Influence of Capping Agents on Silver Nanoparticle (Ag-NP) Toxicity to Nitrifying Bacteria

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June 2012-November 2012
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Acknowledgements

This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2011-38422-31294 from the USDA National Institute of Food and Agriculture. Additionally, I would like to acknowledge my advisor, Dr. Radniecki, and Dr. Pullman for their help and support on the project, and for the students in the lab that contributed: Cameron Mumper, Tanner Houston, Karina Sertich, Lucie Uribe, Vincent Uhde, and Mark Rein.

Executive Summary

Silver has long been used as a powerful microbial agent. With the emergence of nanotechnology, silver nanoparticles (AgNPs) of 1 nm to 100 nm in diameter can be synthesized and have application in an array of industries due to their unique conductive, optical, antibacterial, and magnetic properties. AgNPs have the fastest growing market in nanotechnology, and are increasingly applied as antibacterial and antifungal agents in electronics, optics, catalysis, textiles, water treatment, and consumer products.

As applications of AgNPs increase, the levels of AgNPs and silver ions (Ag+) in wastewater treatment plants (WWTPs) and the environment are of growing concern. Nitrogen removal in WWTPs depends largely on ammonia oxidizing bacteria (AOB), which are sensitive to the antibacterial effects of AgNPs. Inhibition of AOB by AgNPs causes a reduction in nitrogen removal from wastewater. As a result, eutrophication can occur in receiving bodies, leading to algae blooms and oxygen depletion, which severely impact the surrounding ecosystem.

AgNPs begin dissolution once placed in an aqueous solution, releasing Ag+, which are known to have more acute toxic effects on AOB. AgNPs that dissolve at faster rates have, therefore, higher levels of toxicity. The fate and toxicity of AgNPs is likely influenced by the capping agents located on their surfaces to enhance stability. Common capping agents include citrate, tannic acid, and polyvinylpyrrolidone (PVP). Each capping agent has unique chemistries that influence the aggregation and dissolution state of the AgNPs and thus, ultimately, their toxicity. These capping agents may also influence how well the AgNPs sorb to bacteria and may ultimately control their fate in the environment.

This project examines the toxicity of AgNPs to *Nitrosomonas europaea*, the model AOB. The central hypothesis is that the capping agent will determine the toxicity level of the AgNP to *N. europaea* cells and their interactions with macromolecules (e.g. Bovine Serum Albumin (BSA) – a model protein, alginate – a model lipopolysaccharide) divalent cations (e.g. magnesium sulfate (MgSO₄) – representing cations in hard waters) and their behavior in sunlight. These
interactions will greatly influence the fate of AgNPs in both wastewater treatment plants and the natural environment.

**Project Objectives**

The goal of this project was to discover how the underlying chemical structures of AgNP capping agents influence their interactions with light, various components of wastewater and *N. europaea*. Tests were focused on determining the size of the AgNPs made with various capping agents and determining how the size and capping agents influenced their toxicity levels towards *N. europaea*.

Though this project is not associated with any watershed directly, it could prove beneficial to all watersheds. Certain levels of toxicity may result in nitrification failure in WWTPs, which can lead to the release of ammonia and the eutrophication of the receiving watershed. Analyzing the fate and toxicity of a contaminant on the environment and manmade structures could help prepare me for a career in the USDA Forest Service. Also the laboratory experience can be used to aid any research or fieldwork pertaining to tasks as an engineer in the Forest Service.

**Project Approach**

To complete the project objectives, experiments to synthesize AgNPs of varying capping agents and shapes were first conducted. Following these experiments, acute (3 hour) AgNP dissolution experiments and nitrification inhibition experiments were conducted to determine the rate at which Ag⁺ are released from AgNPs and how they interact with *N. europaea* under various controlled environments.

**AgNP Synthesis:** Four capping agents, sodium citrate (NaCit), polyvinylpyrrolidone (PVP), polyvinyl alcohol (PVA), and triethylenetetramine (TEA), were used in this experiment because they represent various structural and chemical groups that may play different roles in the toxicity of AgNPs. For each capping agent, a 1.0 mM stock solution was made in DI water. Additionally, a 1.0 mM stock solution of silver nitrate (AgNO₃) and 5.0 mM solution of sodium borohydride (NaBH₄) were prepared. 7.4 mL of DI water was pipetted into 20-mL vials, along with 1 mL of the capping agent (NaCit, PVP, PVA, or TEA) and 1 mL of the AgNO₃ stock solutions. The vials were placed on a stir plate to be stirred vigorously, while 600 μL of 5.0 mM NaBH₄ were pipetted drop-by-drop into the rapidly stirring solution of silver ions and capping agent. The clear Ag⁺ and capping agent solution
instantly turned a golden amber color upon contact with NaBH₄, signifying the formation of AgNPs.

Once spherical AgNPs were synthesized, additional experiments were carried out exposing these AgNPs to blue, green, red and ultraviolet light. The vials were placed in small boxes containing one of the light colors. The vials were constantly stirred, and small doses of the capping agent were added to the solution each day. The UV-vis spectrums of the AgNPs exposed to the light sources were monitored daily to help characterize their change in shape. After no further changes in the UV-vis spectrums were observed, changes in AgNP shapes were confirmed though transmission electron microscopy (TEM) images.

**AgNP Dissolution:** The dissolution and aggregation rates of the AgNPs of varying capping agents were measured using UV-vis spectrophotometry. Dissolution is determined by a reduction in peak absorbance height and aggregation is determined by a widening of the peak width.

The experiments were conducted in 155-mL glass bottle batch bioreactors containing a total volume of 15-mL solutions. 1 ppm of the 20-nm citrate Ag-NPs was pipetted into DI water in the glass bottles and shook at 250 rpm in the dark for a minimum of 15 minutes. The test media (2.5 mM (NH₄)₂SO₄ and 30 mM HEPES buffer at pH 7.8) was added to the Ag-NP solution along with the desired concentration of BSA, alginate, and MgSO₄. The solutions were shook by hand for 30 seconds before taking the first UV-vis reading (-30 min) and were shaken at 225 rpm in the dark at 30°C between sampling.

Dissolution studies in the presence of sunlight were prepared in the same way, without the addition of BSA, alginate, or MgSO₄. After the first UV-vis reading, the bottles were placed in direct sunlight for 6 hours and sampled at 2-hour intervals.

**Toxicity Tests:** *N. europaea* ATCC 19718 cells were prepared in a minimal growth media and shaken at 150 rpm in the dark at a temperature of 30°C for three days until they reached mid-exponential growth (OD₆₀₀ = 0.070). The cells were spun down in a centrifuge and suspended in 30 mL of 30 mM HEPES buffer (pH 7.8).

The experiments were conducted in 155-mL glass bottle batch bioreactors containing a total volume of 35-mL solutions. 1 ppm Ag-NPs or 0.125 ppm Ag⁺ were pipetted into DI water in 155-mL glass bottle batch bioreactors and shaken at 225 rpm in the dark at 30°C for a minimum of 15 and 5 minutes, respectively. Test media (2.5 mM (NH₄)₂SO₄ and 30 mM HEPES buffer at pH 7.8) was added to the solution along with the desired concentration of BSA or alginate. The batch bioreactors were shaken for 30 minutes before adding *N. europaea* cells (OD₆₀₀ = 0.72).
After addition of *N. europaea*, the batch bioreactors were shaken in the dark at 30°C for 3 hours. Samples were taken at 45-minute intervals. 10 µL of solution were pipetted into 1.5-mL centrifuge tubes containing 890 µL of 1% (w/v) sulphanilamide in 1 M HCl and 100 µL of 0.2% (w/v) N-(1-Naphthyl) ethylenediaminedihydrochloride. The centrifuge tubes were immediately capped and shook. At the end of the experiment, 200 µL was pipetted from each centrifuge into microcentrifuge plates. The nitrite (NO$_2^-$) production of the *N. europaea* cells was measured colorimetrically using a BioTek Synergy HT plate reader to measure the absorbance at 540 nm.

Control bottles containing no Ag-NPs, Ag$^+$, BSA or alginate were run during each experiment. The toxicity of each Ag-NP type/macromolecule combination was determined by comparing the rates of ammonia oxidation (the primary function of *N. europaea*) in the control bottles versus the bottles containing Ag-NPs.

**Project Outcomes**

**AgNP Synthesis:** AgNPs were successfully created using all four capping agent types (NaCit, PVP, PVA, TEA). Experiments were first carried out to discover the ratio of capping agent to reducing agent for a standard 10 ppm solution of AgNPs. The ratio was determined to be equal across all capping agents (1:0.6), regardless of their different molecular structures and electrochemical properties. The resulting AgNPs averaged 10 nm in diameter, as determined by dynamic light scattering (DSL) and TEM.

Exposing AgNPs to light (red, green, blue, or UV) proved to have a profound effect on their shape. As the AgNPs were exposed, the peak absorbance ($\lambda_{max}$) using UV-vis spectroscopy shifted to higher wavelengths, dependent upon the color of light they were exposed to. AgNPs exposed to blue light (Figure 1) underwent a shift in $\lambda_{max}$ from 392 nm to 478 nm and a reduction in peak absorbance from 1.268 to 1.143 and finally 0.903 due to widening in the peak after a period of two weeks. This indicated an increase in size and perhaps shape, which was confirmed using TEM imagery (Figure 2).

For each solution of AgNPs exposed to light, its color changed from amber to the complement color of the light that it was exposed to. For AgNPs exposed to blue light, the solution became orange, while the diameter of the average AgNP increased to approximately 50 nm, and changed in shape from spherical to pentagonal. For AgNPs exposed to red light, the solution turned purple, the average diameter increased to 100 – 300 nm, and the AgNPs developed a triangular shape (Figure 3).
Figure 1: 10-nm citrate coated AgNPs exposed to blue light (450 nm)

10 ppm citrate AgNP exposed to blue light

Figure 2: TEM image of 10-nm citrate AgNPs exposed to blue light
AgNP Dissolution: The dissolution of a 1 ppm solution of 20-nm citrate AgNPs was first studied in test media containing 2.5 mM (NH₄)₂SO₄ and 30 mM HEPES (pH 7.8). The λ_max of the AgNPs was measured at 400 nm and the peak absorption was initially measured at 0.135 and decreased to 0.075 after 3 hours as a result of test media-induced dissolution (Figure 4). The width and shape of the UV-vis spectrum peaks remained constant throughout the experiment suggesting that aggregation of the AgNPs was not occurring.

AgNP dissolution was measured in the presence of BSA at a concentration of 20 ppm (Figure 5). The results indicate minimal reduction in peak absorption from 0.130 to 0.119 after 3 hours, also a slight positive shift in of the maximum wavelength can be observed. This suggests the exposing AgNPs to proteins that are commonly found in WWTPs will reduce the dissolution of the AgNPs and possible reduce their toxicity.

Dissolution experiments were conducted in the presence of alginate at a concentration of 600 ppm (Figure 6). The results show a moderate decrease of the peak absorption from 0.138 to 0.115. This suggests that while alginate does not prevent AgNP dissolution as strongly as BSA, the exposure of AgNPs to alginate does reduce their dissolution and may reduce their toxicity. This phenomenon may help partially explain why biofilms, which contain high concentrations of polysaccharides (e.g. alginate) are more tolerant to AgNPs than suspended cells.
Figure 4: UV-vis absorption spectrum of a 1 ppm 20-nm citrate AgNP suspension in test media (2.5 mM (NH₄)SO₄, 30 mM HEPES buffer at pH 7.8)

Figure 5: UV-vis absorption spectrum of a 1 ppm 20-nm citrate AgNP suspension in test media (2.5 mM (NH₄)SO₄, 30 mM HEPES buffer at pH 7.8) and 20 ppm BSA
Figure 6: UV-vis absorption spectrum of a 1 ppm 20-nm citrate AgNP suspension in test media (2.5 mM (NH₄)SO₄, 30 mM HEPES buffer at pH 7.8) and 600 ppm alginate

![UV-vis absorption spectrum of 1 ppm AgNP + 600 ppm Alginate](image)

Figure 7: UV-vis absorption spectrum of a 1 ppm 20-nm citrate AgNP suspension in test media (2.5 mM (NH₄)SO₄, 30 mM HEPES buffer at pH 7.8) and 600 ppm Alginate and 730 µM MgSO₄

![UV-vis absorption spectrum of 1 ppm AgNP + 600 ppm Alginate + 730µM MgSO₄](image)
Mg\(^{2+}\) is found in natural waters and wastewater and is quantified as hardness. Previous research has shown that the presence of Mg\(^{2+}\) results in a very rapid aggregation of AgNP suspensions. Experiments conducted in the presence of both alginate and MgSO\(_4\) (Figure 7) resulted in peak absorption decrease from 0.135 to 0.080 and the formation of a second peak at approximately 500 nm. This suggests that the presence of polysaccharides may prevent AgNP dissolution and play an important role in the fate and transport of AgNPs in both WWTPs and the natural environment.

Further dissolution tests were performed on 20-nm citrate AgNPs exposing them to sunlight. The experiments were performed for 6 hours in clear bottle batch bioreactors. The results of sunlight exposure (Figure 8) show a more dramatic decline in the peak absorption than can be seen in experimentation with AgNPs in test media not exposed to direct sunlight (Figure 4). In the clear bottle, the peak absorbance dropped from 0.121, 0.039, 0.023, to finally 0.021 after 6 hours. After 2 hours of exposure, the peak of 0.039 is significantly lower than the final peak absorption of 0.076 reached during experimentation with AgNPs exposed to test media in the dark. These results suggest that exposure to sunlight (and perhaps more specifically, UV-light) will accelerate AgNP dissolution. This may have a profound role of the fate and transport of AgNPs in natural environments exposed to sunlight (e.g. rivers and streams) and may increase their toxicity in these environments.

Figure 8: UV-vis absorption spectrum of a 1 ppm 20-nm citrate AgNP suspension in test media (2.5 mM (NH\(_4\))SO\(_4\), 30 mM HEPES buffer at pH 7.8) exposed to sunlight in a clear bottle

1 ppm AgNP in test media exposed to sunlight

![Graph showing absorption spectra over time.]
**Toxicity Tests:** Toxicity tests were carried out with 3-hour nitrification inhibition experiments, from which toxicity levels of AgNPs and Ag⁺ at varying concentrations with various macromolecule interactions could be measured. The percent activity of *N. europaea* in the presence of 120 ppm BSA and 0.25 – 0.60 ppm Ag⁺ was measured (Figure 9). Exposure to 0.25 ppm Ag⁺ resulted in 0% activity of cells, though when exposed to both 0.25 ppm Ag⁺ and 120 ppm BSA, 80% was observed. Higher concentrations of Ag⁺ with 120 ppm BSA resulted in lower percent activity of *N. europaea*. These results suggest that proteins may help protect cells from AgNP exposure by binding dissolved Ag⁺ released from the AgNPs and suggests that environments with high protein content (e.g. wastewater) may significantly reduce the toxicity of AgNPs and Ag⁺.

**Figure 9: Percent nitrification activity of *N. europaea* in test media and varying Ag⁺ concentrations with 120 ppm BSA in 3-hour acute toxicity studies**

Additional toxicity tests were conducted with 0.125 ppm Ag⁺ in the presence of alginate from 100 ppm to 500 ppm (Figure 10). Exposure to 0.125 ppm Ag⁺ resulted in 40% activity. With addition of alginate, percent activity gradually increased to 60% with 1000-ppm alginate in solution. Solutions containing *N. europaea* with 500-ppm alginate without the presence of Ag⁺ yielded 100% activity. These results suggest that once Ag⁺ have been released from AgNPs, the presence of polysaccharides (e.g. in biofilms) will not significantly protect the cells from the Ag⁺.
Toxicity testing was performed using 10-nm citrate AgNPs synthesized in the lab (Figure 11). Results indicate higher levels of toxicity than of the 20-nm citrate AgNPs from Nanocomposix, Inc. Exposed to 0.25 ppm AgNP, percent activity of *N. europaea* cells was 6%, percent activity dropped to 2% when exposed to 0.50 ppm AgNP, and to 0% when exposed to 0.75 ppm AgNP.

Similar testing was done using 20-nm PVP AgNPs from Nanocomposix, Inc. (Figure 12). Results show 85% activity of *N. europaea* cells when exposed to 0.25 ppm AgNP, 34% activity exposed to 0.50 ppm AgNP, 1% activity exposed to 0.75, and 0% activity when exposed to 1.00 ppm AgNP. When toxicity testing was performed using 40-nm triangle shaped citrate AgNPs from Nanocomposix, Inc. (Figure 13). Results indicate 100% when exposed to 0.25 ppm AgNP, 40% activity exposed to 0.50 ppm AgNP, 6% activity exposed to 0.75 ppm AgNP, and 4% activity when exposed to 1.00 ppm AgNP. As expected, as the AgNP diameter decreased, the toxicity of the AgNPs increased. This is due to a larger surface area to volume ratio in smaller AgNPs which leads to a more rapid dissolution rate. However, when triangular AgNPs were explored, their toxicity was very similar to the 20 nm PVP AgNPs even though the triangular AgNPs had a planer diameter of 40 nm. This suggests that the shape of the AgNPs may play a large role in the rate of Ag+ dissolution and toxicity. As shown above, AgNPs can change shape under natural conditions when in the presence of a reducing agent (e.g. citrate) and exposed to
light (Figures 1 – 3). These transformations may be critical in understanding the fate, transport and toxicity of AgNPs in the environment.

**Figure 11:** Percent nitrification activity of *N. europaea* in test media and varying concentrations of 10-nm citrate Ag-NPs

**Figure 12:** Percent nitrification activity of *N. europaea* in test media and varying concentrations of PVP Ag-NPs
Conclusions

Through the course of this project, characteristics of AgNPs and how they interact with their environment were analyzed. While synthesizing AgNPs, we determined that exposure to light has a profound influence on the size and shape of AgNPs. Sunlight was found to enhance the dissolution rate of citrate-coated AgNPs, which could be applied to fate and transport of AgNPs to determine their toxicity upon arrival in a WWTP.

Dissolution studies exposing citrate AgNPs to test media (Figure 4) indicate dissolution due to components of the test media (2.5 mM (NH₄)₂SO₄ and 30 mM HEPES buffer at pH 7.8). Studies exposing the AgNPs to test media and 120 ppm BSA (Figure 5) show a much slower dissolution rate of AgNPs. Additionally, the $\lambda_{\text{max}}$ shifted slightly to higher wavelengths, indicating an increase in particle size. These suggest that BSA prevents the release of Ag⁺ by binding to the surface of the AgNPs.

When exposing citrate AgNPs to test media and 600 ppm alginate (Figure 6), dissolution was slowed and a slight positive shift in $\lambda_{\text{max}}$ was observed. This suggests that alginate, like BSA, binds to the surface of AgNPs to prevent dissolution, though it proves much less effective than BSA. Exposed to 600 ppm alginate, peak
absorbance of AgNPs decreased by 0.055, while testing with 120 ppm BSA resulted in a decrease of 0.011 in peak absorbance. The addition of 730 µM MgSO₄ to AgNPs with test media and 600 ppm alginate (Figure 7) resulted in faster dissolution of AgNPs and the formation of a second peak, indicating agglomeration.

Toxicity testing exposing *N. europaea* to Ag⁺ in test media with 120 ppm BSA (Figure 9) showed increased activity in bacteria cells than without BSA present. Cells exposed to 0.25 ppm Ag⁺ in test media resulted in 2% activity, while those exposed to 0.25 ppm Ag⁺ in test media and 120 ppm BSA had 85% activity. The percent activity of *N. europaea* in the presence of 120 ppm BSA gradually decreased as the concentration of Ag⁺ was increased until reaching 2% at 0.60 ppm Ag⁺.

Toxicity testing exposing *N. europaea* to Ag⁺ in test media with 100 – 1000 ppm alginate demonstrates similar trends. Exposed to 0.125 ppm Ag⁺ in test media without the presence of alginate resulted in 37% activity of *N. europaea* cells. With the addition of 100 ppm alginate, percent activity increased to 50%, though increased slowly with further addition of alginate until reaching a maximum of 59% with 750 ppm alginate (Figure 10).

The results from exposing *N. europaea* cells to 0.25 – 1.00 ppm AgNPs in test media show varying toxicity levels (Figures 11 -13). AgNPs smallest in diameter (10 nm) demonstrate much higher inhibition of cells than AgNPs of larger diameter (20 nm, 40 nm). This suggests that the smaller the AgNP, the more lethal it is due to a higher surface area to volume ratio. However, the shape of the AgNPs appears to also play a key role as the 40 nm AgNP triangles were equally toxic as the 20 nm AgNPs even though the triangles had twice the diameter of the 20 nm spherical AgNPs.

This project has only skimmed the surface of dissolution of AgNPs and effects of wastewater components on toxicity to AOB. Much additional research is needed to fully understand the interactions that occur between AgNPs and their environment. Working on this internship has sparked a great interest in working on remediation projects in the environment. Working as an engineer in the USDA Forest Service could provide such an enriching experience that has become so appealing though my hands-on work.