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Silver Nanoparticles: the Immediate Benefits of Low Bacterial Resistance and the Long-Term Risk of Persistent Stress In Mammalian Cells

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SILVER NANOPARTICLES:
THE IMMEDIATE BENEFITS OF LOW BACTERIAL RESISTANCE AND THE
LONG-TERM RISK OF PERSISTENT STRESS IN MAMMALIAN CELLS

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

By

David H. Ellis
M.S. Wright State University, 2010

2015
Wright State University
I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY David H. Ellis ENTITLED Silver nanoparticles: the immediate benefits of low bacterial resistance development and the long-term risk of persistent stress in mammalian cells, BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy

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Abstract

Ellis, David H. Ph.D. Biomedical Sciences Ph.D. Program, Wright State University, 2015. Silver nanoparticles: the immediate benefits of low bacterial resistance and the long-term risk of persistent stress in mammalian cell

Silver nanoparticles (AgNPs) are the fastest growing sector of nanotechnology, due mostly to their antibacterial properties. The antibacterial effectiveness of AgNPs is well known and derives from the shedding of silver ions which have multiple antibacterial targets in the bacterial cell. Due to their continuous release of ions and demonstrated antibacterial potency, some predict that AgNPs have a low potential for resistance development, which would make them a valuable asset in wound management.

The ability for AgNPs to cause oxidative imbalance in mammalian cells is also well known, but the potential long-term impact of such a stress has not been studied despite its implication for negative outcomes in wound management. In this thesis, I demonstrate by using a stepwise increasing exposure protocol that Pseudomonas aeruginosa, but not Acinetobacter baumannii or Staphylococcus aureus could develop resistance to 10 nm, citrate-coated AgNPs. The potential for resistance development was lower than the antibacterial drug, ciprofloxacin, but not as low as silver nitrate to which none of the bacteria developed resistance. The resistance mechanism is not yet clear but appeared to involve the phenazine pigments produced by P. aeruginosa which can bind and reduce silver ions. In mammalian cells, I demonstrated the persistence and time-dependent oxidative stress of AgNPs in the A549, epithelial cell model, by using specialized
imaging techniques and a common probe for oxidative stress. In addition, I showed that AgNPs can induce a senescent-like phenotype in A549 cells after an exposure that appears non-toxic in the typical viability assays used for assessing cytotoxicity. I confirmed that senescence was induced by showing an increase in senescence-associated, beta-galactosidase activity and the hypertrophic morphology of exposed cells, as well as a decrease in proliferation. The implication of this research for wound management is that AgNPs can be properly applied to wounds in order to inhibit bacterial colonization with little potential for resistant strains to emerge; however, the nanoparticles may persist in wound-associated, mammalian cells. There, the AgNPs will cause persistent oxidative stress with the potential to induce cellular senescence and reduce the long-term health and function of the surrounding tissue.
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INTRODUCTION

Nanotechnology

Nanotechnology is the manipulation of materials at the nanometer scale. A nanometer is $10^{-9}$ meters and nanomaterials (NMs) are defined as having at least one dimension less than 100 nm, which is around one hundred times smaller than the width of a human hair. The impetus for manipulating materials at such a small scale is credited to Richard Feynman from a 1959 presentation entitled, “There’s Plenty of Room at the Bottom” (Feynman 1959). The term nanotechnology, however, did not emerge until 1974 in Norio Taniguchi’s review of semiconductor engineering (Taniguchi 1974). The emergence of the nanotechnology industry might be traced to the Nobel Award winning discovery of the fullerene by Richard Smalley in 1985 (Kroto et al. 1985) or the first reported manipulation of material at the atomic level by Eigler and Schweizer in 1990 (Eigler & Schweizer 1990) or even the first detailed characterization of carbon nanotubes in 1991 by Iijima (Iijima 1991). The industry boom, however, didn’t begin until after the National Science and Technology Council released a report in 1999 on the future of nanotechnology (Roco & Williams 1999). The report spurred formation of the National Nanotechnology Initiative in 2000, aimed at stimulating advancements in the burgeoning field. Just over a decade later, the Initiative reported that annual spending on nanotechnology was nearly 2 billion U. S. dollars in the United States, 44 million in Brazil, 600 million in the European Union, and about 60 million in India (Baucher et al. 2013). According to The Project on Emerging Nanotechnologies, the number of consumer products that claim to use nanomaterials has grown from 54 in 2005 to more
than 1600 in 2013 (Project on Emerging Nanotechnologies 2013). The products come from a broad range of categories including home appliances (laundry, heating, cooling, and kitchen), the automotive industry (parts, maintenance, and lubrication), electronics (batteries, computers, mobile devices, and televisions), foods and beverages (supplements, coloring, storage, and cooking), health and fitness (clothing, cosmetics, filtration, personal care, sporting goods, and sunscreen), as well as general home and garden products (cleaning, construction, and paints). This dramatic increase in research, manufacturing, and use of NMs translates to an increase in human exposure.

Unfortunately, like most novel technologies, the industry grew faster than our knowledge about the health effects of nanomaterials. Great concern has risen over the safety of nanometer sized carbon and inorganic metal particles because the very properties that distinguish them from their bulk counterparts may cause detrimental effects not revealed by historical studies of the bulk materials (Oberdörster et al. 2005). In fact, NMs have some of the same properties of bulk materials but have more surface atoms per unit mass allowing greater influence by quantum mechanics and causing changes in spectral properties, heat and electrical conduction, and their reactivity with other molecules (Feynman 1959). In fact, their minute size and high reactive surface area may promote cellular uptake and deleterious interaction with biomolecules (Powell & Kanarek 2006). While nanometer-sized particulates are probably ubiquitous and humans have been exposed to them throughout history, the increased engineering and purposeful use of nanoparticles has magnified that exposure. The realization of that potential hazard has greatly expanded study into the health effects of NMs and spawned the field of nanotoxicology (Oberdörster et al. 2005).
Silver nanoparticles in wound management

The fastest growing sector of nanotechnology is AgNPs, due mainly to their antibacterial properties which are exploited in over 200 registered consumer products including wound dressings, personal care products, sheets, towels, clothing, toys and products for infants, food containers, laundry products, health drinks, cleaning sprays, air purifiers and humidifiers, and paints and coatings on various large and small appliances like laundry machines, refrigerators, haircare tools, and computer peripherals (Nowack et al. 2011; Project on Emerging Nanotechnologies 2013). The surface of AgNPs are known to become oxidized in solution, which facilitates the release of ionic silver (Ag⁺) at a rate that depends upon the particle’s size and surface coating and the pH and dissolved oxygen content of the solution (Kittler et al. 2010; Liu & Hurt 2010; Maurer et al. 2014; Xiu et al. 2011). The antibacterial properties of Ag⁺ are well recognized and have been exploited for centuries in water purification, wound care, and dietary supplements to bolster immunity, and so the discovery that AgNPs are bactericidal was not surprising (Kim et al. 2007; Silver et al. 2006). The proposed advantage of using AgNPs over the more common sources of Ag⁺, such as silver nitrate (AgNO₃) or silver sulfadiazine, is in the slower release of Ag⁺ from AgNPs which is expected to provides antibacterial protection for an extended period, allowing less frequent changing of the dressing, which prevents disruption of the healing tissue and increases the rate of wound closure (Atiyeh et al. 2007). Counting on that supposition, at least two commercial wound dressings have been produced which contain AgNPs: Acticoat® (Smith & Nephew, Hull, UK) and PolyMem Silver (Ferris Mfg. Corp., Burr Ridge, IL). PolyMem incorporates metallic silver particles into their standard foam dressing while Acticoat is made by depositing
silver atoms directly on a membrane using physical vapor deposition (Dunn & Edwards-Jones 2004). Atomic force microscopy (Bianco et al. 2015) and scanning electron microscopy (Dunn & Edwards-Jones 2004) of the silver layer of Acticoat have revealed an intricate nano-topography on the surface of the membrane with vertical displacements between 16 and 120 nm and horizontal widths of about 15 nm; however, the nano-topology is created as a layer on the membrane and appears in cross-section as projections from the surface, and so the claim of using nanoparticles might be somewhat misleading. Nevertheless, Acticoat, is reported to releases Ag\(^+\) into liquid media for an extended period of time owing to its nano-architecture (Dunn & Edwards-Jones 2004).

The antibacterial efficacy of Acticoat in vitro and its superiority over dressings that contain other forms of silver have been well documented (Castellano et al. 2007; Ip et al. 2006; Ulkür et al. 2005). More importantly, both animal models (Olson et al. 2000; Wright et al. 2002) and human studies (Demling & DeSanti 2002; Dunn & Edwards-Jones 2004) have demonstrated more rapid healing through reduced bacterial colonization, reduced inflammation, and increased re-epithelization. Unfortunately, none of the studies already cited followed the wounds to complete resolution. In one study that did follow wounds to their complete resolution, two patients had to be switched to another dressing because Acticoat failed to induce any signs of wound closure (Innes et al. 2001). In that study, a similar dressing that did not contain silver outperformed Acticoat in every measure of wound healing, and another study (Ozaki et al. 2015) reported that there was no advantage to using Acticoat over a dressing that did not contain silver. In early in vitro studies that were based on cellular metabolic activity, Acticoat was reported to be cytotoxic to keratinocytes and fibroblasts (Paddle-Ledinek et
al. 2006; Poon & Burd 2004), but more recently, metabolic activity was shown to be reduced in fibroblasts while the cells remained viable (Rigo et al. 2013). In other studies, the silver from Acticoat reached systemic circulation without causing any detrimental effects (Vlachou et al. 2007), but caused reversible liver toxicity when the blood concentrations reached 100 μg/ml in a patient with mixed depth burns over 30% of his body (Trop et al. 2006). Mixed results have also been reported for PolyMem Silver, silver-containing hydrogels, and the direct application of silver nanoparticles to experimental wounds (Boonkaew, Kempf, Kimble, Supaphol, et al. 2014; Boonkaew, Barber, et al. 2014; Boonkaew, Kempf, Kimble & Cuttle 2014; Chowdhury et al. 2014; Jain et al. 2009). The mix of positive reports such as inhibiting bacterial colonization and an increased rate of wound healing and negative effects such as cytotoxicity and inhibited wound closure, leaves one to question the benefit of AgNPs in wound management.

The cellular effects of silver nanoparticles

The potential for AgNPs to cause cellular toxicity in vitro has been well documented. The effect most often reported in mammalian cell lines is oxidative stress (Comfort et al. 2011; Foldbjerg et al. 2011; Kawata et al. 2009; Kim et al. 2009) which has been linked to lipid and DNA damage in vitro and also in animal models (Ahamed et al. 2008; Arora et al. 2008; AshaRani, Low Kah Mun, et al. 2009; Ghosh et al. 2012). Interestingly, the typical cytotoxicity study involves a relatively short exposure (up to 24 hours) followed by an immediate assessment for overt signs of toxicity, but animal models have shown that AgNPs distribute to many different tissues regardless of the exposure route, and are persistent in those tissues. Relatively high concentrations of silver (0.1 – 3 μg/g tissue) have been reported in the liver, kidney, lung, brain, and hind-leg
muscle of rats after 28 daily doses by oral gavage (Loeschner et al. 2011). Similar concentrations (0.2 – 0.6 µg/g tissue) were reported in the same tissues 28 days after a single intravenous dose (Dziendzikowska et al. 2012). The concentration of silver in the kidney after an intravenous dose was still high on day 28 even though the urinary excretion of silver was maximal at day 5, which suggests that the silver was not only secreted by the kidney but was sequestered in the tissues. Persistent concentrations of silver were also reported in the lungs (98 ng/g tissue) and liver (3 ng/g tissue), 7 days after a single inhalation exposure to AgNPs (Takenaka et al. 2001). The persistent presence of silver in those tissues is likely a source of persistent oxidative stress, because the particles themselves have been linked to oxidative stress and they can release Ag\(^+\) which also causes stress by binding sulfhydryl groups and creating an imbalance in thiol-mediated, reduction and oxidation (redox) homeostasis (Baldi et al. 1988). In humans, there are few studies into the effects of AgNPs. In the studies already mentioned regarding the use of Acticoat, only two studies measured effects outside the wound area: blood concentrations and liver toxicity associated with high blood concentrations. One clinical study has been reported in which a commercial solution of colloidal silver was administered in daily oral doses to human subjects for up to 14 days (Munger et al. 2014). There was no evidence of toxicity in that study; however, the colloidal solution was made by electrolysis of water using silver electrodes and analytical analysis revealed that Ag\(^+\) accounted for 84% of the total silver, and so the subjects were actually exposed to relatively small doses of AgNPs. Historically, the only negative effect associated with Ag\(^+\) in humans is argyria, a blue and grey discoloration of tissue (Drake & Hazelwood 2005), which was also reported in the Acticoat studies already discussed. The
discoloration is likely produced by deposits of insoluble silver salts, like silver sulfide, which have been found in the biopsies of discolored skin (Westhofen & Schäfer 1986). That silver is presumed to be unavailable for further reaction and probably explains the low incidence of toxicity associated with solutions that are mainly composed of Ag\(^+\). In fact, the difference between Ag\(^+\) and AgNPs is that the Ag\(^+\) can become immobilized immediately upon entering a tissue, but AgNPs can be internalized by the cells and then release Ag\(^+\) in a “Trojan-horse” style of toxicity (Park, et al., 2010). That slow release of Ag\(^+\) may cause persistent oxidative stress and produce long-term toxicity that has not been studied extensively with regard to AgNPs. During wound treatment, cells within the tissue surrounding the wound site are exposed to AgNPs for several days and might internalize an appreciable amount of the particles. Long-term effects, such as redox imbalance, might have serious implications for the ability of that tissue to properly heal and for its long-term health. One potential consequence of long-term oxidative stress is the induction of cellular senescence. Cellular senescence is a natural process by which cells enter a state of permanent growth arrest (Hayflick 1965), but it can also be induced prematurely by oxidative stress (Toussaint et al. 2000). Functional changes associated with cellular senescence include the persistent secretion of inflammatory cytokines, changes in attachment protein expression, and altered cellular differentiation (Coppe et al. 2008; Maier et al. 1993; Saunders et al. 1993). Those changes can cause suboptimal cell or tissue function, inhibit the self-repair of tissues, or even cause chronic inflammation which can contribute to osteoporosis, osteoarthritis, atherosclerosis, and cancer (Campisi et al. 2011; Freund et al. 2010; Wang et al. 2010). Jun and Lau (Jun & Lau 2010a) have postulated a role for senescence in wound healing based on their own
study which suggested that myofibroblast cells were inactivated by the induction of cellular senescence (Jun & Lau 2010b). The study, however, did not convincingly rule out quiescence, which is reversible cell-cycle arrest, instead of senescence, and their theory contradicts a well-established wound-healing model (Desmouliere et al. 1995). In the established model, myofibroblast cells continue to secrete extracellular matrix to close the wound and chemoattractant to recruit immune cells until the myofibroblasts undergo apoptosis. The cells in Jun and Lau’s study would not have entered senescence prior to apoptosis because senescent cells are resistant to apoptosis (Al-Mohanna et al. 2004; Wang 1995). Jun and Lau recognized senescence by senescence associated β-galactosidase activity, which has been reported in quiescent as well as senescent cells (Severino et al. 2000) and by expression of p16\(^{INK4a}\) protein which can be induced by TGF-β (Vijayachandra et al. 2009), but TGF-β is also involved in the differentiation of fibroblast to myofibroblast cells (Xu et al. 2012). Jun and Lau, therefore, may have mistaken the differentiation of fibroblasts into myofibroblasts for cellular senescence. Senescence is more likely to interfere with wound healing than to encourage it because senescence causes chronic inflammation which ultimately inhibits proper wound resolution (Martin & Leibovich 2005). If silver nanoparticles induce senescence, then they might inhibit proper wound resolution. The potential for AgNPs to induce senescence has not been reported to date; however, senescence is a programed response which develops over a period of time and would not be detected in a typical, short-term cytotoxicity assay. Two studies have suggested that AgNPs have a long-term effect on cell proliferation. Zanette, et. al. (Zanette et al. 2011) claimed that AgNPs inhibited HaCaT proliferation for up to 6 days following a 24-hour exposure, but their conclusion
is not convincing because they measured total protein content and metabolic activity to indicate proliferation instead of cell counts or growth rate, and their culture images clearly showed that the cell density was different between treatments. Never-the-less, they did demonstrate that AgNPs had a long-term effect (reduced metabolic activity) that was not apparent until 6 days after the exposure ended. AshaRani, et. al. (AshaRani, Hande, et al. 2009) demonstrated that exposure to AgNPs caused a delay in the time it took normal human lung fibroblast cells and human glioblastoma cells to reach confluence. The delay, however, may have been caused by a dramatic reduction in cell density during exposure because the concentrations were relatively high (≥ 200 μg/ml). Unfortunately, the toxic effect immediately after exposure was not described and the mechanism of inhibition was not studied. The potential for AgNPs to induce cellular senescence would be better evaluated using an exposure that does not cause obvious, or overt toxicity, and that was the goal of the first section of this thesis. In this study, AgNPs induced a senescent-like phenotype in A549 cells, a pulmonary epithelial cell line. The phenotype was induced over time, after an exposure that did not cause overt toxicity (10 μg/ml for 24 hours). These results suggest a potential for AgNPs to induce cellular senescence which is likely to interfere with proper wound resolution; however, that negative effect must be weighed against potential benefits related to their antibacterial activity before speculating on the overall value of AgNPs in wound management.

The antibacterial properties of silver nanoparticles

The use of Ag⁺ as an antibacterial agent has a long history, and some argue that AgNPs have a long history of use but that their presence in silver products was simply not recognized (Nowack et al. 2011). The activity of AgNPs against a broad spectrum of
bacteria including Gram negative and Gram positive organisms, and antibiotic resistant bacteria, has proven consistent regardless of what coating was used to stabilize them, making AgNPs a valuable asset for preventing bacterial infection (Ansari et al. 2011; Khan et al. 2014; Panacek et al. 2006; Pasupuleti et al. 2013; Sharma et al. 2009). The antibacterial potency of AgNPs increases as the particle diameter decreases (Morones et al. 2005; Samberg et al. 2011), which correlates with a greater release of Ag⁺ from smaller particles (Sotiriou et al. 2012; Zhang et al. 2011). Despite the well characterized release of Ag⁺ from AgNPs and recognized importance of Ag⁺ as an antibacterial agent, there is still some debate over the dependence of AgNPs on Ag⁺ for bactericidal activity. The argument against dependence on Ag⁺ is based on AgNPs still being able to inhibit bacteria when the amount of released Ag⁺ that was measured in the medium was not sufficient for antibacterial activity (Amato et al. 2011; Fabrega et al. 2009; Sondi & Salopek-Sondi 2004; Taglietti et al. 2012). The studies which support that statement, however, did not use the same conditions for studying dissolution and bacterial inhibition so that the effects of agglomeration, salt content, reactive surface area, and the bacteria themselves were not taken into account. Another, more convincing study demonstrated that antibacterial efficacy correlated with Ag⁺ release (Xiu et al. 2012). When the AgNPs were synthesized under anaerobic conditions and exposed to oxygen for defined periods of time, the amount of Ag⁺ released correlated directly with how long the AgNPs were exposed to oxygen, and the magnitude to which E. coli was inhibited correlated with the amount of Ag⁺ released. In fact, the AgNPs did not release Ag⁺ and were unable to inhibit the bacteria when maintained under anaerobic conditions. The antibacterial efficacy of AgNPs, therefore, appears to depend on the release of Ag⁺, and that
supposition is further supported by the studies that have identified the same mechanisms of action to both Ag⁺ and AgNPs (see the next paragraph) even though some of the targets are inside the cell where AgNPs have limited access. In mammalian cells, particles are internalized by energy-dependent endocytosis, but bacteria, except for one example in the budding bacterium, *Gemmata obscuriglobus* (Fuerst & Sagulenko 2010), do not have endocytic machinery. The particle must pass through the bacterial cell membrane, but that is unlikely due to their size and surface charge. Gram negative bacteria also have an outer membrane barrier through which sugars, ions, and amino acids can pass using channels formed by outer membrane porin proteins (Opr). The channels can be substrate specific like phage-lambda-receptor-protein (LamB) which is a maltose-specific porin in *Escherichia coli* (Van Gelder et al. 2002), or general like outer-membrane-protein A (OmpA) which has homologs in many bacterial species (Hancock & Brinkman 2002). Most of the general porins provide channels for the passage of 0.5 to 1.5 kDa molecules; however, molecules as large as 3.0 kDa can pass through the *Pseudomonas* OprF (Hancock & Brinkman 2002). The Dalton is a 3-dimensional unit and does not convert directly into the 2-dimensional unit of nanometers, but using Yoon’s approximate correlation (Yoon 2011), the size of particles that can pass through the porins ranges from 1.2 to 2.5 nm. That correlation between Daltons and nanometers is confirmed by sugar-diffusion studies which suggest that OmpAAb from *Acinetobacter baumannii* allows the passage of 1,000 Dalton sugar molecules that are about 2 nm in size (Sugawara & Nikaido 2012; Nikaido et al. 1991). The potential for pores with openings up to 2.5 nm in diameter means that the smallest nanoparticles might pass through the Gram negative outer membrane, but they will still be excluded by the cell
membrane, the only membrane barrier in Gram positive bacteria and the inner membrane barrier in Gram negative bacteria. Ions can cross the cell membrane through ion-specific channels or by facilitated transport, but no studies that address the transport of Ag⁺ into bacterial cells have been reported, and so the transport method for internalization of Ag⁺ is unknown at this time. The Ag⁺ may be able to utilize the same uptake pathway as other cations, or it might be internalized during the protein-mediated uptake of essential metals. For example, both Gram negative and positive bacteria utilize siderophore proteins for the uptake of ferrous iron (Fe²⁺) (Brown & Holden 2002; Cartron et al. 2006), and one such protein, pyochelin from P. aeruginosa, has been shown to bind Ag⁺ (Braud et al. 2009), but the internalization of Ag⁺ bound by siderophores has not been reported. Only the specific export of Ag⁺ has been studied and that has been most extensively studied in association with “Sil” proteins which were discovered on the plasmid pMG101, that conferred silver-resistance to Salmonella typhimurium in silver-treated burn wounds (Gupta et al. 1999; Larkin McHugh et al. 1975). The association of silver uptake with antibacterial efficacy and the ability of some bacteria to accumulate silver from the environment (Slawson et al. 1990) suggests that a pathway for Ag⁺ to cross the cell membrane does exist. On the other hand, the internalization of AgNPs has only been reported in combination with damage that compromised the integrity of the bacterial membrane (Li et al. 2010; Mei et al. 2014).

The reported antibacterial mechanisms for Ag⁺ include membrane damage (Jung et al. 2008; Taglietti et al. 2012), inhibiting aerobic respiration (Bragg & Rainnie 1974; Gordon et al. 2010; Holt & Bard 2005; Yamanaka et al. 2005), the binding and condensation of DNA (Hossain & Huq 2002; Feng et al. 2000; Morones et al. 2005;
Taglietti et al. 2012) and inducing oxidative stress (Park et al. 2009; Gordon et al. 2010). Likewise, AgNPs have been shown to affect membrane integrity (Li et al. 2011; Lok et al. 2006; Mirzajani et al. 2011; Morones et al. 2005; Taglietti et al. 2012), inhibit aerobic respiration (Du et al. 2012), bind to isolated DNA (Yang et al. 2009), and cause oxidative stress (Choi & Hu 2008; Kim et al. 2007; Hwang et al. 2008). As discussed in the preceding paragraph, the bacteria are more likely to internalize Ag⁺ than AgNPs, and so if the AgNPs themselves have an effect on the bacterial cells, they most likely attack the cell membrane while the Ag⁺ that they release may enter the cells and attack intracellular targets. Li, et. al., have shown a concentration-dependent delineation of the effects of AgNPs in E. coli (Li et al. 2010) and S. aureus (Li et al. 2011), indicating that membrane damage is the primary mechanism. Taglietti, et. al. (Taglietti et al. 2012), re-affirmed that conclusion by showing that membrane damage is the most sensitive indicator, occurring at half the minimum inhibitory concentration (MIC) in E. coli. Hwang, et. al. (Hwang et al. 2008), reported that superoxide-based stress and membrane damage were equally important in the antibacterial mechanism of AgNPs. They used E. coli that were transfected with bioluminescent reporters specific for membrane damage, superoxide-based stress, peroxide-based stress, or DNA damage, and suggested that their results indicated the simultaneous action of AgNPs on the membrane and Ag⁺ on the electron-transport enzymes. The researchers strengthened support for their theory by demonstrating that AgNPs do not have synergistic activity with other membrane-active agents (Triton X100 and ampicillin) or with agents that target the ribosomes inside the cell (chloramphenicol and kanamycin), but act synergistically with the energy inhibitors, sodium azide and N,N-9-dicyclohexylcarbodiimide, suggesting that Ag⁺ which can
inhibit the electron transport enzymes was compounding the inhibition of energy production (Hwang et al. 2012). Based on the idea that AgNPs have multiple targets for bactericidal activity and that they are a continuous source of Ag\(^\text{+}\), which has multiple cellular targets, and that resistance which requires multiple changes to the bacterial phenotype is less likely to develop (Hooper 2001), some have proposed that bacteria are less likely to develop resistance to AgNPs than to antibacterial drugs (Pelgrift & Friedman 2013; Percival et al. 2005). Never-the-less, resistance to Ag\(^\text{+}\) has been reported in *Enterobacter cloacae* and *Pseudomonas stutzeri* that were isolated from silver-rich environments (Haefeli et al. 1984; Nakahara et al. 1989), and in *Salmonella typhimurium* and *Pseudomonas aeruginosa* isolated from silver-treated burn wounds (Larkin McHugh et al. 1975; Bridges et al. 1979). Increasing tolerance to Ag\(^\text{+}\) has also been reported in *E. coli* after stepwise exposure (Li et al. 1997). The resistance is typically mediated by efflux of Ag\(^\text{+}\) (Gupta et al. 1999), but the genes known to encode silver-binding and efflux proteins are rare (Percival et al. 2008) and may not be sufficient to protect the bacterium from exposure to silver (Woods et al. 2009). Expression of the plasmid-encoded genes for efflux systems and changes in the permeability of the cell membrane to reduce the amount of silver taken up by cells may both be necessary for effective silver resistance (Starodub & Trevors 1990; Slawson et al. 1990). Moreover, assuming that the AgNPs affect the bacterial membrane directly, they would be unaffected by the efflux systems that offer some protection from silver. If the predicted, low potential for resistance development is accurate, AgNPs would have a high value in wound management, especially in environments where inhibiting bacterial infection during the initial wound treatment may be critical for proper resolution, and where follow-up
treatment to prevent infection is often delayed, for example during military engagements (Murray 2008; O'Shea 2012). The goal of the second section of this thesis was to determine the potential for bacteria to develop resistance to AgNPs compared to ciprofloxacin, a potent, quinolone, antibacterial drug, and benzalkonium chloride, a membrane-active biocide. This study discovered that *P. aeruginosa* could rapidly develop resistance to AgNP or ciprofloxacin but not benzalkonium chloride, and *S. aureus* and *A. baumannii* could only develop resistance to ciprofloxacin. The mechanism of AgNP-resistance is not yet clear but appears to be related to the production of phenazine pigments which can bind or reduce Ag⁺, rendering it biologically unavailable.

These combined studies reveal that AgNPs have advantages and disadvantages for use in wound management. They are effective antibacterial agents with a low potential for bacteria to develop resistance, except for *P. aeruginosa* which can be effectively inhibited by AgNPs, but can develop resistance when the concentration of particles become sufficiently low. The AgNPs also have the potential to cause long-term oxidative stress in mammalian tissue that they penetrate, which can degrade the long-term health of the tissues and induce cellular senescence. Interestingly, the bacteria were unable to develop resistance to Ag⁺ and silver in that form is less likely to cause cellular toxicity due to its rapid salting-out in tissues. Perhaps the key to effective wound management is embedding AgNPs in a dressing so that a sufficient amount of Ag⁺ is released to the wound site to inhibit bacterial growth over an extended period of time, but the particles themselves would not be taken up by mammalian cells. Such an approach might be accomplished by enclosing the particles in a membrane that will allow fluid to enter and only the Ag⁺ dissolved in that fluid to escape.
SECTION 1: THE POTENTIAL LONG-TERM EFFECT OF SILVER NANOPARTICLES ON MAMMALIAN CELLS

Summary:

Silver nanoparticles induce a senescent-like phenotype in A549 epithelial cells following an exposure that does not cause overt cytotoxicity.

In order to determine if AgNPs induce premature cellular senescence, I exposed the pulmonary epithelial cell line, A549 cells, to AgNPs at a dose that did not cause overt toxicity (10 µg/ml) for 24 hours and then the AgNPs were removed. After three days, many of the cells displayed markers for cellular senescence including the expression of senescence associated β-galactosidase activity (SA-β-Gal), a large flat morphology, and a slight decrease in the rate of proliferation. Senescence was most likely induced by oxidative stress which was apparent 2-days after exposure to AgNPs and increased in magnitude thereafter. Less than 50% of the exposed cells exhibited a senescent phenotype, and that was probably due to the cancer-dereived, A549 cells which are resistant to senescence; however, the ability of AgNPs to induce a senescence-like phenotype in cells that are resistant to senescence is highly supportive of their ability to induce premature cellular senescence in normal mammalian cells. If a wound is treated with AgNPs and the cells internalize those particles, they will likely experience long-term oxidative stress and become senescent, which could interfere with wound healing.
Materials and Methods

Chemicals and reagents: The 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and 4′,6-diamidino-2-phenylindole (DAPI) and paraformaldehyde were from Life Technologies (Grand Island, NY). The Live-Dead Cell Staining Kit was from Biovision (Mountain View, CA) and the MTS assay was the CellTiter 96 AQueous One Solution Cell Proliferation Assay form Promega (Madison, WI). The Senescence Cells Histochemical Staining Kit, Doxorubicin (DOX), N-acetyl cysteine (NAC), and hydrogen peroxide (H₂O₂) were was from Sigma (Saint Louis, MO). The Quantitative Cellular Senescence Assay Kit was from Cell Biolabs (San Diego, CA). The RPMI 1640 medium was from ATCC. The Penicillin and Streptomycin solution (Pen/Strep), trypsin solution (0.25%), fetal bovine solution (FBS) and Dulbecco’s Phosphate Buffered Saline (DPBS) were from Hyclone Laboratories (Logan, UT).

The source and characterization of AgNPs: Biopure, citrate-stabilized, 10 nm AgNPs were purchased from nanoComposix (San Diego, CA). The particles were chosen because they typically have a small size distribution and their small size increases the likelihood of observing an effect due to the known, size-dependent cytotoxicity of AgNPs (Braydich-Stolle et al. 2010; Carlson et al. 2008; Gliga et al. 2014). The primary size and shape of the AgNPS were estimated by absorption spectroscopy based on surface plasmon absorption and then more accurately measured by transmission electron microscopy (TEM). The AgNPs were diluted to 50 µg/ml in water and then the absorption spectrum of 0.1 ml was measured using a Synergy HT Spectrophotometer Plate Reader (BioTek Instruments, Winooski, VT) and 0.005 ml was transferred to a Formvar carbon-coated, copper TEM grid (Electron Microscopy Sciences) and allowed
to air dry. Images of the particles were then captured using a Hitachi H-7600 TEM. The diameter of AgNPs were measured using ImageJ software (NIH) which was calibrated to the scale bar generated by the microscope. The hydrodynamic diameter and zeta potential, or relative surface charge, of the particles was measured in water and growth medium at 50 μg/ml using dynamic light scattering (DLS) and laser Doppler micro-electrophoresis in a ZetaSizer Nano (Malvern Instruments).

**The cell model:** The A549 cell line (ATCC CCL-185) was chosen for this study because it represents a population of cells whose entrance into senescence might severely reduce tissue function. The cells were derived from a human, alveolar cell carcinoma and are similar to type II alveolar epithelial cells which have important roles in alveolar function and are progenitor cells involved in repair of the alveolar epithelium (Barar et al. 2007; Fehrenbach 2001; Foster et al. 1998; Lieber et al. 1976). The A549 cells were cultured in RPMI 1640 medium, which was supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics, in a humidified, water-jacketed incubator at 37° C and 5% CO₂.

**The overt cytotoxicity of AgNPs:** The overt cytotoxicity of the AgNPs was assessed by measuring metabolic activity using the MTS assay and by viability using the Live-Dead Cell Staining Kit (Live-Dead kit). The MTS assay was chosen to measure metabolic activity because AgNPs will not interfere with the assay endpoint as long as the cells are thoroughly rinsed prior to adding the reagents (personal experience). The Live-Dead kit uses a fluorescent, membrane permeable, “Live dye” to count the total number of cells and propidium iodide to identify dead cells. The A549 cells were seeded to 96-well culture plates at 7.0 x 10⁴ cell/cm² and allowed to recover overnight, resulting in a confluent cell layer prior to exposure. The medium was then replaced with fresh culture
medium containing AgNPs at 0 – 20 μg/mL (12.5 μg/cm²) and then the cells were cultured for an additional 24 hours. Viability was determined using the Live-Dead kit according to the manufacturer’s recommendations, and the data was normalized to the percent of the mean, control viability. The assay was conducted on three different days with four replicates of each dose. Control viability was greater than 90% in each test.

For metabolic activity, the exposure medium was removed by aspiration and the cells were rinsed with 0.3 ml of DPBS. Fresh growth medium was added to each well (0.1 ml) and then 0.01 ml of MTS reagent was added to each well. The absorbance of MTS-formazan at 490 nm was measured immediately, using the Synergy Spectrophotometer, to represent background absorbance and then the culture plate was incubated for one hour (37° C, 5% CO₂) and the absorbance at 490 nm was measured again. Results were normalized by the percent of the mean, control absorbance after subtracting background. The assays was conducted on three different days with 4 replicates for each dose.

**Measuring oxidative stress:** Redox imbalance was measured by the oxidation of DCFH-DA. Cells were seeded to black-walled, 96-well culture plates at 7.0 x 10⁴ cell/cm² and allowed to recover overnight, resulting in a confluent cell layer prior to exposure. Cells were exposed to AgNPs (10 μg/mL, 6.25 μg/cm²) in complete medium for 12, 24, or 48 hours, rinsed with DPBS, and then incubated with 100 μM DCFH-DA in phenol free RPMI 1640 for 1 hour. The fluorescence of the oxidized probe was then measured at the excitation wavelength of 438 nm and emission of 525 nm (Ex485/Em528), using the well-scan mode of the Synergy HT Spectrophotometer. Cells exposed to H₂O₂ (50 μM) for 30 minutes were used as positive control to confirm the detection of intracellular oxidative stress. The relative fluorescence of treated cells was normalized to that of
untreated control cells and expressed as a multiple of the untreated control cells. The experiment was conducted on 3 different days with 4 replicates per treatment. In a second study, the cells were exposed to AgNPs for only 24 hours at a range of concentrations (0, 5, 10, and 20 µg/ml) and then they were provided fresh, AgNP-free medium daily for 3 days. Each day, eight wells per dose were analyzed for oxidative stress, but thirty minutes before adding the DCFH-DA, NAC (5 mM) was added to half of the wells. The second study was only conducted once with 4 replicates of each exposure.

**The uptake and persistence of AgNPs:** The uptake and persistence of AgNPs in A549 cells was studied using a CytoViva imaging system (CytoViva, Auburn, AL) that can image metal nanoparticles by darkfield microscopy with a specialized condenser to amplify the light diffracted by the particles. The A549 cells were seeded onto an 8-chambered microscope slide and exposed to AgNPs (20 µg/mL, 12.5 µg/cm²) for 2 days, rinsed with DPBS, and then cultured for 3 days in AgNP-free medium. The cells were then rinsed with DPBS, fixed with 2% paraformaldehyde, and counterstained with DAPI, and then examined under the CytoViva Imaging System.

**Measuring senescence-associated β-galactosidase activity (SA-β-Gal):** SA-β-Gal was measured quantitatively using the Senescence Cells Histochemical Staining Kit (histological stain) and qualitatively using the Quantitative Cellular Senescence Assay Kit (fluorescent stain). Cells were seeded to 48-well culture plates at 1.6 x 10⁴ cell/cm² and allowed to recover overnight. The seeding density was adjusted from that of the cytotoxicity and oxidative stress tests to prevent confluence during the 5-day study (overnight recovery, 24-hour exposure to AgNPs, and 3-day post-exposure incubation) because preliminary studies revealed that confluent cells had an increase in SA-β-Gal that
was not related to AgNP exposure (Figure 5e). The cells were then exposed to AgNPs at 10 μg/mL (6.25 μg/cm²) in complete culture medium for 24 hours. Immediately following exposure, cells were rinsed with DPBS and then detached from the culture surface by incubation with 0.25% trypsin solution at room temperature until lifting of the cell layer was obvious (typically 10 minutes) and then complete culture medium was added to neutralize the trypsin. For histochemical staining, the entire volume was transferred to one well of a 12-well plate and the cells were cultured an additional 3 days in complete culture medium. For fluorometric staining the cells were counted using a hemocytometer and seeded to a black-walled, 96-well imaging plate at 1.6 x10⁴ cell/cm² and then cultured an additional 3 days in fresh, complete medium.

The histochemical stain probes for lysosomal β-galactosidase activity at non-optimal pH which is the hallmark staining method developed by Dimri et al. (Dimri, et al., 1995). The manufacturer’s recommendations were followed except staining was shortened to reduce the background staining (Figure 6d). In brief, cells were partially fixed by a 6-minute treatment with 2% formaldehyde and 0.2% glutaraldehyde at room temperature. The cells were rinsed and then stained with a solution containing potassium ferricyanide, potassium ferrocyanide, and X-gal, for 4 hours at 37°C. Three images were captured from each well using a QColor 3 camera (Olympus America, Melville, NY) and QCapture Pro 6 software (QImaging, Surrey, BC, Canada). The number of SA-β-Gal positive (blue) and negative cells in each image were counted using ImageJ software (NIH, Bethesda, MD). For a positive control, cells were treated with 50 μM H₂O₂ for 3-hours and then subcultured and stained in the same manner as AgNP-exposed cells. Each exposure was conducted in triplicate and repeated on at least 3 different days.
The fluorometric stain measured β-galactosidase activity in live cells using a fluorogenic substrate after pre-treating the cells with a lysosomotropic agent in order to increase lysosomal pH and distinguish SA-β-Gal from intrinsic β-galactosidase activity. The manufacturer’s recommendations were followed except for two adjustments: the pretreatment solution was used at 2-fold greater concentration in order to reduce background staining, and the cells were fixed with 4% paraformaldehyde after SA-β-Gal staining, and then counterstained with Hoechst 33342. Fluorescent images were captured using a BD Pathway 435 Confocal Microscope (BD, Franklin Lakes, NJ) and evaluated visually for the presence of SA-β-Gal positive cells. Doxorubicin (DOX) was used as a positive control for fluorometric staining because I found it to be more effective than H₂O₂ at inducing senescence in A549 cells. The cells for DOX treatment were seeded directly to a black-walled, 96-well imaging plate, recovered overnight, and then they were treated with 50 μM DOX for 1 hour. Cells were then rinsed with DPBS and cultured an additional 3 days in fresh, complete culture medium prior to staining. Two studies using the fluorescent stain were conducted on different days with four replicates of each treatment.

**Measuring proliferation and senescent morphology:** Cells were seeded to 48-well culture plates at 1.6 x 10⁴ cell/cm² and allowed to recover overnight. Cells were exposed to 10 μg/mL AgNPs (6.25 μg/cm²) for 24 hours (37° C, 5% CO₂) and then rinsed with DPBS and detached from the culture surface by incubation in 0.25% trypsin solution at room temperature until lifting of the cell layer was obvious (typically 10 minutes). The cells were then detached completely by forcefully pipetting the trypsin solution over the cells. I then counted viable cells using the Countess automated cell counter (Life
Technologies, Grand Rapids, NY) and transferred \(4.4 \times 10^5\) viable cells into a new 100 mm culture dish \((8 \times 10^3 \text{ cells/cm}^2)\) with fresh, AgNP-free medium. DOX was used as a positive control in morphology and proliferation studies because preliminary tests found it more effective at growth inhibition than \(\text{H}_2\text{O}_2\). In order to maintain higher cell numbers for analysis, the cells treated with DOX \((50 \, \mu\text{M for 1 hour})\) were not trypsinized but simply rinsed with DPBS and then remained in the same culture dish with fresh, complete medium. Proliferation rate was measured by tracking the cumulative population doubling (CPD) during 20 days of recovery in AgNP-free medium following the method of Cristofalo, et. al. (Cristofalo, et al., 1998). Briefly, cells were subcultured at 70 – 80% confluence by detaching them with trypsin, counting them using the Countess, and transferring \(4.4 \times 10^5\) viable cells to a new 100 mm culture dish in the same way as described above at the end of the AgNP exposure. The CPD was then calculated using equation 1, in which \(X\) = the number of population doublings since the last subculture; \(N_H\) = the number of cells in the culture at the time it was subcultured; and \(N_I\) = the initial number of cells or the number of cells transferred into the culture dish during the last subculture. The cumulative doubling was calculated by the sum of all \(X\)'s from the time when the exposure to AgNPs ended.

\[
X = \frac{\log_{10} N_H - \log_{10} N_I}{\log_{10} 2}
\]

Since the cells were subcultured based on confluence, AgNP-treated and control cultures were often subcultured on different days. In addition, the culture dishes were coded to partially blind me to their treatment information, and that sometimes resulted in replicate cultures being subcultured on different days. In order to compare the CPD between treatments, all of the replicate CPD results for a particular treatment were pooled.
and the slope of the linear regression of CPD over time was compared by analysis of covariance. I also examined cells for senescent morphology during the CPD study by capturing representative, microscopic images of each culture on the 3 day after exposure ended. I analyzed the images for morphological changes including measuring the surface area of each cell using ImageJ software. The experiment was conducted on three different days with three replicate cultures for each exposure group.

**Statistical analysis:** Statistical analysis was accomplished with a combination of GraphPad Prism 5 for Windows (GraphPad Software, La Jolla, CA) and the data analysis tool pack in Microsoft Excel. Studies with more than two variables were compared by one-way ANOVA and if the difference between means was significant (p ≤ 0.05) individual means were compared by Dunnett’s Multiple Comparison post-test (p ≤ 0.05). The distribution of sizes in the surface area of control and AgNP-treated cells was not normally distributed and so the nonparametric, Mann Whittney test was used to compare the means (p ≤ 0.05).

**Results**

**Characterization of the AgNPs:** The AgNPs were mostly spherical with a mean primary diameter of 9.1 nm (± 1 nm) (Figure 1 and Table 1). The mean hydrodynamic diameter in water was 9.8 nm (± 1 nm) and in culture medium was 66.7 nm (± 0.2 nm), indicating that some agglomeration occurred. The zeta potential was negative 35 mV (± 0.6 mV) in water and negative 9 mV (± 0.45 mV) in culture medium, suggesting a change in solvation or interaction with salts or proteins in the culture medium that reduced the relative surface charge and therefore the electrostatic repulsion between particles, allowing them to agglomerate. The AgNPs had an absorbance maximum at a wavelength of 389 nm, which
is typical for 10 nm silver spheres (data not shown). In a separate study of the dissolution of these same AgNPs, less than 1% of the total silver was released as Ag\(^+\) after a 24-hour incubation in cell-free culture medium (Maurer, et al., 2013).

<table>
<thead>
<tr>
<th>(\lambda_{\text{max}}) (nm)</th>
<th>Mean, Primary Diameter (nm) ± SD</th>
<th>Hydodynamic diameter (nm)</th>
<th>Zeta potential (mV)</th>
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</thead>
<tbody>
<tr>
<td>389</td>
<td>9.1 ± 1.0</td>
<td>9.8 ± 1.0</td>
<td>66.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-35 ± 0.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(-9 ± 0.45)</td>
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</table>

Table 1: Characterization of the citrate-coated AgNPs from nanoComposix. The wavelength of maximum absorbance (\(\lambda_{\text{max}}\)) was determined using a plate reader spectrophotometer. The mean, primary diameter was measured from TEM images using ImageJ software. The hydrodynamic diameter and zeta potential were determined using the ZetaSizer Nano. SD is the standard deviation of the mean, nm is nanometers.
Figure 1: Representative TEM images of the citrate-coated AgNPs from nanoComposix. The particles were dried on a Formvar carbon-coated, copper TEM grid and images were captured using a Hitachi H-7600 TEM at 200,000x magnification.

**Overt toxicity:** The MTS and Live-Dead Cell assays indicated no effect on metabolic activity or viability, respectively, following a 24-hour exposure to AgNPs in the dose range of 5 – 20 μg/ml (Figure 2). Since the AgNPs did not cause an effect after a 24-hour exposure, the dose range tested would be reported as non-toxic in a typical *in vitro*, cytotoxicity study. The concentration of 10 μg/ml was chosen for further study because
that dose would be considered non-toxic based on these results and in a previous test using the same AgNPs and A549 cells (Maurer et al. 2014).

Figure 2: The AgNPs were not cytotoxic using standard MTS and Live-Dead cell assays. Confluent cultures of A549 cells were exposed to AgNPs for 24 hours at the concentrations indicated on the graph in complete growth medium. The cells were then rinsed thoroughly to remove residual silver and then the MTS assay was conducted to measure metabolic activity or membrane integrity was assessed using the Live-Dead cell assay. The tests were conducted on three different days with four replicates for each treatment. There was no significant difference between treatments (p ≤ 0.05), measured by one way ANOVA.

**Oxidative stress:** The AgNPs caused oxidative stress in A549 cells after 48-hours of continuous exposure (Figure 3a). There was no oxidative stress detected after 12 or 24
hours of exposure, and therefore the AgNP-induced oxidative stress did not become apparent until after an exposure that was longer than the typical, *in vitro* cytotoxicity study, and at a concentration that did not cause overt cytotoxicity. The AgNPs also caused oxidative stress after the direct exposure to AgNPs had ended (Figure 3b). At zero hours of recovery (equivalent to the 24 hour time point in Figure 3a) there was no apparent oxidative stress, but after 24 hours of recovery (48 hours after the start of exposure), and 48 and 72 hours of recovery, there was a concentration-dependent increase in stress that was mitigated somewhat by the addition of NAC. The change in oxidative stress between days for each exposure was also statistically significant. The results confirm that even after the AgNPs are removed from the culture medium, oxidative stress continued to increase, suggesting that the AgNPs persist inside the cells and continue to perturb redox homeostasis. Alternatively, the AgNPs could cause irreparable damage to the cells so that their health degrades over time and it became apparent in the redox imbalance measured here.

Figure 3: Oxidative stress was apparent in A549 cells 48 hours after the start of AgNP exposure. (a) A549 cells were exposed continuously to 10 µg/ml AgNPs for 12, 24, or 48 hours and oxidative stress was measured using
DCFH-DA. Stress was only detected after 48 hours. (b) A549 cells were exposed to 0, 5, 10, or 20 μg/ml AgNPs for 24 hours and then while the cells recovered in silver-free medium, the development of oxidative stress was monitored daily using DCFH-DA. Stress was detected after 24 hours of recovery (48 hours after the start of exposure) at each concentration of AgNPs. During recovery (b), half of the wells were treated with NAC (5 mM) in order to confirm that the increased fluorescence was due to oxidative imbalance. The results were analyzed by one way ANOVA with a Dunnett’s Multiple Comparison post-test (p ≤ 0.05). An asterisk (*) denotes statistically significant difference from untreated control cells (a and b) and between each dose group (b). The white dot indicates where the addition of NAC caused a significant change (p ≤ 0.05) in fluorescence from the similar sample without NAC.

**Uptake and persistence of AgNPs:** The A549 cells were exposed to AgNPs, rinsed to remove extracellular nanoparticles, cultured an additional 3 days in medium without AgNPs, rinsed again, fixed with paraformaldehyde and stained with DAPI. Imaging with the CytoViva Imaging System revealed that even after the series of rinses and culturing in AgNP-free medium, the AgNPs were still associated with the A549 cells (Figure 4). The CytoViva system cannot distinguish between external and internal AgNPs, and so while the particles are most likely inside the cells due to their persistence after extensive rinsing, strong attachment to the external cell membrane cannot be ruled out. Likewise, the CytoViva system cannot positively identify the highly reflective particles as silver, but nothing else in the culture is expected to produce such an intense signal.
Figure 4: A representative CytoViva image showing the persistence of AgNPs. The cells were exposed to AgNPs (20 μg/ml) for 24 hours and then rinsed to remove residual particles and allowed to recover for 3 days in AgNP-free medium. The cells were then rinsed again, fixed with paraformaldehyde, stained with DAPI (blue) and then the cells and the AgNPs (white) imaged using a CytoViva imaging system.

**Measurement of SA-β-Gal:** The A549 cells that were exposed to AgNPs at 10 μg/ml for 24 hours or to H₂O₂ (50 μM for 3 hours) had a statistically significant increase in SA-β-Gal compared to the untreated controls (Figure 5). The cells had an intrinsically high SA-β-Gal in preliminary studies (Figure 5d), but once the conditions were optimized by preventing confluence and reducing the incubation time for staining, background SA-β-Gal was reduced to about 20% (Figure 5a). Interestingly, the cell density was also decreased in the cultures exposed to AgNPs (Figure 5b) or H₂O₂ (Figure 5c) compared to control which suggests less proliferation in the treated cultures. The cells were partially fixed and stained in a buffer at pH = 6.0, so the detachment of unhealthy cells in the treated cultures during staining cannot be ruled out.
The conditions for using the fluorescent stain also had to be optimized. Prior to optimization, most of the untreated cells stained positive for SA-β-Gal (Figure 6d), but under optimal conditions, little stain was found in untreated control cells (Figure 6a) but many A549 cells that were exposed to AgNPs were positive for the SA-β-Gal marker (Figure 6b) and virtually all of those treated with the positive control, DOX, stained positive (Figure 6c).
Figure 5: Histochemical staining revealed increased SA-β-Gal in A549 cells. The A549 cells were untreated (a and d) or exposed to 10 µg/ml AgNPs for 24 hours (b) or to the positive control, 50 µM H2O2 for 3 hours (d) and then detached using trypsin and transferred to a larger culture dish. After 3 days of culture in silver-free growth medium, the cells were stained for SA-β-Gal using the Senescence Cells Histochemical Staining Kit. Digital images were captured the number of SA-β-Gal positive (blue in a – d) or negative cells could be counted. There was a high level of intrinsic SA-β-Gal (d), and so the culture and staining conditions had to be optimized in order to reduce the background staining. The experiment was conducted on 3 different days with 3 replicate treatments each day and the difference in the number of positive cells between treatments was compared by one-way ANOVA with a Dunnett’s Multiple Comparison post-test (p ≤ 0.05). The results are graphed as the fold-difference in positive cells from the untreated control and the asterisk (*) denotes significant difference from the untreated control cells.
Figure 6: Increased SA-β-Gal in A549 cells was confirmed by fluorescence staining. The A549 cells were either untreated (a and d) or exposed to 10 μg/ml AgNPs for 24 hours (b) or to the positive control, 50 μM doxorubicin for 1 hour (c) and then transferred to a 96-well culture dish and cultured for an additional 3 days in silver-free medium. The cells were then stained using the Quantitative Cellular Senescence Assay Kit which stained senescent cells with a fluorescent dye (yellow in b – d). The cells were then fixed with paraformaldehyde and counterstained with DAPI (blue in a – d) prior to collecting fluorescent images using a BD Pathway 435 Confocal Microscope. The images were evaluating for visual confirmation that more cells stained positive for SA-β-Gal after treatment with AgNP or Dox. Intrinsic staining was high (d) and so conditions were optimized to reduce the background staining.
**Senescent morphology:** Senescent cells adopt a large, flat morphology due to continued growth and protein synthesis in the absence of cytokinesis, which is termed hypertrophy (Chen & Ames 1994; Matsumura et al. 1979). In this study, hypertrophic A549 cells were readily identifiable by their large surface area and the thin, flat appearance of their cytoplasm (Figure 7, arrows). Some hypertrophic cells appeared in the untreated control cultures (Figure 7a, arrow), but they were uncommon. Hypertrophic cells were more common in cultures exposed to AgNPs (Figure 7b, arrows) and very common after exposure to the positive control, DOX (Figure 7c, arrows). Under greater magnification (Figure 8) the elongated morphology that was frequently observed in hypertrophic cells is readily apparent (arrows). Interestingly, there were some cells noted in the SA-β-Gal, histochemical-stain study that were exceptionally hypertrophic (surface area >20,000 μm²), positive for SA-β-Gal, and multinucleate (Figure 9).

The surface area of A549 cells is highly variable (Table 2). The surface area of each cell was measured from the images captured during the proliferation assay by using ImageJ software, calibrated to a scaled microscope slide. Untreated cells ranged in size from 49 – 3,030 μm² with a median of 236 nm and mean of 275 nm (± 210). Cells treated with AgNPs ranged in size from 40 – 5,113 μm² plus one very large cell at 348,654 μm² which was 38-fold larger than the next largest cell. The median and mean size of the AgNP-treated cells without the very large cell was 241 nm and 327 nm (± 372), respectively. When the largest cell was included the mean was 629 nm (± 10,252) but the median was unchanged. The cell-size distribution was greatly skewed from a normal distribution (Figure 10) and so the non-parametric, Mann Whitney test was used for
statistical comparison, but the p valued (0.076) did not meet the criteria for significance (p ≤ 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of cells measured</th>
<th>Surface area (μm²)</th>
<th>Cells with surface area greater than the median control by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD</td>
<td>Median (range)</td>
</tr>
<tr>
<td>Untreated</td>
<td>1185</td>
<td>275 ± 210</td>
<td>236 (49 – 3,030)</td>
</tr>
<tr>
<td>AgNP</td>
<td>1155</td>
<td>327 ± 372</td>
<td>241 (40 – 5113)</td>
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Table 2: The occurrence of hypertrophic cells in A549 cell cultures exposed to AgNPs. The cells were untreated or exposed to 10 μg/ml AgNPs for 24 hours and then rinsed, detached from the culture plate using trypsin, and transferred to a new culture plate with AgNP-free medium. After 3 days, microscopic images were captured and the surface area of A549 cells was measured using ImageJ software. SD is the standard deviation of the mean. The means were compared by the non-parametric, Mann Whitney test and were not found to have a p-value of 0.076.
Figure 7: Representative images of A549 cells displaying senescent, hypertrophic morphology. The cells were untreated (a) or exposed to 10 μg/ml AgNPs for 24 hours (b) or to 50 mM doxorubicin for 1 hour (c), and then rinsed, detached using trypsin, and transferred to a new culture plate with AgNP-free medium. Hypertrophic cells were clearly visible (arrows) after three days.

Figure 8: Images of untreated and AgNP-treated A549 cells under greater magnification. The cells were untreated (a) or exposed to 10 μg/ml AgNPs for 24 hours (b) and then rinsed, detached from the culture plate using trypsin, and transferred to a new culture plate with AgNP-free medium. After 3 days, microscopic images were captured. Two hypertrophic cells are clearly visible (arrows) in the AgNP-treated culture.
Figure 9: Two examples of exceptionally large hypertrophic cells. The image was from the histochemical-staining study in which A549 cells were exposed to AgNPs (10 μg/ml) for 24 hours, allowed to recover for 3 days in AgNP-free medium, and then stained for SA-β-Gal. The blue, SA-β-Gal stain is readily visible around the multiple nuclei-like structures and the rough edge of the plasma membrane is highlighted by red arrows for one cell and green arrows for the other cell. The cells had surface areas greater than 20,000 μm².
Figure 10: The distribution of cell sizes for untreated and AgNP-treated A549 cells. The cells were untreated (red) or exposed to 10 µg/ml AgNPs (green) for 24 hours, rinsed, and transferred to a new culture plate with AgNP-free medium. After 3 days, microscopic images were captured and the surface area of each cell was measured using ImageJ software. The inset is zoomed in on the same data for cell sizes greater than 800 µm².

**Proliferation:** Senescent cells will not proliferate, even resisting mitogenic stimulation (Chen & Ames 1994). In this study, proliferation was assessed by calculating the cumulative population doubling (CPD) during 20 days of recovery from AgNP exposure (10 µg/mL) or treatment with DOX (50 µM for 1 hour). The cell population treated with DOX failed to double during the 20 days of recovery (data not shown). Cells exposed to
AgNPs for 24-hours had a small but statistically significant \( (p \leq 0.05) \) decrease in the rate of population doubling when compared to untreated cells (Figure 11).

Figure 11: The proliferation of A549 cells was reduced after exposure to AgNPs. The cells were untreated (●) or exposed to 10 \( \mu g/mL \) AgNPs (▲) for 24 hours and then immediately transferred to a new culture dish for recovery in silver-free medium. The cells were subsequently transferred to a new dish each time the culture reached about 70\% confluence. During the transfer, cells were detached using trypsin, counted using Trypan blue dye and a Countess automated cell counter and then \( 4.4 \times 10^5 \) viable cells \( (8000 \text{ cells/cm}^2) \) were transferred to the new culture dish. The number of times that the cell population doubled was calculated after each transfer using Equation 1 in Materials and Methods. The data was analyzed by linear regression of the CPD against the recovery day. The coefficient of determination \( (R^2) \) for each treatment was 0.99 and the slopes of the two lines were significantly different \( (p < 0.0001) \) by analysis of co-variance.
Discussion

The expanding use of AgNPs, especially in wound management, and their persistence in tissues raises concern over their potential to cause long-term effects that are not apparent in typical, *in vitro* toxicity tests. This study found that AgNPs can cause changes in redox homeostasis even 3 days after direct exposure has ended. I also investigated one consequence of potential long-term oxidative stress: premature cellular senescence. I found that AgNPs were able to induce signs of premature cellular senescence after an exposure (24 hours at 10 μg/mL, 6.25 μg/cm²) that did not cause overt signs of toxicity but did cause redox imbalance after 2 days, even if the exposure ended after the first day. Senescence was marked by an increase in SA-β-Gal, a hallmark of senescent cells (Dimri et al. 1995). The increase in this study was about 2-fold over unexposed cells after the assay conditions were optimized to overcome a high background SA-β-Gal in the A549 model used here. High background SA-β-Gal has also been reported in transformed and quiescent cells (Severino et al. 2000), and its utility as a biomarker for senescence has been called to question, mainly because it appears to be greater in all non-dividing cell (Cristofalo 2005; Yang & Hu 2005). The increased enzymatic activity associated with SA-β-Gal is apparently caused by an increase in the concentration of lysosomal β-galactosidase which may result from increased expression or simply from the longer residence of lysosomes in cells that are not dividing to dilute their lysosomal content between daughter cells (Kurz et al. 2000; Lee et al. 2006). It is, therefore, important to analyze additional endpoints to confirm the induction of senescence. In this study, I also studied cellular morphology and proliferation to determine if cellular senescence was induced.
I saw an increase in hypertrophic cells associated with AgNP exposure. In addition to a statistically significant increase in surface area and the more frequent observation of large flat cells in AgNP-treated cultures, some exceptionally enlarged cells were also observed which were also multinucleate. Cells with more than one nuclear body were also observed in less dramatically enlarged cells which were treated with AgNPs. Multinucleation is not considered a biomarker for senescence, but is often reported in senescent cells including serially passaged fibroblasts (Matsumura et al. 1979), 5-Bromo-2-deoxyuridine-treated A549 cells (Masterson & O'Dea 2007), and DOX-treated A549 and colon cancer cells (Litwiniec et al. 2010; Sliwinska et al. 2009). Although I did not observe multinucleated cells after DOX treatment, my observations were conducted 3 days after treatment when the number of cells had already decreased by about half, and the multinucleate cells may have been lost during that time. In line with that theory, Litwiniec, et. al. (Litwiniec et al. 2010), observed multinucleated A549 cells just one day after treatment and Sliwinska, et. al. (Sliwinska et al. 2009), reported a dramatic decrease in multinucleated colon cancer cells between days 1 and 7 post-treatment. The more favorable survival of AgNP-induced multinucleated cells over DOX-induced, may be related to their potency. Both have been linked to DNA damage including double-stranded DNA breaks (Ahamed et al. 2008; Binaschi et al. 1997); however, as I demonstrated in this work, DOX is very potent and acts rapidly while AgNPs require high concentrations and/or long exposures to induce cytotoxic effects.

The induction of cellular senescence results in an enlarged and flattened cellular appearance which has been linked to disruption of the actin cytoskeleton (Alexander et al. 2004; Kwak et al. 2004; Lim et al. 2000). Cytoskeletal changes were not studied in this
work, but another study conducted in the same laboratory, and simultaneous with mine, found that long-term exposure to AgNPs at much lower concentrations than I used here (4 pg/mL daily for 14 weeks) can disrupt the actin cytoskeleton in HaCaT keratinocytes (Comfort et al. 2014). Comfort, et. al., did not study senescence in their work, and they exposed their cells continuously instead of allowing recovery like I did, but they demonstrated that AgNPs can cause a chronic state of cellular stress, further enforcing the potential for AgNPs to induce chronic redox imbalance that could lead to cellular senescence.

Senescent cells undergo permanent cell cycle arrest but in this study, AgNPs had only a moderate effect on proliferation. A549 cells double about every 22 hours, so rapid growth of unaffected cells might mask the effect of senescence. The relative percent of senescent cells would be further reduced during each subculturing event as only a portion of senescent cells were transferred to the new culture dish and would be further diluted by non-senescent, dividing cells. Furthermore, large, flat, senescent cells do not detach from the culture surface as readily as normal cells (Walen 2006) increasing the possibility that fewer senescent cells were transferred during each subsequent subculturing event. Longer exposures or a higher concentration of AgNPs might increase the number of affected cells and more clearly demonstrate the induction of senescence; however, our goal here was to determine if an exposure that appeared to be non-toxic in typical in vitro tests might induce senescence, and so future studies will have to investigate different exposure regimens. In this study, oxidative stress became apparent 48-hours after AgNP-exposure, and so in order to determine if a longer exposure would have a greater effect on proliferation, I tracked the CPD of A549 cells for several days after a 2- or 3-day
exposures to AgNPs, but saw no difference in the outcome from the 24-hour exposure (data not shown). I chose not to test higher concentrations because other studies with similar AgNPs have demonstrated cytotoxicity at 20 μg/mL or greater in various cell lines (Braydich-Stolle et al. 2005; Carlson et al. 2008; Comfort et al. 2011; Gliga et al. 2014), and in this study, the oxidative stress induced by 20 μg/ml AgNPs was dramatically increased over the 10 μg/ml at 3 and 4 days after exposure. Although that dramatic increase in oxidative stress may correlate with a greater number of senescent cells, I felt that the 10 μg/ml exposure was already greater than biologically relevant concentrations, and so increasing the concentration would only artificially inflate the hazards associated with AgNP-exposure. Using Acticoat as an example, the dressing is reported to release about 3.7 μg of silver per cm² of dressing (Wright et al. 2002). The dose I used in this study may already exceed that amount: assuming that all of the AgNPs that were added to the culture medium deposited onto the culture surface, the 10 μg/ml dose would result in 6.25 μg of silver per cm² of culture surface, almost twice the concentration that Acticoat is expected to release, and Acticoat dressing has the highest concentration of silver among silver-containing dressings (Boonkaew, Kempf, Kimble & Cuttle 2014).

Others researchers have also reported an apparent state of senescence without causing permanent growth arrest. A549 cells exposed to pyocyanin (Muller 2006) or particulate pollutants (Sanchez-Perez et al. 2014) displayed a senescent morphology and increased SA-β-Gal, but pyocyanin only decreased proliferation temporarily and particulate pollutants had no effect on proliferation. The limited effect on proliferation in A549 cells reported here and by others is most likely the result of a gene deletion which
prevents A549 cells from expressing the p16\textsuperscript{INK4A} protein (Okamoto et al. 1995). Senescence is typically brought about by a sequential induction of p53, p21\textsuperscript{CIP1}, and p16\textsuperscript{INK4A} proteins (Alcorta et al. 1996). The tumor suppressor protein, p53, initiates cell-cycle arrest in response to DNA damage. If DNA is not repaired, p53 can induce apoptosis, but the cyclin-dependent kinase inhibitor (CDKi), p21\textsuperscript{CIP1}, maintains cell cycle arrest and inhibits p53-induced apoptosis. In senescence, p21\textsuperscript{CIP1} is replaced by p16\textsuperscript{INK4A} which is also a CDKi but it permanently stops the cell cycle and inhibits apoptosis.

Another CDKi, p27\textsuperscript{Kip1} can cause senescent-like growth arrest (Collado et al. 2000) and its level was reported to increase in senescent, 5-Bromo-2-deoxyuridine-treated, A549 cells (Masterson & O’Dea 2007). Interestingly, p27\textsuperscript{Kip1} is less robust at arresting the cell cycle than p16\textsuperscript{INK4A} (Pajalunga et al. 2007). In this study, AgNPs had a modest effect on the proliferation of the p16\textsuperscript{INK4A} deficient A549 cells. If the senescent phenotype induced by AgNPs in this study (or pyocyanin or particulate pollutants in the other studies) was mediated by p27\textsuperscript{Kip1} then the A549 cells may have evaded permanent cell cycle arrest by overcoming that less robust pathway, but that remains to be tested. I confirmed the lack of p16\textsuperscript{INK4A} protein in the A549 cells used in this study by staining untreated, AgNP-treated, and H\textsubscript{2}O\textsubscript{2}-treated cells with a human p16\textsuperscript{INK4A} protein (Abcam) and Alexa Fluor-labeled secondary antibodies (Invitrogen) and visualizing the cells using fluorescence microscopy (data not shown). Although there was no fluorescent signal corresponding to the p16\textsuperscript{INK4A} antibody, I did not employ a positive control to verify that the antibody could detect the intracellular protein, and so those results were inconclusive. Future studies should compare the expression of p53, p21\textsuperscript{CIP1}, p16\textsuperscript{INK4A}, and p27\textsuperscript{Kip1} by measuring gene transcripts and protein products in order to determine if the relevant
proteins are induced, if $p16^{\text{INK4A}}$ is actually missing from the A549 cells, and if $p27^{\text{Kip1}}$ might be induced in place of the $p16^{\text{INK4A}}$ protein.

The effect of AgNPs on proliferation might also be studied by using the Click-iT EdU Assay for Flow Cytometry (ThermoFisher Scientific) which measures the incorporation of 5-ethynyl-2′-deoxyuridine (EdU) during DNA synthesis, using click chemistry to bind a dye-labeled azide to the EdU. Since the cells are not disrupted, flow cytometry can be used to measure dye incorporation in all of the cells, and changes in side scatter can also be used to detect particles present in the cytoplasm (Greulich et al. 2011). The presence of AgNPs could then be correlate with reduced DNA synthesis and indicate whether or not cells with intracellular AgNPs are proliferating.

Additional studies using a human, primary cell model are also necessary to further characterize the potential for AgNPs to induce cellular senescence in a normal human cell. Primary cells are not resistant to senescence and would be a more accurate representative for in vivo exposure. Alternatively, an animal model of wound healing could be employed. The results of this study, however, should bring attention to the potential for AgNPs to cause long-term effects which are not readily apparent in the typical, short-term, in vitro cytotoxicity test.

Another potential explanation for the increased size and reduced proliferation of A549 cells is their differentiation from alveolar type II pneumocyte (AT1)-like cells to alveolar type I pneumocyte (AT2)-like cells. The AT1 cells are smaller, cuboidal epithelial cells, while the AT1 cells are squamous epithelial cells with a more circular shape and larger surface area (Cheek et al. 1989; Ehrhardt et al. 2008). Primary, AT2 cells will differentiate into AT1 cells after several days in culture (Cheek et al. 1989;
Fuchs et al. 2003), but A549 cells do not spontaneously differentiate in culture (Foster et al. 1998), and must be treated with dexamethasone in order to induce differentiation (Barar et al. 2007). If AgNPs induce the differentiation of A549 cells into AT1-like cells then that would also cause an increase in surface area and decrease in proliferation similar to what was observed in this study. I attempted to distinguish the induction of senescence from differentiation by staining untreated, AgNP-treated, and dexamethasone-treated A549 cells with tannic acid and osmium tetroxide which has been described by others for staining the surfactant-storing lamellar bodies that are present in AT2 but not AT1 cells (Foster et al. 1998; Kalina & Pease 1977; Mason et al. 1985); however, there was no apparent staining in any of the cultures (data not shown), rendering the test inconclusive. Future studies should evaluate the presence of lamellar bodies and surfactant proteins in enlarged cells to confirm that they are senescent AT2 cells. Alternatively, the presence of caveolae along with the absence of lamellar bodies would reveal that differentiation to AT1 cells is responsible for the change in cellular morphology.

In relation to wound management, the impact of cellular senescence on the repair and long-term health of tissues is not well understood. Demaria, et. al. (Demaria et al. 2014) claim that senescence has a vital role in wound closure. They used a p16<sup>INK4A</sup> reporter construct to show that senescent cells were transiently present in the wound and suggest that because senescent cells secrete platelet derived growth factor (PDGF), which has an important role in wound closure, that the senescent cells were essential. The researchers did not determine if the expression of p16<sup>INK4A</sup> was actually linked to the induction of senescence. There is, in fact, a complicated interplay between transforming
growth factor beta (TGFβ) and PDGF at the site of the wound (Fischer et al. 2007; Pierce, Mustoe, Lingelbach, Masakowski, Griffin, et al. 1989; Pierce, Mustoe, Lingelbach, Masakowski, Gramates, et al. 1989). In addition, TGFβ has been shown to upregulate p16INK4A (Vijayachandra et al. 2009). It is not clear if TGFβ-induced expression of p16INK4A actually induces senescence, or if senescence-induced expression of p16INK4A would have the same effect on wound resolution as that induced by TGFβ. The research by Demaria, et. al., did show that wounds healed slower in p16/p21 knockout mice than in normal mice, suggesting that p16INK4A expression is important in wound resolution, but its role is unknown. Demaria, et. al., also found that myofibroblasts, which secrete collagen during wound healing, were the most likely source of the increased p16INK4A expression, but myofibroblasts have been shown to undergo apoptotic death at the end of the granulation stage of wound closure (Desmouliere et al. 1995). Since p16INK4A inhibits apoptosis, inducing senescence in those cells may lead to excessive collagen deposition. Senescence is also associated with intrinsic expression of inflammatory factors. In general, inflammation is good for preventing bacterial invasion of a wound, but inhibit the other processes in wound recovery. Alternatively, if the senescence-like phenotype that I observed in A549 cells is actually differentiation, and AgNPs are also able to induce differentiation in myofibroblast cells, then the AgNPs might reduce inflammation and hasten wound healing. They might also cause premature differentiation of the myofibroblasts, thereby reducing collagen deposition and delaying wound closure. Further tests should be conducted to determine the effect of AgNP exposure on myofibroblast cells both in vitro,
to determine if cells differentiate or become senescent, and in an in vivo wound model, to
determine if exposure to AgNPs will hasten or inhibit wound healing.

In summary, it seems clear from my work that AgNPs have the potential to induce
a senescent-like phenotype. Additional studies are necessary, though, to determine if
AgNPs can induce senescence in the cells that are directly associated with wounded
tissues and to evaluate how senescent cells will affect wound resolution.

Conclusion for section 1

This study demonstrated that AgNPs can have a long-lasting effect on mammalian
cells. The AgNPs were persistent in the cell culture and induced oxidative stress which
was not immediately apparent, but increases over time. The senescent-like phenotype
induced in A549 cells after exposure to AgNPs suggests that the long-term effects of
AgNP exposure may be significant; however, additional studies using animal models or
primary cells are necessary in order to support such a conclusion.
SECTION 2: LOW POTENTIAL FOR BACTERIA TO DEVELOP RESISTANCE TO SILVER NANOPARTICLES

Summary:

There is a low potential for bacteria to develop resistance to AgNPs but they are not a silver bullet.

The antibacterial properties of AgNPs are well known. Owing to their proven effectiveness against antibiotic resistant organisms and their continuous, long-term release of Ag⁺, some have proposed that AgNPs have a low potential for resistance development. On the other hand, the slow release of Ag⁺ might expose bacteria to sub-inhibitory concentrations, allowing them to adapt and develop resistance. This study demonstrates that prolonged exposure to ineffective concentrations of AgNPs leads to resistance in only one of the three bacteria tested, Pseudomonas aeruginosa. The mechanism of resistance is not yet clear but appears to derive from the phenazine pigments commonly secreted by that organism.

Material and methods

Chemicals and reagents: The trace-metal grade HCl was from Fisher Scientific; Analytical solutions for mass spectrometry were from Perkin Elmer; Dulbecco’s phosphate buffered saline (DPBS) was from Hyclone; acridine orange and Hoechst 33342 were from Invitrogen; and the remaining chemicals were from Sigma. The water was deionized and filtered by an ELGA system and sterilized in an autoclave (diH₂O).
**Synthesis and characterization of AgNPs:** Citrate-stabilized AgNPs with a nominal diameter of 10 nm were synthesized by a standard, one-pot method. Solutions of diH₂O, 0.4% (w/v) AgNO₃, 1% (w/v) tri-sodium citrate, and 0.5% (w/v) sodium borohydride were stored at 4 °C in order to ensure that a low temperature was maintained during synthesis. The diH₂O was stirred using a magnetic stir bar and then AgNO₃ was added to a final concentration of 0.01% (w/v) followed by sodium borohydride (0.005%) and tri-sodium citrate (0.004%) at one minute intervals. Immediately following synthesis the absorbance spectrum of the solution was measured using a Synergy HT Spectrophotometer Plate Reader (BioTec) and batches with a symmetrical absorbance peak centered near the 390 nm wavelength were chosen for further characterization and used in bacterial studies. The 10 nm diameter was chosen because it has been demonstrated that smaller AgNPs (5 - 15 nm) release more Ag⁺ (Liu et al. 2010; Zhang et al. 2011) and are more effective at inhibiting bacteria (Choi & Hu 2008; Samberg et al. 2011) than larger AgNPs. The citrate coating was chosen because a simple, reproducible synthesis method has been established and the citrate coating provides electrostatic stability in solution and is not expected to affect bacterial growth. The concentration of the AgNPs was determined by dissolving the nanoparticles in 1:3 nitrohydrochloric acid and quantifying the Ag⁺ using inductively coupled plasma mass spectrometry (ICP-MS) with a NexION 300D instrument (Perkin-Elmer). When necessary, the stock solutions were concentrated by using tangential flow filtration (TFF) with a MicroKros 10-kD, 20 cm² filter (Spectrum Labs) on a KrosFlo Research IIi TFF system (Spectrum Labs). The concentration of Ag⁺ in the stock solutions was determined by ICP-MS using the TFF filtrate. The primary size of AgNPs was determined by transmission electron microscopy
(TEM). An aliquot of the AgNP solution (8 µl) was transferred to a Formvar carbon-coated, copper TEM grid (Electron Microscopy Sciences) and allowed to air-dry. Images were captured using a Hitachi H-7600 electron microscope, and the particle diameters were measured using ImageJ software (NIH) which was calibrated to the scale bar that was generated by the microscope. In order to measure the primary size of AgNPs, the particles were dispersed in water and dried on TEM grids. The size of agglomerates were measured by dispersing the particles in MHB2 and then drying them on TEM grids. The diameter of each agglomerate was calculated as the mean of five measurements: the vertical and horizontal lengths through the approximate center of the agglomerate and the lengths from the upper most left to lower most right, and upper most right to the lower most left points. The hydrodynamic diameter and zeta potential of the AgNPs were determined by dynamic light scattering and laser Doppler micro-electrophoresis using a ZetaSizer Nano (Malvern Instruments) and solutions of AgNPs which were diluted to 25 µg/ml in the appropriate media (water or broth).

Organisms and culture conditions: The test organisms were purchased from ATCC and included *Pseudomonas aeruginosa* (ATCC 27853), *Acinetobacter baumannii* (ATCC19606), and *Staphylococcus aureus* (ATCC 29213). Each is an opportunistic pathogen known to interfere with wound healing, especially burns (Church et al. 2006) and wounds from military conflicts in the Middle East (Maragakis & Perl 2008; O’Shea 2012). In addition, each of the organisms is known for their potential to develop resistance to various antibiotics (Hancock & Speert 2000; Maragakis & Perl 2008; Rossolini et al. 2014). The stock cultures of each organism were stored at -80°C in Mueller Hinton Broth 2 (MHB2) supplemented with 10% (v/v) glycerol. Frozen stocks
were prepared for experiments by steaking them on Mueller Hinton agar (agar) which made by adding agarose (2% w/v) to the MHB2 medium prior to sterilization. After culturing overnight at 37° C, representative colonies were transferred to MHB2 and cultured an additional six hours to achieve exponential growth and a sufficient density to conduct experiments.

**Antibacterial efficacy: the MIC and MBC:** I measured antibacterial efficacy by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a standard, microtiter method modified slightly from those described by Amsterdam and by Jeannot and Plesiat (Amsterdam 1996; Jeannot & Plesiat 2014). The agents to be tested were prepared in flat-bottom, 96-well culture plates by serial, 2-fold dilution in diH₂O, leaving 50 μl in each well. The bacterial inoculum was prepared by diluting a culture in the exponential growth phase (after 6-hours at 37° C) to an optical density at 600 nm of 0.10 (OD₆₀₀ = 0.10) using pre-warmed MHB2, which was previously found to correlate with a bacterial density of about 1 x 10⁸ colony forming units (CFU)/ml. The culture was then diluted 1:1000 in pre-warmed MHB2 to make an inoculum density of about 1 x 10⁵ CFU/ml. The inoculum was then added to the test wells (50 μl). I confirmed the bacterial density in the inoculum by dilution-plate counting. The test plate was then incubated at 37° C for 18 to 20 hours and then evaluated for bacterial growth based on turbidity. The MIC was defined as the lowest concentration with no visible turbidity. I determined the MBC by spotting 10 μl from the MIC well and each of the next three greater concentrations onto agar and incubating the agar plate, inverted, at 37° C for at least 18 hours. The concentration which caused a 3-log or greater reduction in bacterial density from the inoculum was defined as the MBC,
and I identified it by the spot with no more than one colony growing. I tested each agent on at least 4 different days with four replicates on each day. The benchmark agents, ciprofloxacin (Cipro) and benzalkonium chloride (BZC), were prepared by dissolving the agents in diH₂O at 50 and 500 μg/ml, respectively. It was necessary to adjust the pH of the diH₂O to 5.0 using HCl in order to dissolve the Cipro, but I found that the pH was 7.0 once the Cipro was diluted in normal diH₂O and mixed with MHB2 for the bacterial studies. The MIC and MBC of Ag⁺ were determined using AgNO₃ dissolved in diH₂O.

**Resistance development:** The potential for *P. aeruginosa*, *S. aureus*, and *A. baumannii* to develop resistance to AgNPs, AgNO₃, BZC, and Cipro was tested by a broth, stepwise-increasing exposure model which was adapted from the one described by Entenza, et. al. (Entenza et al. 1997). I prepared the initial bacterial inoculum at about 1 x 10⁵ CFU/ml in the same way as described for the MIC study. The test agents were prepared in water at twice the target concentrations which were 0.25-, 0.5-, 1.0-, 1.25-, and 1.5-fold multiples of the agent-specific MIC. Equal volumes (1 ml) of the inoculum and the test agent were then mixed in the wells of a 12-well culture plate and incubated for at least 22 hours at 37° C on a slow-speed, rotary shaker. The greatest concentration in which turbid growth occurred was labeled the tolerable concentration, and that culture became the inoculum for the next step by adjusting the OD₆₀₀ to 0.10 and further diluting the culture 1:1000 in fresh, pre-warmed MHB2. In this way, the inoculum was the same for each step of the test (approximately 1 x 10⁵ CFU/ml) in order to avoid falsely identifying resistance due to a high inoculum being able to overcome the antibacterial agent. In preliminary studies I found that the MIC of AgNPs did not change when the inoculum was up to one log greater or less than 1 x 10⁵ CFU/ml. I also found that using 0.25-, 0.5-, 1.0-, 1.25-, and
1.5-fold multiples of the MIC prevented over-growth of a culture if the inoculum was too large and loss of cultures if the inoculum was too small, as long as the inoculum was correct at the next serial transfer. I verified the bacterial density, therefore, in each inoculum by dilution plate counting and discontinued any experiment which had two consecutive inoculums greater than $1 \times 10^6$ CFU/ml or less than $1 \times 10^4$ CFU/ml. The agents were prepared in the same manner for each step, but at multiples of the tolerable concentration instead of the MIC. This procedure was carried out daily for up to nine days. Resistance was indicated by a tolerated concentration that was at least 4-fold greater than the original MIC. Each species of bacteria was tested at least three times on different days against each test agent. Resistant cultures were expanded by culturing them in 50 mL of fresh MHB2, collected by centrifugation at 5,000x relative centrifugal force (rcf) for 10 minutes, re-dispersed in MHB2 which was supplemented with 10% (v/v) glycerol, and frozen at -80°C in aliquots of 400 µl. The AgNP-resistant bacteria were tested for cross-resistance to Cipro, BZC, and AgNO₃ and the stability of the AgNP-resistance was determined by conducting MIC and MBC tests on cultures derived from at least three different frozen aliquots. I then tested the stability of the resistance to AgNPs by MIC and MBC testing after serial passage of 10 µl of bacterial culture to 4 ml of fresh MHB2, daily for 9 days.

**Resistance mechanisms:**

**The efflux of fluorescent dyes:** I examined differences in general efflux between the normal *P. aeruginosa* and the AgNP-resistant strains by using a broth-culture adaptation of the maximum extrudable concentration assay which was originally described by Richmond, *et. al.* (Richmond et al. 2013). In Richmond’s assay, the maximum extrudable
concentration was that which caused a visible increase in the fluorescence of bacteria that were growing on agar which was supplemented with ethidium bromide or acridine orange. I conducted a broth-culture version of the experiment in the same way as the MIC test, but I replaced the antibacterial agents with the fluorescent dyes Hoechst 33342, ethidium bromide, and acridine orange, each at a starting concentration of 5 µg/ml. The microtiter test plate was a black-walled, 96-well culture plate and the fluorescent endpoints were measured using a SpectraMax M2 Multimode Plate Reader (Molecular Devices) after one hour of incubation at 37°C to allow efflux or at 4°C to inhibit efflux. The fluorescent endpoints were measured by the following excitation and emission wavelengths (Ex/Em): Ex502/Em 525 for acridine orange; Ex285/Em605 for ethidium bromide; and Ex361/Em497 for Hoechst 33342. I used four replicates for each concentration and tested each dye one time against the normal strain and 715npRPs strain. In a second test, the efflux of Hoechst 33342 was studied after 10 minutes of incubation using concentration up to 125 µg/ml in the normal strain of P. aeruginosa and up to 150 µg/ml in the resistant strains.

**Biofilm Formation:** Biofilm formation was assessed by a variation of the tissue culture plate method described by Christensen, et. al. (Christensen et al. 1985) with modifications by Oliveira and Cunha (Oliveira & Cunha 2010) and additional changes to the timing, medium, the starting inoculum, and the stain used for quantitative assessment. I prepared cultures of A. baumannii, Ps, 715npRPs, and 718npRPs at 1 x 10^8 CFU/ml as described for the MIC test and transferred 0.1 ml to the wells of a flat-bottom, 96-well culture plate. Since I wanted to determine if the AgNP-resistant strains formed biofilms more rapidly than the normal strain, I measured the attached cells after 0.5, 2, and 4 hours.
of incubation at 37°C. The unattached cells were removed by dumping the culture plate and rinsing the wells three times with diH₂O. I stained the attached cells and extracellular polymeric substance with a saturated iodine solution (Lugol’s solution) which I made by adding iodine crystals, 5% (w/v), to a solution of 10% (w/v) potassium iodide and mixing vigorously by vortex until no solid iodine was visible. The wells were stained with Lugol’s solution at room temperature for ten minutes and then dumped, rinsed once with diH₂O and allowed to air dry. I then measured the absorbance of each well at 450 nm using the SpectraMax plate reader and captured microscopic images using QCapture Pro 6 software (QImaging, Canada) and a QCColor 3 camera (Olympus America, NY) which was attached to an Olympus CKX41 microscope with a 10x objective. I compared the relative extent of biofilm production between each strain of P. aeruginosa at each time point both visually and by their absorbance at 450 nm. The biofilm production by P. aeruginosa was compared to that of A. baumannii as a negative control. The experiment was conducted one time with 8 replicates of each bacterial strain.

**Statistical Analysis:** Central tendencies were analyzed and charts created using either the data analysis tools in Microsoft Excel or Graphpad Prism. Differences between samples were analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison post test using Graphpad Prism. Statistical significance was identified by p-values less than or equal to 0.05 (p ≤ 0.05).

**Results**

**Characterization of AgNPs:** The AgNPs were synthesized in three different batches with maximum light absorbance of 392 nm, 388 nm, and 391 nm (Figures 12e, 2e, and 3e) which corresponded to median diameters of 5.3 nm, 9.4 nm, and 6.3 nm (Table 3,
Figures 12, 13, and 14). With the three batches combined and 1051 particles measured, the diameters ranged from 1.0 nm to 25.6 nm with a median of 5.8 nm. The overall distribution in sizes is displayed graphically in Figure 14f and representative TEM images from each batch are presented in Figures 12a – 12d, 13a – 13d, and 14a – 14d. The hydrodynamic diameter, shown in Table 4, is expressed as the Z-average diameter, which uses the intensity of scattered light from DLS data to calculate a weighted mean. In water, the Z-average for AgNPs was about 21 nm (± 0.65 nm) and it was slightly larger in MHB2, ranging from 26 nm to 34 nm between the batches, suggesting that some agglomeration occurred. The hydrodynamic measurements were obtained using MHB2 with the same composition that was used in the bacterial studies (diluted 1 part stock MHB2 to 1 part water which contained the test agent). When the stock MHB2 was used without dilution, the Z-average diameter increased greatly to 915 nm (± 16 nm), indicating substantial agglomeration in the more concentrated MHB2. This was confirmed by a typical change in the color of the solution from yellow in water (small, individual AgNPs are present) to brown in MHB2 (large agglomerates) (Figure 13f). The zeta potential, or relative surface charge, of the AgNPs was negative 38 mV (± 2.5 mV) in water and negative 13 mV (± 4.6 mV) in concentrated MHB2 (Table 3). The 3-fold change in zeta potential indicates a change in solvation or interaction with salts in the MHB2 which reduced the electrostatic repulsion between nanoparticles and allowed much greater agglomeration in MHB2. The agglomeration was further confirmed by TEM images of samples which were dispersed in concentrated MHB2 prior to being dried on a TEM grid. The TEM analysis revealed agglomerates of particles ranging in size from 714 to 4722 nm (Figure 15). Interestingly, the zeta potential in dilute MHB2
was 2-fold less negative than in water but there was much less agglomeration in the dilute MHB2 which was used for bacterial studies.

The concentration of Ag⁺ in the stock solution which was measured by separating the particles from the ions using TFF was about 0.04 µg/ml which represented about 0.08% of the total silver in the unconcentrated stock solutions or 0.01% of the total silver in concentrated solutions. The absorbance spectrum of the TFF filtrate did not show absorption of light at wavelengths greater than 300 nm, indicating that all of the AgNPs were preserved in the retentate (Figure 12f).

<table>
<thead>
<tr>
<th>Batch number</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Primary Size (nm)</th>
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<tr>
<td></td>
<td>D&lt;sub&gt;Mean&lt;/sub&gt; (SD)</td>
<td>D&lt;sub&gt;Median&lt;/sub&gt;</td>
</tr>
<tr>
<td>1</td>
<td>392</td>
<td>5.6 (2.2)</td>
</tr>
<tr>
<td>2</td>
<td>388</td>
<td>9.9 (4.0)</td>
</tr>
<tr>
<td>3</td>
<td>391</td>
<td>6.3 (2.5)</td>
</tr>
<tr>
<td>Combined</td>
<td>6.2 (2.7)</td>
<td>5.8</td>
</tr>
</tbody>
</table>

Table 3: The primary characteristics of the citrate-stabilized AgNPs. The wavelength of maximum absorbance (λ<sub>max</sub>) was determined by using a plate reader spectrophotometer to measure the absorption spectrum from a 1:10 dilution of the as-synthesized stock solution. The mean and median diameters (D<sub>Mean</sub> and D<sub>Median</sub>) were measured from TEM images using ImageJ software, SD is the standard deviation of the mean and nm is nanometers.
Figure 12: Characterization of the first batch of AgNPs. The representative images from TEM analysis (a, b, c, d) reveal mostly spherical AgNPs with diameters ranging from 2.1 nm to 16.6 nm. The AgNP solution was transferred to a Formvar carbon-coated copper TEM grid and allowed to air dry prior to imaging. The absorbance spectrum of the AgNPs, diluted 1:10 in water immediately after synthesis, was measured using a plate reader spectrophotometer. The spectrum showed a fairly symmetrical peak at the maximum absorbance of 392 nm (e) which was absent when the AgNPs were separated form Ag\(^+\) using TFF (f).
Figure 13: Characterization of the second batch of AgNPs. The representative images from TEM analysis (a, b, c, d) reveal mostly spherical AgNPs with diameters ranging from 2.4 nm to 25.6 nm. The absorbance spectrum showed a fairly symmetrical peak at the maximum absorbance of 388 nm (e). The TEM and absorbance analysis are described in the legend for figure 1. The AgNPs produced a yellow color when dissolved in water but a brown solution when dissolved in MHB2 (f). The vial labeled “W” is water, “NP W” is AgNPs in water (50 µg/ml), “B” is MHB2, “NP B” is AgNPs in MHB2 (50 µg/ml), and “0.1NP B” is AgNPs in MHB2 (50 µg/ml).
Figure 14 Characterization of the third batch of AgNPs. The representative images from TEM (a, b, c, d) reveal mostly spherical AgNPs with diameters ranging from 1.0 nm to 17.4 nm. The absorbance spectrum showed a fairly symmetrical peak at the maximum absorbance of 391 nm (e). The TEM and absorbance analysis are described in the legend for figure 1. The distribution in diameters measured from the TEM images for all three batches of AgNPs had a median value of 5.8 nm (f).
Figure 15 Images of the agglomeration of AgNPs in concentrated MHB2. The AgNPs were diluted to 50 μg/ml in concentrated MHB2 and incubated at room temperature for 10 minutes. The solution was then mixed by vortex and about 10 μl was transferred to a Formvar carbon-coated TEM grid and allowed to air dry. Images of the agglomerated AgNPs were captured by TEM. Images b, c, e, and f are greater magnifications of the areas indicated by the yellow boxes in the adjacent image.
<table>
<thead>
<tr>
<th>Batch number</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Z-ave (SD) in H₂O</td>
<td>PDI (SD) in H₂O</td>
</tr>
<tr>
<td>1</td>
<td>21 (0.65)</td>
<td>0.48 (0.003)</td>
</tr>
<tr>
<td>2</td>
<td>18 (0.12)</td>
<td>0.55 (0.002)</td>
</tr>
<tr>
<td>3</td>
<td>19 (0.94)</td>
<td>0.60 (0.04)</td>
</tr>
</tbody>
</table>

Table 4: The hydrodynamic characteristics of the citrate-stabilized AgNPs.

The Z-average diameter (Z-ave) and the polydispersity index (PDI) in water and MHB2 were measured using dynamic light scattering and the zeta potential was measured by laser Doppler micro-electrophoresis. Each value is the mean and standard deviation (SD) of three tests. NT means not tested, mV is millivolts, and nm is nanometers. The asterisk (*) indicates values that were generated using more concentrated MHB2 (not diluted to the strength used in antibacterial studies). Hydrodynamic measurements were not obtained for batch 2.

**Antibacterial Efficacy (MIC and MBC):** The antibacterial efficacy of AgNPs was assessed by micro-dilution MIC and MBC studies. The Gram negative organisms, *P. aeruginosa* and *A. baumannii*, were more sensitive to AgNPs than *S. aureus*, the Gram positive bacterium (Table 5). The two Gram negative bacteria were also more sensitive to AgNO₃ than *S. aureus* and more sensitive to AgNO₃ than to AgNPs. The Gram positive *S. aureus* was more sensitive to BZC than the two Gram negative organisms. Cipro was highly effective against all three bacteria. The MBC was generally the same or 2-fold greater than the MIC for each test agent. Sodium citrate was also tested and it did not inhibit bacterial growth at concentrations up to 1.7 mM, which is more than 10-fold
greater than the concentration used for AgNP synthesis (data not shown). There was no
difference in the MIC or MBC values between the three batches of AgNPs.

<table>
<thead>
<tr>
<th>Agent</th>
<th>P. aeruginosa</th>
<th></th>
<th>A.baumannii</th>
<th></th>
<th>S. aureus</th>
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<tr>
<td></td>
<td>MIC (µg/ml)</td>
<td>MBC (µg/ml)</td>
<td>MBC/MIC</td>
<td>MIC (µg/ml)</td>
<td>MBC (µg/ml)</td>
<td>MBC/MIC</td>
</tr>
<tr>
<td>AgNPs</td>
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<td>1.88</td>
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<td>2</td>
<td>7.5</td>
<td>30</td>
<td>4</td>
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<td>2</td>
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<td>0.47</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0.94</td>
<td>1.88</td>
<td>2</td>
<td>0.12</td>
<td>0.23</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 5: The MIC and MBC of test agents. The MIC and MBC of
ciprofloxacin (Cipro), benzalkonium chloride (BZC), silver nitrate
(AgNO₃), and citrate-stabilized AgNPs were tested against Pseudomonas
aeruginosa, Acinetobacter baumannii, and Staphylococcus aureus in
micro-dilution assays; µg/ml is micrograms per milliliter and MIC/MBC is
the dividend of the MIC and MBC; each value represents the median of 8
to 30 tests.
Table 6: Stepwise resistance development test results. The development of resistance was tested by a stepwise increasing exposure model. Each pair of bacterium and agent was tested at least twice. The results, relative to the MIC, are listed for exposures that resulted in resistance (bold) or the greatest tolerable concentration after nine experimental steps. Resistance was defined by growth at 400% of the MIC (4-fold greater concentration than the MIC).

**Resistance Development:** The development of resistance to AgNPs, Cipro, BZC, and AgNO₃ was studied using a stepwise increasing exposure model in which the bacteria were exposed to the test agents at 0.25-, 0.5-, 1.0-, 1.25-, and 1.5-fold multiples of the MIC and subsequent tolerable concentrations. Resistance was defined as growth in a concentration that was 4-fold greater than the original MIC. All three of the bacteria tested developed resistance to Cipro, but only *P. aeruginosa* developed resistance to AgNPs (Table 6). Cipro-resistance developed relatively quickly in *P. aeruginosa* (step 3) and *A. baumannii* (step 4) in each of the two tests for each organism, but it did not develop as rapidly in *S. aureus* (step 8). The AgNP-resistance that developed in *P.*
P. aeruginosa was equally as rapid (2 or 3 steps). None of the bacteria developed resistance to BZC or AgNO₃. While S. aureus was able to tolerate AgNO₃ at 22 µg/ml, or 340% of the MIC by step 5 of the test, greater concentration inhibited its growth in steps 5, 6, 7, 8, and 9, which prevented resistance from developing. It is worth noting that in 2 preliminary studies in which 0.5-, 1-, and 2-fold multiples of the MICs and tolerable concentrations, P. aeruginosa was eliminated by AgNO₃ in the second step of the study, and AgNPs eliminated the bacteria by the third step. The bacteria could only develop resistance to silver, therefore, when the concentration was sufficiently low or and the stepwise increase in concentration was relatively small.

When the MIC and MBC for AgNPs, AgNO₃, Cipro, and BZC were tested on at least three different frozen aliquots of the AgNP-resistant strains (715npRPs and 718npRPs), AgNP-resistance was found to be stable. Cross-resistance to AgNO₃ was also discovered in one strain, but there was no cross-resistance to Cipro or BZC (Table 7). Resistance to AgNPs was also stable when the bacteria underwent daily, serial transfer in MHB2 for up to 9 days (Table 8). The highest concentration of AgNPs tested prior to the serial transfer studies was 90 µg/ml, because more concentrated solutions were not available at that time. Since the resistant strains were inhibited at that concentration, the actual MIC and MBC values were greater than 90 µg/ml, but could not be determined with more accuracy. When the serial transfer studies were completed, a more concentrated solution was available and more accurate MIC and MBC values were determined. The MIC decreased slightly after serial passage for both the resistant and normal strains of P. aeruginosa, which could suggest a general change in growth characteristics during serial passage. One change which was noted during the stepwise
increasing exposure experiments was the formation of a thick, mucoid-like substance after 2 to 3 days of passage. Vigorous mixing was required to disperse the substance and preserve the correlation between cell density and optical density which was used to standardize the inoculum for each step. Mucoid secretions are a common characteristic of some *P. aeruginosa* strains and contribute to biofilm formation (Yang et al. 2011). Biofilm production is known to reduce the susceptibility of *P. aeruginosa* to many antibacterial agents by a poorly understood mechanism (Hoiby et al. 2010; Mah & O’Toole 2001). Differences between the three strains of *P. aeruginosa* in the production of a biofilm might, therefore, contribute to resistance; however, there was no obvious difference between the strains. In addition, I noted color differences when *P. aeruginosa* was grown on agar (Figure 16a) or during serial transfer in MHB2 (Figure 16b, c, and d). When cultured overnight on agar and during the first two passages in MHB2, the 718npRPs strain and normal *P. aeruginosa* had a yellow-green color, and 718npRPs maintained that color during serial passage. The normal *P. aeruginosa*, however, produced a blue-green color during passages 3 through 9 in MHB2. The 715npRPs strain produced the blue-green color variation on agar and maintained it throughout the serial passages in MHB2. The different colors originate from phenazine pigments which are another common feature of *P. aeruginosa*. The pigments are involved in nutrient acquisition and quorum sensing, which also contributes to biofilm formation (Adonizio et al. 2008; Evans et al. 1998). The production of phenazine pigments, therefore, might also contribute to resistance, but the color variation was not the same between the two resistant strains, and so there might be different resistant mechanisms involved.
<table>
<thead>
<tr>
<th>Agent</th>
<th>( P. aeruginosa )</th>
<th>( 715\text{npRPs} )</th>
<th>( 718\text{npRPs} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (( \mu g/ml ))</td>
<td>MBC (( \mu g/ml ))</td>
<td>MIC (( \mu g/ml ))</td>
</tr>
<tr>
<td>AgNPs</td>
<td>3.75</td>
<td>7.5</td>
<td>&gt;90</td>
</tr>
<tr>
<td>AgNO(_3)</td>
<td>1.88</td>
<td>3.75</td>
<td>3.75</td>
</tr>
<tr>
<td>Cipro</td>
<td>0.12</td>
<td>0.23</td>
<td>0.12</td>
</tr>
<tr>
<td>BZC</td>
<td>30</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 7: The MIC and MBC of test agents against normal and AgNP-resistant \( P. aeruginosa \). The MIC and MBC of Cipro, BZC, AgNO\(_3\), and AgNPs were tested against the normal and two resistant strains of \( P. aeruginosa \) using at least three different frozen aliquots.

<table>
<thead>
<tr>
<th>Agent</th>
<th>( P. aeruginosa )</th>
<th>( 715\text{npRPs} )</th>
<th>( 718\text{npRPs} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( P_0 ) MIC (( \mu g/ml ))</td>
<td>( P_9 ) MIC (( \mu g/ml ))</td>
<td>( P_0 ) MIC (( \mu g/ml ))</td>
</tr>
<tr>
<td>AgNPs</td>
<td>3.75</td>
<td>0.94</td>
<td>&gt;90</td>
</tr>
<tr>
<td>AgNO(_3)</td>
<td>1.88</td>
<td>0.23</td>
<td>3.75</td>
</tr>
<tr>
<td>Cipro</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>BZC</td>
<td>30</td>
<td>30</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 8: The MICs and MBCs of test agents against \( P. aeruginosa \) after serial passage. The MICs of Cipro, BZC, AgNO\(_3\), and AgNPs were determined against normal and AgNP-resistant \( P. aeruginosa \) before (\( P_0 \)) and after (\( P_9 \)) daily, serial passage in MHB2.
Figure 16: The change in pigments produced by *P. aeruginosa* during serial transfer. Normal (labeled Ps) and AgNP-resistant strains (labeled 715 and 718) were cultured overnight on agar and then transferred to MHB2 for daily, serial passage. The blue-green color of 715 and the yellow-green color of 718 were consistent throughout the experiment, but the color of Ps was similar to 718 on agar (a) and in the first serial passage (b), but by serial passage five (c) and continuing through passage 9 (d), Ps was more similar in color to 715.

**The Mechanism of Resistance:** I tested the potential mechanisms for resistance to AgNP based on the assumption that AgNPs must release Ag$^+$ through dissolution in order to effectively inhibit bacterial growth. The lack of cross-resistance to Cipro (Table 7) suggests that membrane permeability and efflux are not involved in the resistance to AgNP because those mechanisms often confer resistance to quinolone antibiotics like Cipro (Henrichfreise et al. 2007; Lambert 2002; Redgrave et al. 2014). I tested efflux as a mechanism to limit the intracellular dose of Ag$^+$ by measuring the internalization of
membrane-permeable, fluorescent dyes between the normal and AgNP-resistant strains of *P. aeruginosa*. I also did not find a difference in the efflux of fluorescent dyes between the normal and resistant strains, confirming that efflux did not contribute to resistance (Figure 17). The uptake of each fluorescent dye was greater at 4°C than at 37°C, which verified that changes in the intracellular localization of each dye was affected by an energy-dependent process, such as efflux. The efflux of acridine orange was inhibited at 4°C for concentrations up to 1.25 μg/ml, but above 2.5 μg/ml, less dye was internalized at 4°C than at 37°C. The fluidity of the bacterial membrane is presumed to decreases when a bacterium that has acclimated to a warm temperature is moved to low temperatures due to the tight packing of saturated lipids at the lower temperatures (Klein et al. 1999; Mrozik et al. 2004; Sinensky 1974), and low fluidity causes a decrease in permeability (Lande et al. 1995). Since acridine orange was the only dye with an unexpected flip in the relative uptake between 4°C and 37°C, the presumed change in membrane fluidity may have affected its permeability more than the other dyes. Since Hoechst 33342 had the most dramatic change in fluorescence (Figure 17a, between 2.5 and 5 μg/ml), a second study was conducted at higher concentrations to see if differences in efflux between strains would become apparent. There was, however, no difference between the normal and AgNP-resistant strains at the higher concentrations (Figure 18), which suggests that membrane permeability and efflux were not involved in resistance to AgNPs.
Figure 17: The efflux of membrane-permeable, fluorescent dyes by *P. aeruginosa*. Cultures of normal (normal Ps) and AgNP-resistant (715npRPs) strains of *P. aeruginosa* were incubated with different concentrations of (a) Hoechst 33342 (Hoechst) or ethidium bromide (EB), or (b) acridine orange (AO) for 1 hour at 37° C to encourage efflux or at 4° C to inhibit efflux and then their relative fluorescence was measured.
Figure 18: The efflux of membrane-permeable Hoechst 33342 dyes in the different strains of \textit{P. aeruginosa}. Cultures of normal (normal Ps) and AgNP-resistant (715npRPs and 718npRPs) strains of \textit{P. aeruginosa} were incubated with Hoechst 33342 for 10 minutes at 37° C and then their relative fluorescence was measured.

I used a common tissue culture plate method to test for biofilm formation, which could limit the amount of AgNPs and Ag\textsuperscript{+} that reach the bacterial cells. In the method, the bacteria are normally incubated for one or more days to allow biofilm formation, but I chose to use shorter time points because \textit{P. aeruginosa} is already known to form biofilms during longer incubations. In order for the biofilm to contribute to resistance under the condition that I used here to produce and test for resistance, the biofilm must begin to form early, for example as soon as the silver is added to the culture. At the earliest time point (0.5 hours), I observed deposits in each of the wells that contained \textit{P. aeruginosa} and \textit{A. baumannii} (Figure 19b-e), but at 2 hours the deposits in the \textit{A. baumannii} wells were significantly reduced (Figure 19g), and by 4 hours the \textit{A. baumannii} wells (Figure 19l) looked similar to the “no cell” control (Figure 19k), confirming that \textit{A baumannii}
was not forming a biofilm at those time points. The wells containing the normal strain of *P. aeruginosa* appeared similar to *A. baumannii* at the earliest time point (Figure 19c), but thereafter (Figure 19h, m) were similar to the AgNP-resistant strains of *P. aeruginosa* (Figure 19i, j, n, o). The two resistant strains appeared slightly different from each other at 0.5 hours: 718npRPs appeared to have a layer of closely packed cells (Figure 19e, white spots) while the deposits in the 715npRPs wells appeared less densely packed (Figure 19d). In fact, the biofilm deposit appeared to be greater than normal *P. aeruginosa*, but less than 718npRPs. After 4 hours, the bottom of the wells containing *P. aeruginosa* were noticeably darker than the wells in which *A. baumannii* was cultured (Figure 19l-o). Spectral analysis confirmed that there was no biofilm formation associated with *A. baumannii*, but that each of the *P. aeruginosa* strains had significant staining, in other words biofilm formation, by 4 hours (Figure 20). There was no difference, however, between the *P. aeruginosa* strains at any of the time points, suggesting that biofilm formation did not contribute to AgNP resistance. The iodine which I used should preferentially stain the extracellular components of a bacterial biofilm, and so the visual difference in deposits on the bottom of the culture wells (Figure 19) may have been caused by cells which attach to or grow inside of the biofilm. The cells themselves should not be stained by iodine, and therefore the spectral analysis is more descriptive of biofilm formation than the visual observations.
Figure 19: Representative images of biofilms produced by the different strains of *P. aeruginosa*. Normal *P. aeruginosa* (c, h, and m), 715npRPs (d, I, and n), 718npRPs (e, j, and o) and *A. baumannii* (b, g, and l) were seeded to the wells of a tissue culture plate and cultured for up to 4 hours. The wells were stained with iodine, rinsed, and then air-dried in order to determine if the extracellular deposits which are typical of biofilms were present. The wells containing *A. baumannii* appeared to form deposits early (b and g) but appeared the same as the no cell control (a, b, and k) by the 4 hour time point (l). The appearance of deposits formed by the normal and AgNP-resistant *P. aeruginosa* stains were different at 0.5 hours (c, d, and e), but they appeared the same by 2 hours (h, i, and j) and 4 hours (m, n, and o).
Figure 20: The results of the spectral analysis of *P. aeruginosa* biofilms stained with iodine. Early biofilm formation was quantified by culturing *A. baumannii* (negative control) and each strain of *P. aeruginosa* in a tissue culture plate and then staining the wells with iodine after 0.5, 2, or 4 hours. The absorbance of each well at 450 nm was then measured in order to determine how much extracellular material was deposited. Iodine staining for *A. baumannii* was not different from the “No Cells” control at each time point. The *P. aeruginosa* wells were not different from the controls at 0.5 and 2 hours, but they increased at 4 hours. The normal and resistant strains of *P. aeruginosa* did not differ from each other. The difference in absorbance was analyzed by ANOVA with Dunnet’s Multiple Comparison post-test. An asterisk (*) indicates significant difference from the “No Cells” control (p ≤ 0.05).
Discussion

The increased use of AgNPs as antibacterial agents, especially in wound management, has raised concerns over the potential for resistance to develop. This study revealed a realistic potential for *P. aeruginosa* to develop resistance to AgNPs. The bacterium developed a stable resistance to AgNPs as rapidly as it developed resistance to Cipro; however, it required a sufficiently low concentration of silver and relatively small, stepwise increase in concentration in order for *P. aeruginosa* to adapt to the presence of silver. Those conditions may, or may not be relevant in wound treatment situations since wound dressings are changed periodically to optimize healing.

The mechanism by which *P. aeruginosa* developed resistance is not yet clear, but several potential mechanisms exist, including decreased membrane permeability through down-regulation of the outer membrane porins, increased efflux through up-regulation of efflux systems, alteration of the antibacterial target, biofilm formation, and producing molecules that alter the antibacterial agent (Lambert 2002). As discussed in the introduction to this thesis, the antibacterial mechanism for AgNPs appears to depend on the release of Ag\(^+\) (Xiu et al. 2012), and so the mechanisms of resistance to AgNPs is likely based on the ability of *P. aeruginosa* to limit its exposure to Ag\(^+\). Membrane permeability and efflux can each reduce the intracellular dose of Ag\(^+\). I did not find a difference in the internalization of membrane-permeable, fluorescent dyes, which has been used by others to determine the capacity for bacteria to extrude antibacterial agents (Brenwald et al. 1998; Coldham et al. 2010; Martins & Amaral 2012; Martins et al. 2011; Richmond et al. 2013). I assumed from the increased uptake of the dyes at low temperature that I was measuring intrinsic efflux capacity; however, the fluidity of the
bacterial membrane decreases as the temperature decreases which can also reduce the permeability of the membrane to non-polar molecules (Lande et al. 1995; Panja et al. 2008). Efflux should be studied further as a resistance mechanism by measuring the amount of silver taken up by bacterial cells and differences in the rate at which silver is extruded from the cells after being internalized. Chemical inhibitors of efflux such as reserpine or verapamil should be used to strengthen the association between changes in silver content and efflux.

Biofilm formation may protect bacterial communities form bactericidal agents (Hoiby et al. 2010). I did not find a difference in biofilm formation between the normal and AgNP-resistant bacteria; however, biofilm analysis is typically conducted over a longer time period (Christensen et al. 1985; Oliveira & Cunha 2010). Since Ag$^+$ is a relatively potent bactericidal agent, a biofilm would have to form very rapidly to protect the bacteria from exposure. Thick, mucoid-like substances and visible chunks were noted in the cultures of each of the *P. aeruginosa* strains, but only after 6 to 8 hours of culture, and there was no difference noted between strains. The production of exopolysaccharides such as alginate or “polysaccharide from polysaccharide synthesis locus” (Psl) (Colvin et al. 2012) or quorum sensing molecules such as N-(3-oxododecanoyl)-L-homoserine lactone and N-buyanoyl-L-homoserine lactone (Adonizio et al. 2008) should be measured in order to fully reject the involvement of biofilms in silver resistance.

There is a real potential for the phenazine pigments that are produced by *P. aeruginosa* to protect the bacterium from exposure to silver. Some of the phenazine pigments function as siderophores to bind and transport trivalent iron (Fe$^{3+}$) into the bacterial cells, while others are redox active and can reduce Fe$^{3+}$ to the more soluble Fe$^{2+}$.
to facilitate iron uptake (Cornelis & Dingemans 2013). The most common siderophores produced by *P. aeruginosa* are pyochelin, which can bind many metal ions including Ag⁺ (Braud et al. 2009), and pyoverdin, which does not appear to bind Ag⁺ (Bhattacharya 2011). *P. aeruginosa* can switch between its siderophores in response to iron availability in its environment (Cornelis & Dingemans 2013; Dumas et al. 2013), and since only one siderophore can bind Ag⁺, changing which siderophore is produced might alter the sensitivity of the bacterium to silver. In addition, pyocyanin is a redox active phenazine that is commonly produced by *P. aeruginosa*, and Muller and Merrett (Muller & Merrett 2014) recently reported that pyocyanin can reduce Ag⁺ to Ag⁰ and protect the bacteria from silver-mediated inhibition. I noticed a difference in the color produced by each strain of *P. aeruginosa*: the normal *P. aeruginosa* produced a yellow-green color on agar and during the first 2 daily serial passages, which could be pyochelin or pyoverdin, but produced a blue-green color thereafter, which is most likely pyocyanin. The pattern observed is typical of *P. aeruginosa* in culture because pyocyanin production is normally low until the stationary phase of growth when iron availability is low (Dergez et al. 2014; Frank & DeMoss 1959). The 715npRPs strain produced the blue-green color variation on agar and maintained it throughout serial passage, which suggested that the amount of pyocyanin produced by that strain was high throughout its life cycle. If pyocyanin is reducing the Ag⁺ that are released by the AgNPs to a less bioavailable Ag⁰, then increased, early pyocyanin production might be the resistance mechanism for 715npRPs; however, the same mechanism should protect the bacteria form AgNO₃, but that strain was not cross resistant to AgNO₃ (Table 7). The 718npRPs strain maintained a yellow-green color on agar and throughout serial passage, which indicates that it did not switch
to pyocyanin production in stationary phase. If that strain produces more pyochelin than the normal strain and does not switch to an alternate iron-uptake mechanism later in its lifecycle, then the pyochelin could bind Ag⁺ and render it unavailable for bactericidal activity. That strain was cross-resistant to AgNO₃, which would be consistent with a pyochelin-based mechanism; however, since the function of pyochelin is to transport iron into the cell, it seems logical that it would also transport Ag⁺ into the cell and that is counterintuitive toward a resistance mechanism. Additional studies into the production of phenazine pigments and their effect on Ag⁺ bioavailability are necessary in order to understand their potential role in resistance to silver.

**Conclusion for section 2**

This study demonstrated that AgNPs have a lower potential for resistance development than Cipro, but AgNO₃, which was a more potent antibacterial agent than AgNPs, had even less potential for resistance to develop. In wound management, bacteria inhibit wound closure mainly by causing chronic inflammation which increases the expression of matrix degrading enzymes and decreases the expression of growth factors that are necessary for wound closure (Edwards & Harding 2004; Sibbald et al. 2007). Effective elimination of bacteria from the wound will improve healing (Wright et al. 2002). Silver is an effective antibacterial agent, and the 3 species of bacteria tested in this study were relatively sensitive to Ag⁺ from AgNO₃ and to AgNPs, but AgNO₃ was clearly more potent than AgNPs, which has already been described in the literature (Choi et al. 2008; Percival et al. 2007). The Gram negative bacteria (*P. aeruginosa* and *A. baumannii*) were more sensitive to both AgNO₃ and AgNPs than the Gram positive (*S. aureus*), which has also been reported by others (Chudasama et al. 2010; Jung et al. 2008;
Khan et al. 2014; Kim et al. 2007; Sintubin et al. 2011; Szczepanowicz et al. 2010). The difference in susceptibility is believed to be related to the greater susceptibility of the Gram negative outer membrane than the thick proteoglycan layer over the Gram positive bacteria (Feng et al. 2000; Jung et al. 2008). Since both AgNO₃ and AgNPs are effective antibacterial agents, the advantage of one over the other in wound management might derive from their relative potential for resistance development. In this study, none of the bacteria developed resistance to AgNO₃ but P. aeruginosa developed resistance to AgNPs. It seems that the immediate bioavailability of Ag⁺ might be important for efficient bactericidal activity, and so it might be more advantageous to use AgNO₃ instead of AgNPs in wound dressings.
THESIS CONCLUSION

The goal of this thesis was to evaluate the benefit of using AgNPs in wound management based on their potential for resistance development, and the hazard of using them based on their potential to cause long-term stress in mammalian cells. The first section of the thesis demonstrated that AgNPs have the potential to cause long-term redox imbalance and induce premature cellular senescence. While it seems logical that cellular senescence would have a negative impact on the long-term health of the tissue, the impact of senescence on wound resolution is not well understood. Never-the-less, any long-term effects on tissues should have been apparent from the use of commercially available dressings that containing AgNPs, but none have been reported to date. Fong and Wood (Fong & Wood 2006), wrote an extensive review of the dressings available in 2006 that contained AgNPs. They reported that dressings that incorporated AgNPs were superior to other dressings due to less frequent changes of the dressing, less pain reported during dressing changes, and fewer complications during wound resolution. They also pointed out that proper use of the AgNP-containing dressings would prevent resistance development and any potential toxicity involving human cells in the wound. No negative effects on the wound-associated tissues were reported in a more recent review (Wilkinson et al. 2011) which pointed out that silver in any form has a history of minimal in vivo toxicity and that is why it has a long history of use as an antibacterial agent. Still, it is possible that the health of wound-associated tissues was not assessed in follow-up evaluations where silver was used in wound management. Wound resolution was the primary concern and not the long-term impact that the treatment might have on the surrounding tissue. Since the first section of this thesis demonstrated a clear potential for
a long-term, negative effects that could impact the health and function of tissue, caution should still be exercised and additional studies with proper follow up evaluations are necessary. The structure and function of the surrounding tissue should be evaluated in order to reveal any negative impact that the silver-containing dressings may have had on wound closure and tissue remodeling.

The studies discussed in the introduction to this thesis show that the principle hazard associated with AgNPs is not from direct exposure to the particles but from the release of Ag$^+$ after the particles are internalized by a cell. The experiments conducted in section one did not directly test if the particles or the ions were responsible for the cellular changes observed. The studies did demonstrate the persistence of AgNPs in the cells after silver-containing medium was replaced with silver-free medium, and the cellular effects of oxidative stress, increased SA-β-Gal, and hypertrophic morphology were each observed up to three days after the AgNPs were removed. The dissolution of AgNPs over time is well known, and the increased release of Ag$^+$ in the acidic environment of lysosomes, the cellular compartment where AgNPs are sequestered once internalized, has been described for the same AgNPs used for these experiments (Maurer et al. 2014). It is highly likely, therefore, that each of the observed effects was cause by Ag$^+$ that was released after the AgNPs were internalized. The Ag$^+$ is not as toxic to mammalian cells prior to internalization because they form insoluble salts and are no longer bioavailable (Drake & Hazelwood 2005). In order to take full advantage of the antibacterial properties of Ag$^+$ and the extended release properties of AgNPs and avoid the “Trojan horse” (Park et al. 2010) consequence of internalizing AgNPs, the particles should be embedded in the dressing so that mammalian cells at the wound site will only
be exposed to the Ag$^+$ that is released. That can be accomplished by placing the AgNPs behind a membrane through which wound exudate will pass and provide moisture into which Ag$^+$ can dissolve. The dissolved Ag$^+$ would then diffuse from the bandage into the wound area and prevent bacterial invasion, but should precipitate as AgCl or Ag$_2$S prior to affecting the mammalian cells. The effectiveness of such a design would depend upon the amount and nature of exudate produced by the wound. If too little exudate is produced or its fluid content is too low, the amount of silver diffusing from the dressing might be too small to control bacterial growth. Section two of this thesis demonstrated that when the concentration of AgNPs is too low, *P. aeruginosa* can develop a stable resistance to them and complicate the resolution of that wound. The mechanism of resistance which developed in this study was not determined, but appears to be related to silver redox (pyocyanin) or chelation (pyochelin). An organism in the wound that can reduce the bioavailability of Ag$^+$ in a similar way would not only protect itself but would inadvertently protect the other organisms that are present. The potent antibacterial activity of AgNO$_3$ that was observed in this study and the immediate availability of all the Ag$^+$ present make it a better choice for wound management, but since all the Ag$^+$ is released to the wound immediately, it would require more frequent dressing changes in order to maintain an effective Ag$^+$ concentration (Atiyeh et al. 2007). The most effective treatment would most likely result from a dressing that contained both AgNO$_3$ for an immediate bolus of Ag$^+$ and AgNPs (sequestered behind an ion-permeable membrane) to release additional ions over an extended period of time.

In conclusion, AgNPs are an effective antibacterial agent for use in wound management and have a reduced potential for resistance to develop; however, their
potential to cause detrimental effects once internalized by mammalian cells demands that additional studies be conducted to better define those effects and determine how they impact wound resolution and the long-term health of tissues. The more potent antibacterial activity of AgNO$_3$ and its lower potential for resistance development compared to AgNPs make it even better for short-term wound management. The combination of AgNPs whose release from the dressing is limited and AgNO$_3$ which provides a bolus of Ag$^+$ is probably the most advantageous approach for wound management.
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