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Engineering Silver Nanoparticles: Towards a Tunable Antimicrobial

by

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ABSTRACT

Engineering Silver Nanoparticles: Towards a Tunable Antimicrobial

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Hema Lakshmi Puppala

Overwhelming production of commercially available products containing silver nanoparticles (AgNPs) underscores the studies determining their fate in the environment. In order to regulate the use, assess the environmental impact and develop eco-responsible silver products, models that can predict AgNP toxicity based on physicochemical properties are vital. With that vision, this thesis developed well-characterized model libraries of uniform AgNPs stabilized with oleate in the range of 2-45 nm diameter with variable surface coating and investigated the dissolution properties that link AgNP structure to antimicrobial activity. High temperature organic synthesis allowed controlled growth of AgNPs (σ<15%) by an Ostwald ripening mechanism in the first few hours, and followed by size dependent growth rates yielding uniform nanocrystals. Characterization of these materials revealed a crystalline nature, bidentate binding mode of oleate and non-oxidized pristine silver surface. Phase transfer of these AgNPs from organics to water was facilitated by encapsulation and ligand exchange methods using amphiphilic polymers and methoxy poly (ethylene glycol) (mPEGSH) respectively. Among these surface coatings, steric stabilization by mPEGSH not only helped retain their optical properties but also reduced the dissolution (<1(w/w)%) of AgNPs. This
enhanced the stability in various environmentally relevant high ionic strength media (such as Hoaglands, EPA hard water and OECD medium), thereby increasing the shelf life. In addition, size, surface coating, pH of the medium and grafting density of the polymer mediated the dissolution of AgNPs. For instance, the rate of dissolution was decreased by 40% when the polymer coating possessed a mushroom conformation and increased with reducing core size. Analogous to dissolution, physicochemical properties also influenced the antimicrobial activity which were studied by minimum inhibitory concentration (MIC) and bactericidal efficacy assays. For example, surface passivation with mPEGSH prevented the oxidation of active silver atoms on the surface, and resulted in reduced toxicity against *E. coli*. Moreover citrate stabilized AgNPs when surface modified with mPEGSH had reduced toxicity, which was correlated with residual Ag$^+$ in AgNP solution. Therefore this study demonstrates that processes in the environment that increase stability of AgNPs could make them more persistent due to low dissolution.

Furthermore, the size and surface chemistry effects of AgNPs studied here make the intrinsic antimicrobial property of silver tunable and hence more versatile. This work also served as a material support for research on investigating toxicity of AgNPs to *C. elegans, Daphnia Magna, Populus* and *Arabidopsis*. In the future, this data will be used to develop nanomaterial bioavailability & environmental exposure (nanoBEE) models that predict the environmental impact of AgNPs.
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<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>3-MPA</td>
<td>3-mercapto propanoic acid</td>
</tr>
<tr>
<td>11-MUA</td>
<td>11-mercaptoundacanoic acid</td>
</tr>
<tr>
<td>AgClO₄</td>
<td>Silver perchlorate</td>
</tr>
<tr>
<td>AgNP</td>
<td>Silver Nanoparticle</td>
</tr>
<tr>
<td>AgNPs</td>
<td>Silver Nanoparticles</td>
</tr>
<tr>
<td>AgCl</td>
<td>Silver chloride</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>Silver Nitrate</td>
</tr>
<tr>
<td>Ag₂S</td>
<td>silver sulfide</td>
</tr>
<tr>
<td>ABNC</td>
<td>active but nonculturable</td>
</tr>
<tr>
<td>AgI</td>
<td>silver iodide</td>
</tr>
<tr>
<td>AgNP-OA</td>
<td>silver nanoparticles stabilized by Oleate</td>
</tr>
<tr>
<td>AgNP-mPEGSH</td>
<td>silver nanoparticles stabilized by mPEGSH</td>
</tr>
<tr>
<td>AgNP-Citrate</td>
<td>silver nanoparticle stabilized by citrate</td>
</tr>
<tr>
<td>AgNP-Tannic</td>
<td>silver nanoparticle stabilized by tannic acid</td>
</tr>
<tr>
<td><em>C. elegans</em></td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CFC</td>
<td>Chlorofluorocarbons</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium dichloride</td>
</tr>
<tr>
<td>CH₃COOAg</td>
<td>silver acetate</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DOM</td>
<td>Dissolved oxygen matter</td>
</tr>
</tbody>
</table>
DOC Dissolved organic carbon
DLS Dynamic light scattering
DMEM Dulbecco’s modified eagle’s medium
E. coli Escherichia coli
EPA Environmental Protection Agency
EDS Energy dispersive Spectrometer
FIFRA Federal Insecticide, Fungicide, and Rodenticide Act
FC Flow Cytometry
FIFF-ICPMS Flow Field Fraction and Inductively Coupled Plasma Spectrometry
GI gastro intestinal
GA Gum Arabic
HFC hexafluorocarbon
HCH Hexachlorocyclohexane
h hour
ICP-MS Inductively coupled plasma mass spectroscopy
ICP-AES Inductively coupled plasma-atomic emission spectroscopy
LC50 lethal concentration where 50% of the cells viability is observed
LB Luria Broth
MHW Moderately Hard water
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHB</td>
<td>Muller Hinton Broth</td>
</tr>
<tr>
<td>mPEGSH</td>
<td>methoxy poly (ethylene glycol)</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum Inhibitory concentration</td>
</tr>
<tr>
<td>N$_2$H$_4$</td>
<td>hydrazine</td>
</tr>
<tr>
<td>nanoBEE</td>
<td>Nanomaterial Bioavailability &amp; Environmental Exposure</td>
</tr>
<tr>
<td>NPIRS</td>
<td>National Pesticide Information Retrieval System</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>NOM</td>
<td>Natural Organic Matter</td>
</tr>
<tr>
<td>NaBH$_4$</td>
<td>Sodium borohydride</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>ND</td>
<td>not determined</td>
</tr>
<tr>
<td>NA</td>
<td>not applicable</td>
</tr>
<tr>
<td>OA</td>
<td>Oleic acid</td>
</tr>
<tr>
<td>OAmine</td>
<td>Oleylamine</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ODE</td>
<td>1-Octadecene</td>
</tr>
<tr>
<td>OECD</td>
<td>Organization for Economic Co-operation and Development</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
</tbody>
</table>
PAMPS-LA  Poly (2-acrylamido-2-methyl-1-propane sulfonic acid ) -co-lauryl acrylate
PVP       poly -(N—vinyl-2-pyrrolidine)
PAA-OA    Poly (acrylic acid -co-octylamine)
PAA       poly(acrylic acid)
PMAO-PEG  Poly (maleic anhydride-alt-1-octadecene)-graft-
          amine functionalized methoxy poly(ethylene glycol)
Se        selenium
SAS       triethoxysilylpropylsuccinic anhydride
SEM       Scanning electron microscopy
STP       sewage treatment plant
TEM       Transmission Electron microscopy
TWT       Thermal waste treatment
TGA       Thermogravimetric analysis
UV-Vis    Ultraviolet visible spectroscopy
XRD       X-ray diffraction
XPS       X-ray photoelectron spectroscopy
ZOI       Zone of Inhibition
Chapter 1

Introduction

Materials at nanoscale behave different from their bulk counterparts mainly due to their larger surface area, which chemically increases reactivity and may affect mechanical, optical and magnetic properties. Thus, the properties of materials can be tuned by decreasing the size of bulk material to nanodimensions. For example, at nanometer size Au and Ag have distinct optical properties, known since the early Roman era. Figure 1.1(A and B) depicts the picture of elegant Lycurgus Cup, made of ruby glass, which is glass embedded with colloidal gold and silver nanoparticles 5-60 nm in diameter. Due to the presence of nanoparticles the cup appears green in daylight and when lit from inside turns red. Further, nanomagnetite possesses distinct properties than its bulk counterpart, such as its ability to efficiently remove arsenic from water Figure 1.1(C and D). Nanotechnology has triggered advancements in various sciences and technologies including electronics, medicine,
aerospace and military to provide novel and smart materials that serve basic human needs. Pioneering research is in progress to bring safe and innovative nanomaterials to the consumer level.

![Image of Lycurgus Cup](image)

**Figure 1.1 – Unique properties of nanomaterials**

View of the Lycurgus Cup (British Museum; fourth century AD) (A) in reflected light, (B) when light is transmitted through the glass; Magnetite Nanoparticles for Arsenic removal (C) illustration of arsenic remediation from drinking water using magnetite, (D) TEM image of magnetite (average diameter 11.72 ± 1.03 nm) synthesized in Colvin Lab used for As removal.

One such area of research that gained immense importance is nanoparticle-
assisted sterilization, using primarily, silver nanoparticles (AgNPs). These are widely used in commercial products due to their disinfection properties. Currently, there are over 1300 commercial products containing AgNPs which include textiles, additives in personal care products, cosmetics (powdered colors, face powders bath and body products), dietary supplements, paints, food packaging, preservatives, kitchenware, washing machines, household water filters, and drug delivery devices. Figure 1.2. This extensive usage of AgNPs has raised concerns over their environmental and health impacts.

Figure 1.2 – Commercial products containing nanomaterials.

Figure reprinted from Maynard et al.6
Predicting models along with European Union experimental data estimate that 15% of the total silver released into environmental water can be attributed to the plastic and textile industries manufacturing silver goods.\textsuperscript{7} Figure 1.3 denotes various ways Ag impregnated biocidal plastics and textiles can enter the environment.\textsuperscript{8} First, silver released into waste water from biocidal products can either enter into natural waters directly or by sewage treatment plants (STP) through STP effluents. Sewage sludge from STP can distribute AgNPs in 3 different ways; first, disposed as solid waste; second, used in agricultural soils; and finally, incinerated in thermal waste treatment (TWT) plants. These materials can be easily taken up by organisms in soil and water, disrupting the bacterial activities essential to maintain the balance in ecological and biogeochemical cycles.
Figure 1.3 – Major routes of AgNPs entrance into the environment from biocidal plastics and textiles

Arrows denote silver flow; dashed arrows indicate different environmental compartments. Figure adapted from Blaser et al.⁹

In order to make the best use of AgNPs as an antimicrobial, it is necessary to understand their impact on the environment. Lessons learned from harmful chemical usage in the past forewarn us that lack of understanding of how a chemical interacts with the environment can have serious consequences. For example,
dichlorodiphenyltrichloroethane (DDT), an insecticide was banned due to its persistence and bioaccumulation in the food web; Similarly, γ-hexachlorocyclohexane (γ-HCH) commonly known as Lindane, a widely used pesticide was labeled as a neurotoxic and bioaccumulative, was withdrawn by EPA in 2007 after 30 years of review, Further, chlorofluorocarbon (CFC) a widely used refrigerant was banned as it contributed to ozone depletion and was replaced by hydrofluorocarbon (HFC), hydrocarbons and carbon dioxide (CO₂). In this thesis to develop safer AgNP and to understand its impact on the environment, AgNPs coated with methoxy poly (ethylene glycol) (mPEGSH) (AgNP-mPEGSH) were synthesized and its physicochemical properties and toxicity were investigated.

**Scope and objective of the work**

In order to assess and control the risk of AgNPs in the environment it is essential to gather more scientific data based on which nanomaterial exposure models can be developed. To envision this, the Consortium for Manufactured Nanomaterial Bioavailability & Environmental Exposure (nanoBEE) was formed. The work presented in this thesis is a part of the joint US-UK Research Program: Environmental Behavior, Bioavailability and Effects of Manufactured Nanomaterials (EPA-G2008-STAR-RI). As a part of achieving this goal versatile, highly uniform model libraries of engineered AgNPs with defined size and surface chemistry, were produced in this project.
To achieve this the current research priority is to understand the physicochemical properties of AgNPs and predict their transformations in the environment. Existing toxicology studies on AgNPs are based on commercial materials, which lack characterization and are prone to aggregation. These materials have limited value as a biocide and often fail to answer the critical questions regarding the environmental fate. In a broader perspective, some aspects to be addressed are environmental modifications of manufactured nanoparticle such as chemical and structural changes and their effect on physicochemical properties. Models that predict toxicology and inadvertent environmental impacts of AgNPs based on experimental data need to develop. Furthermore, this scientific data would guide policy makers to regulate commercial goods containing AgNPs and help make risk management decisions.

The overall mission of this thesis is to evaluate basic principles of AgNP chemistry and understand the properties that enhance or diminish its toxicity. Chapter 2 gives an overview of literature on AgNPs toxicology, current state of knowledge and detailed discussion on environmental impact. This forms a baseline for the research project and emphasizes the objectives. Chapter 3 is a description on materials and methods used in this study. Further, the specific aims will be discussed in the following order:

- First objective of this project is described in Chapter 4 which focuses on the development of strategies to produce well-characterized, highly uniform
libraries of AgNPs in the range of 2 - 45 nm, optimization of reaction parameters to scale up the synthesis and AgNP growth mechanism

- Second objective discussed in chapter 5 involves further investigation of phase transfer methods that transfer uniform AgNPs from organic to aqueous phases while retaining the optical properties and controlling the dissolution using size and surface chemical principles.

- Third objective described in Chapter 6 comprises of a biological evaluation of AgNPs to assess varying release profiles and disinfectant properties of engineered AgNPs made in-lab and comparison of bactericidal efficacy with the generic silver currently available in the market.

- Finally, Chapter 7 spotlights on implications of the scientific findings discussed in this thesis; a broader perspective of nanobiototoxicology and the environmental impact of AgNPs.
Chapter 2

Silver Nanoparticles – Background

AgNPs exhibit unique optical and antimicrobial properties. There is a long-standing history of silver usage in medicine that dates back to 400 B.C. when Hippocrates, the “Father of Medicine” described the disinfection properties of silver and suggested it to treat ulcers and preserve water. With the advent of antibiotics, silver usage in medicine has declined. Now as bacteria are developing resistance to antibiotics, history is repeating itself and researchers are finding ways to incorporate silver back into medicinal products, either to boost the performance of current antibiotics or as novel antimicrobial products with silver nanotechnology. This chapter provides summary on the current knowledge of AgNP chemistry, details on major developments, environmental impacts and research priorities that preceded this thesis.
2.1. AgNPs: Commercial use

Biocides are substances that can prevent the growth of harmful organisms. Specifically antimicrobial biocides are used to inhibit the growth of bacteria. These can be natural (derived from bacterium and plants) or synthetic (organic or inorganic chemicals). Synthetic biocides are especially used for preserving food and water which dates back to 19th century, with the introduction of antiseptics and the use of chlorine for water disinfection. Later, quaternary ammonium compounds, phenols, aldehydes and biguanides became popular. Inorganic active chemicals mainly constitute of metals such as copper and silver.

According to 2010 database the worldwide production of AgNPs is estimated about 320 tons/year. Specifically, silver has been encountered daily in various different ways such as in algicide for swimming pools. To serve the purpose as antimicrobial agents in a variety of applications, different forms of silver i.e., ionic and zero valent (nanoscale) are impregnated in variety of matrices such as zeolite, glass matrix, and plastics that can control the periodic release of silver ions. Often, silver compounds (especially AgCl and AgNO₃) are directly used. The production of AgNPs dates back to 1889, with the first report on the synthesis of citrate coated silver in the range of 7-9 nm which is similar to the recent reports by Henglein et al.

Due to the extensive usage of AgNPs, the production rate of these materials is exponentially increasing. Statistics from 2006 -2011 reveal a dramatic increase
(~30 times) in silver containing commercial products over other types of nanomaterials.\textsuperscript{18} According to NPIRS (National Pesticide Information Retrieval System (http://ppis.ceris.purdue.edu/npublic.htm) database, in the last 60 years, among EPA registered biocidal silver products, about 53\% are confirmed to have AgNPs, but only 7\% are labeled to contain nanoparticles. The first silver based product approved in U.S.A under FIFRA (Federal Insecticide, Fungicide, and Rodenticide Act) \textsuperscript{19} was the gelatin coated AgNPs (average diameter 2-20 nm) with trade name Algaedyn, an algaecide for swimming pool that has been in use since 1954.

Figure 2.1 – Characterization of AgNPs in commercial products;

TEM analysis of EPA registered silver based products A) AgNPs in carbon-filter, Zodiac Nature2G; brighter spots indicate AgNPs; (B) AgNPs in algicide Algaedyn (8 nm). Figure adapted from Bernd Nowack et al. \textsuperscript{21}
Mainly, EPA-registered biocidal silver can be classified into 3 categories; 1) Silver biocidal additives used in plastic and textile applications (e.g., additive SSB, MicroSilver BG-R, HyGate 4000), 2) Silver impregnated water filters, registered as NATURE2 G45-VC40 Figure 2.1 3) silver algicides and disinfectants (e.g.; Nu-Clo Silvercide, Algaedyn, ASAP-AGX). 20

Furthermore, silver colloids have been used in medication for the last 100 years. In 1884, the first medical use of silver as 1% AgNO₃ eye drops to prevent gonococcal eye infection was reported by C.S.F. Crede. 22 Historically, AgNPs were also used to treat syphilis and other bacterial infections. 23 The first silver based commercial material named Collargol (mean diameter – 10 nm), was produced in 1897 and used for medical purposes. 20, 24 Later, AgNPs loaded carbon (<25 nm) 25, silver colloids with trade names Argyrol and Protargol emerged in the market. A topical cream containing silver sulfadiazine was used in wound dressings and burn treatments. These are still available in the market with trade name Acticoat™. 26, 27 Further, silver impregnated in medical devices such as cardiovascular implants, central venous, 28, neurosurgical catheters 29 and as an additive in bone cement 30 are currently used to prevent biofilm growth as well as catheter related bacterial infections.

The rate of release of silver ions from the above mentioned commercial products could be tuned depending on the form of silver such as size, shape, surface coating, temperature and pH of the matrix. For example, the release rates of ionic silver containing matrices are faster than molecular silver, whereas the latter have
longer life times.\textsuperscript{16} The mechanism of dissolution for AgNPs is more complicated than just ionic silver containing matrices. Moreover, between silver nitrate and silver sulfide, the potential for release would be greater for the former due to high solubility in water. The trends are shown in Figure 2.2. \textsuperscript{21}

![Figure 2.2 - Illustration of silver release capacity from silver containing commercial products](image)

Different forms of silver require different amounts and their release rates vary depending on the matrix. Figure adapted from Bernd Nowack et al. \textsuperscript{21}

**2.2. Biocidal action of AgNPs**

AgNPs are toxic to various organisms, yet the mechanism of biocidal action (particulate vs ionic) is still debatable. Proposed pathways of bactericidal action of silver whether particle or ionic mainly include 1) generating reactive oxygen species (ROS),\textsuperscript{31} 2) binding to the DNA bases and proteins thereby preventing bacterial
reproduction,32 3) disrupting respiratory mechanisms, denaturizing ribosomes, interrupting protein synthesis and promoting plasma membrane degradation33 and, 4) interacting with essential enzymes interrupting vital cellular process such as electron transport chain resulting in cell death. There are three ways of describing the AgNPs toxicity First, AgNP dissolution into ionic silver where residual Ag⁺ in AgNP stock solution causes toxicity. Secondly, by particle effect which can lethally cause irreparable physical damage to the organism from direct contact over time. Finally, by a Trojan-horse mechanism where particle undergoes delayed oxidation inside the cells to release Ag⁺ on site causing the toxicity.34 Previous studies supporting all the three approaches are shown in Figure 2.3 that demonstrates the difference in damaged E. coli membrane treated with silver nitrate and AgNP solution. TEM micrographs indicate the presence of AgNPs inside the membrane.35

![Figure 2.3](image.png)

**Figure 2.3 – TEM images show particulate vs ionic effect on E. coli**

(A) AgNPs inside the membrane (bright spots indicate AgNPs) (B) Elemental analysis of (A) displaying Ag distribution in the membrane (C) E. coli treated with AgNO₃. Figure adapted from Morones et al.35
In another study, the genome-wide library of \textit{E. coli} was exposed to AgNPs shows cell-surface antigen activity (lipopolysaccharides) as a nanoparticle specific response but not associated with ionic silver.\textsuperscript{36} To further understand the difference in silver particle and ion specific stress responses to bacteria, this section dwells into the details to develop an explanation derived from literature and previous experimental studies.

### 2.2.1. Ionic effect

By controlling the pH, ionic strength and surface coating AgNPs oxidize and release ionic Ag, that disrupts bacterial membrane permeability.\textsuperscript{37} The process of releasing ionic silver is termed dissolution. An increase in pH or high ionic strength media can increase the rate of dissolution as well as residual Ag\textsuperscript{+} concentration in AgNP stock solution Equation 2.1. Schematic representation of AgNP dissolution is shown in Figure 7.1

\[
4Ag(O) + O_2 \rightarrow 2Ag_2O \quad (a)
\]

\[
2Ag_2O + 4H^+ \rightarrow 4Ag^+ + 2H_2O \quad (b)
\]

**Equation 2.1 – Dissolution of AgNPs.**
Furthermore, a study of 90 min silver ion exposure to *E. coli* and *S. auereus* showed a decrease in the viability by more than 5 log<sub>10</sub> CFU/ml.<sup>37</sup> Here, reduction in growth rate was observed to be less in flow cytometry (FC) analysis when compared to conventional plate counting method. Authors addressed this observation as state of active but nonculturable (ABNC).

**Figure 2.4 – Schematic of dissolution of AgNPs**

Oxidation of AgNPs releases ionic silver

**Figure 2.5 – TEM analysis of Ag<sup>+</sup> treated *Staphylococcus aureus***

Scale bar is 100 nm for both (A) and (B); (A) untreated cells, black arrow indicates peptidoglycan layer while the white cytoplasmic membrane (B) cells treated with 0.2 ppm of Ag<sup>+</sup> solution. Figure adapted from Jung et al.<sup>37</sup>
In a different study by Schreurs and Rosenberg, Ag\textsuperscript{+} inhibited phosphate uptake and an efflux of phosphate, mannitol, succinate, glutamine and proline was observed.\textsuperscript{38} In both the reports, TEM micrographs showed the disruption of peptidoglycan cell wall and was proposed as the major reason for the cell death Figure 2.5. Recently, electrochemical technique was used to study the effect of silver ion inhibition of the respiratory chain in \textit{E. coli}.\textsuperscript{39} Hence, there is profound damage to cells by ionic silver, from AgNPs, or from pure ionic solution.

\subsection*{2.2.2. Particle specific effect}

Cellular toxicity by particulate effect is ascribed to penetration of Ag into the peptidoglycan cell wall because of its nanodimensions and causing the lysis of the cell membrane.\textsuperscript{32c, 35, 40} Generation of ROS and membrane damage are considered as particle specific action.\textsuperscript{32d, 41} Research indicates that bactericidal activity of AgNPs is not only size dependent but also varies with the surface chemistry. STEM images of the polymerized slices and elemental analysis using X-Ray Energy Dispersive Spectrometer (EDS) revealed the presence of particles (diameter 1-10 nm) were found in the interior of bacterial cell membrane Figure 7.1.\textsuperscript{35} Studies have indicated more evidence related to the disruption of respiratory chain dehydrogenases in \textit{E. coli}.\textsuperscript{42} In another study on AgNPs exposure to \textit{E. coli}, SEM images revealed formation of “pits” on the cell membrane. Figure 2.6 This change in morphology was explained by the damage to the cell membrane due to release of polysaccharide molecules from the membrane.\textsuperscript{43} In another experiment, AgNPs containing varying surface charges (from-mv to +mv) were exposed to \textit{bacillus} (-37 mV under test conditions).
It was observed that higher negative values showed less toxicity. As the magnitude of the negative charge decreased, the toxicity increased which was attributed to the electrostatic barrier between the particle and bacterial membrane surface. Hence, the larger the attraction forces, the greater its interaction between bacteria and particles, resulting in higher toxicity.44

![Figure 2.6 – Particle specific action of AgNPs](image)

**E. coli** Cells treated with 50 ppm of AgNPs in LB medium for 4h (A) SEM image reveal the formation of pits on the membrane (B) TEM image of enlarged view of the E. coli cell membrane indicate the presence of AgNPs, arrow marks point the membrane junction. Figure adapted from Sondi et al.43

In addition to the antibacterial activity, AgNPs also exhibited fungicidal and antiviral property. For instance, pathogenic *Candida* spp 45a (MIC varied from 0.21 – 1.69 mg/L of Ag), phytopathogenic fungi 45b and tacular virus. 46 The inhibition of the virus was highly effective when treated before infection or administered within first 2-4 hours of replication.46 One advantage of using AgNPs over Ag+ in medicinal field
is that $\text{Ag}^+$ becomes less bioactive in the blood stream or in the stomach due to the formation of AgCl and Ag$_3$PO$_4$ salts. On the other hand, AgNPs with the right biocompatible stabilizing agent should be able to sustain the biological condition and still release $\text{Ag}^+$ ions at the targeted site periodically.

### 2.3. Toxicology

The outbreak of Argyria, a bluish gray discoloration of the skin caused by excessive intake of silver called for more research to understand the long-term behavior of silver based products on humans. A 1935 case study on Argyria revealed that 63% of the cases were due to intake of Collargol and Argyrol (AgNPs forms) while the rest were due to the consumption of AgCl and AgI.$^{47}$ Another close evaluation on toxicology of ionic and AgNPs in 1939, revealed that the threshold for Argyria symptoms was 0.9 g of Ag intake over a whole lifetime. This correlates with the current drinking water standards of 100 $\mu$g/L of Ag.$^{48}$ In addition the limits vary for metallic and ionic silver.$^{49}$ For example, according to American Conference of Governmental Industry Hygienists, the limit for metallic silver is 0.1 mg/m$^3$ while for soluble silver compounds, it is 0.01mg/m$^3$.$^{50}$

To understand mechanistic details on skin discoloration in Argyria a recent report has elucidated experiments, where silver nitrate was exposed to UV-light and observed the formation of AgNPs. Authors proposed that argyrial deposits are mainly secondary particles formed $\textit{in vivo}$ due to photo reduction and accumulation of such particulate matter underneath the skin may which may lead to skin
Figure 2.7 – Suggested pathway for Argyrial deposit formation

Proposed mechanism for Argyrial deposits include gastric dissolution, ion uptake, thiol transport and photoreduction resulting in the formation of particulate silver and then exchange reactions by S/Se. Figure adapted from Liu et al. 51

AgNPs are not only toxic to humans but a significant environmental release could be toxic to aquatic and terrestrial organisms as well. 53 Numerous studies were done to understand the modes of AgNPs toxicity in various model organisms.
including *Daphnia magna*, Zebra fish and *C. elegans*, gives an overview of toxicology data for variety of organisms (taken from literature).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Surface coating</th>
<th>Diameter (nm)</th>
<th>Shape</th>
<th>Exposure Time</th>
<th>Nominal concentration / LC50</th>
<th>Major biological Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zebrafish</td>
<td>ND</td>
<td>10-20</td>
<td>Spherical</td>
<td>2-36 days</td>
<td>0.4-40 ppm</td>
<td>Defects in fin regeneration, p53 gene pathway altered, AgNPs penetrated into organelles and cell nucleus</td>
</tr>
<tr>
<td>Zebrafish embryo</td>
<td>ND</td>
<td>20-30</td>
<td>Spherical</td>
<td>72 h</td>
<td>10-20 ppt</td>
<td>AgNP aggregates found in skin and circulatory system</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>Sodium citrate</td>
<td>20-30</td>
<td>Spherical</td>
<td>48 h</td>
<td>7.07 mgL</td>
<td>Toxicity of AgNPs is different at different stages of growth development</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>No coating</td>
<td>3,10, 50, 100</td>
<td>Spherical</td>
<td>120h</td>
<td>3nm - 93μM 10 nm - 126μM</td>
<td>Toxicity and morphological changes are size dependent</td>
</tr>
<tr>
<td>Perch</td>
<td>PVP</td>
<td>81</td>
<td>Elliptical</td>
<td>2 days</td>
<td>50 nm - 127 μM 100 nm - 137 μM</td>
<td>AgNPs attached to gills, tolerance decreased for hypoxic conditions</td>
</tr>
<tr>
<td>Brown trout</td>
<td>Uncoated</td>
<td>10-35</td>
<td>Spherical</td>
<td>10 days</td>
<td>10-100μg/L</td>
<td>AgNPs concentrated in gills, liver and its uptake is size dependent</td>
</tr>
<tr>
<td><em>Daphnia Pulex</em></td>
<td>Sodium citrate</td>
<td>20-30</td>
<td>Spherical</td>
<td>48h</td>
<td>0.04mg/L</td>
<td>Ag(^+) showed high toxicity than AgNPs</td>
</tr>
<tr>
<td><em>Ceriodaphnia</em></td>
<td>Sodium citrate</td>
<td>20-30</td>
<td>Spherical</td>
<td>48h</td>
<td>0.067 mg/L</td>
<td>AgNPs showed high toxicity than Ag</td>
</tr>
<tr>
<td><em>Ceriodaphnia dubia</em></td>
<td>Metal oxide</td>
<td>20-30</td>
<td>Spherical</td>
<td>48h</td>
<td>0.46 mg/L</td>
<td>AgNPs toxicity decreased with increase in NOM</td>
</tr>
<tr>
<td>Organism</td>
<td>Surface coating</td>
<td>Diameter (nm)</td>
<td>Shape</td>
<td>Exposure Time</td>
<td>[Ag] / LC$_{50}$</td>
<td>Major biological Effect</td>
</tr>
<tr>
<td>----------------------------------</td>
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<td>------------------</td>
<td>-----------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><em>Pseudokirchneriella subcapitata</em></td>
<td>Sodium citrate</td>
<td>20-30</td>
<td>Spherical</td>
<td>96h</td>
<td>0.19 mg/L</td>
<td>Ag$^+$ showed high toxicity than AgNPs</td>
</tr>
<tr>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>Carbonate</td>
<td>25+13</td>
<td>Spherical</td>
<td>1-5h</td>
<td>1h-3300nM, 5h-829nM</td>
<td>Trojan - horse mechanism of AgNPs toxicity is observed</td>
</tr>
<tr>
<td><em>Thalassiosira weissflogii</em></td>
<td>PVP</td>
<td>60-70</td>
<td>ND</td>
<td>48h</td>
<td>0.02-0.0002nM</td>
<td>Cell growth was reduced due to toxicity of AgNPs through Trojan - horse route</td>
</tr>
<tr>
<td><em>Paramecium caudatum</em></td>
<td>Tween 80, PEG35000 or PVP 360</td>
<td>30-40</td>
<td>Spherical</td>
<td>1h</td>
<td>Tween NP-16mg/L</td>
<td>TWEEN 80 AgNPs showed increased toxicity</td>
</tr>
<tr>
<td><em>Caenorhabditis elegans</em></td>
<td>ND</td>
<td>14-20</td>
<td>ND</td>
<td>24-72h</td>
<td>0.005-0.5 mg/L</td>
<td>AgNPs accumulated near uterine and oxidative stress increased</td>
</tr>
<tr>
<td>Metplate-Bacterial Bioassay</td>
<td>Metal oxides</td>
<td>20-30</td>
<td>Spherical</td>
<td>1.5 h</td>
<td>47μg/L</td>
<td>Presence of organic matter decreased toxicity</td>
</tr>
<tr>
<td>Nitrifying Bacteria</td>
<td>PVA</td>
<td>15+9</td>
<td>Ellipsoidal and Spherical</td>
<td>18h</td>
<td>1mg/L</td>
<td>AgNPs inhibited respiration by 87% twice the magnitude of Ag$^+$</td>
</tr>
<tr>
<td>Soil bacterial community</td>
<td>ND</td>
<td>56+18.6</td>
<td>ND</td>
<td>30 days</td>
<td>0-1000μg/L</td>
<td>AgNPs accumulated in the top 3 mm, no effect on bacteria</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>Citrate</td>
<td>65+30</td>
<td>Spherical/Triangular</td>
<td>24h</td>
<td>0-200ppb</td>
<td>pH dependent toxicity but NOM decreased the toxicity</td>
</tr>
<tr>
<td><em>Pseudomonas putida biofilm</em></td>
<td>Citrate</td>
<td>65+30</td>
<td>Spherical/Triangular</td>
<td>24h</td>
<td>0-200ppb</td>
<td>NOM increased the uptake of AgNPs by biofilms but decreased their toxicity</td>
</tr>
</tbody>
</table>

Table 2.1 – Toxicity of AgNPs to aquatic organisms.
According to the recent reports oral LD\textsubscript{50} for rats is greater than 1600 \text{mgKg}^{-1}\text{d}^{-1}.\textsuperscript{50} Another study reported median LC\textsubscript{50} (mg/L) of various organisms - fish (1.36), crustaceans (0.01), algae (0.36) and mammalian cells (11.3) while the median MIC (mg/L) for bacteria was 7.1. This revealed an interesting fact that aquatic organisms are more sensitive to AgNPs than bacteria.\textsuperscript{71} As every organism reacts in a unique way with the stress response at different concentrations, it is necessary to understand the release of AgNPs into the environment and its implications.

### 2.4. Environmental impact of AgNPs

The very unique antimicrobial property of AgNPs that made them renowned may also potentially render them toxic towards environmental health and safety. This section provides an overview of the impact of AgNPs on the environment. In general, there are various different routes AgNPs can enter the environment, either through production or unintended processes. For instance, washing AgNP incorporated textiles, incinerating the silver waste products (plastics impregnated with Ag), improper disposal of electronic devices containing Ag, use of aerosol spray containing Ag, manufacturing either at laboratory scale for research or large scale production of commercial products containing Ag of which all of these processes release silver into the atmosphere.\textsuperscript{7,72}

A model of quantified Ag mass flow from silver based biocidal products and other Ag sources, except Ag incorporated plastics and textiles, is shown in Figure 2.8.\textsuperscript{8} In these calculations, mass of Ag from marine ecosystems, leachates and aerial
deposition is excluded. The numerical figures of silver release certainly are alarming as they indicate pollution of soil and ground water that poses delayed risk. For the model studies plastics, that come in contact with water were assumed to release Ag all the time while those in electronics and house hold items were supposed to be 1-4 days per year, via textiles and humans (through sweat) were considered to be 4-87 days per year.\(^8\)

Another study on plastics containing AgNPs in aqueous medium found that the release rates of silver were drastically high when embedded in Zeolites than in polyester and polycarbonates.\(^73\) An experimental study proved that AgNPs released to surface waters could vary from 0.5 to 2 ng/L. A number of review articles based on modeling data and few experimental articles predict an exponential increase in the release of silver to surface waters based on the wide consumption of silver based consumer goods.\(^14\),\(^74\) If the concentration levels exceed limit then there could be ecological risks.\(^75\)
Figure 2.8 – Model representing flow of Ag from Ag based commercial products (tonnes per year).

Numbers on arrows indicate the mass of silver released and number in paranthesis are from biocidal products. Thin arrows denote flow from biocidal plastics and textiles, dashed arrows stand for other Ag sources, thick arrows represent combined flow from biocidal and other Ag sources. Figure adapted from Blaser et al.8
2.4.1. Factors influencing AgNPs transport in the environment

The major driving forces of silver entrance to surface and groundwater's are through sorption and degradation processes. Water with increased organic carbon content increases the sorption of these molecules onto the surface of AgNPs. This in turn alters the properties of the soil surface chemistry. Surfactants mainly influence dissolution, solubility, stability, dissociation of AgNPs which has a major impact on the lifetime, transport, mobility, distribution and degradation processes and hence, the fate of the material. In order to understand the complex behavior of AgNPs, it is necessary to study their physicochemical behavior under realistic conditions. Common physicochemical transformations include, aggregation, dissolution, interaction with the microbes, biofilm, surface modifications, interaction with Natural Organic matter (NOM) Figure 2.9.

On the other hand AgNPs may acquire or lose surface coating by adsorption or desorption of surfactants respectively, or remain intact. This is solely based on the affinity of Ag towards the organic and inorganic ligands prevalent in the environment. For instance due to strong interaction of silver and sulfur most of the silver released into the environment has been found in the form of silver sulfide. In general, conditions that trigger dissolution increase Ag⁺ exposure to the organisms prevalent in that environment, which leads to high toxicity, whereas substances that increase stability of AgNPs increased their life time which can cause bioaccumulation. Furthermore, bioaccumulation also depends on the nature of the
environment, concentration of particles, size, shape, chemical composition, surface chemistry, exposure route and biology of the organism under study.\textsuperscript{61}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2_9}
\caption{Suggested environmental modifications of AgNPs}
\end{figure}

\textit{Figure adapted from Alvarez et al.\textsuperscript{79}}

Unlike the simple chemical molecules engineered AgNPs undergo various physicochemical transformations depending on the environmental conditions, which can reduce or aggravate the toxicity. For instance, studies by Jason M. Unrine indicated that PVP coating prevented AgNP oxidation by the plant derived dissolved oxygen matter (DOM) while gum arabic coating increased the dissolution \textsuperscript{80}. Also, a study on polyvinyl pyrrolidone (PVP) coated AgNPs in freshwater mesocosms simulating an emergent wetland environment, disclosed that AgNPs in terrestrial soils were modified to Ag\textsubscript{2}S (\textasciitilde52\%) and Ag-Sulfhydryl (\textasciitilde27\%) compounds\textsuperscript{81}. A
recent analysis on sewage sludge samples showed that silver released into water was mostly found as Ag₂S (1.3 - 4.4 mg/Kg).\textsuperscript{82} In addition, most parts of UK and US use sewage sludge as a fertilizer in agricultural soil.\textsuperscript{83} Hence developing methods to understand the behavior and properties of these materials in sewage sludge is crucial to avoid the long-term environmental risks.

### 2.4.2. Challenges involved in understanding real time behavior of AgNPs

To understand the behavior of AgNPs in the environment, it is necessary that we have capable scientific tools to characterize them. Some of them include analytical challenges to characterize the materials in environmental matrices that mimic the realistic conditions. For instance, \textit{in-situ} characterization of materials requires high sensitive instruments that can analyze low analyte concentrations such as Flow Field Fraction and Inductively Couple Plasma Spectrometry (FIFF-ICPMS).\textsuperscript{84} Of all the vital importance and crucial factor is to discern particulate and ionic silver toxicity. For that there are limitations in the analytical techniques associated with separating residual ions from AgNP stock solutions. Current methods in literature include membrane filtration and plasmon resonance tracking method.\textsuperscript{85} Often harsh conditions of the sample matrix degrade the membrane and protein adsorption decreases the recovery efficiency. Hence, membrane compatibility is a huge drawback of this technique. Although optical spectra of AgNPs give an estimate of dissolved silver, sensitive nature of surface plasmon to aggregation and change in refractive index of the solvent might influence the values. Hence there is still a need to optimize the analytical techniques to separate the
dissolved silver from AgNP solutions. Silver release experiments of products incorporated with AgNPs would help build accurate silver release models. In a broader perspective, some logical questions that need to be addressed are its effect on dissolution kinetics on transport, differences in the uptake rates of ionic and particulate matter under same environment for a specific species and finally variability in the rate constants between different species, when distinct sizes, shapes, surface coating of the AgNPs were used.  

2.5. Conclusion

Silver in ionic and particulate form is found to be toxic to aquatic and terrestrial organisms. Furthermore, excessive intake of silver colloids by humans is likely to cause Argyria. Core size, surface coating and the sample matrix mediate toxicity of AgNPs. Considering the heavy usage of AgNPs, there is a constant need to test the AgNPs, and its toxicity in a case-by-case method. Therefore correct labeling of commercial products containing AgNPs would benefit both consumers and researchers for risk assessment. Although AgNP release models were developed there are still uncertainties in emission scenarios and biodistribution. Substantial data on research gaps associated with toxicity, biological transformations and control over antimicrobial properties with highly accurate release models of AgNPs, would help design environmentally benevolent silver nanotechnology that consumers can trust.
2.6. References


18. http://www.nanotechproject.org/cpi/search-products/?title=&asmSelect0=&date_created=&date_modified=&nanomaterials%5B%5D=1148&search-products_submit=Search&submitKey=15%3Asearch-products%3A0. In *Consumer product inventory*.


47. Gaul, L.; Staud, A. H., Clinical spectroscopy: Seventy cases of generalized argyrosis following organic and colloidal silver medication, including a biospectrometric analysis of ten cases. *Journal of the American Medical Association* **1935**, *104* (16), 1387-1390.


71. Olesja Bondarenko, K. J., Angela Ivask, Kaja Kasemets, Monika Mortimer, Anne Kahru, Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review. *Archives of Toxicology* **2013**, *87* (7), 1181-1200.


74. (a) Luoma, S. N. *Silver nanotechnologies and the environment: Old problems or new challenges*; Project on Emerging Nanotechnologies: Washington, DC, 2008; (b) Gottschalk, F.; Sonderer, T.; Scholz, R. W.; Nowack, B., Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, fullerenes) for different regions. *Environmental Science and Technology* **2009**, *43* (24), 9216-9222;


79. Alvarez, P. J.; Colvin V Fau - Lead, J.; Lead J Fau - Stone, V.; Stone, V., Research priorities to advance eco-responsible nanotechnology. (1936-086X (Electronic)).

80. (a) Unrine, J. M.; Colman, B. P.; Bone, A. J.; Gondikas, A. P.; Matson, C. W., Biotic and Abiotic Interactions in Aquatic Microcosms Determine Fate and Toxicity of Ag Nanoparticles. Part 1. Aggregation and Dissolution. *Environmental Science & Technology* **2012**, *46* (13), 6915-6924; (b) Bone, A. J.; Colman, B. P.; Gondikas, A. P.; Newton, K. M.; Harrold, K. H.; Cory, R. M.; Unrine, J. M.; Klaine, S. J.; Matson, C. W.; Di Giulio, R. T., Biotic and Abiotic Interactions in Aquatic Microcosms Determine Fate
and Toxicity of Ag Nanoparticles: Part 2, Toxicity and Ag Speciation. *Environmental Science & Technology* **2012**.


Chapter 3

Materials and Methods

This chapter provides the detailed description of materials, experimental methods, sample preparation techniques and instrument settings used for analysis or characterization of samples involved in this research.

3.1. Nanoparticle synthesis and phase transfer

3.1.1. Materials

Silver perchlorate (AgClO₄, anhydrous, 97%), oleic acid (C₁₈H₃₄O₂, premium quality, ≥ 99%(GC)), 1-octadecene (≥ 95%(GC)), and oleylamine (technical grade, 70%) were purchased from Sigma-Aldrich; thiol functionalized methoxy poly (ethylene glycol) (mPEG-SH of MW 3-50 KDa) was purchased from Creative PEGWorks; chloroform, hexane, and ethanol (Reagent grade) were also obtained from Sigma-Aldrich; M9 minimal salts, 5X was purchased from Difco; Amicon
centrifugation filter units (MW 3KDa), Amicon 8400 stirred cell and 0.2 µm nylon syringe filters were purchased from Millipore. All chemicals were used without further purification. For all aqueous samples Millipore ultra pure water (18.2 MΩ) was used.

3.1.2. Synthesis

Oleic acid (OA) protected AgNPs of sizes 2 - 40 nm were synthesized in 1-octadecene (ODE) by reducing AgClO₄ using oleylamine (OAmine). In a typical reaction OA followed by ODE was added to solid AgClO₄ in a round bottom flask. Attached to this flask were a reflux condenser, a thermocouple and a sealed port for injection; before increasing reaction temperature, the solution was stirred at 600 rpm and purged (vacuum followed by a N₂ flush for 15 min). Air-free conditions minimized the formation of silver oxides. Thereafter, OAmine was rapidly injected into the reaction mixture at 100 °C and the temperature increased to 150 °C at the rate of 7 °C/min using a Cole Parmer, Digi-Sense Temperature Controller. The color of the reaction slowly turned from pale yellow to orange, reddish brown, dark brown and finally to black. The reaction was maintained at 150 °C from 4 to 8 h; nanocrystalline silver was synthesized with diameters ranging from 2 to 40 nanometers. After the reaction was complete, the crude reaction mixture was cooled to room temperature and quenched with hexane and ethanol in a ratio of 1:5. Then, the suspension was centrifuged at 11,000 rpm for 20 minutes to separate nanocrystals from unreacted molecular precursors; this purification process was
repeated four times. The resulting nanocrystalline silver stabilized with oleic acid (AgNP-OA) was dried in an inert atmosphere and dissolved in hexane.

### 3.1.3. Phase transfer

#### 3.1.3.1. Ligand exchange

A neutral polymer mPEG-SH of MW 5KDa was dissolved in water and mixed with organic solution of AgNPs. For example, mPEG-SH in CHCl$_3$ (3.5 x 10$^{-7}$ M) was added drop wise to 5 nm AgNPs in hexane (0.5 mM) under stir speed of 6,000 rpm.$^{32}$ soon after the addition, solution becomes turbid as nanocrystals precipitate after pegylation. At this point milli-Q water was added to the reaction mixture to dissolve precipitated nanocrystals and the solution was left stirring for overnight.

Mercaptopropanoic acid 3-MPA (106.14 g/mol) in water was added to AgNPs-OA in hexane and stirred for 5 h. Experimental methodology is same for 11-mercaptoundecanoic acid (MUA) 11-MUA (218.36 g/mol).$^1$ The protocols to phase transfer were modified and adapted from previous article on MPA modified magnetite nanoparticles.$^2$

#### 3.1.3.2. Encapsulation approaches

**Synthesis of amphiphilic polymers**

Synthesis of poly (2-acrylamido-2-methyl-1-propane sulfonic acid) -co-lauryl acrylate (PAMPS-LA): 4 mmol of 2-Acrylamido-2-methylpropane sulfonic acid (AMPS) (MW: 207.25 Da) and 1mmol of Lauryl Acrylate (LA) were dissolved in 15
mL of N,N-Dimethylformamide, (DMF) by stirring for 15 min. To this mixture 0.015 g of Azobisisobutyronitrile (AIBN) was added and stirred well to get homogeneous mixture of reactants. This was put inside the UV reactor (253 nm) for 4 hours. The resulting PAMPS-LA was characterized using NMR.

The synthesis of octylamine modified poly (acrylic acid) was adapted from Zhang et al. method.³

Synthesis of Poly (maleic anhydride-alt-1-octadecene)-graft- amine functionalized methoxy poly (ethylene glycol) PMAO-PEG was adapted from Yu et al. ⁴

**Phase transfer using amphiphilic polymers:**

Poly (acrylic acid -co–octylamine) (PAA-OA) in ethyl ether of concentrations in the range of 0.1 to 50 μmol was added to 2 ml of AgNPs (total silver concentration 5mg/L). The solution was left under stirring for 18 h and the resulting crude reaction mixture transferred into water was purified using ultracentrifugation. The resulting pellet of AgNPs was redispersed in milli – Q water and syringe filtered using Whatman Nylon filters of pore size 0.45 μm.

Experimental methodology to phase transfer using PMAO-PEG and PAMPS-LA was similar to PAA-OA except that chloroform (CHCl₃) was used as a solvent to disperse the polymer.
Methods to synthesize polymers PAA-OA, PMAO-PEG and PAMPS-LA were developed by Dr. Huiguang Zhu and synthesized by Dr. Seung Soo Lee (unpublished work from Colvin Lab, Rice University).

3.1.4. Purification of aqueous AgNPs

After the evaporation of organics such as CHCl₃ and hexane, a thin film of oily layer, which was thought to be detached oleic acid, was observed on top of aqueous layer. To get rid of this, in case of small volumes the solutions were filtered using 0.2μm Nylon syringe filters whereas for large volumes liquid-liquid extraction method was used. The aqueous suspensions of AgNPs were centrifuged in Sorvall Discovery 100 SE Ultracentrifuge using Ti 45 rotor at 43,000 rpm for 5 hours to remove excess polymer; the precipitates after the centrifugation were redispersed in milli-Q water. These steps were repeated twice in order to get nanocrystals with high purity. Resulting solutions were diafiltered (Regenerated cellulose membrane of MW 30K) using Amicon 8400 stirred cell under N₂. The buffer exchange (milli-Q water) was done five times in volumes of 800 ml per run. Purified AgNP solution was purged with N₂ and stored in dark. The efficiency of the phase transfer process was calculated by analyzing the silver concentration before and after phase transfer using ICP-MS. The materials in water absorb at 415 nm similar to those in hexane.
3.2. Nanoparticle characterization

3.2.1. Transmission electron microscopy (TEM)

Transmission electron microscope (TEM) images were acquired by JEOL 1230 High Contrast TEM (HC TEM) and JEOL 2100 field emission gun TEM (JEOL FEG TEM). The FEG TEM was operated at 200 KV with a single tilt holder whereas the HC TEM was operated at 80 KV with a multi-sample holder. All samples were prepared by drying 5 µL of AgNP samples on an ultrathin carbon type-A 400 mesh grid purchased from Ted Pella Inc. The size distribution data was obtained by counting more than 1000 particles using a software called Image-Pro Plus 5.05

3.2.2. Dynamic light scattering (DLS)

Hydrodynamic diameter of the AgNPs both in hexane and water was performed on Dynamic light scattering measurements (Malvern Instruments ZEN-3600 Zetasizer Nano equipped with a HeNe 633 nm laser). Silver nanocrystals with OD 1 at 415 nm were filtered (using 0.2 µm nylon syringe filters) to remove dust particles before taking a DLS measurement. Usually 3 runs per measurement were done. The particle size was taken from the average of the number mean and the standard deviations were obtained from triplicate data.
3.2.3. Spectrophotometer

Optical properties were measured using spectrophotometer (Cary 5000 UV-Vis-NIR spectrophotometer). All samples were measured using a quartz cuvette with 10 mm path length in the range from 200 – 800 nm wavelengths.

3.2.4. AgNPs dissolution method

AgNPs in water ([Ag]_{total} = 5 mg/L) was centrifuged at 4K r.p.m, for 10 min using Amicon centrifugation filter units of molecular weight 3KDa. Supernatant was collected in a 100ml glass beaker. MilliQ water was added to the membrane filter unit and centrifuged at 4K r.p.m, for 10 min. This step was repeated twice to facilitate the release of bound Ag⁺ to AgNP. Supernatant collected from these steps was added to the first one and concentrated by heating the solution at 60° C. Resulting solution was acidified with trace metal grade HNO₃ to get a concentration of 1% HNO₃. These samples were analyzed using ICP-MS to quantify dissolved Ag⁺ in the AgNP solution.

3.2.5. Biologically relevant media

3.2.5.1. S medium

S medium was used for understanding the stability of AgNPs in environmentally relevant biological media. This constitutes, 1 L of S Basal, 10 ml of 1M potassium citrate of pH 6.0, 10 ml of trace metal solution, 3 ml of 1 M CaCl₂, and 3 ml of 1M MgSO₄. S Basal constitutes of 0.1M NaCl, 0.05M potassium phosphate of
pH 6.0, 1 ml of cholesterol (5 mg/ml in ethanol) in a 1 L of H₂O. Trace metal solution constitutes of 5 mM disodium EDTA, 2.5 mM of FeSO₄.7H₂O, 1 mM of MnCl₂.4H₂O and ZnSO₄.7H₂O, 0.1 mM of CuSO₄.5H₂O in 1 L of H₂O.

*Dr. Elizabeth Quevedo Contreras, Colvin Lab, provided s-Medium.

3.2.5.2. EPA hard water

According to 1 L of EPA hard water constitutes of 192 mg of NaHCO₃, 120 mg of CaSO₄.2H₂O, 120 mg of MgSO₄, and 8 g of KCl. pH of the solution after 24 h of aeration varies from 7.6-8.0. Hardness and alkalinity of the solution are 160-180 and 110-120 CaCO₃ mg/L respectively.⁶

*Kim Newton from Clemson University, South Carolina supplied EPA Hard water.

3.2.5.3. OECD medium

A standard OECD (Organization for Economic Co-operation and Development) media ⁷ constitutes: 294 ppm of CaCl₂.2H₂O, 123.25 ppm of MgSO₄.7H₂O, 64.75 ppm of NaHCO₃, 5.75 ppm of KCl and 2 ppm of Na₂SeO₃. Used as a growth medium for Zebrafish.

*Teresa Fernandes research group, Napier University, UK supplied OECD medium.
3.2.5.4. Hoaglands medium

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>202</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>236</td>
</tr>
<tr>
<td>Iron (Sprint 138 iron chelate),</td>
<td>15</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
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<tr>
<td>NH₄NO₃</td>
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<tr>
<td>H₃BO₃</td>
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<td>MnCl₂·4H₂O</td>
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<td>ZnSO₄·7H₂O</td>
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</tr>
<tr>
<td>CuSO₄</td>
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</tr>
<tr>
<td>H₃MoO₄·H₂O</td>
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</tr>
<tr>
<td>Na₂MoO₄.</td>
<td>0.12</td>
</tr>
<tr>
<td>Potassium phosphate (pH 6.0)</td>
<td>136</td>
</tr>
</tbody>
</table>

Table 3.1 – Constituents of Hoaglands medium.
Hoaglands solution is a hydroponic solution used for the growth of plants such as poplar and Arabidopsis.

3.2.6. Thermogravimetric analysis (TGA)

TGA was done using TA instrument’s Q-600 simultaneous TGA/DSC.

Sample preparation: For hexane soluble AgNP stabilized with oleic acid was precipitated using ethanol to remove excess oleic acid and then prepared a really high concentrated sample for TGA measurements. In case of water soluble AgNP stabilized with mPEGSH, lyophiliser was used to prepare solid mass of sample. Lyophilisation is a dehydration technique where the sample is frozen initially and the frozen water was sublimated directly from solid phase to gas phase. This method is also called freeze-drying. The resulting sample was then used directly for TGA analysis.

3.2.7. Optical density measurements

For all the MIC measurements microplate reader TECAN Infinite M200 was used to measure the optical densities of E. coli.

3.2.8. Trace metal analysis

Trace metal analysis of Ag was performed using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) both equipped with an autosampler were purchased from Perkin Elmer.
Sample preparation: For AgNP soluble in organics, 1 ml of the solution was added into a 5 ml beaker, the solution was concentrated to 80% the volume by heating the sample at 45°C under continuous stirring. 100μl of concentrated trace metal grade HNO₃ was added to the above and left stirring at 45°C for 10 min. After the solution evaporated to ¼ of the original volume, 50 μl concentrated trace metal grade HNO₃ was added to make sure the surface coating is digested completely. The resulting solution was diluted with milliQ water until the total concentration of acid in the solution is 1%. This was then filtered using 0.2 μM Whatman Nylon syringe filters. Incase of aqueous AgNP the initial solution was concentrated to 50% of the volume and the rest of steps were followed as it is.

3.2.9. Fourier transform infrared spectroscopy (FT-IR)

The binding energy between nanocrystal and surface stabilizer was analyzed by IR spectroscopy (JASCO FT/IR-660, Fourier Transform Infrared Spectrometer).

3.2.10. Powder X-ray diffraction (XRD)

X-ray diffraction pattern were collected from Rigaku D/Max Ultima II. This was operated at 40KV and 40mA with a Cu Kα radiation (1.54 Å), 2θ (10-80 degrees) and a zero background sample holder was used for all measurements. Scans were collected for 6h. Jade 8.5 software was used to match the Ag peaks.

Sample preparation: AgNP samples coated with either oleic acid or mPEGSH were purified to remove the surface coating. Nanocrystalline silver was precipitated
using ethanol and then centrifuged to remove the supernatant containing free surfactant. This step was repeated twice to remove the surface coating properly. The obtained precipitate of AgNP was dried in a vacuum oven at 45°C for 16 h and the resulting sample was used for XRD analysis.

3.2.11. X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy patterns were obtained on PHI Quantera XPS equipped with a monochromatic aluminum 38.6 W X-ray source and 200.0 μm spot size with pass energy of 26.00 eV at 45.0°.

3.3. Antimicrobial activity

3.3.1. M9 medium preparation

M9 minimal salts, 5X, is a chemically defined dehydrated culture medium. This includes salts such as Na₂HPO₄ (0.3M), KH₂PO₄ (0.1M), NaCl (0.04M) and NH₄Cl (0.09M), which are essential for the growth of E. coli. To the autoclaved solution of 100 ml of M9 media, 10 m of carbon source dextrose 20% and 1ml of MgSO₄ (1M) and 50 μL of CaCl₂ (1M) was added and the resulting solution was diluted to 500 ml.

3.3.2. Preparation of LB agar plate

LB media was prepared by dissolving NaCl (10 g), tryptone (10g) and yeast extract (5g) in 1 L of milliQ water. This was then autoclaved at 120 C for 1 h. For LB media agar plates 15 g of Agar was added to one liter of LB media and then
autoclaved. This was then cooled to 50 C and poured on to the plates. If the agar gets solidified, it was warmed in a microwave for 6 minutes and then used for pouring plates. Each plate contains ~20 ml of LB agar.

3.3.3. Inoculating *E. coli* on a nutrient agar plate

About 4-5 colonies of *E. coli* culture from a previously made plate were picked with a flamed and cooled loop. These colonies were emulsified in a 1 ml of sterile 2mM NaHCO₃. 500ul of this solution was dropped on to the LB media agar plate. This suspension was spreaded on the LB plate using a sterile cotton swab Figure 7.1.

![Image of inoculating process](image)

**Figure 3.1 – Schematic of inoculating *E. coli*.**

(A) represents single colony picking using a sterile loop (B) dispersing the colony in NaHCO₃ (C) spreading the *E. coli* on LB agar plate.

3.3.4. *E. coli* growth in M9 medium

Growth curve of *E. coli* in M9 was performed before studying MIC to understand the growth rate. Here, overnight culture was diluted to hundred fold
with a resulting optical density of 0.02. This was incubated at 37 °C with a shaking speed of 250 rpm. 1 mL of the culture was aliquoted every hour to measure optical density. The culture was monitored until they reached stationary phase.

### 3.3.5. Minimum inhibitory concentration (MIC) assay

For MIC overnight *E. coli* culture with OD$_{600}$ of 1.15 was diluted to 1000 fold using 1X M9 media. This diluted culture was used for study. 9:1 ratio of culture to AgNP or Ag$^+$ was added to micro titer plate (96-well). The resulting total volume in each well was 100 µL. This was incubated at 37 °C for 24 and 48h and OD$_{600}$ of each well were accessed using a plate reader. Also 5 µL of culture from the MIC well was aliquoted and spotted on the LB agar plate to examine the growth of the bacteria. MIC was determined from the concentration where there was no bacterial growth on plates. Each concentration was done in triplicates and each experiment was accompanied with a positive (sterile water) and a negative control (70% Ethanol). All the experiments were repeated three times to obtain an average value with a standard deviation.

### 3.3.6. Zone of inhibition (ZOI) assay

The punching machine was sterilized by wiping it with ethanol and was used to punch holes on to whatman filter paper. These filter paper disks of diameter 10 mm were wrapped in an aluminum wrap and autoclaved to sterilize. These were impregnated with 10µL of AgNP/Ag$^+$ of different concentrations and allowed to dry.
These AgNP/Ag\(^+\) containing disks were placed on the agar surface inoculated with *E. coli*.

![Figure 3.2 – Zone of inhibition assay: Sample preparation](image)

(A) Sterile filter paper discs of 10 mm diameter (B) filter paper disks impregnated with AgNP/Ag \(^+\) (C) digital image of LB agar plate showing inhibition zone around the sample disks.

### 3.3.7. Bactericidal efficacy assay

For time kill experiments log phase cells (Log phase cells are preferred over the stationary phase as they are very sensitive to the antibiotics) of \(\text{OD}_{600} 0.63\) were used. To the 5 ml of this *E. coli* culture, 100 \(\mu\)l of AgNPs solution was added. This was incubated at 37 °C under shaking speed of 250 rpm. 10ul of cells were extracted from the exposed culture at time points 2, 4 and 8h. These were diluted by serial dilution method and plated on to the LB plates, which were then incubated at 37 °C to count the viable cells. Each time point and concentrations were repeated 3 times for median values and standard deviation.
3.4. References


Chapter 4

Synthesis of libraries of AgNPs

Keeping the research priorities in mind to develop a safe silver nanotechnology it is essential to understand and control the chemistry of AgNPs. To achieve this developing a well-characterized library of AgNPs with tunable surface chemistry is indispensable. Moreover, to eliminate complications arising from varying synthetic protocols and eliminate standardization of methodologies, a new approach to produce AgNPs of sizes in the range 2-30 nm is suggested. This chapter introduces a summary on current approaches to synthesize AgNPs, outlines their efficacy for environmental assessment and discusses the need for a new synthetic protocol. Later it focuses on three important subjects first, development and optimization of synthetic methodologies for preparing AgNPs of uniform size and shape. Second, mechanistic details on growth kinetics of uniform crystalline AgNPs
were introduced. Finally, structural and surface characterization of above materials was discussed.

4.1. Existing approaches

Current methods for AgNPs synthesis include laser irradiation,\(^1\) microplasma reduction of silver ions\(^2\), reduction in microwave,\(^3\) electrochemical synthesis,\(^4\) thermal decomposition,\(^5\) radiolytic reduction,\(^6\) reduction by electron beam,\(^7\) sonochemical reduction,\(^8\) sonoelectrochemical,\(^9\) digestive ripening,\(^10\) microemulsion,\(^11\) and photoreduction of silver ions.\(^12\) These can be divided based on nature of the AgNPs produced - hydrophobic or hydrophilic. Hence, the methods are named organic and aqueous synthesis. This section provides an overview of varying protocols, their advantages and limitations for the environmental risk assessment study of AgNPs.

4.1.1. Aqueous

Synthesis of water soluble AgNPs mainly includes reduction of silver salts such as silver nitrate (AgNO\(_3\)) and silver acetate (CH\(_3\)COOAg) with mild to harsh reducing agents such as sodium citrate (C\(_3\)H\(_4\)OH(COOH)\(_2\)COONa),\(^13\) sodium borohydride (NaBH\(_4\)), hydrazine (N\(_2\)H\(_4\)),\(^14\) ascorbic acid(C\(_6\)H\(_8\)O\(_6\))\(^15\) and tollens process.\(^14b\) These methods generally produce aggregated AgNPs. To increase the stability of AgNPs produced from these protocols, stabilizing agents such as cetyl trimethylammonium bromide (CTAB),\(^16\) sodium dodecyl sulfate (SDS),\(^17\)
poly(acrylic acid) (PAA),\textsuperscript{18} poly \text{--}(N\text{---}vinyl\text{-}2\text{-}pyrrolidine) (PVP),\textsuperscript{19} polyols\textsuperscript{20} and amphiphiles\textsuperscript{21} were employed in the literature. Some of the surfactants used here are non-biocompatible. As shown in Figure 4.1, AgNPs prepared using aqueous synthetic approaches lack shape and size selectivity; and they are prone to aggregation\textsuperscript{15a,22}

![Figure 4.1 - Electron micrographs of AgNPs from aqueous synthetic methods](image)

(A) Scanning Electron Micrograph (SEM) of PVP coated Ag nanoplates; TEM image of AgNPs (B) produced from tollens process (C) citrate stabilized. All the images are adapted from literature which elucidate heterogeneity in size and shape.\textsuperscript{13, 14b, 19}

Previous reports reveal that discrete size, shape and surface coating of AgNP influences the rate of oxidation, which in turn affects its toxicity.\textsuperscript{23} Current toxicology studies mostly use AgNPs produced from an aqueous approach leading to inconsistent data that might create issues in regulating AgNP usage. This problem can be solved using organic synthetic methods at high temperatures that produce AgNPs with varying uniform diameters.
4.1.2. Organic

Existing organic synthetic routes include usage of silver precursors such as AgNO$_3$, (PPh$_3$)$_3$Ag-R (where R= -NO$_3$ and -Cl) CF$_3$COO-Ag, CH$_3$COO-Ag and C$_6$H$_5$COO-Ag$^{24}$, mild reducing agents such as oleylamine, tertiary butylamine borane-5 and 1,2-hexadecanediol are commonly used in presence of organic solvents (toluene, 0-dichlorobenzene, liquid paraffin, 4-tertbutyltoluene, ionic liquid, isoamyl ether, hexane, benzene and DMF). Most of the current reports are two-step synthetic routes: synthesis and reduction of silver carboxylate to yield silver nanoparticles$^{25}$. Yamamoto et al has reported the synthesis of 5 nm silver by high temperature decomposition of silver carboxylates.$^{26}$ Osterloh et al and Meng Chen et al have illustrated the synthesis of silver nanoparticles of diameters 12 and 10-14 nm, respectively.$^{27}$

However strategies that produce a wide range of diameters from a single method is relatively unexplored. This criterion is important to minimize contaminants when these materials are used as a model to study AgNPs impact on environment. For instance, materials produced from different processes vary in trace impurities and it is challenging to rationalize the impact of these nanomaterials in the toxicology studies. Moreover, it is cumbersome to run different standardized purification techniques. These concerns have motivated our interest to introduce a standardized protocol to synthesize highly uniform AgNPs that can be used for environmental studies. Here we report AgNPs of diameters ranging from 2-45 nm with $\sigma < 15\%$ through the reduction of silver oleate by oleylamine.
4.2. New approach

A homogeneous mixture of reactants - silver perchlorate, octadecene, oleylamine and oleic acid quickly nucleated AgNPs stabilized by oleate. The strategy for forming nanocrystalline noble metals, particularly silver, in organic solutions was first mentioned over a decade ago. The basic principles of this work guided much of our experimental design. The first challenge is to provide a metal precursor soluble in high concentrations in organic media. Here silver perchlorate is dissolved in oleic acid and octadecene at 50 °C to form a stable Ag (I) oleate complex. Rapidly injected oleylamine at 70 °C reduced Ag (I) to Ag (0). In the reaction shown in Figure 4.2 the silver product does not have to form as nanoparticles, some conditions promoted the formation of micron sized silver particles Figure 4.3. However under the appropriate conditions nucleation is rapid enough to form many small crystallites whose surface is bound to residual oleate.

Figure 4.2 – Schematic of organic synthesis of AgNPs

Silver perchlorate on reduction with oleylamine in presence of oleic acid forms AgNPs stabilized with oleic acid.
Figure 4.3 – Precursor effect: AgNO₃

Color change of reaction after oleylamine injection (A) hazy brown mixture soon after injection (B) reddish brown mixture after 5 min (C) After 15 min reddish brown, due to the increased concentration of nanoparticles the solution seems black in color (D) TEM image of final product AgNPs stabilized with oleate, scale bar in D is 100 nm.

4.2.1. Optimization of reaction parameters

4.2.1.1. Counterion effect

The counter-ion of the silver precursor affects the homogeneity of reactant mixture, which then affects the size distribution of silver nanoparticles. Interestingly, the counterion of the silver salt affects the in situ precursor, silver oleate formation. Silver perchlorate (AgClO₄) and silver cyclohexanebutyrate (C₆H₁₁(CH₂)₃CO₂Ag), formed visually homogeneous suspensions when added to the mixture of oleic acid and octadecene at 50 °C. The latter resulted in formation of smaller size AgNPs of average diameter 2.6±0.7nm. As per my knowledge, the use of this precursor was not reported in the literature to produce AgNPs. Surprisingly, variation in the reaction parameters such as reactant concentrations, temperature and time did not
effect the average diameter Figure 4.4. This could be due to steric effects caused by bulky alkyl chain, limiting the increase in diameter.

![Figure 4.4](image.png)

**Figure 4.4 – Precursor effect: Silver cyclohexanebutyrate**

(A) TEM image of AgNPs fromed from silver cyclohexanebutyrate, Scale bar is 20 nm; (B) Histogram of image (A) with average size 2.6 ± 0.7 nm.

In contrast, AgNO₃ and other organic precursors such as CH₃CO₂Ag, and silver oxalate (Ag₂C₂O₄) formed hazy brown mixtures at 50 °C. This suggested incomplete formation of Silver (I) oleate, which partially reduced to form metallic silver Figure 4.3. These heterogeneous reaction mixtures resulted in broad size distributions because nucleation and growth are not separated ²⁹ Figure 4.5. Among these silver salts, homogeneous precursor solution from AgClO₄ promoted burst of nucleation leading to narrow size distributions (σ < 15%) and provided an opportunity to synthesize various sizes by tuning the reaction parameters.
Figure 4.5 – Counterion effect on AgNPs formation

TEM images of AgNPs when varying precursors were used (A) AgNO₃, 9.4±3.7 (B) CH₃CO₂Ag, 8.4±4.1 and (C) Ag₂C₂O₄, 2.5±0.9 nm; (D), (E) and (F) represent the histograms of TEM images (A), (B) and (C) respectively. Scale bar in (A) is 50 nm which applies to (B) and (C) as well.

The injection of the reducing agent oleylamine triggers the nucleation, which can be clearly seen by change in the color of the solution from colorless to pale yellow, which ultimately turns to reddish brown indicating the growth of AgNPs Figure 4.6. This color change is distinct from the heterogeneous precursor case. In addition, coexistence of excessive counter ions such as perchlorate, promoted aggregation Figure 4.7. Besides molar ratios of precursor and surfactant, reaction temperature and time, stable and visually clear suspensions of silver oleate are necessary for forming uniform and isolated AgNPs.
Figure 4.6 – Illustration of color change in presence of AgClO₄ after oleylamine injection at 70 °C

(A) Before injection of oleylamine; color change After injection of oleylamine (B) after 1 min (C) after 5 min (D) after 10 min.

Figure 4.7 – Excess perchlorate effect on AgNPs

TEM images of AgNPs (A) Before injection of NaClO₄ (B) After injection of NaClO₄. Scale bar in (A) and (B) are 50 and 100 nm respectively.
4.2.1.2. Effect of Molar ratio of the oleic acid and silver salt

Molar ratio of oleic acid to silver salt is the most critical parameter for controlling the silver nanoparticle diameter. Along with optimizing oleic acid to silver perchlorate molar ratio from 1 to 9, careful adjustment of temperature and reaction time ensured good uniformity. By tuning these variables listed in Table 4.1 AgNPs libraries of average diameters 2.5 ± 0.25, 3.9 ± 0.26, 4.7 ± 0.44, 8.9 ± 0.9, 14.5 ± 2.1 and 32.7 ± 8.6 nm were synthesized Figure 4.8.

<table>
<thead>
<tr>
<th>Diameter (nm)</th>
<th>Oleic acid/AgClO$_4$ (Molar ratio)</th>
<th>Oleylamine/silver (Molar ratio)</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5±0.2</td>
<td>9</td>
<td>0.3</td>
<td>4</td>
</tr>
<tr>
<td>3.9±0.2</td>
<td>6</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>4.7±0.4</td>
<td>6</td>
<td>0.3</td>
<td>6</td>
</tr>
<tr>
<td>8.9±0.9</td>
<td>6</td>
<td>1.4</td>
<td>7</td>
</tr>
<tr>
<td>14.5±2.1</td>
<td>6</td>
<td>2.8</td>
<td>8</td>
</tr>
<tr>
<td>32.7±8.6</td>
<td>6</td>
<td>2.8</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 4.1 - Illustration of reaction parameters for the synthesis of AgNPs with σ < 15%. In all cases the reaction temperature was 150 °C except for 35 nm it is 200 °C, 1-Octadecene was used as a solvent and reducing agent oleylamine was injected at 70 °C.
Figure 4.8 – Libraries of AgNPs

TEM images of AgNPs with average diameter of (A) 2.5±0.25, (B) 4.7±0.44, (C) 8.9±0.9 and (D) 14.5±2.1 nm. All scale bars are 50 nm.
Figure 4.9 – Histograms of AgNPs in Figure 4.8

(E), (F), (G) and (H) are histograms of the TEM images shown in (A),(B),(C) and (D) respectively.

Each different oleic acid to silver ratio, and hence nanoparticle diameter, had a different set of optimal reaction times and temperatures. For example, at a molar ratio of oleic acid to silver 6, as oleylamine to silver ratio increases from 0.3 to 2.8, the diameter of nanocrystalline silver increases from 2 to 15 nm. For all conditions the diameter of AgNPs increases linearly with time. While this gives some ability to further tune particle diameter, concurrent change in the particle distributions also
occurred, which will be explained in later part of the discussion. Similar to many high temperature nanocrystal growth process, an increase in the amount of surfactant here oleic acid, led to larger particle diameter.\textsuperscript{31} This is counterintuitive if the surfactant is thought to limit the growth of the particles. However, in case of iron oxide and quantum dot growth excess surfactant promotes the dissolution of particles thereby increasing the availability of monomer at longer reaction times.\textsuperscript{32}

4.2.1.3. Temperature effect

A narrow range of reaction temperature and time led to uniform silver nanoparticles. The optimum temperature to obtain narrow size distributions is 150 °C; Temperatures below 120 °C led to broad size distributions and those over 250 °C triggered aggregation of nanoparticles. Figure 4.10 provides a schematic of the reaction product quality as a function of reaction temperature (T) and reaction time (t). At temperatures below 70 °C, the reaction mixture did not change color prior to injection of the reducing agent and no products were observed. Temperatures from 70 to 150 °C led to noted color changes after the addition of oleylamine. However, the particles maintained a broad size distribution throughout the reaction Figure 4.11. Between 130 and 150 °C, the particle size distributions began to notably evolve with reaction time. The initial products are polydisperse, then narrowed after several hours, later broadened after several hours more. For most sizes, 150 °C offered the best particle uniformity. Structural and chemical analysis of products obtained above 250 °C revealed a presence of silver oxide, suggesting that at high temperatures, oxidation of the silver surface becomes active Figure 4.13.\textsuperscript{23d}
Figure 4.10 – Schematic of AgNP product quality

Temperature vs Time map provides map of reaction conditions to obtain uniform AgNPs: Temperatures ≤ 70 °C represent no nucleation zone, 150 °C optimum temperature to obtain uniform AgNPs, ≥ 250 °C indicates nonuniform particle zone.

One additional parameter that can affect particle uniformity is the injection temperature of the reducing agent, oleylamine. Rapid injection at 70 °C induces burst of nucleation which helps in decreased polydispersity of particles. Injection at temperatures above 120 °C then no conditions tested in this experiment could provide size distributions below 30%. There are distinct advantages for lower temperature injection.
Figure 4.11 – Temperature effect on degree of uniformity of AgNPs

TEM images of AgNPs obtained from three different oleic acid to silver molar ratios (M) and temperatures (T): Top column (A), (D) and (G) represent T=120°C and M=3, 6 and 9 respectively; middle column (B), (E) and (H) denote T=150 °C and M=3, 6 and 9 respectively and right column (C), (F) and (I) represent T= 250 °C and M=3, 6 and 9 respectively. In all the cases reducing agent, oleylamine was injected at 70 °C, 1-octadecene was used as solvent and reaction time was 6h. Scale bar in (A) is 50 nm which applies to images from (B) to (I) in this figure.
Figure 4.12 – Histograms of Figure 4.11

Histograms (J) 6.6±3.5, (K) 7.1±3.2, (L) 3.5±2.1 (M)4.7±0.5 (N) 10.2±3.4 and (O) 5.5±3.4 nm belong to Images in Figure 4.11 (A), (B), (D), (E), (G) and (H) respectively.
Figure 4.13 – XRD of AgNPs obtained at 250 °C.

Structural analysis using XRD indicates the presence of silver oxides Ag$_2$O and AgO in addition to fcc Ag(0) phase.

Injection at temperatures below and above 70 °C resulted in broad size distributions. Specifically silver was reduced at temperatures greater than 70 °C resulting in grey colored solutions which is nothing but silver oxide later converted to AgNPs of varying shape and size. This phenomenon of reduction of silver by temperature has been observed in the literature.\textsuperscript{33} Here, silver oleate in the presence of ODE resulted in stable micelles. Silver is also thought to form complexes with ODE.\textsuperscript{34} The high affinity of silver towards the solvent and surfactant prevents reduction of silver at temperatures less than 70 °C.
4.2.2. Large scale synthesis of AgNPs

Scaling up nanoparticle synthesis is challenging in terms of controlling the diameter and size distributions. Reaction parameters such as uniform heating and mixing of the reactants play a crucial role in addition to precursor, surfactant and injection rates of reducing agent. Here the reactions parameters were tuned in order to get uniform size of AgNPs Table 4.2. Major difference was the reactants were allowed to mix well for 30 min at 50 °C before raising the temperature to 70 °C to form homogeneous reactant mixture which is key for uniform growth of AgNPs.

<table>
<thead>
<tr>
<th>Diameter (nm)</th>
<th>AgClO₄ (mM)</th>
<th>Oleic acid (mM)</th>
<th>Oleylamine (mM)</th>
<th>Temperature (°C)</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>150</td>
<td>4</td>
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<td>7</td>
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</tr>
<tr>
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<td>2.1</td>
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<td>7</td>
<td>200</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Table 4.2 – Illustration of reaction parameters for the synthesis of AgNPs in large scale. In all cases ODE was used as a solvent and reducing agent oleylamine was injected at 70 °C.

The resulting AgNPs formed are shown in Figure 4.14 with the associated histograms in Figure 4.12.
Figure 4.14 – AgNPs synthesized in large scale

Average dimaters of TEM images are (A) 2.5±0.4 (B) 4.7±0.5 (C) 10.5±1.8 and (D) 44.0±6.0 nm.
Figure 4.15 – Histograms of Figure 4.14

Size histograms (E), (F), (G) and (H) belong to TEM images shown in Figure 4.14 (A), (B), (C) and (D) respectively.

4.2.3. Mechanism of AgNPs formation

Measurement of monomer concentration in the reaction mixture and the polydispersity index of AgNPs at one-hour time intervals imply that a size-focusing phenomenon occurs leading to high uniformity in size. To better understand particle
growth, free silver was measured Figure 4.16. Nucleation occurs rapidly right after injection of oleylamine indicated by color change in the reaction. Aliquots taken from the first moment of silver nucleation reveal that the concentration of soluble (e.g. monomeric) silver over the first hour drops to levels that are barely detectable. After monomer depletion, the particle diameters and size distribution continue to change markedly Figure 4.17. The source of monomer for these changes is likely due to the dissolution of the smaller particles, in a manner consistent with Ostwald ripening. Interestingly, size distributions ($\sigma$) remained broad with an increase in average diameter but at 8 h, size focusing was observed with a significant decrease in $\sigma$ (15%) with time.

![Figure 4.16 - Depletion of monomer concentration with time](image)

**Figure 4.16 – Depletion of monomer concentration with time**

Monomer concentration in the crude reaction mixture decreases with time suggests completion of nucleation of AgNPs
Figure 4.17 – Variation of AgNP diameter with time

Average diameter of AgNPs with time illustrating particle growth kinetics.

The narrowing of particle size with time is the hallmark of the growth process governed by “Ostwald ripening”. The driving force for this process is the difference in the solubility of the nanocrystalline silver ($S_r$) and the bulk solid solubility ($S_b$). This can be explained by Gibbs-Thomson equation (Equation 4.1) where $\sigma$ is the specific surface energy of the particle; $V_m$ is the molar volume of the solid; $r$ is the radius of the nanocrystal; $R$ is the gas constant and $T$ is the temperature of the reaction.

\[
S_r = S_b \exp \left( \frac{2\sigma V_m}{r RT} \right)
\]

Equation 4.1 – Gibbs-Thomson equation
Here, the solubility difference gives raise to concentration gradiation between small and large particles. This leads to preferential dissolution of smaller particles while larger particles grow at the expense of the smaller particles. Figure 4.18 represents the schematic where particles with $r < r^*$ dissolve and acts as monomer source for particles with $r > r^*$. Here in a specific time a particle with average diameter of 10 nm grows less compared to 5 nm leading to uniform AgNP formation.

**Figure 4.18– Schematic of particle growth**

Particles below critical radius dissolve and act as a precursor for larger particles to grow. Figure provided by Vicki L. Colvin, Rice University.

More difficult to understand is that as reaction times increase, the size distribution broadened again. For a classic Ostwald ripening process, the particle uniformity continually improves with time albeit at a less noticeable rate. It may be that the phenomenon observed parallels the “size focusing” of quantum dots...
reported for similar kinds of reactions \(^{31a}\). In this case, Ostwald processes led to preferential dissolution of smaller materials and the initial narrowing of the particle size distributions. Figure 4.19 is the graph of particle growth rate considering thickness of diffusion as infinite. This model explains the negative growth rates of smaller nanoparticles. However, once particles are large enough, their dissolution rates are in effect similar then, size-dependent growth rates will dictate the distribution.\(^{36}\)

![Graph of particle growth rate](image)

**Figure 4.19 – Variation of the growth rate with size of nanoparticle**

Figure adapted from Peng et al (a model by Sugimoto)\(^{31a, 37}\).
4.2.4. Characterization of AgNPs -OA

Further, structural and optical characteristics of these isolated, uniform silver nanomaterials are consistent with crystalline, non-oxidized silver. AgNPs absorb at 414 nm and were size dependent as shown in Figure 4.20. As the particle diameter increased, surface plasmon wavelength red shifted indicating the change in the active surface of the nanoparticles.

![Graph showing optical properties of AgNPs](image)

**Figure 4.20 – Optical properties of AgNPs -OA**

*Size dependent optical properties, red shift in the wavelength was observed with increase in diameter.*

Oleic acid stabilizes face centered cubic (FCC) silver through most stable bidentate fashion and finds no significant evidence for silver oxide on nanocrystal surfaces. X-
ray diffraction of powders recovered from purified suspensions of uniform AgNPs, reveals the presence of Ag (0) in the FCC lattice structure Figure 4.21.

![Figure 4.21 – Characterization of AgNPs – OA](image)

(A) X-ray diffraction pattern; XPS spectra of AgNPs (B) Ag 3d (C)Ag O1S and Ag C1S.

Samples produced at 150 °C contain no diffraction peaks from oxidized forms of silver present (e.g. AgO₂ or Ag₂O). In addition, surface characterization
data from XPS and FTIR suggest that oleic acid binds to the silver surfaces as a bidentate ligand Figure 4.21. Typically oleic acid can bind to nanocrystalline silver in three different ways: as a monodentate ligand, a chelated ring (bidentate) or a bridged structure. Here, the infrared spectra of pure oleic acid C=O stretching frequency occurs at 1709 cm$^{-1}$ while in case of AgNPs stabilized with oleic acid (AgNPs-OA) it is shifted to 1640 and 1549 cm$^{-1}$. These peaks can be assigned to a resonated carboxylate anion, which further validates the oleic acid binding mode as a bidentate oleate ligand.\textsuperscript{51} The XPS spectrum has a notable shift in the oxygen 1S peak that is consistent with a bidentate structure. This data correlates well with XRD confirming the presence of metallic silver and finds no sign of oxidation. The high resolution TEM image of AgNPs-OA in Figure 4.22 reconfirms the crystalline nature of silver.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{TEM_image.png}
\caption{High resolution TEM of AgNPs-OA}
\end{figure}

TEM image representing the crystallinity of AgNP-OA
4.3. Conclusion

A new approach to synthesize highly uniform libraries of AgNPs stabilized with oleic acid in the range of 2-44 nm was developed. Growth kinetics of AgNPs were explored and were in agreement with Ostwald ripening in first few hours, it was later dominated by size dependent growth rates yielding highly monodisperse nanocrystals. Characterization of these materials reveals crystalline nature, bidentate binding mode of oleate and non-oxidized pristine silver surface was produced in organic media.

4.4. References


Dependent on Dissolved Silver and Surface Coating in Caenorhabditis elegans. 


Chapter 5

Enhanced stability and controlled dissolution of AgNPs-mPEGSH

In order to better understand the behavior of AgNPs in complex matrices such as that prevail in the environment, systematic studies are crucial. The mission of this chapter is to evaluate the kinetics and stability of AgNPs in various biological matrices with a scope to predict its fate in the environment. For this purpose, the model libraries of highly uniform AgNPs-OA synthesized in chapter 4 were used. This chapter emphasizes on three important features:

1. Phase transfer of AgNPs-OA from organic to aqueous phase
2. Studies on colloidal stability of AgNPs encapsulated with mPEGSH (AgNPs-mPEGSH) in biologically relevant matrices
3. Studies on dissolution properties of AgNPs-mPEGSH
5.1. Phase transfer

In order to explore the antimicrobial nature of the AgNPs-OA, these particles first require surface modifications that do not change the morphology, size homogeneity or solubility in water. The process of phase transfer of NPs from hydrophobic to hydrophilic solvents is widely applied in nanochemistry.¹ These procedures often require biocompatible phase transfer agents, as the ultimate use requires application in biological fluids. Ideally, the best phase transfer agent should have a high transfer efficiency of nanomaterial from polar to nonpolar solvents and prevent aggregation and dissolution.

Existing methods for phase transfer mainly include encapsulation and ligand exchange. Encapsulation approaches involve interaction of hydrophobic tail of the surfactant with the hydrophobic part of original surface coating in NPs.² Examples of encapsulation using amphiphilic polymers include lipids, pluronic block copolymers, α-cyclodextrin, polyacrylic acid (PAA) and polyethylene glycol (PEG).³ This process of steric stabilization could be challenging due to formation of nanoparticle clusters stabilized by polymers as shown in Figure 5.1. On the other hand, ligand exchange method replaces the original surface coating with a new hydrophilic polymer that can stabilize the NPs in water without aggregation. Often during the process of stripping the original surface coating, NPs optical properties are changed. Choosing a biocompatible surfactant that can eliminate these issues is crucial. Successful examples of surfactants used in the literature include functionalized PEG polymers.⁴
Figure 5.1 – Schematic of aqueous transfer of magnetite nanoparticles.

Phase transfer via (A) addition of IGEPAL CO 630 surfactant leads to formation of magnetite clusters (B) addition of oleic acid results in bilayer formation. Figure adapted from Prakash et al.\textsuperscript{5}

Since the purpose of this study is to understand the transformations in surface chemistry of the AgNPs in the environment, it becomes essential to find a surfactant that can enhance the stability of AgNPs in biologically relevant media and control dissolution at high ionic strengths. In this study, PEG was chosen as the backbone of the polymer due to its ability to eliminate nonspecific binding interactions with biological molecules.\textsuperscript{6, 7} This concept has been well studied with gold NPs. Previous studies have shown that PEG tend to form densely packed
random coils on the surface of gold shielding the other ligands from binding in a highly rich nutrient media with competing ligands.\textsuperscript{8, 9} These have been highly preferred over other moieties for biomedical applications as PEG exhibits less toxicity and longer circulation times when compared to NPs with other surface coatings. The focus here is to prepare colloidally stable water soluble AgNPs without interrupting the intrinsic optical and antimicrobial properties of silver.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Quantum dot encapsulated with an amphiphilic polymer}
\end{figure}

QD stands for quantum dot, F implies functional group such as –OH, -COOH, etc. Figure adapted from William Yu et al.\textsuperscript{3b}
5.1.1. Versatility of oleic acid coating

Oleic acid is a versatile surface coating and allows the use of both hydrophilic and amphiphilic polymers for phase transfer.\textsuperscript{3b, 5, 10} The goal here is to find a suitable surfactant that can transfer AgNPs into water while preserving the optical properties and avoiding aggregation. The following are the approaches used in this study.

5.1.1.1. Encapsulation method

Experimental strategies employed for the preparation of aqueous AgNPs by encapsulation are different compared to magnetite and quantum dots. For example, probe sonication is a key step that facilitates the bilayer formation of oleic acid, highly efficient for phase transfer of magnetite nanocrystals and quantum dots however when applied to AgNP, it resulted a change in particle size distributions.\textsuperscript{5} Here small nuclei were introduced to the system due to disruption of original AgNPs. Therefore mild stirring was used instead of sonication.

As shown in Figure 5.3, phase transfer efficiency for the encapsulation approach ranges from 45-70% with PMAO-PEG on the high end. In case of PAA-OA and PAMPS-LA optical properties were not retained (characteristic surface plasmon peak at 414 nm was quenched) and wavelength was red shifted along with peak broadening suggesting aggregation of AgNPs, which was confirmed by TEM. On the other hand AgNPs modified by PMAO-PEG (AgNP-PMAO-PEG) retained the optical properties but lacked long-term stability (with shelf life 48h).
Figure 5.3 – Phase transfer of AgNPs via polymer encapsulation.

(A) UV-Vis of Ag stabilized with PMAO-PEG, PAA-OA and PAMPS-LA polymers; Transfer yield of Ag stabilized with (B) PAMPS-LA (C) PMAO-PEG & (D) PAA-OA

5.1.1.2. Ligand exchange method

Thiol functionalized PEG polymers (MW 300 – 50KDa) and small thiol containing acids (3-MPA, 11-MUA of MW 100-100KDa) were used as phase transfer agents.\textsuperscript{11} Here, strong interaction between silver and sulfur is the key to facile oleate
In case of MUA and MPA, AgNPs were supposedly phase transferred to water but TEM images reveal aggregation. Optimizing the concentration of phase transfer agent and AgNP–OA did not improve the particle state. Among thiol containing ligands, neutral mPEGSH (MW 3-50KDa) gave promising results without aggregation. Schematic to phase transfer reaction was shown in Figure 5.4.

Figure 5.4 – Schematic of phase transfer of AgNPs

AgNPs-OA react with mPEGSH (left) to form AgNPs-mPEGSH (right).

mPEGSH coating effectively saturated the silver surface to produce stable aqueous dispersions of AgNPs with 95% transfer yield. However, increased concentrations of mPEGSH led to agglomeration at the interface of organic and aqueous layer. Further, the optical properties of silver colloids were retained after phase transfer Figure 5.5.
Figure 5.5 – Retention of optical properties after phase transfer.

(A) Optical spectra of AgNPs-OA in hexane and AgNPs-mPEGSH in water; TEM images of (B)AgNPs-OA and (C) AgNPs-mPEGSH.

Infrared spectra of AgNPs before and after phase transfer confirm the thiol functionalized PEG Figure 5.6. Polymer shell thickness on AgNPs was obtained from DLS measurement. For instance, silver with 10 nm core diameter has a hydrodynamic radius of 15 nm. TGA data reveals that silver with 10 nm diameter constitutes $1.18 \times 10^4$ molecules of oleic acid per nm$^2$, whereas in case of mPEGSH it has 1.45 molecules per nm$^2$. Since oleic acid (MW 282.4 g/mol) is a small molecule as compared to mPEGSH (MW 5KDa), a 10 nm silver can accommodate 2600 more oleic acid molecules.
Figure 5.6 – Infrared spectra of AgNPs.

Infrared spectra (A) Oleic acid (B) mPEGSH; AgNPs stabilized with (C) Oleic acid (D) mPEGSH.

5.2. Colloidal stability of AgNPs-mPEGSH

The central idea behind studying the stability is to predict the behavior and fate of AgNPs in the environment. Many studies in the literature have prioritized this subject in their experiments.\textsuperscript{14} In general, AgNPs can be stabilized either
through electrostatic forces or steric hindrance. Typically, electrostatic repulsive forces preclude aggregation of NPs in biological media either due to neutralization of surface charges or interaction with proteins via electrostatic forces. Protein fouling in general leads to precipitation of particles due to massive increase in the particle size. Hence, long term stability of NPs in rich nutrient media is extremely challenging especially in case of silver. PEG was shown to improve the NP stability by providing steric hindrance around the NP and preventing interaction with proteins.\textsuperscript{15} It was observed that iron oxide NPs modified with triethoxysilylpropylsuccinic anhydride (SAS) and amine functionalized PEG retained stability in Dulbecco’s modified eagle’s medium (DMEM) with 10% of fetal bovine serum (FBS) for 16 days.\textsuperscript{16} Authors attributed the unusual nanoparticle stability to strong interaction between SAS and iron oxide surface and the steric hindrance provided by amphiphilic PEG chain. In another study, oligo(ethylene glycol) functionalized gold NPs depicted stability in varying ionic strengths (0-0.1M), temperatures (5-70 °C) and pH (1.3-12.4).\textsuperscript{6,17} Therefore, PEG was used to enhance the stability of AgNPs in biologically relevant media in this study.

5.2.1. Stability in biologically relevant media

AgNPs can undergo various physicochemical changes such as dissolution and aggregation when subjected to high ionic strength media containing competing organic/inorganic ligands.\textsuperscript{18} This may cause changes in morphology or stability. Hence, it is essential to understand their surface chemistry before employing these materials for biological applications. Here biologically relevant media were selected
to evaluate these parameters. These include EPA hard water, 2mM NaHCO₃, ¼\textsuperscript{th} strength hoaglands, LB, M9, S medium, and OECD media. Inorganic ligands such as phosphate, chloride, and macromolecules such as proteins present in these media may compete with the current coating on AgNPs. Change in material properties were monitored by TEM, DLS and optical spectroscopy. The optical properties of the materials provided one measure of the silver stability. If the materials dissolved, the resonance feature should disappear, meanwhile significant red shifts would be observed if aggregated. \textsuperscript{19}

5.2.1.1. Generic AgNPs

Generic AgNPs includes Citrate and Borate stabilized AgNPs. These were synthesized in the lab using literature reported methods.\textsuperscript{20} Average diameters of these particles from TEM were observed to be 24.1±18.2 and 19.5±11.3 nm. Size distributions of these samples were greater than 57%. Since these particles are widely used for toxicology studies in current literature, the efficiency of highly uniform engineered AgNPs was compared with the generic AgNPs. Figure 5.7 shows the libraries of AgNPs used for evaluating stability.

In the presence of highly rich nutrient media such as M9, LB and S media, AgNPs were either instantaneously precipitated or dissolved and this was confirmed by loss of characteristic surface plasmon features. In the presence of sodium bicarbonate buffer, EPA hard water and ¼\textsuperscript{th} strength Hoaglands, surface
plasmon peak at 433 nm of citrate stabilized particles was reduced by greater than two fold.

**Figure 5.7 – TEM images of generic AgNPs.**

(A) lab synthesized citrate 24.1±18.2 nm, (B) lab synthesized borate 19.5±11.3 nm, (C) commercial citrate 3.23±2.2 nm, (D) commercial tannic acid 3.03±2.5 nm.
indicating spontaneous dissolution. However LB medium promoted aggregation of citrate stabilized particles indicating a redshift by 37 nm and a three fold reduction in the optical density. In case of borate stabilized AgNPs, even the slight change in the ionic strength such as EPA hard water caused an instantaneous thirteen fold decrease in the optical density at 398 nm. After 24 h the surface plasmon peak is

---

**Figure 5.8 – Size histograms of AgNPs in Figure 5.7.**

(E), (F), (G) and (H) are the size histograms of TEM images (A), (B), (C) and (D) in Figure 5.7 respectively.
reduced to 225 fold suggesting fast kinetics in the absence of strong stabilizing agent. When dispersed in bicarbonate buffer (2 mM), \(\frac{1}{4}\)th strength Hoaglands and M9 the particles were rapidly destabilized Figure 5.9.

![Figure 5.9](https://example.com/figure59.png)

**Figure 5.9 – Stability of generic AgNPs measured through optical spectroscopy.**

Stability of generic AgNPs in biologically relevant media (A) citrate stabilized after 0h (B) Borate stabilized after 0, 24 h in EPA hard water

**5.2.1.2. Enhanced colloidal stability of AgNPs-mPEGSH**

The dense polymer shell of mPEGSH coating enhanced the colloidal stability of silver in various different media with high ionic strengths.\(^{21}\) Over a period of five months there was no change in the peak position whereas the peak height difference of OD 0.1 was observed in case of Hoaglands medium. On a shorter timescale the DLS results on these suspensions was also explored.
Figure 5.10 – Media stability tests of AgNPs-mPEGSH.

Stability of AgNPs-mPEGSH in biologically relevant media through optical spectroscopy (A) after 0h (B) after 24h (C) monitored for 5 months (OD$_{414}$); (D) hydrodynamic radius monitored for 5 months is a measure of aggregation.
<table>
<thead>
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<th>Surface coating</th>
<th>Diameter (nm)</th>
<th>EPA hard water</th>
<th>1/4&lt;sup&gt;th&lt;/sup&gt; Hoaglands</th>
<th>M9</th>
<th>LB</th>
<th>S</th>
<th>OECD</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3.23±2.2</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24.1±18.2</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<tr>
<td><strong>Borate</strong></td>
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<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td>2.5±0.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>8.9±0.9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>15.2±2.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Citrate-exchanged with mPEGSH</strong></td>
<td>3.23±2.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>PMAO-PEG</strong></td>
<td>4.7±0.4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Table 5.1 – Stability chart for AgNPs with varying surface stabilizers. “+” indicates stable while “-” unstable.*

DLS can only be applied to those solutions that are simple buffers since the presence of proteins and organic matter contributes to scattering. However, the data is consistent with the optical response meaning no particle aggregation or dissolution.
was observed. Therefore AgNPs-mPEGSH remained fully intact and non-aggregated in a wide range of biological and environmental media.\textsuperscript{56} Table 5.1 further validates the efficiency of mPEGSH coating over the generic AgNPs in a variety of biological media.

**5.2.2. Stability in high ionic strength solutions**

Here AgNPs-mPEGSH (10 nm) and AgNPs-Citrate (3.23±2.2 nm) were exposed to varying concentrations of monovalent, divalent salt solutions of NaCl and CaCl\textsubscript{2} (0.1 to 0.95M). In presence of CaCl\textsubscript{2}, AgNPs-Citrate dissolved right away whereas for NaCl it took 24 h for 100% dissolution. In contrast, AgNPs-mPEGSH showed a gradual increase in dissolution with increasing salt concentration. Salt solutions of 0.95 to 0.75 M resulted in 97% of AgNPs dissolution within a couple of seconds. Divalent cations stimulated a 5-10% higher dissolution than NaCl. For the lowest salt concentration of 0.1 M, only 41% silver was dissolved and showed no changes with increase in exposure times Figure 5.11.
Figure 5.11 – AgNPs-mPEGSH stability in high saline solutions

(A) NaCl and (B) CaCl₂

5.3. Dissolution

Dissolution is a vital parameter that can elevate the toxicity of AgNPs. In a study of long term transformation and fate of manufactured AgNPs stabilized with PVP (AgNPs-PVP), it was found that NPs were transported from soil to sediments which indicated that erosion and runoff as a potential pathway for AgNPs to enter waterways. This experiment also demonstrated that 70% of the weight of Ag was present in the soils and sediments, most of it prevailed in the injected compartment. Further, Ag in the terrestrial soils converted to Ag₂S (55%), Ag-cysteine (27%), 3% in the terrestrial plant biomass and 0.5-3.3ug/g in mosquito, fish and chironomids was found.²² It was demonstrated that silver release is independent of the synthesis method and can be varied depending on the surface oxidation. Authors found that
70% of the Ag was leached into solution as ions but washing the nanoparticle has reduced the oxidation and hence silver release was negligible. Studies from wastewater treatment plant indicate that the AgNPs that are being used commercially will ultimately end up sulfidized. These sulfidized NPs are shown to reduce toxicity in a model study using E. coli. This study was done using varied levels of sulfide layers on NPs and it indicated that inhibition to E. coli is inversely proportional to the extent of sulfidation. Even 0.03 mol% of sulfur reaction with AgNP had a huge impact and resulted in decrease of toxicity.

Previous studies also showed size as an important factor to control the dissolution of AgNPs. Similarly, dissolved silver in AgNPs-PVP (18 nm) was found 2.5 times higher than 30 nm particles. Moreover, stable dispersions of AgNPs exhibited higher toxicity compared to the aggregated particles. For instance introducing humic acid into the system relatively decreased the nanoparticle toxicity due to adherence of ionic silver by humic acid. Further, relative increase in dissolution was observed with the decrease in size of the ZnO but found no exponential increase. This was explained using aggregation, surface coating and shape of the material. In the past studies, it was shown that decrease in dissolution of the NP can decrease its toxicity. Hence measuring dissolved silver content and understanding the fundamental chemistry that control the dissolution of AgNPs is very critical to account for the toxicity. In this study, controlled dissolution of engineered AgNPs was demonstrated by tuning surface coating and size and showed
ways to trigger the dissolution using lower pH and peroxide. Further, reduction in surface oxidation controlled dissolution.

Surface passivation of AgNPs using mPEGSH reduces the rate of oxidation of surface silver atoms. More sensitive measures of particle dissolution in pure water found that less than 1 (w/w) % of these materials dissolved to provide molecular silver. For these experiments, purified surface stabilized particles were kept in water, under air and in the dark. Aliquots of the solution were collected over several months and subjected to centrifugation using Amicon centrifugation filters (MW 3KDa at 4000 r.p.m for 10 min) to separate NPs and collect any molecular forms of silver in the filtrated solution. ICP-MS of the silver content of the filtrate found a slow release of silver over thirty days. For an unpurified solution, dissolved silver percentage for 2,9 and 15 nm were 14, 11 and 5.8% respectively Figure 5.12.

![Graph](image)

**Figure 5.12 – Size dependent dissolution of AgNPs-mPEGSH.**

(A) Dissolution of partially purified AgNPs of 2, 9 and 15 nm  (B) Illustration of available surface area/g of silver
Higher surface area of 2 nm favored faster dissolution over larger sizes of silver. Also data suggested a less significant difference in rate of dissolution among 9 and 15 nm. This can be explained by the fact that as particle size increases the surface area differences become less pronounced. However, for effectively purified aqueous silver dispersions in the range of diameter 2 to 15 nm, the total amount dissolved corresponded to less than one percent by weight of the total silver in the nanocrystalline suspensions. Therefore, silver dissolution was found negligible for all purified samples. We speculate that these samples may contain very small amounts of the more soluble silver oxides at 1 (w/w) %. This impurity would not be detected by either X-ray diffraction or X-ray photoelectron spectroscopy. Yet, it would slowly dissolve into molecular silver.

5.3.1. pH dependent dissolution

Here, AgNPs-mPEGSH of size 5 nm was tested in the pH range 4-8. It was found that acetate buffer of pH 4 induced a 50% decrease in the optical density with a prominent blue shift in wavelength (5 nm) indicating a spontaneous dissolution. For buffers of phosphate pH 6 and 8, phosphate citrate pH 6 and 7, acetate pH 5.6, 1-2 nm blue shift was observed Figure 5.13.
Figure 5.13 – pH dependent dissolution of AgNPs-mPEGSH.

(A) dissolution of 2nm Ag stabilized with mPEGSH of 10 and 20KDa in citrate buffer pH4. pH dependent stability of Ag-mPEGSH in phosphate citrtae buffer of pH 6,7 and 8; phosphate buffer of pH 6,7 and 8; acetate buffer of pH 4 and 5.6.

In general, as the pH of the solution increased from 4 to 8 surface plasmon peak of AgNPs blue shifted from 414 nm to 409nm and a decrease in dissolution was observed Table 5.2. On the other hand, 2 nm AgNPs- mPEGSH of Mwt-10, 20KDa in citrate buffer (pH 4) showed an increase in dissolution where 85% of Ag was dissolved in 45 days. Further lowering the MW (<5KDa) facilitated faster dissolution rates.
<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Blue (nm)</th>
<th>Shift</th>
<th>*Decrease in OD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>4.0</td>
<td>5</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.6</td>
<td>2</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Phosphate-citrate</td>
<td>6.0</td>
<td>2</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>1</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.0</td>
<td>2</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>2</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>1</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2 – Stability of AgNPs-mPEGSH (5 nm) in phosphate citrate buffer of pH 6 and 7; phosphate buffer of pH 6,7 and 8; acetate buffer of pH 4 and 5.6; *To calculate the decrease in absorbance OD at $\lambda_{\text{max}}$ 414nm was used.

The active surface area of silver available for oxidation can explain this behavior. Lower molecular weights favored brush confirmations of polymer, leaving the surface atoms exposed for oxidation Figure 5.16. Therefore, these data suggest that, smaller dimensions (<5 nm) in combinations with low molecular weight polymers (< 5KDa) would be more effective to deliver a more soluble silver.
5.3.2. **Accelerated dissolution in presence of peroxide**

For AgNPs to be used as an effective antimicrobial, its dissolution needs to be tuned according to applications. As shown in Figure 5.12, mPEGSH coating reduced the dissolution. To better understand the surface chemistry of these particles, ways to trigger the dissolution of AgNPs-mPEGSH were explored.

5.3.2.1. **Size effect**

AgNPs-Citrate of 3 and 24 nm size were subjected to peroxide exposure and found that the rate of dissolution of 24 nm Ag is 1600 times slower than that of 3 nm Ag.

![Graph showing the dissolution of AgNPs-Citrate at different sizes](image)

**Figure 5.14 – Peroxide triggered dissolution: Size effect.**

(A) Citrate 3.23 ± 2.2 nm (B) 24.0 ± 18.5 nm.
In other words, to get 95% dissolution for a 24 nm Ag, it took 20 days whereas for a 3 nm just 20 min Figure 5.14. This can be explained by surface area per gram of silver, for a 3 nm it is 188m², which is 8 times higher than the 24 nm particle. Energetic surface atoms available on the 3 nm AgNP favored the dissolution than 24 nm. Similarly stabilized AgNP with larger surface area is more prone to dissolution.

5.3.2.2. Surface chemistry effect

AgNPs-citrate of 3 nm was reacted with FITC-PEG-SH of 3KDa to prepare FITC-PEGSH stabilized AgNPs. These were exposed to peroxide and were found that 95% of the silver was dissolved in 18 min for citrate coating whereas 78% was dissolved for FITC-PEGSH Figure 5.15.

![Graph showing dissolution of AgNPs over time](image)

Figure 5.15 – Peroxide triggered dissolution: Surface Chemistry effect.

Dissolution of 3 nm Ag was significantly reduced when citrate coating (MW-192 Da) was exchanged with FITC-PEGSH (MW-3KDa)
This observation correlates well with previous studies proving that high affinity Ag-S bond and surface saturation decelerated the rate of dissolution.$^{26a,31,33}$

5.3.2.3. Polymer molecular weight effect

Grafting density and the confirmation of the polymer on the particle surface define the surface coverage efficiency. In this study, AgNPs-mPEGSH (2 nm) of 10KDa dissolved 95% in 20 min. As the molecular weight of the PEG doubled only 75% of the silver was dissolved in 300 min. This can be explained in terms of grafting density. Grafting density is number of PEG molecules per nm$^2$ of the Ag Figure 5.16. It was found that as the molecular weight of the polymer increases, grafting density decreases and the polymer adapts mushroom confirmation rather

![Diagram](image.png)

**Figure 5.16 – Grafting density dependence on polymer molecular weight.**

Plot of grafting density Vs MWt of mPEGSH and a schematic representation of polymer confirmation on NPs. Figure adapted from Denise Benoit Thesis.$^{34}$
Figure 5.17 – Peroxide triggered dissolution: Polymer MW effect.

(A) Dissolution of 2 nm Ag stabilized with mPEGSH of 10 and 20KDa (B) dissolution of 2 nm Ag stabilized with mPEGSH of 10 KDa

than brush.\(^\text{34}\) Mushroom confirmation effectively blocks the active Ag surface preventing oxidation. This not only reduces the dissolution but also protects against competent inorganic/organic ligands preventing exchange of the polymer and therefore reduces aggregation.

5.4. Conclusion

Ligand exchange of oleate by mPEGSH resulted in non-aggregated water soluble AgNPs. Steric stabilization by the thick polymer shell assisted AgNPs to sustain highly saline conditions and rich nutrient biological media. It was observed that dissolution of these NPs is not only size and surface dependent but also on the chain length of polymer coating. Conditions explored in this study prove that
delivery of molecular silver can be tuned by varying size and surface chemistry principles. These basic principles of dissolution would be beneficial in silver antimicrobial applications that may require short/long time spans.

5.5. References


15. (a) Sun, C.; Lee, J. S.; Zhang, M., Magnetic nanoparticles in MR imaging and drug delivery. *Advanced drug delivery reviews* **2008**, *60* (11), 1252-65; (b)


33. (a) Levard, C.; Reinsch, B. C.; Michel, F. M.; Oumahi, C.; Lowry, G. V.; Brown, G. E., Sulfidation processes of PVP-coated silver nanoparticles in aqueous solution:

Chapter 6

Reduced toxicity of AgNPs-mPEGSH

AgNPs are a well-known bactericide, which gained focus as a nano-disinfectant in the commercial world and are currently used in a variety of antimicrobial applications. AgNPs are not that different from molecular silver; they exhibit toxicity via two different pathways, particle-specific activity and through dissolution of silver. Dissolution is considered the main route that determines the toxicity. In order to develop Eco-responsible AgNPs containing biocides without losing the performance, it is essential to understand the physicochemical factors that contribute to the toxicity. Here, the antimicrobial property of organically produced finest AgNPs is evaluated against *E. coli*. Differentiating and qualitatively assigning the AgNPs toxicity to either particle or ions was challenging. Here the surface modified materials provide an opportunity to understand this by separating the particle and ions from the AgNPs stock solutions. In brief, this chapter starts with a
concise discussion on AgNPs toxicity against *E. coli*, based on current literature and then focuses on three important topics:

1. Evaluation of AgNPs-mPEGSH toxicity via minimum inhibitory concentration (MIC) and Kirby-Bauer diffusion assays
2. Influence of core size and surface coating on AgNP toxicity
3. Comparison of bactericidal efficacy of engineered AgNPs to the generic particles used in the market

**6.1. Antimicrobial activity of AgNPs**

AgNPs are a well-known antimicrobial biocide, Table 1.1. provides a glimpse of its toxicity to various different organisms. In addition, AgNPs were found to be toxic to various different strains of bacteria. Here, the focus is on *E. coli*. Previous reports have shown that their toxicity can be influenced by structural and surface properties such as shape, size, exposure medium and solubility. Although bactericidal action of AgNPs is still debatable, recent reports have shown that both particle and ionic silver routes include cell membrane degradation as a major pathway for cell death. Moreover, ionic silver is more toxic than AgNPs. Table 6.1 illustrates AgNPs of various surface coatings and size and its effect on toxicity. From the literature data, nominal concentration of AgNPs ranges from 0.1-100 ppm.
<table>
<thead>
<tr>
<th>Surface coating</th>
<th>Diameter (nm)</th>
<th>Shape</th>
<th>Media</th>
<th>Exposure Time</th>
<th>Tested silver concentration</th>
<th>Major biological Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND²</td>
<td>10</td>
<td>Spherical and truncated</td>
<td>Culture media</td>
<td>24h</td>
<td>0.1-1 mg/L</td>
<td>Cell membrane was damaged and DNA was unaffected</td>
</tr>
<tr>
<td>Citrate³</td>
<td>39</td>
<td>Rod, truncated triangular and spherical</td>
<td>Difco nutrient broth</td>
<td>0-26h</td>
<td>0.1-10 µg/ml</td>
<td>Truncated triangular AgNPs are more toxic compared to spherical</td>
</tr>
<tr>
<td>Citrate⁴</td>
<td>9.3±2.8</td>
<td>Spherical</td>
<td>HEPES buffer and M9</td>
<td>30 min</td>
<td>0.4-0.8 nM</td>
<td>AgNPs more toxic than Ag⁺. Outer membrane was destabilized and plasma membrane potential was disturbed</td>
</tr>
<tr>
<td>Citrate⁴</td>
<td>9.3±2.8</td>
<td>Spherical</td>
<td>HEPES buffer and M9</td>
<td>24h</td>
<td>0-100 µg/ml</td>
<td>AgNPs toxicity was mediated by dissolution of particles. Smaller Nps found to be more toxic than larger ones</td>
</tr>
<tr>
<td>Daxad 19⁵</td>
<td>12</td>
<td>Spherical</td>
<td>Aqueous dispersion</td>
<td>10</td>
<td>0-100 µg/ml</td>
<td>Cell wall damaged</td>
</tr>
</tbody>
</table>

Table 6.1 – Toxicity of AgNPs to *E. coli* (summarized from literature).
6.2. Minimum inhibitory concentration (MIC)

MIC is the lowest concentration of the disinfectant that can inhibit the growth of bacteria. This technique is commonly used to identify the efficiency of a new antimicrobial agent against bacteria. In general, MIC is considered to be the ‘gold standard’ to determine the resistance of a microorganism to antibiotic.\(^6\) It is often used to confirm the marginal result from other testing methods. MIC can be determined either by agar or broth dilution method with a ±2 fold dilution as an accepted dilution range. Table 6.2 illustrates the MIC of various different forms of silver tested in the literature. From the data, MIC of Ag\(^+\) varies from 0.84 – 22 ppm whereas AgNPs ranges from 0.77-75 ppm; Clearly, the MIC is dependent not only on the structural and surface properties of silver (surface coating and size) but also on the exposure medium, which reveals the environmental significance.

Most importantly, all these parameters can be weighed by understanding the bioavailable silver. Bioavailability is the free-floating nanomaterial (in this case silver) available to interact and cross the cellular membrane of the organism from the environment causing either benefit or harm.\(^7\) Presence of inorganic /organic ligands decreases the bioavailability by binding silver. For ionic silver, the difference can be attributed to an exposure medium, since a rich nutrient medium (LB) decreased the bioavailability of silver requiring a large quantity of silver to inhibit the growth of same cell density. This implies that exposure medium is critical in the cytotoxicity studies.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Silver type</th>
<th>Surface</th>
<th>Diameter (nm)</th>
<th>MIC (ppm)</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli (ATCC 25922)&lt;sup&gt;8&lt;/sup&gt;</td>
<td>AgNPs</td>
<td>Hexadecylamine</td>
<td>9.8</td>
<td>12.5</td>
<td>MH</td>
</tr>
<tr>
<td>E. coli (ATCC8739)&lt;sup&gt;9&lt;/sup&gt;</td>
<td>AgNPs</td>
<td>ND</td>
<td>5</td>
<td>10</td>
<td>MH Muller–Hinton (MH)</td>
</tr>
<tr>
<td>AgNPs</td>
<td>Carbon matrix</td>
<td>21 ± 18&lt;sup&gt;1a&lt;/sup&gt;</td>
<td>75</td>
<td></td>
<td>LB (Luria-Bertani)</td>
</tr>
<tr>
<td>E. coli (CCM 3954)&lt;sup&gt;10&lt;/sup&gt;</td>
<td>AgNPs</td>
<td>Glucose</td>
<td>44</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Maltose</td>
<td>25</td>
<td>3.38</td>
<td>MH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lactose</td>
<td>35</td>
<td>27.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>No coating</td>
<td>25-50</td>
<td>1.69</td>
<td></td>
</tr>
<tr>
<td>Ag+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>E. coli (CCM3954)&lt;sup&gt;11&lt;/sup&gt;</td>
<td>AgNPs</td>
<td>Maltose</td>
<td>25</td>
<td>1.69</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SDS</td>
<td>25</td>
<td>1.69</td>
<td></td>
<td>MH</td>
</tr>
<tr>
<td></td>
<td>tween 80</td>
<td>25</td>
<td>1.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PVP 360</td>
<td></td>
<td>3.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli (CIP 53126)&lt;sup&gt;12&lt;/sup&gt;</td>
<td>AgNO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>22</td>
<td>MH /HEPES buffer</td>
</tr>
<tr>
<td>Organism</td>
<td>Silver type</td>
<td>Surface</td>
<td>Diameter (nm)</td>
<td>MIC (ppm)</td>
<td>Medium</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------</td>
<td>---------</td>
<td>---------------</td>
<td>-----------</td>
<td>--------------</td>
</tr>
<tr>
<td><em>E. coli (ATCC8739)</em>&lt;sup&gt;13&lt;/sup&gt;</td>
<td>AgNO₃</td>
<td>-</td>
<td>-</td>
<td>2.35</td>
<td>LB</td>
</tr>
<tr>
<td></td>
<td>AgNPs</td>
<td>PMMA</td>
<td>7</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td><em>E. coli (ATCC 25922)</em>&lt;sup&gt;8&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lysozyme-3K</td>
<td></td>
<td></td>
<td>3.5±1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lysozyme-25K</td>
<td></td>
<td>8±3</td>
<td>1.7±0.7</td>
<td>MH</td>
</tr>
<tr>
<td></td>
<td>Lysozyme-SDS</td>
<td></td>
<td></td>
<td>2.5±0</td>
<td></td>
</tr>
<tr>
<td><em>E. coli (ATCC 25922)</em>&lt;sup&gt;14&lt;/sup&gt;</td>
<td>Ag⁺</td>
<td>-</td>
<td>-</td>
<td>5.6±2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ag⁺</td>
<td>-</td>
<td>-</td>
<td>&lt;10</td>
<td>Liquid gause media</td>
</tr>
<tr>
<td><em>E. coli (ATCC 25922)</em>&lt;sup&gt;15&lt;/sup&gt;</td>
<td>Myramistin</td>
<td></td>
<td>10.0±1.8</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Citrate</td>
<td></td>
<td>9±0.9</td>
<td>10</td>
<td>MH agar plates</td>
</tr>
<tr>
<td><em>E. coli (ATCC43890)</em>&lt;sup&gt;16&lt;/sup&gt;</td>
<td>AgNPs</td>
<td>Borohydride</td>
<td>13.4±2.6</td>
<td>&gt;33nM</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.2 –MIC of AgNPs against *E. coli* (summarized from literature). ND implies not determined.
6.2.1. Growth medium selection

In this study, M9 medium was used for all the bactericidal experiments, which contains essential nutrients for the bacterial growth and did not interfere with the AgNPs solubility. Stability of AgNPs in M9 was studied using optical spectroscopy, DLS and TEM. The results were shown in Figure 5.9. For further microbial diagnostic experiments *E. coli* MG1655 growth kinetics in M9 were studied. From Figure 6.1, *E. coli* in M9 certainly had slower kinetics than the rich nutrient media LB, and this correlates with previous observations. For example, the optical density at 600 nm (OD\textsubscript{600}) in LB medium was seven at 7h where as to reach saturation in M9 with OD\textsubscript{600} of 1.5, it took 20h Figure 6.2. In other words, it took 5h for bacteria in M9 to reach the exponential phase, the same phenomenon happens in the first hour of exposure for LB. Further, biological evaluation of the engineered AgNPs was done using MIC.

Here, MIC was measured via turbidity of a suspension of bacteria in mixture of M9 media and AgNPs after 24 and 48h exposure times. Two fold dilutions of AgNPs solutions were prepared, and all the experiments were performed in a microtiter plate Figure 6.3
Figure 6.1 – *E. coli* growth curve in M9 medium

Figure illustrating the growth phases of *E. coli* in M9 medium.

Figure 6.2 - *E. coli* growth curve in LB medium

Figure adapted from Baev et al.\(^\text{18}\)
Figure 6.3 – Digital image of microtiter plate: MIC assay

Arrow marks indicate (A) MIC concentration where there is no growth of *E. coli* while the next two minimal concentration show positive growth of *E. coli* (B) positive control (milli-Q water)(C) Negative control (70% ethanol).

The developed protocol was validated with well-documented antibiotic ampicillin. MIC of ampicillin was found to be 6.25±1.5 ppm which is in agreement with literature. MIC in M9 media was found to be equivalent to that in literature. MIC assay was performed for AgNPs-mPEGSH (2.5, 4.7 and 11.0 nm), AgNPs-Citrate (3.5, 24, 40, 60 and 80 nm), AgNPs-tannic acid (20,40,60 and 80 nm). TEM images of these particles are shown in Figure 5.7. Dilution ranges were mPEGSH (877-0.0134 ppm), citrate and tannic acid (20-0.02 ppm), borohydride (28.2-0.05 ppm) and silver salts (343 - 3.4X10⁻⁷ ppm).
<table>
<thead>
<tr>
<th>Surface coating</th>
<th>Diameter (nm)</th>
<th>MIC ([Ag] in ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgClO₄ (Salt)</td>
<td>-</td>
<td>2.9</td>
</tr>
<tr>
<td>AgNO₃ (Salt)</td>
<td>-</td>
<td>3.4</td>
</tr>
<tr>
<td>Citrate</td>
<td>40</td>
<td>15.3</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>32.4</td>
</tr>
<tr>
<td></td>
<td>3.23±2.2</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>24.1±18.2</td>
<td>16.4</td>
</tr>
<tr>
<td>Citrate</td>
<td>40</td>
<td>15.6</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>31.3</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>62.8</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>19.5±11.3</td>
<td>88.0</td>
</tr>
<tr>
<td></td>
<td>2.5±0.3</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>4.7±0.5</td>
<td>31.2</td>
</tr>
<tr>
<td></td>
<td>11.0±1.1</td>
<td>43.0</td>
</tr>
</tbody>
</table>

Table 6.3 – MIC of AgNPs of varying surface coatings and size.
The data suggest that silver salts were more toxic than AgNPs with lowest MIC’s 2.9-3.4 ppb. This implies that ionic silver is 1800 times more toxic to *E. coli* than AgNPs.

### 6.2.2. Size and surface coating dependent MIC

The moderate bactericidal nature of AgNPs was size dependent; the smaller particles on per particle basis and on per weight basis were more toxic to bacteria than larger particles. A 9 nm increase in the diameter leads to three-fold increase in MIC. On the other hand, tannic acid stabilized AgNPs exhibited a substantial increase in MIC as diameter increased from 3 to 80 nm. Particles of 80 nm were found to be on the high end with a MIC of 193 ppb; this was less toxic of all the particles tested in this study. In contrast AgNPs-citrate showed a less significant increase in MIC with diameter due to broad particle size distributions.

The presence of dense polymer shell reduced the bactericidal property of AgNPs.\textsuperscript{20} It was observed that surface saturation of citrate stabilized AgNPs by mPEGSH resulted in a twenty-fold increase in the hydrodynamic radius compared to citrate coating. This in turn led to four-fold increase in MIC suggesting that surface passivation by mPEGSH-SH limits the reactivity of silver compared with citrate. This can be attributed to first, highly stable dense polymer coating that hinders the complete exposure of active silver surface to interact with bacteria. Second, strong Ag-S interaction impedes the most common bactericidal route of silver interaction to sulfur containing bacterial proteins.\textsuperscript{55} Competition between two alike binding molecules weakens the cell destruction pathways. Therefore, bactericidal efficacy is
not only the intrinsic nature of the core material but also mediated by surface coating.

6.3. Bactericidal efficacy

Presence of seemingly low amounts of dissolved silver associated with AgNPs elevated the toxicity. Similarly sized AgNPs of same total silver concentration of (3 mg/L) but with different surface coatings citrate (3.05 nm), tannic acid (3.23 nm), and tannic acid replaced with mPEGSH (3.23 nm) were exposed to E. coli culture in M9 media for 6h. 10 µl of these exposed solutions were diluted by serial dilution method (10^6, 10^7, and 10^8 dilutions) and inoculated onto LB Agar plates. It was found that there was a substantial decrease in the colony forming units (CFU) in case of citrate and tannic acid Figure 6.4. This was in correlation with the dissolved silver content, 30% for tannic acid, 10 % for citrate and negligible amounts for borate and mPEGSH. In a similar study when the tannic acid coating was replaced with mPEGSH cell viability increased by 40% suggesting a reduction in toxicity due to decrease in residual Ag⁺ associated with AgNPs.
Figure 6.4 – Effect of dissolved silver on CFU formation

AgNPs of varying surface coatings with varying dissolved silver (A) control with no AgNPs (B) tannic acid with 900 μg/L Ag⁺ (C) citrate with 300 μg/L Ag⁺ (D) mPEGSH with negligible Ag⁺. In all cases, dilution factor is 10⁶ fold.

Surface passivation reduced the toxicity of AgNPs where AgNPs-mPEGSH suppressed the cell division while citrate-AgNPs effectively caused cell death.

AgNPs-citrate of 3.23 nm (total silver concentration-6, 24 and 98 ppb) and AgNPs-mPEGSH of 10 nm (total silver concentration - 4, 16 and 67 ppb) were exposed to E. coli for 8 h. In case of mPEGSH, for all the tested concentrations, cell division was
suppressed at 2, 4, and 8h while the control sample without AgNPs showed increase in cell density Figure 6.5. On the other hand citrate coating exhibited a 3 fold decrease in the cell density after 2h and showed no further significant reduction until 8h exposure time. This can be attributed to increase in bioavailable silver; insufficient coverage of silver surface by citrate coating promoted further oxidation resulting in high toxicity.

Inspite of surface saturation by mPEGSH, reduction in the AgNP core size elevated the toxicity. AgNPs- mPEGSH (5nm) were tested in the concentration range from 22.8 to 0.95 ppm. AgNP stock solutions of 7.6 - 22.8 ppm were lethal to bacteria whereas 70% of the cells were found viable at 3.8 ppm after 2 h exposure; with increase in exposure time, cells were more susceptible and resulted in 100% cell death after 4h Figure 6.6. Interestingly, 0.95-1.9 ppm increased the cell density which can be explained by hormetic effect.21 This phenomenon of unusual stimulatory bacterial growth at sublethal concentrations of silver was previously noted. In this study, minimum lethal concentration of silver ions was found to be 0.025 mg/L. From these observations, lethal dosage of AgNPs is atmost 150 times higher than that of ionic silver.
Figure 6.5 – Time dependent bactericidal efficacy

AgNPs of varying concentrations stabilized with (A) citrate showed 3 fold reduction in cell density (B) mPEGSH suppressed cell division
Figure 6.6 – Bactericidal efficacy of 5 nm silver

AgNPs-mPEGSH (5nm) at 3.8 ppm suppressed the cell division after 4h of incubation.

6.4. Kirby-Bauer diffusion method

In the previous section bactericidal efficacy of AgNPs was diagnosed using MIC in a liquid media. For the point of application of AgNPs as a disinfectant on a solid surface, here Kirby-Bauer test (also known as disk diffusion test) was selected as a diagnostic tool. The efficiency of this test is dependent on mobility of the antibiotic on the solid medium and creates a bacteria free zone of inhibition (ZOI) around the antibiotic. The larger the diameter of the ZOI, more active is the antibiotic.
6.4.1. Protocol development using ampicillin

Here commonly used antibiotic ampicillin was tested to validate the protocol. The inhibition zone diameter was in correlation with the literature reported values. Zone diameter was measured after 24 h incubation at 37 °C. As shown in the Table 6.4, increase in the zone diameter was observed with an increase in the concentration of ampicillin.

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Disk Potency (µg)</th>
<th>Susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>375</td>
<td>+</td>
</tr>
<tr>
<td>25</td>
<td>187.5</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>75</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>37.5</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>22.5</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>7.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 6.4 – Zone of inhibition of ampicillin. “+” sign indicates presence of zone whereas “-” indicates an absence of zone.
Figure 6.7 – Zone of inhibition of ampicillin

Illustration of increase in zone diameter with increase in concentration of ampicillin.

Figure 6.7 exemplifies the significant increase in zone diameter with increase in ampicillin concentrations. In case of AgNPs and ionic silver, significant difference in the zone diameter was not observed with an increase in concentration. This can be attributed to bioavailable silver. Diffusion of silver is possibly restricted due to binding with free ligands in the LB medium and hence did not have significant effect on the zone diameter with a two-fold increase in the concentration of Ag stock solutions Figure 6.8. Further the susceptibility of AgNPs to *E. coli* on LB Agar plate is shown in Table 6.5.
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration (ppm)</th>
<th>Susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNO₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AgNPs-mPEGSH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>AgNPs-citrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.5 – Zone of inhibition test for silver. “+” sign indicates presence of zone where as “-” indicates an absence of zone.
Figure 6.8 – Susceptibility of *E. coli* to silver

Illustration of growth free zone in presence of (A) AgNO₃ (B) AgNPs stabilized with mPEGSH in the top quadrant and citrate coated AgNPs in the bottom quadrant.

6.5. Conclusion

In this study, organically produced AgNPs retained the intrinsic antimicrobial nature of silver even after chemical modification of surface. Surface saturation of AgNPs with a dense polymer shell prevented the oxidation of active silver atoms on the surface, which resulted in reduced toxicity. In addition, size dependent antimicrobial activity was observed both in MIC and bactericidal efficacy tests; smaller size AgNPs were prone to oxidation and hence more toxic to bacteria on a particle basis in the order of 2nm>4nm >9nm>15 nm. Further, residual Ag⁺ associated with AgNPs elevated the toxicity of AgNPs. Moreover, highly stable AgNPs exhibited antimicrobial activity both in liquid and solid medium opening a
new arena to explore the incorporation of nano-disinfectant in variety of applications. The basic chemical modifications of AgNPs explored here make the very inherent antimicrobial property of silver tunable and hence more versatile. All the experiments in this study were done with model organism *E. coli* but these experimental methods can be applied to other strains of bacteria.

### 6.6. References


Chapter 7

Implications of the Research

In order to assess and control the risk of AgNPs in the environment it is essential to gather more scientific data based on which nanomaterial exposure models can be developed. As a part of achieving this goal here versatile, highly uniform model libraries of engineered AgNPs with defined size and surface chemistry, were produced in this project. This research continued as a material support, and supplied engineered AgNPs to the research groups lead by Dr. Colvin, Dr. Pedro Alvarez and Dr. Janet Braam from Rice University, Dr. Stephen J. Klaine from Clemson University and Dr. Jerald L. Schnoor from The University of Iowa. This chapter highlights on the research findings on toxicity of AgNPs-mPEGSH produced in this thesis against *E. coli*, *Daphnia Magna*, Poplars, Arabidopsis and *C. elegans*. 
7.1. Bacteria - *E. coli*

This work was done in collaboration with Dr. Zooming Xiu, Dr. Qingbo Zhang and Dr. Pedro Alvarez at Rice University.

The narrow size distribution of AgNPs allowed us to observe a distinct pattern on size dependent toxicity AgNPs against *E. coli*. Here AgNO₃ and AgNP-mPEGSH of sizes 2.8 ±0.47, 4.7±0.20 and 10.5±0.59 nm were exposed to resting *E. coli* cells in 2 mM NaHCO₃. AgNO₃ was toxic at 25 ppb Figure 7.1(A), while for AgNP-mPEGSH, toxicity was in the order of 2.8>4.7>10.5nm with toxic concentrations of 4.1, 5.2 and 8.2 ppm respectively Figure 7.1(B). Authors explained the increase in cell viability at sub lethal concentrations of silver as a phenomenon called hormetic effect. This was caused by residual ionic silver in the AgNP-mPEGSH stock solutions.

Dense polymer shell coating of mPEGSH on AgNPs promoted, controlled dissolution in aerobic and anaerobic conditions and allowed to discern the particle and ion specific antimicrobial activity. Here AgNPs–mPEGSH of size 5 and 11 nm were exposed to *E. coli* in anaerobic and aerobic conditions (exposed to air for 48 h before treating with *E. coli*). Particle toxicity significantly increased in aerobic conditions due to upsurge in dissolution of AgNPs, which released more ionic silver Figure 7.2. This study states that ionic silver released from the AgNP is directly proportional to the increased toxicity of AgNPs. Thus this supports the mechanism of negligible particulate specific antimicrobial activity of AgNPs.
Figure 7.1 – Cell viability after exposure to AgNO$_3$ and AgNPs-mPEGSH.

Cell viability of E. coli cells in NaHCO$_3$ buffer after 6 exposure to (A)AgNO$_3$ (B) AgNPs-mPEGSH. One asterisk represents significant decrease in cell viability and rectangular box containing data with two asterisks represents hormesis effect. Figure adapted from Xiu et al.$^1$
Figure 7.2– Influence of aerobic and anaerobic conditions on cell viability

Cell viability of *E. coli* cells in NaHCO₃ buffer after 6h exposure. AgNP-mPEGSH aerated for 48 h was more toxic than unaerated particles for both 5 and 11 nm. Figure adapted from Xiu et al.¹
7.2. Terrestrial plants - *Populus* and *Arabidopsis*

This work was done in collaboration with Jing Wang, Dr. Anne Alexander, Dr. Qingbo Zhang, Dr. Yeonjong Koo, Dr. Pedro Alvarez, Dr. Jerald L. Schnoor and Dr. Janet Braam.²

Current environmental model studies on silver predict the accumulation of AgNPs in sewage sludge, which implies silver entrance into the terrestrial environment. Hence, there is a significant possibility that they can have effect on plants. Therefore, in this study two different terrestrial model plants - *Arabidopsis* and *Populus* were selected to study toxicity of AgNPs. Here, both the model plants were exposed to AgNPs in the concentration range of 0.01 to 100 mg/L (total silver concentrations) Figure 7.3. Similar to toxicity studies on *E. coli* both plant species showed stimulatory effects at low concentrations of AgNP-mPEGSH. In addition, AgNPs not only affected the root growth of the plants but NP deposition was observed on the roots.

Again the highly uniform AgNPs-mPEGSH of distinct sizes synthesized in this thesis allowed understanding size dependent toxicity to plant species where 5 nm were more toxic than 10 nm. Further, mPEGSH coating preserved the AgNP morphology in hydroponic plant growth solution and retained its stability in the duration of experiments. These experiments illustrate that engineered AgNPs that are resistant to aggregation in high saline conditions tend to be more toxic than commercially available materials.
Figure 7.3– Growth enhancement of Arabidopsis after exposure to AgNPs-mPEGSH.

AgNP-mPEGSH of size 10 nm with total silver concentrations of 0.01 – 0.1 ppm exhibited stimulatory effect while 1 pppm inhibited the growth of arabidopsis plant. Figure adapted from Jing Wang et al.²

The synthesis of stable model libraries of AgNP-mPEGSH enabled this research on phytotoxicity, which is a stepping-stone to understand the most important part of the ecosystem that can influence the fate of AgNP in the environment.

7.3. Invertebrates - Daphnia Magna

This work was done in collaboration with Kim M. Newton and Dr. Stephen J. Klaine from Clemson University.³
In this study AgNPs-mPEGSH of 5 nm were exposed to *Daphnia Magna* and the results indicate that toxicity was a function of dissolved silver present in the samples. Here the nanoparticle toxicity in EPA hard water was in the order of AgNO$_3$ > AgNPs-gumarabic > AgNPs-mPEGSH > AgPVP. Further addition of dissolved organic carbon from Suwanne River decreased the toxicity of NPs due to decrease in bioavailability of silver. Therefore this research demonstrated that toxicity of engineered AgNPs can be mitigated by the ligands present in the environment.

### 7.4. Nematodes - *C. elegans*

This work was done in collaboration with Dr. Elizabeth Quevedo from Dr. Colvin research group, Rice University.  

![Image of *C. elegans*](image)

**Figure 7.4– An image of *C. elegans***
In this study *C. elegans* Figure 7.4 were exposed to AgNPs of three distinct sizes (2, 5 and 10 nm). It was found that 10 nm particles adversely affected the lifespan while the 2 nm particles affected reproduction. Figure 7.5 reveals the size effect of AgNP-mPEGSH uptake on nematodes.

**Figure 7.5– AgNPs-mPEGSH uptake by *C. elegans***

Size dependent uptake by *C. elegans*. The internalized silver concentration per nematode follow the order 2 nm < 5 nm < 10 nm. These were quantified by ICP-MS. Figure adapted from
Here the synthesis of distinct sizes of AgNP-mPEGSH and the dense polymer coating allowed understanding the toxicity of AgNPs on multigenerational response of nematodes which would have been difficult to assess with the commercial AgNPs as they are unstable and leach ionic silver that might destroy the nematode food source.

7.5. Conclusion

The synthesis of model libraries of AgNP-mPEGSH presented in this thesis allowed understanding the toxicity of AgNPs against *E. coli, Daphnia magna, Poplar, Arabidopsis* and *C. elegans*. The distinct sizes allowed the size dependent toxicity study while the mPEGSH coating enhanced the stability of AgNPs in biological media and promoted to discern the particle and ion specific toxicity of AgNP. This research succeeds in understanding the dissolution of AgNPs in harsh to mild conditions of media and allowed to study exposure of manufactured AgNPs to various organisms that are cornerstones of ecosystem. This scientific data precludes the generalities regarding the toxicity of AgNPs and incites further research on mechanistic aspects to avoid inadvertent environmental impacts on a long-term basis.

7.6. References

