aggregation. No losses in the Plasmon resonance were observed at 24 hours compared to 6 hours indicating that aggregation resulted in the formation of small Ag NP aggregates that did not settle out of suspension. These findings are in good agreement with previous results suggesting that cystine induces aggregation of PVP-coated Ag NPs^{21,25}.

Environmental and Nanotoxicological Implications

This research represents the first systematic study of the effect of cystine concentration on the aggregation of Ag NPs at ecotoxicologically relevant and near environmentally relevant Ag NP concentrations. Cystine significantly impacts the stability of citrate, PEG and PVP coated Ag NPs, resulting in Ag NP aggregation in a concentration-dependent manner, with a shift in the CCC toward lower concentrations of cystine at lower concentration of Ag NPs. SRFA reduced the cystine induced aggregation of Ag NPs within the limit of cystine solubility in water, likely due to the steric effect of SRFA. Consequently, in natural surface waters, it is likely that cystine will play an important role in determining the fate and behavior of cit-Ag NPs, and further research is required to investigate the interplay between cystine, electrolytes and NOM on the stability of Ag NPs. Due to the higher affinity of the cysteine thiol groups to Ag⁺ and Ag surfaces compared with the cystine disulfide group, a similar concentration-dependent aggregation of Ag NPs in the presence of cysteine is expected. Several studies have demonstrated that cysteine induces aggregation of Ag NPs²⁵, but none has investigated the concentration-dependent aggregation of Ag NPs in the presence of cysteine.

In ecotoxicological studies of Ag NPs, cysteine is typically used to scavenge the dissolved ions and thus to differentiate dissolved vs. NP effects assuming no interaction occurs between Ag NPs and cysteine. Several papers have reported lower toxicity of Ag NPs in the presence of cysteine, and consequently concluded that silver ions are the major cause of their antimicrobial activity. Our results suggest that the reduced toxicity in the presence of cysteine widely observed in the literature could be due to a combined effect of Ag⁺ scavenging by cysteine and Ag NP aggregation. The concentration-dependent aggregation of Ag NPs induced by cystine (a similar behavior is expected with cysteine) can alter the nature of the dose (toxicant) in a typical dose-response experiment; that is Ag NPs may form aggregates at low concentration and stay suspended as individual NPs at high Ag NP concentration.

This work underscores the importance of understanding the impact of biogeochemical molecules on NP stability in both ecotoxicological media as well as in the natural environment, and thus the fate and effects of NPs. This study also underscores the importance of investigating NP interactions at environmentally and toxicologically relevant NP concentrations.

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Figure 1. Uv-vis spectra of cit-Ag NP reacted with different concentrations of cystine at different concentrations of cit-AgNPs: (a) 125 $\mu\text{g L}^{-1}$ Ag, (b) 250 $\mu\text{g L}^{-1}$ Ag, (c) 500 $\mu\text{g L}^{-1}$ Ag, and (d) 1000 $\mu\text{g L}^{-1}$ Ag

Figure 2. Zeta potential of cit-Ag NPs reacted with cystine (a) at different concentrations of cystine and cit-Ag NPs, and (b) at fixed concentration of cystine (225 μM) and different concentrations of cit-Ag NPs (1-3.5 mg L^{-1}). (c) Uv-vis spectra of cit-Ag NPs 24 hours post interaction with 225 μM cysteine.

Figure 3. Attachment efficiency of cit-Ag NPs reacted with cystine at different concentrations of cit-Ag NPs.

Figure 4. (a) rate loss of the UV-vis absorbance at 394 nm as a function of cystine concentration in the presence of 1 mg/L^{-1} SRFA, (b) zeta potential of cit-Ag NPs reacted with cystine in the presence of 1 mg/L^{-1} SRFA, and (c) zeta potential of cit-Ag NPs reacted with fixed concentration of cystine (225 μM) and different concentrations of cit-Ag NPs (1-3.5 mg/L^{-1}). (d) normalized Uv-vis spectra of cit-AgNPs 24 post reaction with cystine.

Figure 5. UV-vis spectra of AgNPs (225 µg L⁻¹) with different surface coatings (a-c) citrate, (d-f) PVP, and (g-i) PEG at different time points (a, d, g) 0, (b, e, h) 6 and (c, f, i) 24 hours following reaction with different concentrations of cystine (7-133 µM)