INFLUENCE OF AMMONIA AND MACROMOLECULES ON THE TOXICITY AND ADSORPTION OF SILVER NANOPARTICLES TO THE AMMONIA OXIDIZING BACTERIA NITROSOMONAS EUROPaea

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Influence of Ammonia and Macromolecules on the Toxicity and Adsorption of
Silver Nanoparticles to the Ammonia Oxidizing Bacteria

Nitrosomonas europaea

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DEDICATION

I dedicate this thesis to my wife, who has remained steadfast during times of great distress and given me the strength and wisdom to face life’s challenges with patience and a generous amount of laughter.
ABSTRACT OF THE THESIS

Influence of Ammonia and Macromolecules on the Toxicity and Adsorption of Silver Nanoparticles to the Ammonia Oxidizing Bacteria *Nitrosomonas europaea*

by

Cameron Kostigen Mumper
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Due primarily to their remarkably effective antimicrobial properties, silver nanoparticles (AgNPs) are commonly incorporated into a myriad of consumer products. The rapid and unregulated introduction of these products to the consumer market has raised concerns over the potential for AgNPs to impair essential biological processes within wastewater treatment plants. *Nitrosomonas europaea*, a model ammonia oxidizing bacteria, plays an important role in the removal of organic nitrogen from wastewater. In the studies presented herein, the toxicity and adsorption of 20 nm citrate capped AgNPs to *N. europaea* was investigated in the presence and absence of surrogate wastewater and natural water constituents, including bovine serum albumin (BSA), alginate, and Suwannee River Humic Acid (SRHA).

The AgNPs were found to be highly susceptible to rapid dissolution to Ag\(^+\) in the presence of NH\(_3\), resulting in their increased toxicity to *N. europaea*. The presence of 100 ppm BSA yielded relatively uniform AgNP dissolution and subsequently enhanced toxicity to *N. europaea* independently of NH\(_3\) concentrations, suggesting that the BSA concentration was a better predictor of AgNP dissolution than NH\(_3\). Unlike BSA, the high concentration of alginate (600 ppm) used in these studies did not result in uniform dissolution of the AgNPs. However, the protective coating afforded by the adherence of alginate to the surface of the AgNPs was compromised by NH\(_3\) and is reflected by the enhanced dissolution of the AgNPs and the subsequent increase in their toxicity to *N. europaea*. In addition, NH\(_3\) compromised the protective coating endowed by 15 ppm SRHA, resulting in increased dissolution and toxicity of the AgNPs to *N. europaea*.

Furthermore, results from these studies indicate a strong affinity for the AgNPs to adsorb to cells. At lower concentrations (40 ppm), BSA appeared to coat the surface of the AgNPs and reduced their adsorption to *N. europaea* cells through electrosteric hindrances. When compared with BSA, a significantly higher concentration of alginate (800 ppm) was required to saturate the surface of the AgNPs and reduce their adsorption potential to cells through electrosteric hindrances, indicating that polysaccharides may, in general, interact weakly with the AgNPs surface. Similar to BSA, a relatively low concentration of 10 ppm SRHA was required to saturate the surface of the AgNPs and significantly reduce their adsorption to cells. However, the electrostatic stabilization afforded by SRHA was quickly compromised in waters with a high electrolyte concentration.
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Figure A.22. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 40 ppm BSA, and 1.8 mM MgSO₄ (Top Row) and 2.4 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.

Figure A.23. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 1,000 ppm alginate (Top Row) and 0.35 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.

Figure A.24. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 1,000 ppm alginate, and 0.73 mM MgSO₄ (Top Row) and 1.2 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.

Figure A.25. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 1,000 ppm alginate, and 1.8 mM MgSO₄ (Top Row) and 2.4 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.

Figure A.26. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA (Top Row) and 0.35 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.

Figure A.27. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA, and 0.73 mM MgSO₄ (Top Row) and 1.2 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.

Figure A.28. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA, and 1.8 mM MgSO₄ (Top Row) and 2.4 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.
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CHAPTER 1

INTRODUCTION

Nanotechnology is a rapidly growing interdisciplinary field that strives to exploit the unique physical, chemical, and biological properties exhibited by matter at a scale ranging from 1-100 nm (National Nanotechnology Initiative 2012). At the nanoscale, the properties of matter can differ greatly from those of bulk materials, molecules, and single atoms as a result of an increased relative surface area per unit mass and the dominance of quantum effects. It is the very same unique properties of nanoscale materials (nanomaterials) that complicates their toxicity to biota and fate and transport in the natural environment and engineered systems. In particular, there is growing interest in the potential impacts of nanomaterials to sensitive bacteria that play an essential role in nutrient recycling within wastewater treatment plants (WWTPs).

1.1 NANOAMATERIALS

Nanomaterials can be categorized into two broad categories; (1) carbon-based nanomaterials (e.g. fullerenes, carbon nanotubes) and (2) inorganic nanoparticles, including metal oxides (e.g. zinc oxide, iron oxide, titanium dioxide, etc.), metals (e.g. gold, silver and iron), and quantum dots (e.g. cadmium sulfide and cadmium selenide) (Ju-Nam and Lead 2008). Compared with their macroscopic counterparts, nanomaterials exhibit unique physical, chemical, and biological properties that are incorporated into electronics, optics, information technology, healthcare, biotechnology, food and agricultural, and environmental remediation (Prasad, Kambala, and Naidu 2011). Nanomaterials can take many shapes, but their structures can be generally classified as particles or crystals; tubes, wires, or rods; dendrimers (branched structures), and composites (Sellers 2009).

Nanoparticles (NPs), a subcategory of nanomaterials, are defined as ultrafine particles with lengths in two or three dimensions in the range of 1-100 nm ($10^{-9} - 10^{-7}$ m), which may or may not exhibit size-related intensive properties (American Society for Testing and Materials [ASTM] 2012). As a reference, matter at this scale is about one ten-thousandth the
diameter of human hair, a thousand times smaller than a red blood cell, or about half the size of the diameter of DNA (U.S. Environmental Protection Agency [EPA] 2007). Manufactured NPs represent an intermediate supramolecular state of matter between bulk and molecular material (Hoet, Bruske-Hohlfeld, and Salata 2004).

Reducing the size of a particle to the nanoscale effectively increases its surface area to mass ratio. Since the reactive portion of a particle is at its surface, increasing the relative surface area will therefore increase the particles reactivity for a given amount of material. Both classical and quantum physics can govern the behavior of a NP, and the influence of quantum effects can change essential material characteristics, such as optical, magnetic, and electrical properties. For example, optical properties of NPs change from that of their bulk material because as the particle becomes smaller the quanta of light energy that can interact with them increases. Consequently, gold NPs can be purple, green, orange, or red depending on their size (Sellers 2009). In its bulk state, solid carbon (graphite) is a poor conductor of electricity. However, at the nanoscale a sheet of graphite that is one atom thick can act as a semi-conductor with significantly less resistance.

The use of NPs dates back to the 4th century B.C., where they were used as a decorative means for staining glass (National Nanotechnology Initiative 2012). In modern times, the nanotechnology market is undergoing explosive growth and new and novel applications of NPs are emerging constantly. The accelerated growth in the production of nanomaterials and the rapid increase of their application in many diverse areas has captured the attention of researchers, government agencies, and various industries worldwide (Ju-Nam and Lead 2008).

According to the Nanotechnology Consumer Products Inventory, between March 2006 and March 2011, at least 303 new products containing some form of nanotechnology were added to the global market. This brought the total product number to 1,317, equivalent to a 270% increase since 2006 (Figure 1.1). In overall production of nanomaterials, the U.S. outcompetes both Asia and Europe (PEW Charitable Trusts [PEW] 2013). Investments in the nanotechnology industry grew from $13 billion in 2004 to $50 billion in 2006, and are projected to reach $2.6 trillion by 2014 (Aitken et al. 2006). Consequently, large amounts of materials and products will be marketed and may come in contact with the environment, either during production, transport, use, or as waste.
1.2 Nanomaterial Regulation

In response to the rapid increase in nanomaterial production, in 2004 the British Royal Society and the Royal Academy of Engineering recommended that factories and research laboratories treat manufactured nanomaterials as hazardous waste and seek to reduce or remove them from the waste streams (The Royal Society and the Royal Academy of Engineering 2004). In the U.S., current regulatory and oversight efforts are divided among several agencies. The most prominent player is the EPA, which regulates the manufacturing of engineered nanomaterials under the Toxic Substance Control Act (TSCA) (Gibson and Pula 2009). In January 2008, the EPA began collecting data from manufacturers using the voluntary Nanoscale Materials Stewardship Program in an attempt to aid in determining how and whether certain nanomaterials present risks to human health and the environment. The program was discontinued in December 2009 due to a limited response and the EPA developed regulatory approaches under Sections 5 and 8(a) of the TSCA.

Under Section 5 of the TSCA, any chemical not already included in the TSCA inventory database is considered ‘new’ and the manufacturing company is required to submit pre-manufacture notification (PMN). Under the definition of ‘new’, a chemical that is
composed of the entirely same components but is one already included in the database but differs in structure is considered ‘new’ and is subject to regulation. Even if a chemical is already listed in the TSCA inventory database, the EPA can require the company to file a PMN if it plans to engage in a “Significant New Use.” Currently, the EPA has only recognized carbon nanotubes as substances meriting regulation under the TSCA, as they are recognized as being distinct from graphite or other allotropes of carbon listed in the TSCA inventory database.

Although these regulations are beneficial in tracking current nanomaterials they do not establish exposure limits. Furthermore, an internal review conducted by the Office of Inspector General in 2011 found that the EPA does not currently have sufficient information or processes to effectively manage the human health and environmental risks of nanomaterials because it currently lacks the environmental and human health exposure and toxicological data to do so effectively (EPA 2007).

1.3 SILVER NANOPARTICLES

Approximately 20% of commercially available products are those containing nanoscale silver, or silver nanoparticles (AgNPs), making them the fastest growing market segment of nanomaterials (Figure 1.2) (PEW 2013). In 2008, the use of AgNPs was estimated to be around 500 tons per year (Mueller and Nowack 2008). The widespread manufacturing of AgNPs is attributed to their unique metal properties, including conductivity, chemical stability, catalytic activity, nonlinear optical behavior, and antimicrobial activity (Capek 2004). AgNPs are being rapidly incorporated into numerous consumer products, such as wound dressings, detergents, surgical instruments, hand sanitizers, clothing, food containers, and appliances, including washing machines, refrigerators, water purification, and HVAC filters (Rejeski and Lekas 2008).

The methods used in the production and synthesis of AgNPs are widely varied and include electrochemical (Tang et al. 2001), thermal decomposition (Kim et al. 2005), laser ablation (Bae, Nam, and Park 2002), microwave irradiation (Patel et al. 2005), and sonochemical synthesis (Zhang et al. 2004). Inasmuch, the method of synthesis influences
several AgNP properties, including the mean particle diameter, size distribution, shape, stability, and the chemical yield of the reaction (Ju-Nam and Lead 2008).

The most commonly used method in the synthesis of AgNPs is the chemical reduction of a silver salt, e.g. silver nitrate (AgNO$_3$). In this method, AgNP synthesis is achieved by reducing AgNO$_3$ to its zero-valent state with a strong reducing agent, e.g. sodium borohydride, as shown in Equation 1.1:

$$AgNO_3 + NaBH_4 \leftrightarrow Ag + \frac{1}{2}H_2 + \frac{1}{2}B_2H_6 + NaNO_3$$ (1.1)

The reduction of silver ions (Ag$^+$) to zero-valent silver (Ag$^0$) results in the formation of the AgNPs. However, the lifetime of the synthesized AgNPs is very short as they tend to coalesce into large aggregates due to van der Waals forces following the neutralization of electrostatic repulsive forces during the reduction phase of synthesis (Ju-Nam and Lead 2008; Kittler et al. 2010). Classical Derjaguin, Laudau, Verwey, and Overbeek (DLVO) theory describes colloidal particles as being stabilized in solution by an electrical double layer (EDL), and colloidal stability as an interplay between electrostatic repulsive and van
der Waals attractive forces (Akaighe et al. 2012). Increases in ionic strength of AgNP suspensions will result in neutralization of counter-ions in the diffusion layer, resulting in contraction of the EDL and increasing the likelihood of attractive particle-particle interactions leading to agglomeration and eventual particle sedimentation (Chen, Mylon, and Elimelech 2006; Cumberland and Lead 2009; El Badawy et al. 2012).

Consequently, an essential step in the synthesis process is the addition of a capping agent that controls aggregation by coating the particles and reducing their high surface energies (Ju-Nam and Lead 2008). Capping agents are often organic ligands with various functional groups that coordinate strongly with the surface atoms of the growing AgNP and provide either electrostatic repulsive forces or steric hindrances when integrated into the AgNP structure (Daniel and Astruc 2004; Templeton et al. 1998). One of the most commonly used capping agents is sodium citrate, which provides stability through electrostatic repulsion and is weakly bound to the core silver.

An important property of electrostatically stabilized AgNPs is the zeta-potential, which reflects the electrical charge on the particle surface. The zeta-potential varies in charge and magnitude for each capping agent and as a result influences their interaction with charged colloidal particles, including clays and cells (MacCuspie 2011). Polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG) are common polymeric capping agents that provide steric hindrances, are strongly bound to the core of the NP, and may potentially be permeable to solutes and solvents (Tejamaya et al. 2012). Other capping agents include bovine serum albumin (BSA) and humic acid. Capping agents that provide stability through steric hindrances have been shown to be more effective in high ionic strength solutions than AgNPs electrostatically stabilized (Tejamaya et al. 2012; MacCuspie 2011). This is most likely due to the interaction of the negatively charged electrostatic stabilizing capping agents with other charged chemical species present in natural aquatic systems or test media.

The synthesis of AgNPs can be documented fairly easily by UV-vis spectrophotometry due to the presence of surface plasmon resonance (SPR), or the collective excitation (oscillation) of electrons in the metal conduction band near the surface of the NP by the electromagnetic field of light. It is the presence of the SPR that imparts the unique colors to NPs, and is the reason that AgNPs appear yellow and gold nanoparticles (AuNPs).
red in solution. The surface of a metal is like plasma, having free electrons in the conduction band and a positively charged nucleus. When the particle is illuminated, the electromagnetic field of the light exerts a force on these conduction electrons moving them towards the NP surface and creating an electric dipole on the surface of the NP. This dipole generates an electric field inside the NP opposite to that of the light that will force the electrons to return to the equilibrium position. If the electrons are displaced from the equilibrium position and the field is removed later, they will oscillate with a certain resonant frequency (Garcia 2011) as depicted in Figure 1.3A.

![Figure 1.3](image)

**Figure 1.3.** (A) Schematic of surface plasmon resonance where the free conduction electrons in the NP are driven into oscillation due to strong coupling with incident light (B) Extinction spectra of silver nanoparticles. Source: Oldenberg, Steven J. n.d. "Silver nanoparticles: Properties and applications." Accessed June 12, 2013. [http://www.sigmaaldrich.com/materials-science/nanomaterials/silver-nanoparticles.html](http://www.sigmaaldrich.com/materials-science/nanomaterials/silver-nanoparticles.html).

The resonant frequency of these oscillations corresponds to UV–vis light and consequently creates absorption bands in this region of the spectrum that are subsequently detected by spectrophotometric instruments (Wang et al. 2007). The characteristic resonance of NPs depends on the surface chemistry, size, shape, and aggregation state of the NP (Junnam and Lead 2008). Shifts in the position of SPR peak denote varying particle size (Mulvaney 1996), with shifts towards longer wavelength (red shift) indicating an increased particle size (Henglein 1993) and state of aggregation (Smitha et al. 2008), and shifts to shorter wavelengths (blue shift) indicating a decrease in size (Figure 1.3B). In general, the SPR for the AgNPs absorbs in the wavelength region of 390 - 420 nm (Zook et al. 2011).
1.4 Nanomaterial Ecotoxicology

Material properties at the nanoscale vary considerably from those of their macroscale counterparts, leading to unique and revolutionary applications in a wide variety of electronic, engineering, medical, and personal care sectors. Inasmuch, there is growing concern among environmental toxicologists and pathologists that manufactured metallic NPs may present living systems with a novel challenge, since such materials were not generally encountered by organisms during the course of their biological evolution (Moore 2006). Furthermore, material that is normally inert to living organisms when encountered in its bulk phase has the potential to become highly toxic at the nanoscale, presumably due to the proportionately higher surface area of NPs (Poole and Owens 2003). For example, titanium dioxide (TiO$_2$) is a relatively inert substance at the microscale, but nanoscale TiO$_2$ has been shown to produce considerable cellular damage in both prokaryotic and eukaryotic cell cultures (Long et al. 2006; Adams, Lyon, and Alvarez 2006). While toxicity mechanisms have not yet been completely defined for most nanomaterials, some possible mechanisms include disruption of membranes or membrane potential, oxidation of proteins, genotoxicity, interruption of energy transduction, formation of reactive oxygen species, and release of toxic constituents (Klaine et al. 2008).

1.4.1 Silver Nanoparticle Ecotoxicology

Silver has long been recognized as an effective antimicrobial agent. Cyrus the Great, King of Persia, reportedly kept water fresh in the sixth century B.C. by boiling the water and then storing it in silver flagons (Baker 1948). In 78 A.D., Pliny the Elder wrote that silver slag, “has healing properties as an ingredient in plasters, being extremely effective in causing wounds to close up” (Etris 2001). Most commercially available products exploit AgNPs for their antimicrobial properties (Sellers 2009). The unregulated increase in AgNP production has raised concerns over their inevitable release into the environment, and AgNPs are now being considered an emerging contaminant that merit special attention and rigorous study to mitigate their potential ecotoxicity (Fabrega et al. 2011; Marambio-Jones and Hoek 2010). During the evolution of most organisms the necessity to regulate high concentrations of silver was absent due in part to its relatively low concentration in most hospitable
environments. Consequently, the anthropogenic addition of only a small mass of silver to aquatic environments has the potential to cause disproportionately large devastation to biota.

Once in an aquatic environment, whether natural or engineered, AgNPs have been shown to undergo a particle-to-ion conversion, resulting in the dissolution of the AgNP and the release of Ag\(^+\) during the process of oxidation (Zhang et al. 2011) as represented by Equation 1.2 and depicted in Figure 1.4:

\[
Ag + \frac{1}{2} O_2 + 2H^+ \leftrightarrow 2Ag^+ + H_2O
\]  

(Figure 1.4. Representation of AgNP dissolution to Ag\(^+\) in an aquatic system. Source: Zhang, W., Y. Yao, N. Sullivan, and Y. S. Chen. 2011. "Modeling the Primary Size Effects of Citrate-Coated Silver Nanoparticles on Their Ion Release Kinetics." Environmental Science & Technology 45 (10):4422-4428. doi:10.1021/es104205a.

Since AgNPs coexist with Ag\(^+\), the rate of dissolution has significant implications in understanding and assessing AgNP toxicity (Zhang et al. 2011). Several factors, including size and shape, aggregation states, surface coatings, and solution chemistry, contribute to the overall toxicity of AgNPs as they each influence the dissolution rate of AgNPs in unique ways (Fabrega et al. 2011; Marambio-Jones and Hoek 2010). It is plausible that each factor occurs to some degree and that the existence of one does not necessarily preclude that of the other. However, the contribution of each factor to the overall AgNP toxicity is likely to be strongly dependent on the media in which they reside.

AgNPs and Ag\(^+\) exhibit different physiochemical properties and subsequent biological toxicity, with the latter species being more toxic than the former in most cases (Fabrega et al. 2009; Liu and Hurt 2010). In general, Ag\(^+\) toxicity occurs primarily through their interaction proteins and subsequent inactivation of vital enzymes (Matsumura et al. 2003); disrupt bacterial membrane integrity and permeability (Feng et al. 2000); and likely
affect DNA replication (Neal 2008). Whereas, AgNPs have been shown to cause cell death by pitting bacterial cell membranes and increasing their permeability (Shahverdi et al. 2007) (Figure 1.5).

![Figure 1.5. Possible mechanisms of nanomaterial toxicity to bacteria. Different nanomaterials may cause toxicity via one or more of these mechanisms. CYP = cytochrome. Source: Klaine, S. J., P. J. J. Alvarez, G. E. Batley, T. F. Fernandes, R. D. Handy, D. Y. Lyon, S. Mahendra, M. J. McLaughlin, and J. R. Lead. 2008. "Nanomaterials in the environment: Behavior, fate, bioavailability, and effects." Environmental Toxicology and Chemistry 27 (9):1825-1851. doi:10.1897/08-090.1.](image)

Environmental toxicologists are interested in determining if AgNPs released into the environment should be considered as a completely new contaminant or as a new release of a legacy contaminant (Ag⁺) that will complex with various ligands (Luoma and Phillips 1988; Hogstrang and Wood 1998). It is still unclear if the nanoscale form of silver presents the risk or if the AgNPs increase the percentage of soluble species of Ag⁺ that are responsible for the toxic effects (Liu and Hurt 2010; Lok et al. 2007; El Badawy et al. 2011; Levard et al. 2012). Nevertheless, there is a growing consensus that the toxicity of AgNPs is predominantly related to the release of Ag⁺ (Radniecki et al. 2011; Arnaout and Gunsch 2012).

The toxicity of AgNPs to microorganisms is well documented in the literature (Fabrega et al. 2011; Marambio-Jones and Hoek 2010). Studies have shown that toxicity increases as the AgNP diameter decreases (Lok et al. 2007) due to higher specific surface areas and higher enthalpies of formation compared to the same amount of bulk silver (Marambio-Jones and Hoek 2010). When *E. coli* bacteria were exposed to a range of AgNP sizes (1-100nm), NPs in the 1-10 nm range preferentially inhibited the gram-negative
bacteria by attaching to the surface of the cell membrane and disturbing its proper function, penetrating inside the bacteria and causing further damage by interacting with sulfur- and phosphorus-containing compounds such as DNA, and by releasing Ag\(^+\) (Morones et al. 2005; Sondi and Salopek-Sondi 2004; Lok et al. 2007).

Additional research indicates that the size-dependent toxicity of AgNPs is the result of increased dissolution rate, which has been shown to be directly proportional to the primary size and concentration of the AgNP (Luo, Hu, and Xiao 2008; Gilbert et al. 2004; Zhang, Rittmann, and Chen 2011). The toxicity of AgNPs to the ammonia oxidizing bacteria (AOB) *Nitrosomonas europaea* has been linked to particle stability and the dissolution of Ag\(^+\), which was inversely proportional to the AgNP size (Radniecki et al. 2011). In another study, solutions containing AgNPs under anaerobic conditions, which preclude the oxidative dissolution of Ag\(^0\) to Ag\(^+\), were reportedly 20 times less toxic to *E. coli* bacteria than Ag\(^+\) (Xiu, Ma, and Alvarez 2011).

The aggregation state also has the potential to influence the overall toxicity of AgNPs. One study showed that AgNPs aggregate in media with high electrolyte content due to the neutralization of repulsive electrostatic surface charges, resulting in a loss of toxicity (Lok et al. 2007; Prathna, Chandrasekaran, and Mukherjee 2011). One possible explanation for the reduced toxicity of AgNP aggregates is that decreasing the surface to volume ratio effectively reduces the dissolution rate of the AgNPs to Ag\(^+\). Aggregates could also settle out of solution and become no longer bioavailable. To complicate matters further, the concentration at which silver becomes toxic varies among available experimental data. This is likely due in part to complex solubility issues affecting the bioavailability of Ag\(^+\) through formation of various complexes, such as silver chloride (AgCl) and silver sulfide (Ag\(_2\)S) (Chopra 2007).

### 1.4.2 Ammonia Oxidizing Bacteria

Microorganisms are fundamental components of many engineered systems as they provide essential services, ranging from primary production to nutrient cycling and waste decomposition. Nitrification is an essential process in WWTPs that relies on the biological conversion of ammonia (NH\(_3\)) to nitrogen gas (N\(_2\)) by nitrifying bacteria through a series of complex redox reactions. Nitrification is accomplished in a two-step process depicted in
Figure 1.6. The first step involves the conversion of NH$_3$ to nitrite (NO$_2^-$) by AOB, and NO$_2^-$ to nitrate (NO$_3^-$) via nitrate oxidizing bacteria (NOB). In the second step, NO$_2^-$ is converted to N$_2$ via denitrifying bacteria. When comparing the two processes, the oxidation of NH$_3$ is believed to be the rate limiting step since AOB have slower growth rates than NOB.


*N. europaea* is a model AOB commonly found in WWTPs that facilitates the oxidation of NH$_3$ to NO$_2^-$ through a series of redox reactions depicted in Figure 1.6. *N. europaea* obtains all of its energy and reductant for growth from the oxidation of NH$_3$, and can obtain all its carbon for biomass from carbon dioxide (CO$_2$). The conversion of NH$_3$ to NO$_2^-$ is a two-step process catalyzed by ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO). AMO catalyzes the oxidation of NH$_3$ to hydroxylamine (NH$_2$OH) and HAO catalyzes the oxidation of NH$_2$OH to NO$_2^-$. During this process, the bacterium gains a net of 2 mole electrons for every mole of NH$_3$ that are used for maintenance and cell growth (Arp, Sayavedra-Soto, and Hommes 2002). Consequently, *N.*
**Europaea** has a relatively slow growth rate of 8-12 days (Radniecki and Lauchnor 2011). In comparison, *E. coli*, a model heterotroph gains 24 moles of electrons from each mole of glucose and has a cell doubling-rate of 20-40 min (Blanch and Clark 1996).

AOB are highly sensitive to a variety of contaminants (Choi and Hu 2008). Studies have shown that *Nitrosomonas spp.* displayed a 10-fold increase in toxicity from organic contaminants when compared to aerobic heterotrophs (Blum and Speece 1992). In particular, heavy metals can adversely inhibit nitrifying bacteria, and unlike organic compounds can bioaccumulate. Although the exact mechanism by which heavy metals interfere with nitrifying activity is unclear, it is believed that the first step in the microbial response to toxic heavy metals is the uptake of free metal cations via a nonspecific metal inorganic transport system (Nies 1999). Within the cell, heavy metals can interact with thiol groups and destroy protein structure and function. In addition, heavy metals may interfere with physiologically important ions and inhibit the function of the respective physiological cations (Nies 1999).

Consequently, the release of AgNPs into WWTPs may significantly inhibit the nitrification process, leading to the potential discharge of nutrient rich effluent into receiving bodies of water. The high nitrogen content can subsequently lead to eutrophication of receiving waters, resulting in toxic algal blooms, dissolved oxygen depletion, and large-scale fish mortality (EPA 1993). Due to their high sensitivity, AOB can be used for predicting the impact of contaminants on nitrification disruption and ultimately WWTP failures (Arnaout and Gunsch 2012).

Using pure cultures of *N. europaea* to examine AgNPs toxicity has several advantages over using mixed cultures of nitrifying activated sludge, including the ability to image the interactions of AgNPs with *N. europaea*, as well as the ability to methodically test how the aqueous chemistry in a variety of test media affects Ag⁺ and AgNP inhibition. Compared to the varying consistency of activated sludge, the purity of the water chemistry of pure culture media provides further advantages, including the ability to study gene expression and to dissect the biochemistry of the organism.

A wide range of environmental parameters influences the stability, and thus resulting toxicity, of AgNPs necessitating the need for toxicity studies using more complex media. Wastewater is a complex media; therefore, it is beneficial to deconstruct it to understand how various aqueous constituents influence the fate and behavior of AgNPs. Some of the primary
model components of wastewater consist of cations and anions (e.g. Mg$^{2+}$, Ca$^{2+}$ and Cl$^{-}$), natural organic matter (NOM) and biological macromolecules, including proteins (e.g. bovine serum albumin [BSA]) and polysaccharides (e.g. alginate).

Humic acid is a large macromolecule that is the main component found in NOM and is derived from the slow microbial decomposition of plants and bacterial biomass (Dubas and Pimpan 2008). The Suwannee River, GA, is a prolific source of humic acid, which has been purified for scientific application and is commonly referred to as Suwannee River Humic Acid (SRHA). According to data from the International Humic Substance Society (IHSS), SRHA is primarily composed of various amounts of carboxylic, phenolic, carbonyl, quinone, aromatic, acetal, heteroaliphatic, aliphatic, and amino components (IHSS 2013). Numerous functional groups have the ability to interact with metallic cations, thereby regulating their bioavailability and acting as potential reservoirs of metal cations (Wen et al. 1997). In particular, the functional groups that contribute most to surface charge and reactivity of humic substances are phenolic and carboxylic groups (Stevenson 1994). NOM can modify AgNP properties by adsorption onto the surface and formation of surface coating (Baalousha 2009; Chappell et al. 2011), which enhances the stability of AgNPs in aqueous dispersion and decreases their aggregation rate (Lin and Xing 2008; Chappell et al. 2009). Adsorption of NOM to AgNPs is also believed to inhibit the release of Ag$^{+}$ from AgNPs (Liu et al. 2010).

BSA is a large globular model protein derived from cows. It has a bulky macromolecular structure that encompasses domains of negatively and positively charged amino acids, allowing BSA to adsorb at positively and negatively charged surfaces (Kudelski 2003, 2006). BSA can adsorb on the AgNP surface by van der Waals and electrostatic forces forming a BSA-AgNP complex (Liu et al. 2009). The adsorbed BSA functions as a capping agent and provides steric stabilization which increases the retention of single AgNPs in different aqueous solutions (MacCuspie 2011).

Alginate is derived from brown algae and is a linear polysaccharide containing 1,4-linked β-D-mannuronic (M) and α-L-guluronic (G) acid residues arranged in non-regular intervals along the chain (Fourest and Volesky 1997). The principal mechanism of metallic cation sequestration involves the formation of complexes between a metal ion and functional groups (carboxyl, carbonyl, amino, amidio, sulfonate, phosphate) present on the surface or inside the porous structure of the biological material (Fourest and Volesky 1997). Recently,
polysaccharides have been used in the synthesis of AgNPs as they act as both a reducing and stabilizing agent (Park et al. 2011; Ghaseminezhad, Hamedi, and Shojaosadati 2012).

1.5 Environmental Fate and Transport of Silver Nanoparticles

In any type of environmental risk assessment, it is essential to have an understanding of the behavior of a target contaminant under environmentally relevant conditions. It is also equally important to describe the manner in which the contaminant can enter the natural environment and eventually affect public health, either directly or indirectly. The rapid and unregulated production of consumer products containing AgNPs has highlighted the need for additional studies documenting their fate and transport in the natural environment.

The primary release of AgNPs into the environment is believed to be through wastewater, since an increasing number of consumer products contain some form of nanoscale silver. One study found that the washing of socks containing AgNPs resulted in their liberation from the fabric matrix into test media (Benn and Westerhoff 2008). Similar studies have also shown that AgNPs are released as byproducts of their use in toothpastes, shampoos, or swimming pool disinfectants (Benn et al. 2010; Blaser et al. 2008).

The lowest concentrations of dissolved silver are found in the open ocean, with an estimated range of 0.03-0.1 ng L\(^{-1}\) (Ranville and Flegal 2005). As such, the presence of elevated silver concentrations in the natural environment is commonly an indication of anthropogenic influences, typically from municipal and industrial waste discharge. In the late 1970s and early 1980s, silver concentrations as high as 189 ng L\(^{-1}\) were detected in the lower South San Francisco Bay, California (Luoma and Phillips 1988). The elevated concentrations were linked to depressed reproductive activity among the bivalve *Corbula amurensis* in the Bay (Brown et al. 2003). Following upgrades to treatment facilities discharging directly into the Bay, and the closure of a large photographic facility, silver concentrations decreased significantly to 2-8 ng L\(^{-1}\) (Squire et al. 2002). Similarly, in the late 1980s elevated silver concentrations were detected along the Pacific Coast stretching from San Diego, California, into Mexican waters as a result of discharges from the Point Loma WWTP, San Diego, California (Flegal and Sanudowilhelmy 1993).

Due to the complexity of wastewater, the interaction of AgNPs with biosolids has not been well documented. Nevertheless, the potential remains for the AgNPs to sorb onto the
biosolids and settle-out of solution, only to reenter the environmental through the application of the organics as fertilizer for agricultural purposes. On the other hand, if the AgNPs are not removed with the biosolids they can be released into surface waters with unknown consequences to aquatic biota (Benn and Westerhoff 2008). The potential release of AgNPs into wastewater may have broad reaching implications for the health of various ecosystems that receive direct or indirect inputs from engineered treatment processes that are not specifically designed to remove AgNPs or their constituents. Potential pathways for the introduction and release of AgNPs are depicted in Figure 1.7.

![Figure 1.7](image_url)


Considerable effort has been allocated to studying the mechanism by which AgNPs adsorb to different bacteria, primarily for the purposes of drug carrier, delivery vehicle,
nonviral transfection vector, imaging label, or therapeutic agent (Wilhelm et al. 2002). The process of adsorption is essentially an interfacial interaction mediated by the balance between interparticle forces arising from a combination of van der Waals, electrostatic, and acid-base forces (He, Wan, and Tokunaga 2008; Snoswell et al. 2005). Surface interactions of NPs with cells have been shown to begin with adsorption and progress into internalization. Adsorption kinetics are governed by factors such as interaction energy barrier, concentrations of adsorbent and adsorbate, diffusivity, hydraulic conditions, and temperature (Zhang, Rittmann, and Chen 2011). Several studies have successfully established a pseudo first order kinetic model (Langmuir) for NP binding on cell surfaces (Cho et al. 2009; Wilhelm et al. 2002; Zhang, Rittmann, and Chen 2011).

To date, research into the potential impacts of AgNPs to microorganisms commonly found in WWTPs is scarce, owing no doubt in part to the inherent difficulties in differentiating AgNPs and Ag⁺ in complex media. Nevertheless, a study on the fate and transport of titanium dioxide NPs (TiO₂-NPs) in a WWTP found that TiO₂-NPs (>700 nm) were not removed by primary treatment. Rather, the majority were found to be strongly associated with the biosolids in secondary treatment (Kiser et al. 2009). In another study, simulated primary and secondary treatment processes were establish to determine the influence of treatment processes on the fate of AgNPs. Approximately 90% of AgNPs were retained in the primary effluent and entered directly into secondary treatment where they readily adsorbed to biosolids. AgNP concentrations in secondary effluent were indistinguishable from background levels present in control samples (Hou et al. 2012).

Similar results were found for Ag⁺ in studies conducted at several publicly owned treatment works, wherein the majority of Ag⁺ sorbed onto particles that are removed during secondary treatment by settling/filtration processes. Despite high removal efficiency, effluent Ag⁺ concentrations were detected several orders of magnitude above those typically found in streams and rivers. However, effluent concentrations were significantly reduced in the receiving water body due to dilution and incorporation into stream sediments (Shafer, Overdier, and Armstrong 1998). While there still remains a deficit of studies reporting on the fate and transport of AgNPs in WWTPs, these studies suggest that AgNPs can enter WWTPs where they will readily adsorb to biosolids that may ultimately be applied to crop fields as fertilizer.
Despite the modest advances in modeling the adsorption of AgNPs to various bacteria, little effort has been allocated to understanding how the presence of organic macromolecules typically encountered in WWTPs can influence the adsorption kinetics of the AgNPs and how these might influence their environmental fate and transport. Studies indicate that organic macromolecules, such as BSA, alginate, and SRHA readily interact with the surface of AgNPs, forming protective coatings that can reduce dissolution and aggregation rates, and consequently their overall toxicity to microorganisms (Liu and Hurt 2010; Liu et al. 2010; Ostermeyer 2012; T. Radniecki, unpublished data). Protective coatings can decrease adsorption by several different mechanisms, including electrostatic and steric repulsion (Figure 1.8). For the case of electrostatic repulsion, surface charges adsorbed macromolecule functional groups form an energy barrier that repels surfaces with a like charge. Large uncharged macromolecules may endow steric repulsions by inhibiting close contact between the particles and the cells. Large charged macromolecules afford both an electrostatic and steric repulsion, known as electrosteric repulsion, that is stronger than either electrostatic repulsion or steric repulsion (Phenrat et al. 2008), and its repulsive force is less sensitive to changes in ionic strength or pH than electrostatic repulsions that would occur using bare (uncoated) particles (Li et al. 2010).


Adsorption of organic macromolecules to the AgNPs surface is expected to affect particle dynamics depending on the thickness of surface coverage and sorbed conformation
of the compounds. In the former case, the affinity of the organic molecules for the surface and the degree of packing will determine the resultant properties and magnitude of surface coverage. Complete coverage of the AgNP surface will potentially shield the particle surface from van der Waals attractive forces (which promote aggregation), and modify the surface of the particle toward the properties of the sorbed organic, particularly in terms of charged organic compounds. In the latter case, the sorbed configuration of organic compounds determines which groups will be available to interact with the surrounding aqueous media. If strongly hydrophilic groups are left to interact with the aqueous media, then sorption of the organic compounds will facilitate stability in the AgNPs, thus slowing aggregation kinetics (Chappell et al. 2011).

AgNP dissolution and aggregation have been shown to be reduced in the presence of certain macromolecules, such as BSA, alginate, and SRHA, which holds the potential to alter the fate and transport of silver once released into the environment (Ostermeyer 2012; Akaighe et al. 2012; Chen, Mylon, and Elimelech 2006; Ravindran et al. 2010). These macromolecules are believed to stabilize the AgNPs either through steric hindrances, electrostatic repulsion, or a combination of the two known as electrosteric repulsion (Li et al. 2010; Phenrat et al. 2008). The same forces that safeguard AgNP aggregation and dissolution may also influence their adsorption to bacteria. Therefore, understanding the mechanism by which each macromolecule stabilizes the AgNPs is an important step in predicting the fate of AgNPs upon their release into the environment.

1.6 RESEARCH OBJECTIVES

The objective of this research was to obtain a better understanding of how NH₃ and macromolecules influence both the toxicity and adsorption of AgNPs to *N. europaea*. The specific research objectives were fourfold and are as follows:

1. Investigate the influence of NH₃ concentrations typically found in WWTPs on the inhibitory effects of AgNPs to *N. europaea* in the presence and absence of the macromolecules BSA, alginate, and SRHA.

2. Investigate the influence of NH₃ on the dissolution of AgNPs in the presence and absence of BSA, alginate, and SRHA, and how this relates to observations in *N. europaea* inhibition.

3. Investigate the adsorption of AgNPs to *N. europaea* in the presence and absence of the macromolecules BSA, alginate, and SRHA.
4. Determine whether the macromolecules influencing dissolution and adsorption do so through electrostatic repulsive forces or steric hindrances.
CHAPTER 2

EXPERIMENTAL APPROACH, METHODS AND MATERIALS

In this chapter, the experimental approach, analytical methods, and materials used in this research are presented in a step-by-step procedure. The experimental approach includes the culturing and harvesting protocols for *N. europaea*, the setup of the batch bioreactor for inhibition experiments, and the preparation of the UV-vis studies for AgNP dissolution and adsorption to cells. The analytical methods include a nitrite assay and the UV-visible absorbance measurements.

2.1 EXPERIMENTAL APPROACH

The following sections describe the protocols for culturing and harvesting *N. europaea*, setting up the batch bioreactors for the inhibition experiments, and preparing the UV-vis studies for AgNP dissolution and cell adsorption. Each section describes the step-by-step procedure involved in each study, and includes the reagents and instrumentation used.

2.1.1 *N. europaea* Culturing and Harvesting Protocol

*N. europaea* ATCC 19718 cells were inoculated in a 4 L Erlenmeyer flask containing 2 L of autoclaved minimal growth media at a 1:100 dilution, capped with a foam stopper and aluminum foil and shaken in the dark at 115 rpm (MaxQ3000, Thermo Scientific, Madison, WI) and 30°C. Inoculation procedures were performed under aseptic conditions within a laminar flow hood (Thermo Scientific 1300 Series A2, model number 1323). Aseptic conditions were achieved by spraying the surfaces of the laminar flow hood with 70% ethanol followed by at least 15 min exposure to UV light.

The minimal growth media for *N. europaea* contained 25 mM (NH₄)₂SO₄, 43 mM KH₂PO₄, 3.92 mM NaH₂PO₄, 3.77 mM Na₂CO₃, 730 μM MgSO₄, 200 μM CaCl₂, 18 μM FeSO₄, 17 μM EDTA Free Acid and 0.6 μM CuSO₄. For optimal growth, the pH of the growth media was adjusted to 7.8 with 10 N NaOH (pH meter Denver Instrument Model 250
with high performance glass-body pH/ATC electrode 300729-1, Denver Instrument
Company, Arvada, CO). After 3 days, *N. europaea* cells were in mid-exponential growth
with an approximate optical density at 600 nm (OD\textsubscript{600}) of 0.072.

Cells were harvested approximately 3-4 days after inoculation (mid-exponential
growth phase) by dividing the contents of the 4 L inoculated flask into 1 L polypropylene
Nalgene bottles and centrifuging at 9,000 rpm (5,625 RCF) for 30 min in an Sorvall RC6+
centrifuge (Thermo Scientific, Madison WI). The supernatant was immediately decanted at
the end of the cycle and the cell pellet was resuspended in 30 mL of 30 mM HEPES buffer
(pH 7.8). This process was repeated until all cell pellets were resuspended. The resultant
solution was then transferred into a 50 mL Falcon tube and centrifuged at 9,000 rpm (5,625
RCF) for 20 min in an Aleegra X-30R centrifuge with a conical C0650 rotor (Beckmann
Coulter, Brea, CA 92821). Again, the supernatant was immediately decanted, the cells
resuspended in 20 mL of 30 mM HEPES and stored in the dark until used in the batch
experiments. The process of cell washing was necessary to remove any residual nitrite and
trace metals from solution prior to the cells being used in experiments.

### 2.1.2 *N. europaea* Batch Inhibition Studies

Organic macromolecules, including BSA, alginate, and SRHA have been shown to
enhance the stability of AgNPs by coating their surface and preventing dissolution
(MacCuspie 2011; Ravindran et al. 2010; Chen, Mylon, and Elimelech 2006; Akaighe et al.
2012; Gao et al. 2012), which in turn decreased the overall toxicity to *N. europaea*
(Ostermeyer 2012; T. Radniecki, unpublished data). However, recent studies have shown that
the presence of NH\textsubscript{3} leads to the dissolution of AgNPs (Ostermeyer 2012), which is primarily
believed to be the result of the formation of silver amine complexes with NH\textsubscript{3}. Therefore, the
possibility exists for AgNPs entering environments with high NH\textsubscript{3} concentrations (*i.e.*
WWTPs) to undergo dissolution to the more toxic Ag\textsuperscript{+} species. Consequently, the purpose of
the inhibition studies was to determine the influence of NH\textsubscript{3} concentrations on the inhibitory
effects of 20 nm citrate caped spherical AgNPs to *N. europaea*.

Inhibition studies were performed over a 3 hr period in 155 mL glass bottle batch
bioreactors containing a total volume of 35 mL. Aliquots from the 1,000 ppm AgNP stock
solution were initially placed into DI water (15 M\(\Omega\)) and dispersed via shaking at 250 rpm on
a Thermo Scientific MaxQ3000 shaker table in the dark at 30°C for at least 15 min prior to the addition of test media. Dispersing AgNPs prior to the addition of test media is essential to reduce the potential for AgNP aggregation upon introducing test media that may alter the electrostatic potential of the solution (Lok et al. 2007; Prathna, Chandrasekaran, and Mukherjee 2011).

Test media, consisting of NH₃ and HEPES buffer (pH 7.8), was added at the -30 min time point at 15X concentrate to final concentrations ranging from 0.04 mM to 1.6 mM NH₃ and 30 mM HEPES. A time designation of -30 min was used as this signified the amount of time the AgNPs were exposed to the test media (30 min) prior to the addition of the cells. NH₃ was added to the batch reactors as (NH₄)₂SO₄ and the final concentrations are representative of those typically found in WWTPs (Tchobanoglous et al. 2003). Concentrated stock solutions of BSA, alginate, and SRHA were added simultaneously to the batch bioreactors to varying final concentrations.

The batch bioreactors were then capped and shaken under the same conditions described above for 30 min at which time *N. europaea* cells were added (0 min time point) to a final concentration of 5 mg protein L⁻¹ (OD₆₀₀ ≈ 0.072). Samples were collected every 45 min, and all conditions were carried out in triplicate. To account for potential differences in growth rates due to variation in NH₃ concentrations, control batch bioreactors that contained the same NH₃ concentrations but no AgNPs were run alongside the treatment conditions.

### 2.1.3 AgNP Dissolution Studies

These studies were conducted separately from those described in Section 2.1.2 and sought to investigate whether an increase in NH₃ corresponded to an increase in AgNP dissolution in the presence and absence BSA, alginate, and SRHA. AgNPs possess surface plasmon resonance (SPR) absorbance spectrum with a λₘₐₓ around 400 nm, which can shift depending on the AgNPs aggregation state, surface coating, size, and shape (Malinsky et al. 2001). As such, the characteristic absorbance at λₘₐₓ can be used to measure the concentration of monodispersed AgNPs in solution (Zook et al. 2011). Batch reactors were prepared as described in Section 2.1.2 with the exception that no *N. europaea* cells were added at the 0 min time point and the total volume equaled 15 mL. All treatment conditions
were carried out in triplicate and samples were collected according to the same schedule described in Section 2.1.2.

### 2.1.4 AgNP Adsorption Studies

The potential for AgNPs to adsorb onto cells holds important consequences for the fate and transport of silver in the environment as discussed in Section 1.5. Therefore, the purpose of these studies was to determine the influence of the macromolecules BSA, alginate, and SRHA on the adsorption of AgNPs to cells and how this relates to their fate and transport in the natural environment.

Batch reactors were prepared as described in Section 2.1.2 with two main exceptions. The first of which was the omission of NH$_3$ to control for potential AgNP dissolution. The second exception was the addition of 50 mg L$^{-1}$, which was 10X more concentrated than the inhibition studies. Cells were harvested using the same protocol described in Section 2.1.1. Samples were collected every 45 min for 3 hrs. All conditions were carried out in triplicate.

### 2.1.5 Macromolecule Stabilizing Mechanism

To better understand whether the macromolecules used in these studies imparted additional AgNP stability against dissolution and adsorption through either electrostatic repulsive forces or steric hindrance, batch reactors were prepared as described in Section 2.1.2 with a few exceptions. Following the addition of either BSA, alginate, or SRHA to the batch reactors at the -30 min time point, the batch bioreactors were hand shaken for 30 sec to allow the macromolecules to coat the AgNPs before a concentrated solution of anhydrous magnesium sulfate ($\text{MgSO}_4$) was added to final concentrations ranging from 0.35 - 2.4 mM $\text{MgSO}_4$. The final concentrations of $\text{MgSO}_4$ selected for these studies correspond to EPA hardness definitions of very soft to very hard water (United States Geological Survey [USGS] 2012). After 30 min of shaking at 250 rpm in the dark at 30°C, *N. europaea* cells were added to the batch reactors to a final concentration of 50 mg L$^{-1}$, as described in Section 2.1.4. Samples were collected every 45 min for 3 hrs and measured on the UV-vis to quantify the concentration of AgNPs remaining in solution. Should the macromolecule in question impart electrostatic repulsive forces, the addition of $\text{Mg}^{2+}$ as $\text{MgSO}_4$ would act to neutralize the negative charge of the macromolecule and result in the enhanced adsorption of the AgNPs to the cells. On the other hand, sterically hindered macromolecules would
theoretically remain unencumbered by the divalent cation and AgNP adsorption would remain relatively linear across the range of Mg\(^{2+}\) concentrations.

### 2.2 Analytical Methods

The analytical methods used in these studies are described in detail in the following sections. They include step-by-step discussions of a colorimetric nitrite assay used to quantify the percent nitrification activity of *N. europaea* during the inhibition experiments, as well as the UV-visible spectrophotometer measurements used to quantify AgNP dissolution and adsorption to cells.

#### 2.2.1 *N. europaea* Batch Inhibition Studies

Nitrite production was measured colorimetrically at 45 min intervals over a 3 hr period and was the main indicator of *N. europaea* activity. Given that the doubling time of *N. europaea* is approximately 8-12 hrs it is unlikely that significant bacterial growth occurred during the experimental period. At each time interval, a 0.5 mL aliquot was removed from the batch bioreactor and placed into a 1.5 mL polypropylene microcentrifuge tube. Ten microliters of sample was then transferred to an additional 1.5 mL polypropylene microcentrifuge tube containing 890 µL of 1% (w/v) sulphanilamide in 1 M HCl and 0.1 mL of 0.2% (w/v) N-(1-Naphthyl) ethylenediaminedihydrochloride. The samples were thoroughly mixed by inverting them several times and vortexing for 10 sec. After at least ten min but less than 4 hrs, 0.2 mL of sample was transferred to a 96-well plate and the absorbance of the nitrite assay was measured at 540 nm (A\(_{540}\)) using a BioTek Synergy HT plate reader. The absorbance values were then compared with those from a standard curve to arrive at the concentration of NO\(_2^-\) produced in the batch bioreactors during the 3 hr experimental period. In addition, the OD\(_{600}\) of the batch bioreactors were collected at the end of the experiment to normalize the NO\(_2^-\) production to the mg of protein of cells. This reading was then entered into a previously created standard curve equation relating OD\(_{600}\) to protein concentrations. The standard curve was created by measuring the protein content and the OD\(_{600}\) of *N. europaea* cultures at various cell densities and is linear for readings ranging from 0 to 0.14. The OD\(_{600}\) of the bottles containing AgNPs was not accurate due to the light scattering properties of the AgNPs. Therefore, the average OD\(_{600}\) of the control bottles was used as the OD\(_{600}\) for the bottles containing AgNPs.
Nitrification activity was defined as the linear average of μmol of NO$_2^-$ produced per mg of protein per min. Over the 3 hr experimental period, the average nitrite production rate (ANPR) per mg of protein was calculated for each batch bioreactor. The averages and standard deviations for each triplicate group of batch bioreactors were calculated and the percent nitrification inhibition (%NI) and percent nitrification activity (%NA) were computed from Equations 2.1 and 2.2, respectively.

\[
\%NA = \frac{[ANPR]_{trt}}{[ANPR]_{con}} \times 100\%
\]

(2.1)

\[
\%NA = 100\% - \%NI
\]

(2.2)

Where, \([ANPR]_{con}\) is the average nitrite production rates of the triplicate control bottles and \([ANPR]_{trt}\) is the average nitrite production rates of the triplicate treatment bottles.

The standard deviation of the % NA was computed from the product standard deviation:

\[
\sigma = \%NA \times \frac{\sigma_{con}^2}{[ANPR]_{con}^2} + \frac{\sigma_{trt}^2}{[ANPR]_{trt}^2}
\]

(2.3)

Where \(\sigma\) is the standard deviation of the calculated %NA (product standard deviation), \(\sigma_{con}\) is the standard deviation of the nitrite production rates of the triplicate control bottles, \(\sigma_{trt}\) is the standard deviation of the nitrite production rates of the triplicate treatment bottles. The product standard deviation was used to calculate the 95 % confidence intervals, which were used as error bars.

### 2.2.2 AgNP Dissolution Studies

Batch bioreactors were set up as described in Section 2.1.3, and the start of the experimental period was staggered by 3 min for each bottle to ensure that the AgNPs were exposed to the test media for equal amounts of time. After the addition of the test media, the batch bioreactors were hand shaken for 30 sec and the UV-vis spectrum was immediately measured at the -30 min time point. A 600 μL aliquot was placed into a 1-cm quartz cuvette and the absorption spectrum was measured from 300 nm to 700 nm at 2 nm intervals on a BioMate 3S UV-vis spectrophotometer. Prior to sample measurements, a baseline was
collected from a standard solution that contained the same concentration of the macromolecule under study to correct for their potential interference. An additional sample was measured after 30 min at the 0 min time point, and subsequently every 45 min for the remainder of the 3 hr experimental period to remain consistent with the procedures described in Section 2.2.1. After each reading, the sample was discarded to reduce the potential for cross-contamination. The quartz cuvette was rinsed three times with DI water (15 MΩ) in between each sample, and was washed with 10% nitric acid (HNO₃) at the end of each experiment to remove any residual AgNPs.

To quantify the concentration of AgNPs remaining in solution, a standard curve was created from the UV-vis peak absorbance values for varying concentrations of AgNPs in DI water (15 MΩ) and is presented in Figure A.1 in the Appendix. The percent dissolution of the monodispersed (i.e. non-aggregated) AgNPs was calculated using Equation 2.4:

\[
% \text{ Dissolution} = 1 - \frac{[AgNP]_{trt}}{[AgNP]_{con}} \times 100\% (2.4)
\]

Where \([AgNP]_{trt}\) is the average concentration of monodispersed AgNPs in triplicate treatment bottles measured at the 180 min time point, and \([AgNP]_{con}\) is the average concentrations of monodispersed AgNPs in the triplicate control bottles measured at the -30 min time point. Treatment samples contained NH₃, whereas control samples did not.

The standard deviation of the % Dissolution was computed from the product standard deviation:

\[
\sigma = % \text{ Dissolution} \times \frac{\sigma_{\text{con}}^2}{[AgNP]_{\text{con}}} + \frac{\sigma_{\text{trt}}^2}{[AgNP]_{\text{trt}}} \quad (2.5)
\]

Where \(\sigma\) is the standard deviation of the calculated % Dissolution (product standard deviation), \(\sigma_{\text{con}}\) is the standard deviation of the AgNP concentration of the triplicate control bottles (-30 min time point), \(\sigma_{\text{trt}}\) is the standard deviation of the AgNP concentration of the triplicate treatment bottles (180 min time point). The product standard deviation was used to calculate the 95 % confidence intervals, which were used as error bars.

A common method to quantify changes in absorbance or emission spectra is the full width at half maximum (FWHM) (National Optical Astronomy Observatory 2013). This
technique allowed for the differentiation between AgNP aggregation and dissolution. For example, a broadening of the absorbance peak generally indicates aggregation of the AgNPs (Smitha et al. 2008; Oldenberg n.d.) and corresponds to an increase in FWHM values. As such, FWHM values were computed for each time point during the 3 hr experimental period.

2.2.3 AgNP Adsorption Studies

Batch bioreactors were prepared as described in Section 2.1.4, and the addition of test media at the -30 min time point was staggered by 7 min for each triplicate. Following the addition of the test media, each bottle in the triplicate was hand shaken for approximately 30 sec before 600 uL of sample was transferred to a 1-cm quartz and measured on a BioMate 3S UV-vis spectrophotometer at 2 nm intervals over the range of 300-700 nm. Prior to measurement, a baseline was collected from a standard solution that contained the same concentration of the macromolecule under study to correct for their potential interference with the AgNP absorption spectrum.

After 30 min of AgNP exposure to the test media, cells were added (0 min time point) and each batch bioreactor was hand-shaken for 10 sec before a 1 mL aliquot was pipetted into a 1.5 mL polypropylene microcentrifuge tube and centrifuged for 1 min at 13,300 rpm (17,000g) in a Sorvall Legend Micro 17R microcentrifuge with a 75003424 Micro-liter rotor. At the end of the centrifugation, 600 µL of supernatant was pipetted into clean 1.5 mL microcentrifuge tubes. The supernatant was subsequently transferred into a 1-cm quartz cuvette and the absorbance spectrum was measured by UV-vis spectrophotometry. After each reading, the sample was discarded to reduce the potential for cross-contamination. The quartz cuvette was rinsed three times with DI water (15 MΩ) in between each sample, and washed with 10% HNO₃ at the end of each experiment to remove any residual AgNPs.

Additional information regarding the procedure for determining the optimal centrifugal forces and time required to achieve the maximum removal of cells from solution without the significant pelleting of AgNPs is included in the Appendix. The same standard curve described in Section 2.2.2 was used to quantify the concentration of the AgNPs in solution throughout the experimental period.

The percent of AgNPs remaining in solution at the end of the experimental period was calculated from the following equation:
\[ \text{% AgNP in Solution} = \frac{[\text{AgNP}]_{\text{trt}}}{[\text{AgNP}]_{\text{con}}} \times 100\% \] (2.6)

Where \([\text{AgNP}]_{\text{trt}}\) is the average concentration of monodispersed AgNPs in triplicate treatment bottles that contained cells and were measured at the 180 min time point. \([\text{AgNP}]_{\text{con}}\) is the average concentration of monodispersed AgNPs in a triplicate control measured at the 0 min time point that did not contain cells or macromolecules, which allowed for the correction of AgNP concentrations by accounting for reductions due to both centrifugation and the initial high rates of adsorption that occurred once cells were added.

The standard deviation of the percent of AgNPs remaining in solution at the end of the experimental period was computed from Equation 2.5.

### 2.2.4 Macromolecule Stabilizing Mechanism

Batch bioreactors were prepared as described in Section 2.1.5, and the analytical methods used were identical to those described in Section 2.2.3. During each experiment, two controls were run alongside each treatment condition. Control 1 consisted of AgNPs in test media that was not centrifuged and did not contain any cells. Control 2 consisted of AgNPs in test media that was centrifuged but did not contain any cells. The Treatment condition consisted of AgNPs in test media containing cells that were added at the 0 min time point and was therefore centrifuged prior to UV-vis readings to remove any absorbance interference from the cells. The percent of AgNPs in solution at the end of the experimental period was computed from Equation 2.6, where \([\text{AgNP}]_{\text{trt}}\) was the concentration of monodispersed AgNPs remaining in solution at the 180 min time point, and \([\text{AgNP}]_{\text{con}}\) was the concentration of monodispersed AgNPs in solution at the -30 min time point immediately after the addition of the test media but prior to the addition of cells. The standard deviation of the percent of AgNPs remaining in solution at the end of the experimental period was computed from Equation 2.5.

### 2.3 MATERIALS

All chemicals were purchased from commercial sources and used without further purification. The various instruments used throughout the studies presented herein are included in Table 2.1.
Table 2.1. Instrumentation

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BioSafety Cabinet Thermo Scientific 1300 Series A2, Model Number 1323</td>
<td>Thermo Scientific, Madison, WI</td>
</tr>
<tr>
<td>Sorvall RC6+ centrifuge with conical SN 6114074</td>
<td>Thermo Scientific, Madison, WI</td>
</tr>
<tr>
<td>Allegra X-30R centrifuge with conical C0650 rotor</td>
<td>Beckmann Coulter, Brea, CA 92821</td>
</tr>
<tr>
<td>Sorvall Legend Micro 17R microcentrifuge with 75003424 Micro-liter rotor</td>
<td>Thermo Scientific, Madison, WI</td>
</tr>
<tr>
<td>UV-Visible Spectrophotometer Biomate 3S</td>
<td>Thermo Scientific, Madison, WI</td>
</tr>
<tr>
<td>Plate reader BioTek Synergy HT</td>
<td>Winooski, VT</td>
</tr>
<tr>
<td>Shaker table Thermo Scientific, MaxQ3000</td>
<td>Thermo Scientific, Madison, WI</td>
</tr>
<tr>
<td>pH meter Denver Instrument Model 250 with high performance glass-body pH/ATC electrode 300729-1</td>
<td>Denver Instrument Company, Arvada, CO</td>
</tr>
<tr>
<td>Shimadzu TOC-VWS</td>
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</tr>
</tbody>
</table>

20nm Citrate BioPureTM Silver nanoparticles were purchased from NanoComposix, Inc. (San Diego, CA). The AgNPs were synthesized through aqueous reductions with sodium borohydride using citrate as a capping agent. The AgNPs were characterized by NanoComposix to have a Ag mass concentration of 1 mg ml\(^{-1}\) (1,000 ppm), an Ag atomic molarity of 9.25 mM, particle diameters of 20.8 nm (± 2.7 nm), hydrodynamic diameters of 32 nm, a zeta potential of -21.4 mV, a particle concentration of 2x10\(^{13}\) particles ml\(^{-1}\), dispersed in 20 mM citrate buffered solution with a final pH of 6.8.

The following reagents used in this study were purchased through EMD Millipore Chemicals: Bovine serum albumin fraction V (BSA, CAS 9048-46-8), calcium chloride dehydrate (CaCl\(_2\) 2H\(_2\)O, CAS 10035-04-8), ethylenediamine teraacetic acid (EDTA, CAS 60-00-4), ferrous sulfate heptahydrate (FeSO\(_4\) 5H\(_2\)O, CAS 7782-63-0), HEPES free acid (CAS 7365-45-9), hydrochloric acid (HCl, CAS 7647-01-0), magnesium sulfate anhydrous (MgSO\(_4\), CAS 7487-88-9), N-(1-naphthyl) ethylenediamine dihydrochloride (NED, CAS 1465-25-4), sodium chloride (NaCl, CAS 7647-14-5), sodium phosphate monobasic (NaH\(_2\)PO\(_4\), CAS 10049-21-5), and sodium nitrite (NaNO\(_2\), CAS 7632-00-0).

The following reagents used in this study were purchased through VWR International LLC (West Chester, PA): Ammonium sulfate ((NH\(_4\))\(_2\)SO\(_4\), CAS 7783-20-2), nitric acid (HNO\(_3\), CAS 7697-37-2), potassium phosphate dibasic (K\(_2\)HPO\(_4\), CAS 7758-11-4),
potassium phosphate monobasic (KH$_2$PO$_4$, CAS 7778-77-0), sodium hydroxide beads (NaOH, CAS 1316-73-2).

Cupric sulfate, 5-hydrate (CuSO$_4$ 5H$_2$O, CAS 7758-99-8) was purchased from J.T. Baker (Avantor Performance Materials, Inc., Center Valley, PA). Ethylalcohol anhydrous (CAS 64-17-5) was obtained through Fisher Scientific, Rochester, NY. Sulfanilamide, 98% (CAS 63-74-1) came from Alfa Aesar, Lancaster, PA. Spectrum Chemical MFG Corporation, Gardena, CA delivered the sodium alginate (CAS 9005-38-3). Calibrations standards for the pH meter were purchased through Denver Instruments (Reagecon, certified traceable to N.I.S.T standards). The Suwannee River Humic Acid (SRHA, 1R101N) was purchased from the International Humic Substances Society.
CHAPTER 3

EXPERIMENTAL RESULTS

This chapter presents the experimental results from the inhibition, dissolution, and adsorption studies described in Sections 2.1 and 2.2. The chapter is divided into four primary sections to compliment the research objectives outlined in Section 1.6.

3.1 N. europaea Inhibition Studies

As mentioned in Section 2.1.2, the purpose of these studies was to determine the potential influence of NH$_3$ on the inhibitory effect of 20 nm spherical citrate capped AgNPs to *N. europaea* in both the presence and absence of the macromolecules BSA, alginate, and SRHA. The presence of these macromolecules has been shown to reduce the inhibitory effects of AgNPs to *N. europaea* primarily by coating the AgNPs and decreasing their dissolution to Ag$^+$ (Ostermeyer 2012). However, previous studies have relied on a minimal growth concentration of 0.16 mM NH$_3$ without consideration for higher NH$_3$ concentrations that could be present in WWTPs (Ostermeyer 2012). Since NH$_3$ has been shown to trigger AgNP dissolution (Ostermeyer 2012), these investigations sought to determine whether higher concentrations of NH$_3$ would compromise the protective capacity of BSA, alginate, and SRHA and result in increased inhibition of *N. europaea*.

Previous studies have shown that 20 nm citrate capped AgNPs added to a final concentration of 0.6 ppm in batch bioreactors with test media consisting of 30 mM HEPES buffer (pH=7.8) and 0.16 mM NH$_3$ resulted in approximately 60% nitrification activity of *N. europaea* over a 3 hr period (Ostermeyer 2012). As such, identical AgNPs were selected for these studies to serve as a means of comparison and build upon the current knowledge base. The results from a 3 hr inhibition study conducted with 0.6 ppm AgNPs in test media containing 0.04 to 1.6 mM NH$_3$ are shown in Figure 3.1. Over the 3 hr experimental period, the percent nitrification activity decreased significantly from 81% ± 9.3% at 0.04 mM NH$_3$ to
Figure 3.1. Percent nitrification activity of *N. europaea* during 3 hr inhibition studies in batch bioreactors containing 0.6 ppm of 20 nm citrate capped AgNPs, 30 mM HEPES buffer (pH=7.8), varying concentrations of NH$_3$, and 5 mg L$^{-1}$ cells. Batch bioreactors without AgNPs were run as controls. Error bars represent 95% confidence intervals.

18% ± 4.1% at 1.60 mM NH$_3$. The percent nitrification activity of 65% ± 7% at 0.16 NH$_3$ condition correlate well with those from previous studies (Ostermeyer 2012).

Figure 3.2 shows the results from batch inhibition studies conducted with 1 ppm AgNPs in test media containing 30 mM HEPES buffer (pH=7.8), 100 ppm BSA, and 0.04 to 1.6 mM NH$_3$. Results indicate a high degree of inhibition despite the presence of a relatively high concentration of BSA, which has been shown in previous studies to reduce inhibition. Percent nitrification activity varied slightly from 15% ± 3% to 28% ± 5%, which is in stark contrast with previous studies conducted with a similar concentration of BSA that report a percent nitrification activity of approximately 50% at 0.16 mM NH$_3$ (Ostermeyer 2012).

Figure 3.3 shows the results from batch inhibition studies involving 1 ppm AgNPs in test media consisting of 30 mM HEPES buffer (pH=7.8), 0.04 to 1.6 mM NH$_3$, and 600 ppm alginate. Overall, the percent activity decreased from 86% ± 3.8% at 0.04 mM NH$_3$ to 48% ± 8.8% at 0.80 mM NH$_3$, with an increase to 67% ± 10% at 1.60 mM NH$_3$. Percent nitrification activity at 0.16 mM NH$_3$ was found to be 61% ± 3.1%, which is reasonably consistent with those reported during previous studies (75%) conducted under the same conditions. The trend in Figure 3.3 is similar to that reported in Figure 3.1 and shows an overall decrease in percent
Figure 3.2. Percent nitrification activity of *N. europaea* during 3 hr inhibition studies in batch bioreactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 100 ppm BSA, varying concentrations of NH₃, and 5 mg L⁻¹ cells. Batch bioreactors without AgNPs were run as controls. Error bars represent 95% confidence intervals.

Figure 3.3. Percent nitrification activity of *N. europaea* during 3 hr inhibition studies in batch bioreactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 600 ppm algiane, varying concentrations of NH₃, and 5 mg L⁻¹ cells. Batch bioreactors without AgNPs were run as controls. Error bars represent 95% confidence intervals.
nitrification activity with increasing NH$_3$ concentration, despite the slight increase at 1.6 mM NH$_3$.

The results from a batch inhibition study involving 1 ppm AgNPs, in test media consisting of 30 mM HEPES buffer (pH=7.8), 0.04 to 1.6 mM NH$_3$, and 15 ppm SRHA are shown in Figure 3.4. Similar to Figures 3.1 and 3.3, there is a decreasing trend in percent nitrification activity with increasing NH$_3$ concentrations. Overall, the percent nitrification activity decreased from 54% ± 3% at 0.04 mM NH$_3$ to 17% ± 2.6% at 1.6 mM NH$_3$. Percent nitrification activity at 0.16 mM NH$_3$ was 46% ± 3%. Previous studies have shown approximately 80% nitrification activity under similar experimental conditions. Despite the difference between the results, the overall trend is similar to that reported in Figures 3.1 and 3.3 and indicates a 37% decrease in percent nitrification activity of _N. europaea_ between 0.04 and 1.6 mM NH$_3$.

![Figure 3.4. Percent nitrification activity of N. europaea during 3 hr inhibition studies in batch bioreactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA, varying concentrations of NH$_3$, and 5 mg L$^{-1}$ cells. Batch bioreactors without AgNPs were run as controls. Error bars represent 95% confidence intervals.](image)

Given that the primary mechanism of AgNP inhibition is believed to be the delivery of Ag$^+$, and that Ag$^+$ can complex with NH$_3$ to form AgNH$_3^+$ and Ag(NH$_3$)$_2^+$, a series of 3 hr experiments were conducted with 0.115 ppm Ag$^+$ and NH$_3$ concentrations ranging from 0.04
to 1.6 mM to determine whether NH₃ influenced the toxicity of Ag⁺. As shown in Figure 3.5, the average percent nitrification activity remained relatively constant around 47% ± 7% for all concentrations of NH₃, suggesting that NH₃ does not influence the inhibitory effects of dissolved Ag⁺ to *N. europaea*.

![Figure 3.5. Percent nitrification activity of *N. europaea* during 3 hr inhibition studies in batch bioreactors containing 0.115 ppm Ag⁺, 30 mM HEPES buffer (pH=7.8), varying concentrations of NH₃, and 5 mg L⁻¹ cells. Batch bioreactors without AgNPs were run as controls. Error bars represent 95% confidence intervals.](image)

3.2 **Silver Nanoparticle Dissolution Studies**

One of the primary mechanisms by which AgNPs inhibit microbial activity is through the aqueous dissolution to Ag⁺ as depicted in Figure 1.4 (Radniecki et al. 2011; Arnaout and Gunsch 2012). In previous studies, the dissolution of AgNPs was linked to the presence of NH₃ in the test media used during batch inhibition studies. However, the AgNP dissolution rates in the same studies were significantly decreased by the addition of BSA, alginate, and SRHA, which likely interacted with surface sorbed Ag⁺ and formed protective coatings around the AgNPs (Ostermeyer 2012; T. Radniecki, unpublished data). Consequently, these studies sought to investigate the potential for greater concentrations of NH₃ to compromise the protective coating endowed by the macromolecules and how this might explain the results from the inhibition studies presented in Section 3.1.
3.2.1 AgNP Dissolution in Test Media

Select UV-vis absorbance spectra from abiotic dissolution studies conducted with 1 ppm 20 nm citrate capped AgNPs in test media consisting of 30 mM HEPES buffer (pH=7.8) and NH3 concentrations ranging from 0 – 1.6 mM NH3 are shown in Figure 3.6. A control condition without NH3 is shown in Figure 3.6A and indicates minimal dissolution over the experimental period (15% ± 3%). In all cases, \( \lambda_{\text{max}} \) occurred between 400 to 402 nm, which is characteristic of the monodispersed AgNPs used in these studies. The gradual decrease in absorbance peak height shown in Figures 3.6B-F indicates that AgNP dissolution occurred proportional to the concentration of NH3 in solution. As shown in Figures 3.6E-F, AgNP dissolution at higher NH3 concentrations occurred rapidly after the addition of test media at the -30 min time point and gradually decreased over the duration of the experimental period. A complete dataset of the absorbance spectra is presented in Figures A.6-A.7 in the Appendix.

The dissolution rate of AgNPs was found to vary depending on the concentration of NH3 present in batch reactors (Figure 3.7). For example, AgNP dissolution correlated strongly with nonlinear logarithmic regressions at 1.1 mM NH3 (\( R^2=0.90 \)) and 1.6 mM NH3 (\( R^2=0.99 \)), with steep slopes occurring within the initial 30 min of the experimental period that gradually leveled-off. Whereas, dissolution rates were primarily linear at the lower concentrations of 0.08 mM NH3 (\( R^2=0.94 \)) and 0.16 mM NH3 (\( R^2=0.98 \)).

Figure 3.8 indicates a strong linear correlation between dissolution and NH3 concentration (\( R^2=0.89 \)). Dissolution of AgNPs reached approximately 96% ± 2% by the end of the experimental period under conditions containing 1.8 mM NH3. It is possible that the dissolution of the AgNPs in the other treatment conditions would have reached completion if the experimental period was extended beyond 3 hrs given that the slopes of the rate curves remained slightly positive (Figure 3.7).

FWHM measurements derived from the UV-vis absorbance spectra are shown in Figure 3.9. The high degree of overlap between datasets irrespective of NH3 concentrations suggests that aggregation of AgNPs did not occur during the experimental period. Therefore, the reduction in the absorbance peak height was caused by AgNP dissolution rather than sedimentation of large aggregates. FWHM values for the treatment condition containing 1.6 mM NH3 are absent from Figure 3.9 after the 0 min time point due to the inability of the UV-
Figure 3.6. UV-vis absorbance spectra from 3.5 hr abiotic dissolution studies in batch reactors containing 1 ppm spherical 20 nm citrate AgNPs in test media consisting of 30 mM HEPES (pH=7.8) (A), 0.08 mM NH₃ (B), 0.3 mM NH₃ (C), 0.8 mM NH₃ (D), 1.1 mM NH₃ (E), and 1.6 mM NH₃ (F). Test media was added at the -30 min time point.
Figure 3.7. Percent AgNP dissolution rates from a series of 3.5 hr abiotic studies in batch reactors containing 1 ppm 20 nm AgNPs, 30 mM HEPES (pH=7.8), and varying concentrations of NH3. Test media was added at the -30 min time point.

Figure 3.8. Percent AgNP dissolution following a series of 3.5 hr abiotic studies in batch reactors containing 1 ppm 20 nm citrate AgNPs, 30 mM HEPES buffer (pH=7.8), and varying concentrations of NH3. Error bars represent 95% confidence intervals.
Figure 3.9. FWHM measurements of 1 ppm 20 nm citrate AgNPs from a 3.5 hr abiotic study in test media consisting of 30 mM HEPES buffer (pH=7.8), and varying concentrations of NH₃. Error bars represent 95% confidence intervals. Test media was added at the -30 min time point.

vis spectrophotometer to resolve a viable absorbance peak upon which to measure the FWHM (Figure 3.6F).

### 3.2.2 AgNP Dissolution Studies with BSA

Select UV-vis absorbance spectra from 3 hr batch experiments conducted with 1 ppm 20 nm citrate AgNPs in test media consisting of 30 mM HEPES buffer (pH=7.8), 100 ppm BSA, and NH₃ concentrations ranging from 0 – 1.6 mM NH₃ are shown in Figure 3.10. A control condition without NH₃ is shown in Figure 3.10A and indicates only minimal dissolution of the AgNPs (15% ± 3%). Figure 3.10B represents an additional control condition containing 100 ppm BSA but no NH₃, and indicates that a moderate amount of AgNP dissolution (30% ± 7%) occurred over the course of the experimental period. In all cases, the λₓₙₐₓ shifted from 400 nm to 406-408 nm, which is characteristic of a red-shift to higher wavelengths and indicates that BSA interacted with the AgNPs by substituting weakly bound citrate to form a BSA-AgNP complex (Liu et al. 2009). Unlike Figure 3.6, the decrease in absorbance peak height for the conditions depicted in Figure 3.10B-F remained relatively consistent across the range of NH₃ concentrations used in the studies, suggesting
Figure 3.10. UV-vis absorbance spectra from 3.5 hr abiotic dissolution studies in batch reactors containing 1 ppm 20 nm citrate AgNPs in test media consisting of 30 mM HEPES (pH=7.8) (A), 100 ppm BSA (B), and 0.04 mM NH₃ (C), 0.16 mM NH₃ (D), 0.32 mM NH₃ (E), and 1.6 mM NH₃ (F). Test media was added at the -30 min time point.
that dissolution occurred independently of NH$_3$ and may have been catalyzed by BSA. A complete dataset of the absorbance spectra is presented in Figures A.8-A.9 in the Appendix.

The percent dissolution of AgNPs is presented in Figures 3.11 and 3.12. The dissolution rate primarily followed a linear regression (R$^2$=0.92) for all NH$_3$ concentrations (Figure 3.11). Figure 3.12 shows that percent AgNP dissolution increased only slightly from 32% ± 2% at 0.04 mM NH$_3$ to 39% ± 1% at 1.6 mM NH$_3$. However, the best fit linear regression suggests a weak correlation between percent AgNP dissolution and NH$_3$ concentration (R$^2$=0.55) and may be better explained by the presence of a high concentration of BSA in the batch reactors.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0 mM NH$_3$</th>
<th>0.04 mM NH$_3$</th>
<th>0.08 mM NH$_3$</th>
<th>0.32 mM NH$_3$</th>
<th>0.8 mM NH$_3$</th>
<th>1.6 mM NH$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30</td>
<td>60</td>
<td>90</td>
<td>120</td>
<td>150</td>
<td>180</td>
</tr>
<tr>
<td>% AgNP Dissolution</td>
<td>0%</td>
<td>10%</td>
<td>20%</td>
<td>30%</td>
<td>40%</td>
<td>50%</td>
</tr>
</tbody>
</table>

Figure 3.11. Percent AgNP dissolution rates from a series of 3.5 hr abiotic studies in batch reactors containing 1 ppm 20 nm AgNPs, 30 mM HEPES (pH=7.8), 100 ppm BSA, and varying concentrations of NH$_3$. Test media was added at the -30 min time point.

FWHM measurements derived from the UV-vis absorbance spectra are shown in Figure 3.13. Overall, FWHM values remained relatively consistent and varied only slightly between 60 and 71 nm over the course of the experimental period. The high degree of data overlap indicates that significant AgNP aggregation did not occur during the experimental period, which further suggests that the reduction in peak height was caused by AgNP dissolution rather than sedimentation of large aggregates.
Figure 3.12. Percent AgNP dissolution following a series of 3.5 hr abiotic studies in batch reactors containing 1 ppm 20 nm citrate AgNPs, 30 mM HEPES buffer (pH=7.8), 100 ppm BSA, and varying concentrations of NH$_3$. Error bars represent 95% confidence intervals.

Figure 3.13. FWHM measurements of 1 ppm 20 nm citrate AgNPs from a 3.5 hr abiotic study in test media consisting of 30 mM HEPES buffer (pH=7.8), 100 ppm BSA, and varying concentrations of NH$_3$. Error bars represent 95% confidence intervals. Test media was added at the -30 min time point.
3.2.3 AgNP Dissolution Studies with Alginate

Select UV-vis absorbance spectra from 3 hr batch experiments conducted with 1 ppm 20 nm citrate AgNPs in test media consisting of 30 mM HEPES buffer (pH=7.8), 600 ppm alginate, and NH₃ concentrations ranging from 0 – 1.6 mM NH₃ are shown in Figure 3.14. A control condition in which no NH₃ was present is shown in Figure 3.14A and indicates only minimal dissolution (15% ± 3%). Figure 3.14B represents an additional control condition containing 1,000 ppm alginate but no NH₃, and indicates that a moderate amount of AgNP dissolution (15% ± 3%) occurred over the course of the experimental period. In all cases, the λ max shifted from 400 nm to 404 nm, which is characteristic of a red-shift to higher wavelengths and suggests that alginate interacted with the AgNPs by substituting weakly bound citrate to form a alginate-AgNP complex similar to that formed by BSA (Liu et al. 2009; Chen, Mylon, and Elimelech 2006; Sangeetha et al. 2012).

A slight degree of aggregation in the treatment condition containing 1.6 mM NH₃, indicated by the appearance of a secondary absorbance peak around 500 nm, occurred almost instantaneously after the addition of NH₃ at the -30 min time point and persisted throughout the course of the experimental period (Figure 3.14F). The formation of the aggregates may have been partially responsible for the slight deviation in peak absorbance at the -30 min time point (0.114 ± 0.004 abs) from the average of the remainder of treatment conditions (0.129 ± 0.006 abs).

Quantification of the unknown aggregate concentration was not possible during these experiments due to the inability to generate a standard curve on which to compare the absorbance of the unknown aggregates with that of known aggregates. Nevertheless, the dissolution of the monodispersed AgNPs continued through the remainder of the experimental period as indicated by the disproportionately larger decease in peak absorbance when compared to that of the aggregates. Furthermore, if the persistent reduction in peak absorbance was the result of the formation of aggregates rather than dissolution it would be expected to coincide with an increase in absorbance for the aggregates at 500 nm. A complete dataset of the absorbance spectra is included in Figures A.10-A.11 in the Appendix.

The dissolution rate of the AgNPs was found to vary depending on the concentration of NH₃ present in batch reactors (Figure 3.15) and correlate well with those from the control studies where no macromolecules were present (Figure 3.7). For example, the AgNP
Figure 3.14. UV-vis absorbance spectra for abiotic dissolution studies in batch reactors containing 1 ppm 20 nm citrate AgNPs in test media consisting of 30 mM HEPES buffer (pH=7.8) (A), 600 ppm alginate (B), and 0.08 mM NH$_3$ (C), 0.3 mM NH$_3$ (D), 0.8 mM NH$_3$ (E), and 1.6 mM NH$_3$ (F). Test media was added at the -30 min time point.
dissolution rate at 1.6 mM NH$_3$ correlated reasonably well with a nonlinear logarithmic regressions ($R^2=0.89$), with a steep slope occurring within the initial 30 min of the experimental period that gradually leveled-off. However, the initial dissolution rate appears to have been reduced by the presence of alginate. The AgNP dissolution rates were primarily linear at the lower concentrations of 0.8 mM NH$_3$ ($R^2=0.95$), 0.16 mM NH$_3$ ($R^2=0.93$) and below.

Figure 3.16 indicates a strong linear correlation between the percent of AgNP dissolution and NH$_3$ concentration ($R^2=0.99$). Overall, alginate appeared to stabilize the AgNPs and reduce their dissolution in the presence of NH$_3$ concentrations that otherwise resulted in 96% ± 2% dissolution (Figure 3.8). Nevertheless, it is apparent from Figure 3.16 that the protective capacity of alginate was compromised in the presence of relatively high concentrations of NH$_3$. Furthermore, the slightly positive slopes of the rate curves presented in Figure 3.15 suggest the possibility of complete AgNP dissolution over a long enough experimental period.
Figure 3.16. Percent AgNP dissolution following a series of 3.5 hr abiotic studies in batch reactors containing 1 ppm 20 nm citrate AgNPs, 30 mM HEPES buffer (pH=7.8), and varying concentrations of NH$_3$. Error bars represent 95% confidence intervals.

FWHM measurements derived from the absorbance spectra are shown in Figure 3.17 and indicate that aggregation of AgNPs did not occur during the experimental period under the conditions of NH$_3$ concentrations ranging from 0 – 0.8 mM NH$_3$. The exception is the slight increase in FWHM values for the treatment condition containing 1.6 mM NH$_3$, in which the appearance of a secondary peak around 500 nm indicated minor aggregation. Even still, the slight deviation in FWHM shown in Figure 3.17 indicates minimal aggregation.

### 3.2.4 AgNP Dissolution Studies with SRHA

Select UV-vis absorbance spectra from 3 hr batch experiments conducted with 1 ppm 20 nm citrate AgNPs in test media consisting of 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA, and NH$_3$ concentrations ranging from 0 – 1.6 mM NH$_3$ are shown in Figure 3.18. Figure 3.18A represents a control condition containing only AgNPs in HEPES buffer, and indicates minimal dissolution of the AgNPs (15% ± 1%) over the course of the experimental period. Figure 3.18B represents an additional control condition containing 15 ppm SRHA but no NH$_3$, and indicates that a moderate amount of AgNP dissolution (22% ± 0%) occurred over the course of the experimental period.
Figure 3.17. FWHM measurements of 1 ppm 20 nm citrate AgNPs from a 3.5 hr abiotic study in test media consisting of 30 mM HEPES buffer (pH=7.8), 600 ppm, alginate, and varying concentrations of NH$_3$. Error bars represent 95% confidence intervals. Test media was added at the -30 min time point.

The $\lambda_{\text{max}}$ for both the control and select treatment conditions occurred between 400 to 402 nm, which is characteristic of the monodispersed AgNPs (Figure 3.18). The slight red-shift of $\lambda_{\text{max}}$ to 402 nm likely indicates that SRHA interacted with the surface of the AgNPs by substituting weakly-bound citrate to form SRHA-AgNP complexes (Liu et al. 2009; Dubas and Pimpan 2008; Gao et al. 2012). Minor aggregation occurred in the condition containing 1.6 mM NH$_3$ similar to alginate (Figure 3.14F), as indicated by the appearance of a secondary absorbance peak around 500 nm that gradually decreased over the course of the experimental period (Figure 3.18F). The minor aggregation did not appear to have significantly influenced the AgNP dissolution rates. For a complete dataset of the absorbance spectra refer to Figures A.12-A.13 in the Appendix.

The percent dissolution rates of the AgNPs for various NH$_3$ concentrations are presented in Figure 3.19. The initial dissolution rate of the AgNPs was the highest for the treatment condition containing 1.6 mM NH$_3$, and correlated moderately well with a nonlinear logarithmic regression ($R^2=0.88$) similar to those shown in Figures 3.7 and 3.15. The AgNP dissolution rates were significantly lower for NH$_3$ concentrations less than 1.6 mM NH$_3$ and
Figure 3.18. UV-vis absorbance spectra for abiotic dissolution studies in batch reactors containing 1 ppm 20 nm citrate AgNPs in test media consisting of 30 mM HEPES (pH=7.8) (A), 15 ppm SRHA (B), and 0.08 mM NH$_3$ (C), 0.16 mM NH$_3$ (D), 0.8 mM NH$_3$ (E), and 1.6 mM NH$_3$ (F). Test media was added at the -30 min time point.
Figure 3.19. Percent AgNP dissolution rates from a series of 3.5 hr abiotic studies in batch reactors containing 1 ppm 20 nm AgNPs, 30 mM HEPES (pH=7.8), 15 ppm SRHA, and varying concentrations of NH₃. Test media was added at the -30 min time point.

correlated more strongly with a linear regression for treatment conditions containing 0.32 mM NH₃ (R²=0.77) and 0.08 mM NH₃ (R²=0.95). The exception to this trend was observed in the treatment condition containing 0.8 mM NH₃, which correlated more strongly with a nonlinear logarithmic regression (R²=0.92) despite having an overall percent dissolution lower than treatment conditions containing less NH₃.

As shown in Figure 3.20, the linear correlation between the AgNP percent dissolution and NH₃ is not as strong (R²=0.49) as those in Figures 3.8 and 3.16. This is attributed to the high percent of the AgNPs dissolution in the control condition (22% ± 0%), as well as at 0.04 mM NH₃ (20% ± 3%) and 0.08 mM NH₃ (22 ± 6%), when compared to the treatment conditions containing 0.16 mM NH₃ (9% ± 1%), 0.32 mM NH₃ (15% ± 8%), and 0.8 mM NH₃ (15% ± 3%). SRHA appeared to reduce the overall dissolution of the AgNPs when compared to the conditions where no SRHA was present (Figure 3.8). Nevertheless, the protective coating afforded by SRHA appeared to have been compromised at the higher concentrations of NH₃, leading to increase AgNP Dissolution (Figure 3.20).
Figure 3.20. Percent AgNP dissolution following a series of 3.5 hr abiotic studies in batch reactors containing 1 ppm 20 nm citrate AgNPs, 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA, and varying concentrations of NH$_3$. Error bars represent 95% confidence intervals.

FWHM measurements derived from the UV-vis absorbance spectra are shown in Figure 3.21. Overall, FWHM values remained relatively consistent and varied only slightly between 60 and 72 nm over the course of the experimental period. The high degree of dataset overlap indicated that significant aggregation of the AgNPs did not occur during the experimental period, which confirms that the reduction in the absorbance peak height was caused by the dissolution of the AgNPs rather than the formation and subsequent sedimentation of large aggregates.

3.3 Silver Nanoparticle Adsorption Studies

The results from a series of experiments designed to determine the potential for the 20 nm citrate capped AgNPs to adsorb onto *N. europaea* in the presence and absence of the macromolecules BSA, alginate, and SRHA are presented in this section. Similar to the AgNP dissolution studies, UV-vis spectrophotometry was the primary technique used to quantify the adsorption of AgNPs to cells. Based on the results presented in Section 3.2, NH$_3$ was omitted from the batch reactors to control for AgNP dissolution.
Figure 3.21. FWHM measurements of 1 ppm 20 nm citrate AgNPs from a 3.5 hr abiotic study in test media consisting of 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA, and varying concentrations of NH$_3$. Error bars represent 95% confidence intervals. Test media was added at the -30 min time point.

### 3.3.1 AgNP Adsorption to Cells in Test Media

Prior to the incorporation of cells and/or macromolecules to the experimental design, baseline experiments were conducted to determine the potential influence of dissolution and centrifugation on the stability of the AgNPs in solution (Figures A.2 through A.5 in the Appendix). In Figure 3.22, the results from an experiment conducted with 1 ppm AgNPs in 30 mM HEPES buffer (pH=7.8) indicated that the AgNPs remained relatively stable with only 15% ± 1% dissolution by the end of the 3 hr experimental period as measured by the decrease in peak absorbance. In comparison, the results from a similar experiment in which the contents of the batch reactor were identical to those presented in Figure 3.22 but were subjected to 17,000g centrifugation for 1 min indicate a percent decrease in peak absorbance of 26% ± 3% (Figure 3.23). As such, the 11% difference between the peak absorbance is attributed to the removal of the AgNPs from solution during centrifugation. Nevertheless, Figure 3.23 served as a baseline upon which the remaining adsorption studies involving cells and macromolecules were measured against.
Figure 3.22. UV-vis absorbance spectra from batch bioreactor containing 1 ppm 20 nm citrate AgNPs and 30 mM HEPES buffer (pH=7.8).

Figure 3.23. UV-vis absorbance spectra of supernatant collected from batch bioreactors containing 1 ppm 20 nm citrate AgNPs and 30 mM HEPES buffer (pH=7.8) subjected to 17,000g centrifugation.
As shown in Figure 3.24, the AgNP peak absorbance decreased rapidly following the addition of cells to the batch reactors at the 0 min time point, and by the end of the 3 hr experimental period the percent of the AgNPs remaining in solution appeared to have been reduced to a concentration below the detection limit of the UV-vis. Considering dissolution and centrifugation resulted in a combined reduction in peak absorbance of 26% between the -30 min and 0 min time points, the additional decrease in peak absorbance is attributed to the adsorption of the AgNPs to cells. The peak absorbance values from Figure 3.24 were used to generate the adsorption curve shown in Figure 3.25, and indicate that AgNP adsorption occurred rapidly following the introduction of cells to the batch reactors.

![Figure 3.24. UV-vis absorbance spectra of supernatant samples collected from batch bioreactors containing 1 ppm 20 nm citrate AgNPs, 30 mM HEPES (pH=7.8), and 50 mg L⁻¹ cells subjected to 17,000g centrifugation.](image)

### 3.3.2 AgNP Adsorption Studies with BSA

Select UV-vis absorbance spectra from a series of adsorption experiments containing 1 ppm AgNP in 30 mM HEPES buffer (pH=7.8) and BSA concentrations ranging from 0-50 ppm are shown in Figure 3.26. A red-shift in $\lambda_{\text{max}}$ values from 400 nm to 406-408 nm occurred in all treatment conditions containing BSA and is associated with the substitution of
weakly bound citrate on the AgNP surface with BSA (Liu et al. 2009). Figure 3.26A represents a control containing 1 ppm AgNPs in 30 mM HEPES buffer (pH=7.8) that was subjected to centrifugation despite the absence of cells to remain consistent with the treatment conditions. Figure 3.26B represents a treatment condition where cells were added at the 0 min time point to batch reactors containing the AgNPs in test media with no BSA present. As shown in Figures 3.26C-F, the peak absorbance increased with BSA concentrations and appeared to reach an upper limit. A complete dataset of the absorbance spectra is presented in Figures A.14-A.15 in the Appendix.

The percent of the AgNPs that adsorbed to the cells over the course of the experimental period is shown in Figure 3.27. Error bars from 95% confidence intervals were omitted from Figure 3.27 for clarity, but their values are presented in Table A.1 of the Appendix. Nonlinear logarithmic regressions were fitted with a high degree of agreement to several of the rate curves in order to identify potential trends and provide explanations of the AgNPs adsorption rates in the presence of BSA. Due to centrifugation, a fraction of the AgNPs in the control (10% ± 2%) were removed from solution and produced a false positive
Figure 3.26. Select UV-vis absorbance spectra from supernatant samples collected from a series of biotic studies conducted in batch reactors containing 1 ppm 20 nm citrate AgNPs and 30 mM HEPES buffer (pH=7.8) (A), with 50 mg L\(^{-1}\) cells (B), and 2.5 ppm BSA (C), 5 ppm BSA (D), 10 ppm BSA (E), and 50 ppm BSA (F).
Figure 3.27. Percent AgNP adsorption rates during a series of 3 hr biotic studies conducted in batch reactors containing 1 ppm 20 nm citrate AgNPs, 30 mM HEPES buffer (pH=7.8), 50 mg L\(^{-1}\) cells, and varying concentrations of BSA. Cells were added at the 0 min time point.

Figure 3.28 shows the percent of the AgNPs remaining in solution at the end of the 3 hr experimental period as a function of BSA concentration. As shown, the percent of the AgNPs remaining in solution increased nonlinearly with BSA concentrations and appeared to reach an upper limit of 66\(\pm\)3\%. The initial steep slope of the curve between 0 and 10 ppm BSA indicates that adsorption was readily decreased by small incremental increases in BSA concentrations within the batch bioreactors. Increases in BSA concentrations above 10 ppm did not enhance the stability of the BSA-AgNP complex and a significant amount of adsorption still occurred.

FWHM measurements shown in Figure 3.29 remained relatively consistent between 66 and 72 nm in batch bioreactors containing 2.5 to 50 ppm BSA. BSA interacted with the
Figure 3.28. Percent of AgNPs remaining in solution after a series of 3 hr biotic studies conducted in batch reactors containing 1 ppm 20 nm citrate AgNPs, 30 mM HEPES buffer (pH 7.8), 50 mg L$^{-1}$ cells, and varying concentrations of BSA. Error bars represent 95% confidence intervals.

Figure 3.29. UV-vis studies with FWHM trends of 1 ppm 20 nm citrate AgNPs in 30 mM HEPES buffer (pH=7.8), 50 mg L$^{-1}$ cells, and varying concentrations of BSA. Error bars represent 95% confidence intervals.
AgNP surface, as indicated by the slight increase in FWHM values for conditions containing 2.5 to 50 ppm BSA when compared to baseline conditions where no BSA or cells were present. The FWHM values in batch bioreactors containing no BSA and 1 ppm BSA increased rapidly after 90 min from approximately 80 to 195 nm. The increase in FWHM for both conditions is attributed to the adsorption of AgNPs to cells rather than aggregation, which is supported by the absorbance spectra shown in Figure 3.26B. For example, as the AgNPs continue to adsorb to cells, indicated by the gradual decrease in peak absorbance to near completion by 3 hrs, the FWHM approaches infinity as the UV-vis spectrophotometer can no longer resolve a viable absorbance peak.

### 3.3.3 AgNP Adsorption Studies with Alginate

Selected absorbance spectra for a series of experiments conducted using 1 ppm 20 nm citrate AgNPs in 30 mM HEPES buffer (pH=7.8) and various concentrations of alginate are shown in Figure 3.30. A shift in $\lambda_{\text{max}}$ values from 402 to 408 nm occurred in treatment conditions containing 100 to 1,200 ppm alginate, respectively. The shift is indicative of the interaction of alginate with the surface of the AgNPs, which likely resulted in the substitution of weakly bound citrate (Chen, Mylon, and Elimelech 2006; Sangeetha et al. 2012). The gradual increase in $\lambda_{\text{max}}$ with the addition of relatively high concentrations of alginate suggests that alginate interacted weakly with the AgNP surface. In comparison with BSA, it took a proportionately greater amount of alginate to produce the same shift in $\lambda_{\text{max}}$.

Figure 3.30A represents a control containing 1 ppm AgNPs in test media consisting of 30 mM HEPES buffer (pH=7.8) that was subjected to centrifugation despite the absence of cells to remain consistent with the treatment conditions. Figure 3.30B represents a condition where cells were added to batch bioreactors containing AgNPs in test media with no alginate. As shown in Figures 3.30C-F, the decrease in absorbance peak was reduced with increased alginate concentration and appeared to reach an upper limit similar to the studies conducted with BSA. A complete dataset of the absorbance spectra is presented in Figures A.16-A.17 in the Appendix.

The percent of the AgNPs that adsorbed to the cells over the course of the experimental period is shown in Figure 3.31. Error bars from 95% confidence intervals were
Figure 3.30. Select UV-vis absorbance spectra from supernatant samples collected from a series of biotic studies conducted in batch reactors containing 1 ppm 20 nm citrate AgNPs and 30 mM HEPES (pH=7.8) (A), with 50 mg L⁻¹ cells (B), and 50 ppm alginate (C), 200 ppm alginate (D), 400 ppm alginate (E), and 1,000 ppm alginate (F).
Figure 3.31. Percent AgNP adsorption rates during a series of 3 hr biotic studies conducted in batch reactors containing 1 ppm 20 nm citrate AgNPs, 30 mM HEPES buffer (pH=7.8), 50 mg L⁻¹ cells, and varying concentrations of alginate. Cells were added at the 0 min time point.

omitted from Figure 3.31 for clarity, but their values are presented in Table A.2 of the Appendix. Nonlinear logarithmic regressions were fitted with a high degree of agreement to several of the rate curves in order to identify potential trends and provide explanations of the AgNPs adsorption rates in the presence of alginate. Due to centrifugation, a fraction of the AgNPs in the control (10% ± 2%) were removed from solution and produced a false positive for adsorption that was consistent with the BSA studies. Nevertheless, the control served as the metric upon which the adsorption rates were measured. The results indicate that in all cases AgNP adsorption was highest immediately after the addition of the cells at the 0 min time point, and gradually declined over the reminder of the experimental period. Adsorption of the AgNPs to cells appeared nearly complete by the 135 min time point for most treatment conditions containing alginate. However, the slightly positive slope of the adsorption rate curves (Figure 3.31) suggests that adsorption may continue over a longer experimental period.

Figure 3.32 shows the percent of AgNPs remaining in solution at the end of the 3 hr experimental period. As shown, the percent of AgNPs remaining in solution increased with
alginate concentrations and approached an upper limit around 68% ± 6%. Increases in the concentration of alginate in batch reactors above 800 ppm had minor influence on the stability of the alginate-AgNP complex and a significant amount of adsorption still occurred. The amount of alginate required to reduce adsorption was significantly greater when compared with BSA. In addition, the mild slope in Figure 3.32 indicates that greater concentrations of alginate were required to impart the same level of stability when compared with BSA.

FWHM values presented in Figure 3.33 remained relatively consistent in batch bioreactors containing 200 to 1,200 ppm alginate across the experimental period, suggesting that significant aggregation did not occur. The slight deviation of FWHM in treatment conditions from the control condition further indicates the interaction of alginate with the surface of the AgNPs. The exception to this trend is noted at the 180 min time interval for the sample containing 50 ppm alginate, which showed a slight increase from 88 nm to 121 nm between the 135 min and 180 min time interval, respectively. FWHM values in samples containing cells but no alginate increased rapidly after the 45 min time interval from 89 nm
to 212 nm. Similarly, FWHM values for the samples containing 25 ppm alginate increased rapidly after the 90 min time interval from 81 nm to 206 nm at the 180 min time interval. The increase in FWHM in both cases is attributed to the adsorption of AgNPs to cells rather than aggregation, which is supported by the absorbance spectra in Figures 3.30B-C.

### 3.3.4 AgNP Adsorption Studies with SRHA

Select absorbance spectra from a series of experiments conducted with 1 ppm 20 nm citrate AgNPs in 30 mM HEPES buffer (pH=7.8) and various concentrations of SRHA are shown in Figure 3.34. Figure 3.34A represents a control containing 1 ppm AgNPs in test media consisting of 30 mM HEPES buffer that was subjected to centrifugation despite the absence of cells to remain consistent with the treatment conditions. Figure 3.34B represents a condition in which cells were added to batch bioreactors containing AgNPs in test media with no SRHA. As shown in Figure 3.34C-F, the absorbance peak increased with SRHA concentration and appeared to reach an upper limit similar to the studies conducted with BSA (Figure 3.26) and alginate (Figure 3.30). As with BSA, a relatively low concentration of SRHA was required to produce a large reduction in the adsorption of the AgNPs to cells. A
Figure 3.34. Select UV-vis absorbance spectrum from supernatant samples collected from a series of biotic studies conducted in batch reactors containing 1 ppm 20 nm citrate AgNPs and 30 mM HEPES buffer (pH=7.8) (A), with 50 mg L\(^{-1}\) cells (B), and 1 ppm SRHA (C), 2.5 ppm SRHA (D), 15 ppm SRHA (E), and 25 ppm SRHA (F).
complete dataset of the absorbance spectra for all conditions is presented in Figures A.18-A.19 in the Appendix.

The percent of the AgNPs that adsorbed to the cells over the course of the experimental period is shown in Figure 3.35. Error bars from 95% confidence intervals were omitted from Figure 3.35 for clarity, but their values are presented in Table A.3 of the Appendix. Nonlinear logarithmic regressions were fitted with a high degree of agreement to several of the rate curves in order to identify potential trends and provide explanations of the AgNPs adsorption rates in the presence of SRHA similar to BSA and alginate. The results indicate that the AgNP adsorption rates were highest immediately after the addition of the cells at the 0 min time point, and gradually declined over the remainder of the experimental period. However, the slope remained slightly positive in all cases, suggesting that the adsorption of the AgNPs may go to completion over a long enough time period. A fraction of the AgNPs in the control were removed from solution during centrifugation and produced a false positive for adsorption. Nevertheless, the control served as the metric upon which the adsorption rates were measured.

Figure 3.36 shows the percent of the AgNPs remaining in solution at the end of the 3 hr experimental period. As shown, the percent of AgNPs remaining in solution increased with SRHA concentration and appeared to reach an upper limit of approximately 66% ± 1%. Inasmuch, increases in the concentration of SRHA above 5 ppm had a minor influence on the stability of the SRHA-AgNP complex and a significant amount of adsorption still occurred. The exception to this trend was observed in the samples containing 10 ppm SRHA, which showed a higher percent of AgNPs remained in solution when compared to the treatment conditions containing 15 and 25 ppm SRHA. This appears to be the result of minor cell interference at the 180 min time point as indicated by the slight increase in absorbance around 320 nm as shown in Figure A.18 in the Appendix.

The stability and aggregation state of AgNPs throughout the experiments were measured using the FWHM technique and the results are presented in Figure 3.37. FWHM values remained relatively consistent between 68 and 90 nm in batch bioreactors containing 1 to 25 ppm SRHA. After the 90 min time point, FWHM values for all treatment conditions increased slightly above the control condition, further suggesting that SRHA interacted with the surface of the AgNPs. FWHM values for the batch bioreactors containing 10 ppm SRHA
Figure 3.35. Percent AgNP adsorption rates during a series of 3 hr biotic studies conducted in batch reactors containing 1 ppm 20 nm citrate AgNPs, 30 mM HEPES buffer (pH=7.8), 50 mg L^{-1} cells, and varying concentrations of SRHA. Cells were added at the 0 min time point.

Figure 3.36. Percent of AgNPs remaining in solution after a series of 3 hr biotic studies conducted in batch reactors containing 1 ppm 20 nm citrate AgNPs, 30 mM HEPES buffer (pH 7.8), 50 mg L^{-1} cells, and varying concentrations of SRHA. Error bars represent 95% confidence intervals.
Figure 3.37. UV-vis studies with FWHM trends of 1 ppm 20 nm citrate AgNPs in 30 mM HEPES buffer (pH=7.8), 50 mg L$^{-1}$ cells, and varying concentrations of SRHA. Error bars represent 95% confidence intervals.

were detected slightly higher (116 nm) than average values (92 nm) at the 180 min time point and are again attributed to cell interference. After the 90 min time point, the FWHM values for the condition containing AgNPs and cells with no SRHA increased rapidly and signified adsorption of the AgNPs to cells rather than aggregation, which is supported by the absorbance spectra in Figure 3.34.

3.4 Macromolecule Stabilization Studies

The results from the macromolecule stabilization studies described in Section 2.1.5 are presented in Sections 3.4.1 through 3.4.3 for BSA, alginate, and SRHA, respectively. The concentrations of BSA, alginate, and SRHA used in these studies were selected based on the results from Section 3.3 in order to achieve the minimal amount of AgNP adsorption to cells prior to the addition of MgSO$_4$. Similar to the adsorption studies presented in Section 3.3, UV-vis spectrophotometry was the primary technique used to quantify the percent of AgNPs that remained in solution at the end of the 3 hr experimental period. The potential for AgNP dissolution in these studies was regulated by the omission of NH$_3$ from the batch bioreactors.
3.4.1 AgNP Stabilization Studies with BSA

Figure 3.38 shows the results from the AgNP stabilization studies conducted with 1 ppm AgNPs in test media consisting of 30 mM HEPES buffer (pH=7.8), 40 ppm BSA, and concentrations of MgSO₄ ranging from 0 to 2.4 mM. Each data point represents the percent of the AgNPs that remained in solution at the end of the 3 hr experimental period as a function of MgSO₄. The percent of AgNPs remaining in solution for both Control 1 (81% ± 3.5%) and Control 2 (73% ± 5.4%) remained relatively constant within their respective datasets over the range of MgSO₄ concentrations and are consistent with the findings presented in Figures 3.22 and 3.23 of Section 3.3.1. The percent of AgNPs in control samples subjected to centrifugation (Control 2) was on average approximately 8% less than that from control samples not subjected to centrifugation (Control 1). This indicates that centrifugation elicited only minor influence on the concentration of AgNPs in solution, which is consistent with findings presented in Figures 3.22 and 3.23. Similarly, the percent of AgNPs remaining in solution within the treatment condition remained relatively constant (52% ± 5.1%) over the range of MgSO₄. The percent of AgNPs remaining in solution for the treatment condition containing cells was significantly lower when compared to the controls and is consistent with the results in Figure 3.28, which show a significant amount of adsorption despite have attained saturation of the AgNP surface with BSA. A complete dataset of the UV-vis absorbance spectra is presented in Figures A.20-A.22 in the Appendix.

3.4.2 AgNP Stabilization Studies with Alginate

The results from the AgNP stabilization studies conducted with 1 ppm AgNPs in test media consisting of 30 mM HEPES buffer (pH=7.8), 1,000 ppm alginate, and MgSO₄ concentrations ranging from 0 to 2.4 mM are presented in Figure 3.39. The results indicate a trend similar to that observed in the BSA studies, in that the average percent of AgNPs remaining in solution for both the control (80% ± 1.9%; 68% ± 2.5%) and the treatment conditions (53% ± 4%) remained relatively constant within their respective datasets and appear to be independent of the concentration of MgSO₄. The variation within datasets over the range of MgSO₄ concentrations was notably less than that of BSA. The percent of the AgNPs in Control 2 samples was on average 12% less than that from Control 1 samples,
Figure 3.38. Percent of AgNPs remaining in solution following a series of 3 hr adsorption studies in batch bioreactors containing 1 ppm 20 nm AgNPs, 30 mM HEPES buffer (pH=7.8), 40 ppm BSA, various concentrations of MgSO₄, and 50 mg L⁻¹ cells. Error bars represent 95% confidence intervals. Control 1 – no cells or centrifuge. Control 2 – centrifuge and no cells. Treatment – cells and centrifuge.

Figure 3.39. Percent of AgNPs remaining in solution following a series of 3 hr adsorption studies in batch bioreactors containing 1 ppm 20 nm AgNPs, 30 mM HEPES buffer (pH=7.8), 1,000 ppm alginate, various concentrations of MgSO₄, and 50 mg L⁻¹ cells. Error bars represent 95% confidence intervals. Control 1 – no cells or centrifuge. Control 2 – centrifuge and no cells. Treatment – cells and centrifuge.
indicating that centrifugation elicited only minor influence on the concentration of the AgNPs in solution and is consistent with findings presented in Figures 3.22 and 3.23. The influence of centrifugation was slightly greater in these studies when compared to the studies conducted with BSA. The percent of the AgNPs remaining in solution within the treatment condition remained relatively constant at 52% ± 3.0% as the concentration of MgSO₄ increased, which is consistent with results from Figure 3.32. A complete dataset of the UV-vis absorbance spectra is presented in Figures A.23-A.25 in the Appendix.

3.4.3 AgNP Stabilization Studies with SRHA

Figure 3.40 shows the results from the stabilization studies conducted with 15 ppm SRHA. When compared with the results from the studies conducted with BSA and alginate, the percent of AgNPs remaining in solution after 3 hr exposure to various concentrations of MgSO₄ varied considerably for both the control and treatment conditions. In all cases, the percent of AgNPs in solution declined at MgSO₄ concentrations greater than 0.35 mM. The decline in percent AgNP within Control 1 was less significant in comparison with Control 2 and the treatment condition over the range of MgSO₄ concentrations. Initially, the percent AgNPs in solution decreased from 82% at 0.35 mM MgSO₄ to 62% at 1.2 mM MgSO₄ before leveling out and remaining relatively constant for 1.8 and 2.4 mM MgSO₄. The percent of AgNPs in Control 2 declined initially from 76% ± 7% at 0.35 mM MgSO₄ to 68% ± 4% at 0.73 mM MgSO₄ and then to 35% ± 11% at 1.2 mM MgSO₄ and eventually to a concentration below the detection limits of the UV-vis spectrophotometer at 1.8 and 2.4 mM MgSO₄. The treatment condition containing the cells followed a similar trend, with an initial decrease from 57% ± 6% at 0.35 mM MgSO₄ to 27% ± 6% at 0.73 mM MgSO₄, and then to 20% ± 6% at 1.2 mM MgSO₄ and eventually to a concentration below the detection limits of the UV-vis spectrophotometer at 1.8 and 2.4 mM MgSO₄. A complete dataset of the UV-vis absorbance spectra is presented in Figures A.26-A.28 in the Appendix.
Figure 3.40. Percent of AgNPs remaining in solution following a series of 3 hr adsorption studies in batch bioreactors containing 1 ppm 20 nm AgNPs, 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA, various concentrations of MgSO$_4$, and 50 mg L$^{-1}$ cells. Error bars represent 95% confidence intervals. Control 1 – no cells or centrifuge. Control 2 – centrifuge and no cells. Treatment – cells and centrifuge.
CHAPTER 4

INHIBITION AND DISSOLUTION SUMMARY

Given that the AgNP inhibition and dissolution studies were conducted separately, the synthesis of the two datasets merits further investigation in order to better understand the potential interplay between the two processes. This chapter aims at accomplishing this goal by combining the results from Sections 3.1 and 3.2 into a more coherent dataset.

Figure 4.1 combines the percent nitrification activity and percent dissolution datasets initially presented in Figures 3.1 and 3.8, respectively, as a function of NH$_3$ concentration. In both cases, batch bioreactors contained 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), and varying concentrations of NH$_3$, with the exception of the batch bioreactors used to determine the inhibition of *N. europaea* from AgNPs in the absence of macromolecules. A final concentration of 1 ppm AgNPs was used in the abiotic dissolution studies due to detection limits of the UV-vis spectrophotometer. Cells were added to the batch bioreactors used to calculate percent nitrification activity but were omitted from those used to calculate percent dissolution of the AgNPs due to their potential interference with UV-vis spectrophotometry readings. No macromolecules were added at any point during the experimental period.

As shown, the rapid decline in percent nitrification activity coincides with the equally rapid dissolution of the AgNPs, suggesting that Ag$^+$ was a stronger determinant of *N. europaea* inhibition than the AgNPs alone. The decrease in percent nitrification activity followed a nonlinear logarithmic regression suggesting the possibility of a rate limiting step that may have somehow been associated with AgNP dissolution. As a metric for comparison, previous studies reported approximately 60% nitrification activity and 34% dissolution when 0.16 mM NH$_3$ was added to the batch reactors (Ostermeyer 2012).

Figure 4.2 is similar to Figure 4.1 with the exception that BSA was added to a final concentration of 100 ppm in the batch bioreactors. The uniformity of the AgNP dissolution correlated well the percent nitrification activity. In general, the slight increase in AgNP
Figure 4.1. Correlation between percent nitrification activity of *N. europaea* and percent dissolution of AgNPs as a function of NH$_3$ concentrations in batch reactors containing 1 ppm AgNPs and 30 mM HEPES buffer (pH=7.8). Cells were only present in the batch reactors used to calculate percent nitrification activity. Error bars represent 95% confidence intervals.

Figure 4.2. Correlation between percent nitrification activity of *N. europaea* and percent dissolution of AgNPs as a function of NH$_3$ concentrations in batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), and 100 ppm BSA. Cells were only present in the batch reactors used to calculate percent nitrification activity. Error bars represent 95% confidence intervals.
dissolution at 1.6 mM NH₃ was reflected by a slight decrease in percent nitrification activity. In this case, the lack of enhanced dissolution suggests that BSA concentration was a stronger determinant of AgNP dissolution than NH₃. The average percent AgNP dissolution of 34% ± 3% and subsequent average percent nitrification activity of 20% ± 5% corresponded well with the remainder of data that showed similar dissolution and inhibition (Table 4.1). This suggests that BSA catalyzed the dissolution of the AgNPs while simultaneously protecting the NP structure from further NH₃-induced dissolution.

**Table 4.1. Percent Nitrification Activity (% Nit) and Percent AgNP Dissolution (% Diss) Data from a Series of Experimental Conditions Presented in Sections 3.1 and 3.2. 95% Confidence Intervals are Presented as ±%**

<table>
<thead>
<tr>
<th>NH₃ (mM)</th>
<th>0.6ppm AgNPs</th>
<th>1 ppm AgNPs</th>
<th>1 ppm AgNPs</th>
<th>1 ppm AgNPs</th>
<th>0.115ppm Ag⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Nit</td>
<td>% Diss*</td>
<td>% Nit</td>
<td>% Diss</td>
<td>% Nit</td>
</tr>
<tr>
<td>0</td>
<td>---</td>
<td>15±1%</td>
<td>---</td>
<td>30±7%</td>
<td>---</td>
</tr>
<tr>
<td>0.04</td>
<td>80±12%</td>
<td>16±0%</td>
<td>22±7%</td>
<td>32±2%</td>
<td>86±4%</td>
</tr>
<tr>
<td>0.08</td>
<td>73±4%</td>
<td>16±0%</td>
<td>21±7%</td>
<td>35±3%</td>
<td>65±3%</td>
</tr>
<tr>
<td>0.16</td>
<td>65±7%</td>
<td>38±7%</td>
<td>15±3%</td>
<td>33±3%</td>
<td>61±3%</td>
</tr>
<tr>
<td>0.32</td>
<td>36±4%</td>
<td>27±4%</td>
<td>28±5%</td>
<td>36±2%</td>
<td>49±6%</td>
</tr>
<tr>
<td>0.80</td>
<td>19±2%</td>
<td>32±7%</td>
<td>17±2%</td>
<td>33±1%</td>
<td>48±9%</td>
</tr>
<tr>
<td>1.10</td>
<td>---</td>
<td>68±5%</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1.60</td>
<td>18±3%</td>
<td>96±2%</td>
<td>16±3%</td>
<td>39±1%</td>
<td>67±10%</td>
</tr>
</tbody>
</table>

*1 ppm AgNP was used for all dissolution studies due to detection limits of the UV-vis.

Similar to Figure 4.1, an increase in NH₃ concentration resulted in a linear increase in AgNP dissolution in the presence of 600 ppm alginate that corresponded with a nonlinear decrease in percent nitrification activity (Figure 4.3). However, the relatively high concentration of alginate reduced the overall percent of AgNPs dissolution when compared with conditions where no alginate was present. The heightened percent nitrification activity
Figure 4.3. Correlation between percent nitrification activity of *N. europaea* and percent dissolution of AgNPs as a function of NH$_3$ concentrations in batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), and 600 ppm alginate. Cells were only present in the batch reactors used to calculate percent nitrification activity. Error bars represent 95% confidence intervals.

at 1.6 mM is attributed to the aggregation of the AgNPs (Figure 3.14F), which occurred immediately after the NH$_3$ was added to the batch bioreactors and has been reported to reduce the toxicity of AgNPs to microorganisms (Marambio-Jones and Hoek 2010). Overall, the results correlate well with those presented in Figure 4.1 and suggest that dissolution of AgNPs is a main contributor to *N. europaea* inhibition. Furthermore, it appears that the protective coating provided by alginate was compromised by NH$_3$, suggesting that alginate interacted weakly with the AgNPs surface.

Figure 4.4 combines the percent nitrification activity with the percent AgNP dissolution datasets for the treatment conditions containing 15 ppm SRHA presented in Figures 3.4 and 3.20, respectively, as a function of NH$_3$ concentration. As with Figures 4.1 and 4.3, the percent nitrification activity decreased nonlinearly as NH$_3$ concentrations increased linearly and lead to enhanced AgNP dissolution. However, a decrease in percent activity did not always correspond with an increase in percent dissolution (Table 4.1). For example, the percent AgNP dissolution of 22% ± 6% at 0.08 mM NH$_3$ corresponded with a percent nitrification activity of 46% ± 11%, whereas the percent AgNP dissolution at 0.16
mM NH$_3$ decreased to 9% ± 1% while the percent activity remained relatively constant at 46% ± 3%. Furthermore, at 0.32 mM NH$_3$ the percent nitrification activity decreased further to 30% ± 2%, but the percent dissolution (15% ± 8%) was less than that at 0.04 (20% ± 3%) and 0.08 (22% ± 6%) mM NH$_3$. In addition, a significant amount of inhibition was observed for a relatively low amount of dissolution, when compared with the results presented in Figure 4.1, suggesting the possibility of interaction between cells and the AgNPs that was not captured by these mutually exclusive experiments.

Figure 4.5 represents the results from a mathematical model used to quantify the speciation of Ag$^+$ contributed during AgNP dissolution as a function of NH$_3$ concentration. As shown, free silver in the form of Ag$^+$ readily complexes with NH$_3$ and forms two main silver amine complexes, the major complex being Ag(NH$_3$)$_2^+$. These results could then be compared with those from the inhibition studies presented in Figure 3.5 to make certain inferences on the effect of silver complexes to N. europaea toxicity that are further discussed in Section 5.1.
Figure 4.5. Predicted equillibrium concentrations of silver-amine complexes in batch reactors containing 0.115 ppm Ag\(^+\), 30 mM HEPES buffer (pH=7.8), and various concentrations of NH\(_3\). Visual MINTEQ was used to calculate species concentrations.
CHAPTER 5
DISCUSSION

The experimental results presented in Chapter 3 are discussed in further detail within this chapter. The chapter is divided into two primary sections that discuss the correlation between AgNP dissolution and _N. europaea_ inhibition in the presence and absence of NH$_3$ and macromolecules, as well as the adsorption of the AgNPs to cells and the influence of macromolecules.

### 5.1 Ammonia Influence on the Inhibition of AgNPs to _N. europaea_

The 20 nm citrate capped AgNPs used throughout the experimental studies presented in Section 3.1 succumbed to rapid and nearly complete dissolution in the presence of NH$_3$ concentrations above 0.8 mM (Figure 3.7; Figure 3.8). The AgNPs dissolution rate was strongly dependent on the concentration of NH$_3$ in solution, with 0.8 and 1.6 mM NH$_3$ inducing rapid dissolution within the first 30 min that gradually leveled off along nonlinear logarithmic regressions (Figure 3.7). The NH$_3$-induced dissolution of the AgNPs coincided with increased inhibition of _N. europaea_ (Figure 4.1).

The mechanism behind the observed AgNP dissolution is believed to be the complexation of surface-sorbed Ag$^+$ with free NH$_3$, resulting in the formation of silver-amine complexes including AgNH$_3^+$ and Ag(NH$_3$)$_2^+$ (Figure 4.5). The complexation depends primarily on the concentration of NH$_3$ initially present, and the Ag$^+$ stripped from the surface of the AgNPs during the complexation process eventually reaches an equilibrium position where the formation of the complex is balanced by its dissolution to Ag$^+$ and NH$_3$. It is possible that the stripping of the surface sorbed Ag$^+$ destabilized the AgNP structure and resulted in enhanced dissolution. This is one possible explanation as to why the initial dissolution rates were much greater at the higher concentrations of NH$_3$.

As shown in Figure 4.5, the distribution of silver-amine complexes favors the formation of Ag(NH$_3$)$_2^+$ above Ag(NH$_3$)$_3^+$ and results in the precipitous decline of free Ag$^+$ across a relatively narrow range of NH$_3$ concentrations (0.04 – 0.22 mM NH$_3$). Despite the
formation of the silver-amine complexes predicted by the model, the average percent nitrification activity of *N. europaea* remained relatively consistent at 47% ± 7% across the range of NH$_3$ concentrations (Figure 3.5), suggesting that the complexed silver retained its biocidal properties.

### 5.1.1 AgNP Inhibition and Dissolution Studies with BSA

In the results presented in Section 3.2.2, it can be seen that the presence of a relatively high concentration of BSA (100 ppm) induced approximately 34% ± 3% dissolution of the AgNPs over the range of NH$_3$ used in these studies (Figure 3.12). The nearly uniform dissolution of the AgNPs correlated with a nearly uniform inhibition of *N. europaea* (Figure 3.2; Figure 4.2), suggesting that the concentration of BSA was a stronger determinant of AgNP dissolution in this case. Further evidence of the BSA-induced dissolution is indicated by the relatively linear dissolution rates ($R^2$=0.84) across the range of NH$_3$ concentrations (Figure 3.11), which is unique when compared to those of alginate (Figure 3.15) and SRHA (Figure 3.19).

The percent dissolution of the AgNPs not exposed to BSA was much greater for NH$_3$ concentrations above 0.32 mM (Figure 3.8), which suggests that BSA also imparted a degree of stability to the AgNPs over the experimental period. The interaction of BSA with the AgNPs surface was indicated by a red-shift of $\lambda_{max}$ from 400 nm to 406-408 nm. However, trends in Figure 3.11 imply that dissolution rates may remain fixed and eventually lead to complete dissolution of AgNPs given a long enough time period.

The high degree and uniformity of the AgNPs dissolution is attributed to the sequestration of surface-sorbed Ag$^+$ to thiol groups present in BSA, such as cysteine, which are known to have a high binding affinity for metal cations (Kittler et al. 2010; Liu et al. 2009; MacCuspie 2011). Since AgNPs consist of Ag$^0$ nanoparticles and surface-sorbed Ag$^+$ in equilibrium with each other (Liu and Hurt 2010; Liu et al. 2010), it is possible that the binding of Ag$^+$ to BSA caused instability within the NP structure and induced further AgNP dissolution. The propensity for BSA to catalyze the AgNPs dissolution appeared to be concentration dependent. For example, controls containing 40 ppm BSA used during the adsorption studies presented in Section 3.3.2 indicated minimal dissolution of 13%
(Figure 3.26A) when compared to those with 100 ppm BSA present that resulted in 34% ± 3% dissolution (Figure 3.10; Table 4.1).

### 5.1.2 AgNP Inhibition and Dissolution Studies with Alginate

For the experimental conditions containing 600 ppm alginate, the percent nitrification activity of *N. europaea* initially decreased from 86% ± 4% to 48% ± 9% between 0.04 mM and 0.80 mM NH₃, respectively, followed by an increase to 67% ± 10% at 1.6 mM NH₃ (Figure 3.3). The decrease in percent nitrification activity is attributed to an increase in AgNP dissolution across the range of NH₃ concentrations used in these studies (Figure 4.3). Dissolution was the primary mechanism behind the decrease in $\lambda_{\text{max}}$ values as FWHM values did not show signs of aggregation (Figure 3.17). As shown in Figure 3.14F, AgNP aggregation occurred at 1.6 mM NH₃, which explains the observed increase in percent nitrification activity at this concentration of NH₃. Previous studies have indicated similar results, wherein an increase in the aggregation state of NPs resulted in a decrease in their overall toxicity to microorganisms as a result of a decreased surface to volume ratio and the masking of surface charges that occur during the process of aggregation (Marambio-Jones and Hoek 2010).

Unlike BSA, the presence of 600 ppm alginate in the batch bioreactors did not induce additional dissolution of the AgNPs (Figure 3.3; Table 4.1) despite the apparent interaction of the alginate with the AgNPs surface as indicated by the red-shift in $\lambda_{\text{max}}$ from 400 nm to 404 nm (Figure 3.14). The protective coating afforded by the alginate was compromised by NH₃, which likely stripped surface-sorbed Ag⁺ from the AgNPs to form silver-amine complexes that ultimately resulted in a cascading effect of enhanced dissolution as described in Section 5.1. Consequently, NH₃ was a stronger determinant of AgNP dissolution than the alginate itself and was concentration dependent (Figure 3.16). Nevertheless, the overall percent dissolution of the AgNPs at 1.6 mM NH₃ (61% ± 2%) was more than 30% less than the 96% ± 2% observed in the treatment condition containing no macromolecules, indicating that alginate imparted ephemeral stability to the AgNPs.

A more complex mechanism may also be at play. For instance, the principal mechanism of metallic cation sequestration involves the formation of complexes between a metal ion and functional groups (carboxyl, carbonyl, amino, amido, sulfonate, phosphate)
present on the surface or inside the porous structure of the biological material (Fourest and Volesky 1997). Therefore, in addition to providing stability to the AgNPs, alginate may also sequester free Ag\(^+\) that have reached equilibrium within the system after being stripped from the AgNPs surface by NH\(_3\) during the formation of silver-amine complexes thereby reducing the inhibitory effects of the AgNPs to \(N.\) europaea when compared to control conditions. Furthermore, recent studies have reported that polysaccharides were used in the synthesis of AgNPs as they act as both a reducing and stabilizing agent (Park et al. 2011; Ghaseminezhad, Hamedi, and Shojaosadati 2012), indicating that AgNP synthesis could be occurring alongside dissolution resulting in an overall decrease in inhibition.

The dissolution rate of the AgNPs in the presence of alginate was strongly dependent on the concentration of NH\(_3\) in solution, with 1.6 mM NH\(_3\) inducing rapid dissolution within the first 30 min of the experimental period that gradually leveled off along a nonlinear logarithmic regression \((R^2=0.89; \text{Figure 3.15})\). Linear regressions were fitted to the remainder of dissolution rate curves, and their positive slopes suggest that dissolution of the AgNPs may have gone to completion over a long enough period of time. Similarly, the slight positive slope of the nonlinear regression rate curve for the treatment condition containing 1.6 mM NH\(_3\) also suggests the potential for complete AgNP dissolution over a long enough period of time.

### 5.1.3 AgNP Inhibition and Dissolution Studies with SRHA

Similar to both BSA and alginate, SRHA can modify AgNP properties by adsorption onto the surface and formation of a surface coating (Baalousha 2009; Chappell et al. 2011), which enhance AgNP stability and decrease the aggregation rate by shielding the effects of van der Waals forces (Lin and Xing 2008; Chappell et al. 2009). Adsorption of SRHA is also believed to inhibit Ag\(^+\) release from citrate stabilized AgNPs (Liu and Hurt 2010). The interaction of SRHA with the surface of the AgNPs in these studies was confirmed by the slight shift in \(\lambda_{\text{max}}\) from 400 nm to 402 nm (Figure 3.18).

The results from the dissolution studies presented in Section 3.2.4 indicate a general trend similar to those presented above, wherein an increase in NH\(_3\) concentrations resulted in an increase in dissolution of the AgNPs (Figure 3.20). In general, the increase in the AgNPs dissolution coincided with a decrease in percent nitrification activity of \(N.\) europaea
similar to those presented in the cases above, with the exception of BSA
(Figure 4.2). However, the linear correlation between dissolution and NH₃ was not as strong
($R^2=0.49$) as those from the other dissolution studies. This is no doubt due in part to the
anomalous decrease in percent dissolution of the AgNPs between 0.16 to 0.8 mM NH₃ to
levels below those observed for lower NH₃ concentrations. For instance, the percent
dissolution of the AgNPs between 0 and 0.08 mM NH₃ averaged 21% ± 1%, whereas the
percent dissolution between 0.16 and 0.8 mM NH₃ averaged 13% ± 3%. Furthermore, a
larger decrease in percent nitrification activity was observed for relatively lower percent of
AgNP dissolution for 0.32 and 0.8 mM NH₃. Overall, the results indicate that the protective
capacity of the SRHA was compromised by the presence of a relatively high (1.6 mM)
concentration of NH₃, which likely stripped surface-sorbed Ag⁺ from the AgNPs to form
silver-amine complexes that ultimately resulted in a cascading effect of enhanced dissolution
as described above.

5.2 Silver Nanoparticle Adsorption and Macromolecule Stabilization Studies

In the absence of any macromolecules, the AgNPs readily adsorbed to the cells
(Figure 3.24) at a rate that was dependent on the concentration of AgNPs in solution
(Figure 3.25). Nearly complete adsorption was observed after 3 hr exposure of the AgNPs to
the cells, with the vast majority of adsorption occurring within the first 30 min of the
experimental period. The high degree of adsorption was likely due to the high surface to
volume ratio and high surface energy of the AgNPs (Liu et al. 2009). On the other hand, the
adsorption of the AgNPs to cells was significantly reduced by in the presence of BSA
(Figure 3.28), alginate (Figure 3.32), and SRHA (Figure 3.36), as indicated by the nearly
uniform 66% of AgNPs remaining in solution at the end of the experimental period.

5.2.1 BSA Adsorption Studies

BSA had limited coverage over AgNPs at low concentrations ranging from 1 to 10
ppm BSA, resulting in a linearly increasing percent of AgNPs remaining in solution at the
end of the 3 hr experimental periods. The steep slope over this low concentration range of
BSA indicates that saturation of the AgNP surface with BSA occurred rapidly and reduced
the adsorption of AgNPs to cells. As the concentration of BSA increased, it is likely that the
AgNPs became more densely coated with BSA and the magnitude of the AgNP surface charge was reduced.

Consequently, the rate at which BSA adsorbed to the surface of the AgNPs was reduced, as indicated by the reduced slope in Figure 3.28. The increase in BSA concentration above 10 ppm BSA did not result in an increase in percent AgNPs in solution above 66% ± 4%, indicating that a significant amount of AgNPs adsorbed to cells despite the reported stabilizing properties of the protein (MacCuspie 2011; Ravindran et al. 2010). This suggests that even though BSA acts as a stabilizing agent to AgNP dissolution, its capacity to reduce the adsorption of AgNPs to cells is limited. This is likely the result of disproportionately greater affinity of AgNPs for certain cell components relative to the stabilizing mechanism provided by BSA.

The isoelectric point, the pH at which a protein carries no net charge, of BSA is 4.7 (Watanabe et al. 1988). Therefore, at pH 7.8 the BSA molecules have a significant number of negatively charged functional groups, which have an attractive coulombic interaction with AgNPs that generate a BSA-AgNP complex held together by van der Waals and electrostatic forces (Liu et al. 2009). Even though these charged functional groups are considered weakly nucleophile (Ravindran et al. 2010), the BSA-AgNP complexes stabilized the AgNPs enough to reduce their adsorption over the 3 hr experimental period in the presence of a high concentration of cells (50 mg L⁻¹) that otherwise result in rapid adsorption (Figure 3.25).

The mechanism by which BSA acted to stabilize the AgNPs and reduced their adsorption to cells is likely steric hinderance (Figure 3.38), as the increase in MgSO₄ concentration did not result in adsorption of AgNPs to cells greater than that reported in the adsorption studies (Figure 3.28). As AgNP-BSA complexes formed, the net negative charge held by BSA functional groups at a pH above the isoelectric point was reduced and steric hindrances accounted for a larger percent of repulsion. It is likely that BSA retained some of its electrostatic stabilizing properties (MacCuspie 2011), and for this reason can be considered slightly electrosteric. However, the main stabilizing mechanism appears to have been steric hindrances as neither aggregation nor adsorption were observed as shown in the graphs presented in Figures A.20-A.22 in the Appendix.


5.2.2 Alginate Adsorption Studies

Alginate most likely substituted weekly-bound citrate as a capping agent on the AgNP surface similar to BSA, resulting in an AgNP-alginate complex with enhanced stability (Liu et al. 2009; Ghaseminezhad, Hamedi, and Shojaosadati 2012; Sangeetha et al. 2012). The interaction of alginate with the AgNP surface resulted in a slight broadening of the SPR absorbance spectrum as depicted by a red-shift in $\lambda_{\text{max}}$ from 400 to 408 nm (Figure 3.30), as well as an increase in FWHM values above the conditions without alginate present (Figure 3.33).

Alginate had limited surface coverage of AgNPs up to 400 ppm alginate, as indicated by the linearly increasing percent of AgNPs remaining in solution at the end of each experimental period (Figure 3.31). The concentration at which saturation of the AgNP surface with alginate occurred (800 ppm) was significantly higher relative to BSA (10 ppm) and SRHA (15 ppm). The gradual increase in the percent of AgNPs remaining in solution with alginate concentration indicates that alginate interacted weakly with the AgNP surface, which is likely due to a limited number of binding sites for AgNPs per molecule of alginate. Similar to BSA, the maximum amount of AgNPs that remained in solution at the saturation concentration of alginate was approximately 65% ± 7%, suggesting that a significant amount of adsorption still occurred.

The mechanism by which alginate stabilized the AgNPs initially appeared to be steric hindrances, as an increase in MgSO$_4$ concentration did not result in increased adsorption of AgNPs to cells (Figure 3.39). Furthermore, the percent of AgNPs in solution across the range of MgSO$_4$ concentrations correlated well with the percent of AgNPs adsorbed to cells once the AgNP surface was saturated with alginate (Figure 3.32). However, AgNP aggregation was observed for AgNPs at MgSO$_4$ concentrations of 1.2 mM to 2.4 mM, suggesting that alginate also imparts stabilization through electrostatic repulsions as shown in Figures A.23-A.25 in the Appendix.

Polyelectrolytes with large molecular weights, such as alginate, can endow both electrostatic and steric repulsion (electrosteric repulsion) that is stronger than either electrostatic repulsion or steric repulsion alone (Phenrat et al. 2008). The resultant electrosteric repulsive forces are less sensitive to changes in ionic strength or pH than electrostatic repulsions that would occur using bare NPs (Li et al. 2010). It is therefore
concluded that electrosteric repulsion was the primary mechanism through which the alginate-AgNP complexes reduced adsorption.

### 5.2.3 SRHA Adsorption Studies

The concentration of SRHA required to reduce the adsorption of AgNPs to cells was similar to that of BSA, indicating that SRHA has a high affinity for AgNPs and likely forms complexes through substitution of the weakly bound citrate. The formation of the complexes is likely due to the presence of both oxygen rich functional groups such as carboxyl (COOH), carbonyl (C=O), hydroxyl (OH), and hydrophobic aliphatic and aromatic groups (Dubas and Pimpan 2008; Bae, Nam, and Park 2002). Adsorption saturation was achieved for concentrations of 5 ppm SRHA and greater.

Similar to the studies conducted with BSA and alginate, at the adsorption saturation point approximately 66% ± 1% of the initial amount of AgNPs were detected in solution, indicating that a significant portion of the AgNPs adsorbed to the cells. AgNP adsorption was quickly reduced at low concentrations of SRHA, as indicated by the steep slope in Figure 3.36. In addition, the adsorption rate was highest within the first 30 min of the experimental period and gradually leveled off along nonlinear regression similar to the studies conducted with BSA and alginate. Nevertheless, the slightly positive slope of the rate curves suggests that adsorption may eventually go to completion over a longer experimental period.

Systematic studies have demonstrated that adsorbed humic acids can have a profound effect on the stability of colloidal particles, and can stabilize NPs via steric or electrostatic repulsion (Chen, Mylon, and Elimelech 2006). Based on the results presented in Figure 3.40, the enhanced stability afforded by the SRHA appeared to be primarily electrostatic repulsive forces, given that an increase in MgSO$_4$ concentrations resulted in a decrease in the percent of AgNPs remaining in solution at the end of each experimental period. Unlike BSA and alginate, a significant amount of aggregation was observed at MgSO$_4$ concentrations of 1.2 and above as shown in Figures A.26-A.28 in the Appendix, complicating the differentiation between whether adsorption or aggregation was the predominant mechanism behind the reduction in the AgNPs concentration within the batch bioreactors. For example, as AgNPs aggregate and increase in size they become increasingly susceptible to the centrifugal forces.
required to remove the cells from suspension, and they may even fall out of solution depending on their size.

Consequently, the absence of AgNPs from a solution where significant aggregation has been observed may not always be the most effective technique for quantifying their adsorption to cells and caution must be taken during interpretation of the data. Nevertheless, the Control 2 and Treatment conditions for 0.35, 0.73, and 1.2 mM MgSO₄ presented in Figures A.26-A.28 in the Appendix show that the peak absorbance for the monodispersed AgNPs in the Treatment conditions is less than that for the Control 2 condition with the only difference being the presence/absence of cells. Therefore, we can conclude that increased adsorption of the AgNPs to cells occurred upon addition of MgSO₄ despite the simultaneous occurrence of AgNP aggregation. In addition, the increased amount of aggregation observed during the studies also indicates that electrostatic repulsive forces were the primary mechanism behind the enhanced stability afforded by SRHA.
CHAPTER 6
CONCLUSION

The 20 nm citrate capped AgNPs used in these studies were found to be highly susceptible to rapid dissolution in the presence of NH₃, resulting in their increased toxicity to *N. europaea*. The primary mechanism behind the observed dissolution is believed to be destabilization of the AgNP that likely occurred when NH₃ sequestered surface-sorbed Ag⁺ during the formation of silver amine complexes, mainly Ag(NH₃)₂⁺. Consequently, AgNPs entering WWTPs, or other highly eutrophic environments, have the potential to become more toxic to bacteria responsible for reducing nutrient loads to receiving water bodies that could ultimately result in discharge violations and/or large-scale plant failures. Furthermore these studies suggest a strong potential for AgNPs entering WWTPs to sorb onto biosolids during secondary treatment processes, only to potentially be released into the natural environment through the application of fertilizer. Nevertheless, the adsorption of the AgNPs to biosolids can be significantly reduced in the presence of surrogate wastewater constituents (BSA and alginate), indicating the potential for the enhanced mobility and eventual discharge of AgNPs into receiving water bodies.

The presence of relatively high concentrations of BSA (100 ppm) yielded unexpectedly large amounts of AgNP dissolution and subsequent toxicity to *N. europaea*, despite previous reports of its stabilizing properties (Liu et al. 2009; MacCuspie 2011; Ravindran et al. 2010) and high affinity for Ag⁺ (Ostermeyer 2012). BSA-induced dissolution has been observed in other studies (Ostermeyer 2012), and may be caused by the sequestration of Ag⁺ that exist on the AgNP surface in equilibrium with Ag⁰ and the subsequent destabilization of the AgNP complex. The high degree and relatively uniform amount of AgNP dissolution occurred independently of NH₃ concentrations, suggesting that the BSA concentration was a better predictor of AgNP dissolution. At lower concentrations (40 ppm), BSA appeared to coat the surface of the AgNPs and reduce their adsorption to *N. europaea* cells, thereby enhancing their potential mobility through engineered or natural systems. BSA imparted enhanced AgNP stability through electrosteric hindrances, which,
Unlike electrostatic repulsive forces alone, are generally more robust and resilient to changes in environmental conditions, such as pH, temperature, and conductivity (Phenrat et al. 2008; Li et al. 2010). The extent to which BSA protects the AgNPs is unknown, though positive slopes on the rate curves suggest the eventual acquiescence to adsorption.

Unlike BSA, the high concentration of alginate used in these studies did not result in uniform dissolution of the AgNPs. However, the protective coating afforded by the adherence of alginate to the surface of the AgNPs was compromised by NH$_3$ and is reflected by the enhanced dissolution of the AgNPs and the subsequent increase in their toxicity to N. europaea. When compared with BSA, a significantly higher concentration of alginate (800 ppm) was required to saturate the surface of the AgNPs and reduce their adsorption potential to cells, indicating that polysaccharides may, in general, interact weakly with the AgNPs surface. Nevertheless, alginate coatings are likely to be more resilient to changes in environmental conditions as they appear to impart electrosteric hindrances. This suggests that in engineered systems (i.e. WWTPs), where polysaccharides are more or less superfluous, there exists the potential for enhanced AgNP transport through these systems and subsequent deposition in receiving water bodies. In addition, the protective capacity endowed by alginate could explain why biofilms, which often possess extracellular polymeric substances, have been found to be more resilient to AgNPs than planktonic cells (Flores et al. 2013).

In regards to SRHA, it can be seen from these studies that NH$_3$ compromises the protective coating endowed by SRHA, resulting in the potential enhanced toxicity of the AgNPs should they enter highly eutrophic natural water bodies. Similar to BSA, a relatively low concentration (10 ppm) of SRHA was required to saturate the surface of the AgNPs and significantly reduce their adsorption to cells, indicating the potential for AgNPs entering water bodies with heavy organic compositions to have enhanced mobility. However, the electrostatic stabilization afforded by SRHA is quickly compromised in waters with high electrolyte concentrations (hard water), suggesting that the AgNPs would rapidly aggregate upon entrance to brackish estuarine systems and possibly accumulate in benthic sediments.
REFERENCES


APPENDIX

SUPPLEMENTAL MATERIAL
**AgNP Standard Curve**

A standard curve was created in order to quantify the concentration of 20 nm citrate capped AgNPs in solution during the dissolution, adsorption, and stabilization studies presented in Sections 3.3 through 3.5, respectively. The standard curve was created by dispersing AgNPs in DDI water for 15 min on a shaker table rotating at 250 rpm in a dark room set to 30°C. After the AgNPs were dispersed, 600 uL of sample were transferred into a 1-cm quartz cuvette and measured on the UV-vis spectrophotometer. All conditions were carried out in triplicate within 155 mL sealed glass bottles. The peak absorbance values for each condition within the triplicates were then averaged and plotted for the various AgNP concentrations, and the results are shown in Figure A.1. A linear regression analysis indicates a strong correlation between peak absorbance and AgNP concentration (R²=0.99), with an increase in AgNP concentration eliciting a proportional increase in peak absorbance. The equation of the line was used to quantify the concentration of AgNPs remaining in solution during experimental trials.

![Graph showing AgNP Standard Curve](image)

**Figure A.1.** Standard curve of UV-vis absorbance versus concentration of 20 nm citrate AgNPs in DDI water. Error bars represent 95% confidence interval.
**Adsorption Optimization Centrifuge Study**

The results from a series of experiments designed to determine the optimal centrifugal force and time are presented in Figures A.2 through A.5. The goal of the optimization study was to determine the amount of time and centrifugal force required to remove the most cells from solution while retaining the maximum amount of AgNPs in solution.

The experiments were carried out in 155 mL glass batch reactor. Aliquots from the 1,000 ppm AgNP stock solution were added to DDI water and dispersed by placing the batch reactors on a shaker table at 25 rpm in the dark at 30°C for at least 15 min. HEPES buffer (pH=7.8) was then added at 15X concentrate to a final concentration of 30 mM and the batch reactors were placed back on the shaker table for an additional 30 min. After 30 min, a 1 mL aliquot was collected from the batch reactor and placed into a 1.5 mL centrifuge tube and centrifuged over a range of forces for either 1 or 2 min. At the end of the centrifuge cycle, 600 uL of sample was removed and placed into clean 1.5 mL microcentrifuge tubes and then transferred to a 1-cm quartz cuvette and measured on the UV-vis. This process was repeated for all samples, and batch reactors were run in triplicate. The average absorbance values of the triplicates are depicted in Figures A.2 through A.5.

The absorbance spectra for a series of experiments conducted with 1 ppm AgNPs in HEPES buffer (pH=7.8) subjected to varying centrifugal forces for 1 min is shown in Figure A.2. Similarly, Figure A.3 shows the absorbance spectra for 1 ppm of 20 nm AgNPs subjected to varying centrifugal forces for 2 min. The centrifugal forces chosen for Figure A.3 were based on the results from Figure A.2. For comparison, the absorbance spectra of cells subjected to the same centrifugal forces as Figures A.2 and A.3 are shown in Figures A.4 and A.5.

Based on the results from Figures A.2 through A.5, it was decided that an RCF of 17,000g at 1 min would remove the maximum amount of cells from solution while retaining the maximum amount of AgNPs. These conditions were subsequently applied to experiments described in Sections 3.2 through 3.4.
Figure A.2. UV-vis absorbance spectrum for 1 ppm 20 nm citrate AgNPs in 30 mM HEPES buffer (pH=7.8) subjected to various centrifugal forces for 1 min. Control samples were not centrifuged.

Figure A.3. UV-vis absorbance spectrum for 1 ppm 20 nm citrate AgNPs in 30 mM HEPES buffer (pH=7.8) subjected to various centrifugal forces for 2 min. Control samples were not centrifuged.
Figure A.4. UV-vis absorbance spectra for 50 mg L$^{-1}$ cells in 30 mM HEPES buffer (pH=7.8) subjected to various centrifugal forces for 1 min. Control samples were not centrifuged.

Figure A.5. UV-vis absorbance spectra for 50 mg L$^{-1}$ cells in 30 mM HEPES buffer (pH=7.8) subjected to various centrifugal forces for 2 min. Control samples were not centrifuged.
Figure A.6. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8) (A), 0.04 mM NH$_3$ (B), 0.08 mM NH$_3$ (C), 0.16 mM NH$_3$ (D), 0.3 mM NH$_3$ (E), and 0.8 mM NH$_3$ (F).
Figure A.7. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), and 1.1 mM NH₃ (G), and 1.1 mM NH₃ (H).
Figure A.8. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8) (A), 100 ppm BSA (B), and 0.04 mM NH$_3$ (C), 0.08 mM NH$_3$ (D), 0.16 mM NH$_3$ (E), and 0.3 mM NH$_3$ (F).
Figure A.9. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 100 ppm BSA, 0.8 mM NH$_3$ (G), and 0.16 mM NH$_3$ (H).
Figure A.10. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8) (A), 600 ppm alginate (B), and 0.04 mM NH$_3$ (C), 0.08 mM NH$_3$ (D), 0.16 mM NH$_3$ (E), and 0.3 mM NH$_3$ (F).
Figure A.11. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 600 ppm alginate, 0.8 mM NH$_3$ (G), and 0.16 mM NH$_3$ (H).
Figure A.12. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8) (A), 15 ppm SRHA (B), and 0.04 mM NH$_3$ (C), 0.08 mM NH$_3$ (D), 0.16 mM NH$_3$ (E), and 0.3 mM NH$_3$ (F).
Figure A.13. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA, 0.8 mM NH$_3$ (G), and 0.16 mM NH$_3$ (H).
Figure A.14. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8) (A), 50 mg L$^{-1}$ (B) cells, and 2.5 ppm BSA (C), 5 ppm BSA (D), 10 ppm BSA (E), 25 ppm BSA (F).
Figure A.15. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 50 mg L\(^{-1}\) cells, and 50 ppm BSA (G). An additional control condition containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), and 50 ppm BSA is shown in (H).
Figure A.16. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8) (A), 50 mg L⁻¹ (B) cells, and 25 ppm alginate (C), 50 ppm alginate (D), 100 ppm alginate (E), 200 ppm alginate (F).
Figure A.17. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 50 mg L$^{-1}$ cells, and 400 ppm alginate (G), 800 ppm alginate (H), 1,000 ppm alginate (I), and 1,200 ppm alginate (J); control condition containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), and 1,000 ppm BSA (K).
Figure A.18. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8) (A), 50 mg L\(^{-1}\) (B) cells, and 1 ppm SRHA (C), 2.5 ppm SRHA (D), 5 ppm SRHA (E), 10 ppm SRHA (F).
Figure A.19. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 50 mg L\(^{-1}\) cells, 15 ppm SRHA (G), and 25 ppm SRHA (H). An additional control condition containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), and 15 ppm SRHA is shown in (I).
Figure A.20. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 40 ppm BSA (Top Row) and 0.35 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.
Figure A.21. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 40ppm BSA, and 0.73 mM MgSO$_4$ (Top Row) and 1.2 mM MgSO$_4$ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.
Figure A.22. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 40 ppm BSA, and 1.8 mM MgSO₄ (Top Row) and 2.4 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.
Figure A.23. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 1,000 ppm alginate (Top Row) and 0.35 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.
Figure A.24. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 1,000 ppm alginate, and 0.73 mM MgSO\textsubscript{4} (Top Row) and 1.2 mM MgSO\textsubscript{4} (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.
Figure A.25. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 1,000 ppm alginate, and 1.8 mM MgSO$_4$ (Top Row) and 2.4 mM MgSO$_4$ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.
Figure A.26. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA (Top Row) and 0.35 mM MgSO₄ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.
Figure A.27. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA, and 0.73 mM MgSO\(_4\) (Top Row) and 1.2 mM MgSO\(_4\) (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.
Figure A.28. UV-vis absorbance spectra for batch reactors containing 1 ppm AgNPs, 30 mM HEPES buffer (pH=7.8), 15 ppm SRHA, and 1.8 mM MgSO$_4$ (Top Row) and 2.4 mM MgSO$_4$ (Bottom Row). Column 1 - No cells or centrifugation. Column 2 - No cells with centrifugation. Column 3 – Cells and centrifugation.
Table A.1. Percent Adsorption of 1 ppm AgNPs to Cells over a 3 hr Experimental Period in Batch Bioreactors Containing Test Media Consisting of 30 mM HEPES Buffer (pH=7.8), 50 mg L\(^{-1}\) Cells, and Varying Concentrations of BSA. 95% Confidence Intervals are Indicated as ± #%

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Table A.2. Percent Adsorption of 1 ppm AgNPs to Cells over a 3 hr Experimental Period in Batch Bioreactors Containing Test Media Consisting of 30 mM HEPES Buffer (pH=7.8), 50 mg L\(^{-1}\) Cells, and Varying Concentrations of Alginate. 95% Confidence Intervals are Indicated as ± #%

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<td>77% ± 5%</td>
<td>75% ± 5%</td>
<td>66% ± 1%</td>
<td>49% ± 6%</td>
<td>40% ± 13%</td>
<td>43% ± 4%</td>
<td>34% ± 7%</td>
<td>31% ± 2%</td>
<td>26% ± 2%</td>
</tr>
<tr>
<td>180</td>
<td>91% ± 1%</td>
<td>88% ± 4%</td>
<td>77% ± 8%</td>
<td>78% ± 3%</td>
<td>53% ± 4%</td>
<td>50% ± 5%</td>
<td>44% ± 3%</td>
<td>34% ± 3%</td>
<td>36% ± 8%</td>
<td>27% ± 3%</td>
</tr>
</tbody>
</table>
Table A.3. Percent Adsorption of 1 ppm AgNPs to Cells over a 3 hr Experimental Period in Batch Bioreactors Containing Test Media Consisting of 30 mM HEPES Buffer (pH=7.8), 50 mg L⁻¹ Cells, and Varying Concentrations of SRHA. 95% Confidence Intervals are Indicated as ± #%.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>No SRHA</th>
<th>1 ppm SRHA</th>
<th>2.5 ppm SRHA</th>
<th>5 ppm SRHA</th>
<th>10 ppm SRHA</th>
<th>15 ppm SRHA</th>
<th>25 ppm SRHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>45</td>
<td>43% ± 6%</td>
<td>29% ± 3%</td>
<td>23% ± 6%</td>
<td>16% ± 2%</td>
<td>5% ± 2%</td>
<td>16% ± 3%</td>
<td>12% ± 5%</td>
</tr>
<tr>
<td>90</td>
<td>63% ± 7%</td>
<td>38% ± 2%</td>
<td>32% ± 7%</td>
<td>22% ± 2%</td>
<td>7% ± 6%</td>
<td>14% ± 6%</td>
<td>26% ± 1%</td>
</tr>
<tr>
<td>135</td>
<td>77% ± 4%</td>
<td>47% ± 6%</td>
<td>43% ± 5%</td>
<td>27% ± 9%</td>
<td>14% ± 4%</td>
<td>24% ± 3%</td>
<td>28% ± 0%</td>
</tr>
<tr>
<td>180</td>
<td>86% ± 1%</td>
<td>56% ± 6%</td>
<td>51% ± 1%</td>
<td>35% ± 3%</td>
<td>10% ± 5%</td>
<td>33% ± 2%</td>
<td>34% ± 3%</td>
</tr>
</tbody>
</table>