Exposure to pathogenic organisms, and the potential for subsequent infectious disease acquisition, concerns everyone on a daily basis. The ever-increasing incidence of antibiotic resistance has resulted in renewed interest in products containing silver (Ag), and its innate antimicrobial activity is drastically increased at the nanoscale. Accordingly, Ag-nps have been incorporated into hundreds of personal and professional products ranging from surgical and food handling tools, water purifiers, textiles, cosmetics, contact lens cases, wound dressings, implantable devices and catheters, children’s toys, and most recently engineered scaffolds designed for tissue regeneration. Since skin is in constant, direct contact with the environment, it is often the site of unintentional or deliberate exposure to surfaces and products containing Ag-nps.

In this report, Ag-nps of various sizes, surface conditions, and synthesis methods were evaluated for their cytotoxic and inflammatory potential to skin cells, human epidermal keratinocytes (HEK), and their penetrating capacity into porcine skin dosed daily for 14d in vivo. Ag-nps were not toxic to HEK at concentrations up to 1024µg/ml, but residual solution contaminants present in freshly synthesized Ag-nps solutions were highly toxic to HEK. This revealed the importance of Ag-nps solution characterization and purity prior to their use. Ag-nps caused focal intercellular and intracellular edema to porcine skin, and were localized within intracytoplasmic vacuoles of HEK. Electron energy loss spectroscopy was used to examine the surface transformation of Ag-nps following HEK internalization. It was revealed that initially purely metallic Ag-nps acquired up to 8 oxide monolayers on their
surface as a result of cellular internalization. The antibacterial efficacy of the Ag-nps was tested against a range of Gram-negative, Gram-positive, and antibiotic resistant bacterial strains. Ag-nps were capable of inhibiting the growth of all strains starting at 64µg/ml.

Elastomeric and bioresorbable skin scaffolds were successfully electrospun with a 50:50 poly(L-lactide-co-epsilon-caprolactone) (PLCL) copolymer incorporated with 20nm Ag-nps. The scaffolds supported the confluent growth of HEK for up to 14d of culture. Lastly, the 10nm and 20nm Ag-nps were evaluated for their potential cellular uptake, toxicity, and affect on the differentiation of human adipose derived stem cells (hASC). Exposure of hASC to Ag-nps resulted in no significant cytotoxicity to hASC, and minimal dose-dependent toxicity to adipogenic and osteogenic cells at antimicrobial concentrations. Each of the hASC, adipogenic and osteogenic cells showed cellular uptake Ag-nps, without causing significant ultrastructural alterations, and exposure did not influence the intended differentiation of the cells. Therefore, the general biocompatibility and antibacterial efficacy of Ag-nps establishes their suitability for incorporation into tissue engineered graft scaffolds.
Biological Interactions of Silver Nanoparticles

by
Meghan Samberg

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Biomedical Engineering

Raleigh, North Carolina

2012

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DEDICATION

To my family.
BIOGRAPHY

Meghan Samberg was born in Annapolis, Maryland. In May, 2006 she received her B.S. cum laude in Biological Engineering from the University of Georgia. On May 28, 2007 she received her M.S. in Biomedical Engineering from Yale University, and married Joshua Samberg. To complete her education, she joined the Joint Department of Biomedical Engineering at North Carolina State University’s Department of Engineering and the University of North Carolina Chapel Hill’s School of Medicine. She expects to complete her Ph.D. in December 2011 under the direction of Dr. Nancy Monteiro-Riviere.
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1. Introduction

Silver (Ag) is the single most used material in all of nanotechnology; the desirable antimicrobial property activity of Ag is drastically increased at the nanoscale. Accordingly, Ag-nps have been incorporated into hundreds of personal and professional products ranging from surgical and food handling tools, water purifiers, textiles, cosmetics, contact lens cases, wound dressings, implantable devices and catheters, children’s toys, and most recently engineered scaffolds designed for tissue regeneration [www.nanotechproject.org]. Since skin is in constant, direct contact with the environment, it is often the site of unintentional or deliberate exposure to surfaces and products containing Ag-nps. Therefore, it is imperative that the safety of Ag-nps is thoroughly evaluated.

The evaluation of the Ag-np safety requires the use of many different in vitro and in vivo models for toxicity and inflammation. These models, and predictions derived from them, are useful for a relatively simple assessment of toxicity; but, are often not successful when applied to the immensely variable conditions of real-world exposure to nanomaterials. The continued and unwavering evaluation of Ag-nps in different settings, under many variables is needed to fully evaluate the suitability and cost-versus-benefit of Ag-np usage.

In this dissertation, the uses of specific in vitro and in vivo models were used to evaluate the biological interactions of Ag-nps:

- Toxicity and inflammatory potential of Ag-nps to skin cells in vitro, and their penetrating capacity to porcine skin in vivo (Chapter 3);
- The surface transformations of Ag-nps following human cell internalization (Chapter 4);
- The antibacterial efficacy of Ag-nps against a range of both Gram-negative, Gram-positive, and multi-drug resistant bacterial strains (Chapter 5);

- Performance of a degradable polymer skin tissue engineered scaffold incorporated with Ag-nps (Chapter 6); and,

- The effects of Ag-nps on adipose-derived stem cells used in tissue engineering scaffolds (Chapter 7).

To evaluate these biological interactions, Ag-nps of varied sizes, surface conditions, and synthesis methods were used. It is hoped that these studies will contribute to the accurate assessment of risk following Ag-nps exposure and to the development of safe and effective guidelines for Ag-nps use in both personal and professional healthcare products.
2. Review of Literature

Nanotechnology

Nanomaterials are composed of structures with at least one dimension of 1-100 nanometers (nm) [www.nano.gov]. They may be subdivided into different basic geometric structures: zero-dimensional basic nanostructures are smaller than 100nm in all three dimensions, applicable to most point-shaped objects such as nanoparticles; one-dimensional basic structures include tubes, rods, fibers, and wires; two-dimensional basic structures are represented by layers or films with a thickness less than 100nm [Liu et al. 2008]. These structures are either nanoscale variants of conventional materials such as metals and metal oxides, or new material classes such as fullerenes and carbon nanotubes. As of March 10, 2011, The Project on Emerging Nanotechnology consumer products inventory contained 1317 manufacturer-identified products that incorporate nanomaterials in some form; there is a small set of materials commonly used in products, and the most frequent is silver (Ag), followed by carbon nanotubes and fullerenes, titanium and titanium dioxide, silica, zinc and zinc oxide, and gold [nanotechproject.org]. Various other materials include organic lipids and polymers, inorganic silica and quantum dots and ceramics, and other metals or metal oxides such as aluminum and aluminum oxide, copper and copper oxide, and iron oxide, as well as hybrids [Vollath 2008, Cao and Wang 2011, Kumar 2010].

Nanotechnology is the study and application of technology conducted at the nanoscale, and is utilized across an array of science fields, particularly chemistry, biology, physics, materials science, and engineering. Nanomaterials often exhibit properties and behaviors drastically different to their corresponding bulk materials of the same chemical composition. Unlike bulk materials, electrons in a nanomaterial can only adopt specific
quantized energy states, which are influenced by the number of atoms that are free to react [Gammon 2000]. Nanostructuring greatly increases surface reactivity due to the amplified ratio of reactive surface atoms to inert core atoms [Auffan et al. 2009]. For example, the percentage of surface atoms for a particle of 10nm diameter is only 10%, where as a particle with 1nm diameter is entirely composed of surface atoms and can result in increases in surface energy upwards of seven orders of magnitude [Shih et al. 1976]. Therefore, current nanotechnology focuses on deliberately fabricating nanomaterials to take advantage of their enhanced magnetic, electrical, optical, mechanical, or biological properties [Nel et al. 2006].

Silver Nanoparticles

Ag is a naturally occurring metallic chemical element with the chemical symbol Ag and atomic number 47; a soft, white, lustrous transition metal, it has the highest electrical conductivity of any element and the highest thermal conductivity of any metal [Nordberg and Gerhardsson 1988]. In its bulk form, Ag and Ag compounds have been used for thousands of years in jewelry, tableware, currency, dental alloys, photography, electrical conductors, mirrors, and most importantly, as an antimicrobial agent [Harmata 2010]. Historically, ancient Phoenicians were known to place silver coins into their water jugs as a preservative [Angelotti and Martini 1997], doctors administered Ag nitrate solutions to the eyes of newborns for the prevention of neonatal conjunctivitis [Crede 1881], and Ag sulfadiazine creams have long been considered the gold standard for the prevention of widespread bacterial growth on burn patient’s denuded skin [Moyer et al. 1965]. Although the majority of Ag-np predecessors such as Ag nitrate and Ag sulfadiazine were supplanted by the advent of penicillin and other modern antibiotics, advanced research into the medical potential of Ag
continues, most recently at the nanoscale [Monafo and Moyer 1968, Crede 1881, Yin et al. 1999, Wright et al. 1998].

Currently, Ag nanoparticles (Ag-nps) are the single most manufacturer-identified material used in all of nanotechnology products [www.nanotechproject.org]. As is the case for most nanoparticles, Ag-nps exhibit unique physicochemical properties that differ from, and provide distinct advantages over, those of their bulk metallic constituent. While Ag-nps exhibit unique optical and electrical properties at the nanoscale, the enhanced antibacterial activity of Ag-nps has been the most valuable. The professional and personal healthcare fields have taken advantage of this augmented property through the incorporation of Ag-nps into hundreds of products, specifically surgical and food handling, packaging and storage tools, water purifiers, textiles, cosmetics, contact lens cases, wound care products, implantable devices and catheters, and even children’s toys [www.nanotechproject.org].

**Synthesis**

One of the most challenging goals in nanomaterial research is to develop successful protocols for the large-scale, simple and low-cost preparation of morphologically pure nanomaterials. Synthesis methods must possess tight control over key properties such as size, shape, crystal structure, morphological purity and should generally be easily stored and manipulated without the loss of their properties [Cioffi et al. 2010]. Depending on the method of synthesis, Ag-nps may take on a variety of different shapes such as spheres, rods, cubes, hollow tubes, multifacets, and films (Figure 1) [Sun and Xia 2002, Sajanlal et al. 2011].
Figure 2.1: Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images depicting various Ag-nps morphologies. (A) Large area SEM image of Ag nanowires. Inset shows a cross-sectional TEM image of a microtomed nanowire, revealing its fivefold twinned crystal structure and pentagonal profile. (B) SEM image of Ag nanocubes. (C) SEM image of the Ag nanobars (D) SEM of nanorice. (E) SEM images of Ag bipyramids. (F) SEM images of Ag nanoplates. Image from: Sajanlal PR, Sreeprasad TS, Samal AK, Pradeep T. 2011. Anisotropic nanomaterials: structure, growth, assembly, and functions. Nano Reviews 2, 5883-946.

The generation of Ag-nps may be achieved by several different methods including spark discharging, electrochemical reduction, chemical reduction, solution irradiation, laser
ablation, and cryochemical synthesis [Chen and Schluesener 2008]. Generally, nanoparticle fabrication may be differentiated into two broad approaches: 1) the top-down approach, and 2) the bottom-up approach.

In the top-down approach a solid mass is milled into smaller portions. One such method, the mechanical technique of ball milling involves controlled erosion of a solid mass to produce finer structures, or irregularly shaped nanopowders. However, this method usually results in nanomaterials with quite broad grain sizes between 200-300nm, and contamination from milling media or atmosphere [Koch 2003]. Another method utilizes electrochemistry whereby an anode is made from the metal to be dispersed as the nanomaterial and when the applied potential is sufficiently high the anode dissolves into metal ions that subsequently are precipitated [Yu et al. 2008].

In the bottom-up approach the nanomaterial is built atom by atom in four steps, in which a precursor is first condensed to solid phase, initiates the formation of multiple nuclei, growth is achieved on the nuclei, and finally stabilization terminates the procedure to yield a desired size [Cioffi et al. 2010]. Examples of this method include metal vapor condensation and metal-organic chemical vapor deposition approaches, as well as the simplest approach of solution-based reduction of a precursor in an appropriate aqueous solution. This method results in the synthesis of very small nanomaterials with dimensions less than 5nm of several different morphologies, including compact or hollow nanocubes and nanospheres, nanorods, and ultra long nanowires [Ren et al. 2005, Chang et al. 2005, Pileni 2002]. However, chemical approaches do not always produce high purity nanoparticles, and the requirement of strong reducing agents such as hydrazine, formaldehyde and sodium borohydride may create local variations in the rates of nucleation and growth that eventually results in polydisperse
and contaminated solutions of nanomaterials [Jana 2005]. Tangential flow filtration of polydisperse nanomaterial solutions through sequentially smaller filter pore sizes may eliminate polydispersion issues as well as contaminant concentration; an alternative is the use of “green” reducing agents such as starch, glucose, and supercritical CO₂ for the fabrication of nanoparticles [Raveendran et al. 2003].

**Functionalization**

Nanoparticle functionalization is an important process that is often necessary to eliminate polydispersion, aggregation, and nonspecific binding issues. For many applications such as drug delivery and *in vitro* and *in vivo* imaging, functionalized nanoparticles must be able to provide strong analytical signals, bind targets with high affinity and specificity, and be adaptable [Nakamura 2010]. There are two main strategies for the preparation of functionally modified nanoparticles: grafting or internalization.

The first approach is the grafting of functional groups to synthesized nanoparticles, known as either surface or post-synthesis functionalization, and has the advantage of altering the interfacial properties without affecting the bulk characteristics of materials. Methods of surface functionalization include plasma treatment, lipid self-assembly, silanization, and physical adsorption [Bayer and Wilchek 1980]. In the case of Ag-nps synthesized in liquid phase, size and shape are often controlled by addition of organic stabilizers such as thiols, phosphates, phosphines, amines, carboxylates, polymers, and/or surfactants, which are in some cases chemically bonded to the particle or in other cases simply adsorbed to its surface [Masala and Seshadri 2004].
The second approach to functionalization is internalization, or *in situ* functionalization, whereby the functional group of interest is introduced during the nanoparticle synthesis; this method is particularly useful for fluorescent dye and drug incorporation for use as biomarkers. A typical preparation method for dye or drug incorporation into nanoparticles such as polystyrene or polymethyl methacrylate involves the swelling of polymeric nanoparticles in an organic solvent solution doped with the molecule of interest [Van Blaaderen and Vrij 1992, Nakamura 2010].

**Characterization**

Nanomaterials can present characterization challenges due to their decreased size and increased surface reactivity, compared to traditional materials and chemicals. The most widely used technique for nanoparticle characterization is that of electron microscopy [Williams and Carter 2009]. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) may be employed to visualize the physical size, shape, morphology, uniformity, and topography of nanoparticles. However, both internal and surface functionalization may not always be visible by these techniques, depending upon the electron density of the functional group. Alternatively, dynamic light scattering (DLS) can be used to measure the hydrodynamic size and surface charge of suspended nanoparticles in different vehicles. DLS does not directly visualize individual nanoparticles; rather, the size is derived from the time dependent fluctuations of the scattering intensity caused by interference between the relative Brownian movements of multiple nanoparticles in suspension [Berne and Pecora 1976]. Through analysis of these fluctuations, average particle size and polydispersion may be calculated. Additionally, the surface charge of the
nanoparticles can give a measure of the suspension stability, and can be measured by DLS via the nanoparticle zeta potential.

Purchased nanoparticles are often referred to by the nominal size declared by the supplier, and then only in the as-synthesized dispersant. Obviously, it is poor practice to assume this value to be precise or accurate; however, it is equally hazardous to rely solely on one method of characterization to derive the nanoparticle size. Additionally, it is important to consider the size of not only a newly synthesized nanoparticle, but also following storage prior to use and in various application dispersants. The hydrodynamic diameter is typically larger than that of the physical diameter due to both intentional surface functionalization and unintentional adsorption of molecules, as well as agglomeration and insolubility factors that affect the calculation of the hydrodynamic diameter [Murshed et al. 2011]. Additionally, the various morphologies of nanoparticles may affect their mobility and diffusion in medium, as well as the deposition and adsorption kinetics in biological media [Park et al. 2003].

Furthermore, Ag-nps have a specialized chemical surface that can be reflected by light, whereby individual Ag-nps can be visualized by dark filed microscopy and in the reflection mode by confocal laser scanning microscopy (CLSM) [Sherry et al. 2005]. Other methods of nanoparticle characterization include UV-visible spectroscopy to examine the unique surface plasmon resonance in the visible range, X-ray diffraction to examine the average size of nanoparticles, Raman spectroscopy to characterize materials, and BET analysis of nanoparticle specific area [Hassellöv and Kaegi 2009].

Analytical tools used in conjunction with TEM to identify the elemental composition of a sample include energy dispersive X-ray (EDX) microanalysis, electron energy-loss spectroscopy (EELS), and X-ray absorption spectroscopy [Williams Carter 2009]. The
advantage of using EELS over EDX lies in its higher resolution and ability to identify the atomic composition, chemical bonding, valence and conduction band electronic properties, and surface properties of a specimen. When TEM-EELS is performed on a biological specimen, the chemical elements of a compound localized within a cellular or intracellular compartment at the ultrastructural level may be studied [Leapman et al. 1994].

The chemical composition and purity of a nanomaterial is an intrinsic property, and consequently an important physicochemical parameter to characterize. The use of analytical chemistry techniques such as high performance liquid chromatography (HPLC), inductively coupled plasma mass spectroscopy (ICP-MS) should be used to confirm the composition and purity of nanoparticles, as well as their dispersant solutions. The synthesis of nanomaterials often requires the use of other materials and chemicals; common impurities include metals and chemicals such as iron, nickel and cobalt, and alcohols, acids, surfactants [Zuin et al. 2007]. During application, these impurities could affect the nanoparticle interactions and ultimately mask the nanoparticle behavior with the intrinsic toxicity of the impurities [Maynard et al. 2004, Pulskamp et al. 2007].

Biological Applications of Ag-nps

Antibacterial Activity

Microscopic organisms are ubiquitous in nature, the majority of which are harmless to humans. However, a small percentage of microbes are pathogenic and result in infectious disease. Gram-negative bacterial strains are responsible for many nosocomial infections and include Acinetobacter, Escherichia, Pseudomonas, Salmonella, and Vibrio. Gram-positive bacterial strains include Bacillus, Clostridium, Enterococcus, Listeria, Staphylococcus, and
Streptococcus. Antibiotic-resistant bacteria include strains such as Enterococcus faecium, and methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus (VRE).

Any and all surfaces are capable of harboring pathogens capable of survival for many hours after their initial arrival on a surface. These organisms are often found on high contact surface public areas such as computer keyboards, ATM buttons, indwelling catheters, handrails, and doctor and nurse uniforms. Contact with contaminated surfaces results in bacterial spreading 92% of the time, depending on the bacterial strain [Harvey 1998, Noskin et al. 1995], and healthcare uniforms have been shown to transfer MRSA bacteria 65% of the time when leaning over an infected patient [Boyce et al. 1997]. Up to 70% of carefully cleaned hospital rooms of infected patients were found to still contain MRSA isolates [Sexton et al. 2006]. It has been estimated by the CDC that approximately 1 in 20 hospital patients would acquire an infection from their stay alone, resulting in approximately 1.7 million hospital-acquired infections each year, and approximately 90,000 deaths [Klevens et al. 2002].

Given that we are in an era where antibiotic resistance is a growing concern, there is a renewed interest in developing products containing Ag for use as antimicrobials. As the most used nanomaterial, Ag-nps are used almost exclusively for their enhanced antibacterial activity. They are coated onto the surfaces of medical products such as catheters and surgical tools to provide immediate antibacterial activity, as well as embedded into products such as contact lens cases to provide controlled and sustained antimicrobial activity [www.nanotechproject.org]. Ag-nps of various sizes, morphologies, surface conditions, and synthesis methods have proven to be effective against a broad spectrum of both Gram-

The antimicrobial activity of Ag-nps may be explained by several mechanisms: 1) excessive binding of Ag ions and Ag-nps may prevent the uptake of essential nutrients to the bacterial cell, ultimately leading to cell death [Schreurs and Rosenberg 1982]; 2) Ag ion entry into the cell by competitive binding with essential metals such as Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$ [Ghandour et al. 1988]; or 3) Ag transport and irreversible accumulation in the cell could occur by complexation with ligands or substrates to inhibit respiration, or bind and condense DNA [Yahya et al. 1990]. Ultimately, the biocidal activity of Ag ions is likely caused by a synergistic effect between the binding of Ag ions to the cell wall, Ag uptake and subsequent accumulation in the cell, and Ag interference with critical biomolecules within the cell. The Ag ions released from Ag-nps, as well as the Ag-nps themselves, bind to the electron donor receptors present on the bacterial cell membranes such as disulphide, amino, imidazole, carbonyl, and phosphate. Inactivation of membrane-related enzymes like phosphomannose isomerase results in denaturation of the bacterial cell envelope and its functional capacity to regulate the inward diffusion of nutrients and limits the effusion of essential electrolytes and metabolites. The predominant intracellular effect of Ag probably lies in its ability to impair key intracellular enzyme systems by replacing trace metals and electrolytes, leading to defective respiratory pathways and RNA and DNA replication [Yahya et al. 1990]. The formation of reactive oxygen species has been implicated in bacterial toxicity, and these are thought to damage DNA and proteins, as well as perturb cell membrane integrity [Su et al.}
For maximal antimicrobial efficacy, it is therefore a critical function of Ag-nps to release a steady amount of Ag ions from their surface.

**Tissue Engineering**

Every year, millions of people suffer tissue loss or end-stage organ failure; treatments include grafting, transplantation, surgical reconstruction, or implantation of medical devices [Langer and Vacanti 1993]. While autologous donor tissue for grafting is ideal, the availability is significantly limited; allograft tissue is similarly limited, and carried the inherent risk of transferring disease to the host. Bone autografts and allografts have resulted in donor site morbidity [Younger and Chapman 1989, Ahlmann et al. 2002, Sasso et al. 2005], chronic pain, nerve damage, infection, fracture, pelvic instability, hematoma, and tumor transplantation [Seiler and Johnson 2000]. Similarly, skin grafts derived cadaver skin, pig skin, and neonatal skin substitutes provide good temporary coverage, but are hindered by limited supply, tonal mismatch, and immunological complications [Burd and Chiu 2005]. Additionally in the case of skin, the resulting severe scarring and deformity continues to be one of the greatest challenges facing skin wound victims. Alternatively, surgical reconstruction or replacement of diseased organs with medical devices has its limitations due to risk of infection, low patency rates, material failure, and the use of immunosuppressant drugs.

Therefore, the field of tissue engineering and regenerative medicine with its mission to “develop biological substitutes that restore, maintain, or improve tissue function” has been revolutionary in terms of attempting to resolve these shortcomings [Langer and Vacanti 1993]. The requirements for suitable tissue engineered scaffolds is extensive; the scaffold
should facilitate cell adhesion, promote cell growth, have a high porosity, be mechanically strong, be capable of being fabricated into a desired shape, have a suitable degradation rate to meet the requirements of new tissue growth, be biocompatible, easily handled and processed during manufacture, and have nontoxic degradation products [Tateishi et al. 2002]. More specific requirements are necessary on a per tissue basis; for example, skin replacements are required to be bacteriostatic, semi-permeable to water, and cosmetically acceptable. For successful use, tissue engineered scaffolds should mimic the extracellular environment, which differs depending on the target tissue, as this structure greatly influences many aspects of cell behavior such as morphology, functionality, and cell-cell interactions. Additionally, scaffolds should be elastic enough to withstand cyclic mechanical strains without any significant permanent deformation or creep, which is especially important in dermal and vascular scaffold design [Chung et al. 2010, Jeong et al. 2004a, Jeong et al. 2005, Kim et al. 1999, Kim et al. 2000].

Different methods, such as particle leaching, fiber bonding, phase separation, rapid prototyping, and electrospinning have been used for the fabrication of porous scaffolds [Yang et al. 2001]. However, degradable polymer electrospun nanofibrous scaffolds are increasingly being favored since they provide a three-dimensional structure similar to the natural environment and are easily tailored to provide the necessary requirements such as mechanical properties, surface topography and chemistry, and degradation rate in vivo [Pham et al. 2006]. Successful in vitro cell culture on ultrafine fibrous scaffolds have been demonstrated on a number of natural and synthetic biodegradable polymers and their copolymers such as collagen, chitosan, poly(glycolic acid) (PGA), poly(ε-caprolactone) (PCL), and poly(L–lactic acid) (PLA), for regeneration of various soft tissues such as bone,

For many tissue engineering applications, the seeding of patient-derived stem cells helps to optimize the scaffold outcome; these cells have unique properties that are important for the development of engineered tissue constructs, including high proliferation rates and self-renewal capacity, maintenance of the unspecialized state, and specialized differentiation under given conditions [Moore and Lemischka 2006]. Both human bone marrow stromal cells (hBMSCs) and human adipose-derived stem cells (hASCs) possess a high capacity for self-replication and have the potential to differentiate into osteogenic, chondrogenic, adipogenic, and tenogenic lineages when placed in an appropriate environment [De Ugarte et al. 2003, Endres et al. 2003, Engler et al. 2006, Guilak et al. 2006, Halvorsen et al. 2001, Im et al. 2005, Mauney et al. 2004, Pittenger et al. 1999, Strem et al. 2005, Sumanasinghe et al.
nanoparticles composed of osteogenic factors have shown remarkable improvements in stem cell adhesion, attachment, and differentiation on tissue engineered bone scaffolds [Lock and Liu 2011]. Similarly, a patient’s skin cells may be seeded onto a degradable membrane to allow for skin regeneration. In contrast to conventional grafting where autologous donor skin is expanded 1:4, the rapid growth of cells in vitro has been shown to heal approximately 60 times the area of the initial biopsy [Boyce et al. 2006].

Despite advances in scaffold design and cell seeding, tissue engineered scaffolds currently in clinical use often still suffer unnecessary failure due to contamination during the in vitro culture step and also surgical site infection during implantation [Langer 2007]. The incorporation of antimicrobial Ag-nps into tissue engineering scaffolds would be pivotal in the reduction of implant associated, and surgical site infection incidences. Fortunately, there are a number of studies that have shown that internalized nanoparticles have no apparent deleterious effects on the morphology, cell proliferation, cell viability, and differentiation efficiency of mesenchymal stem cells [Chung et al. 2007, Huang et al. 2008, Lewin et al. 2000, Lorenz et al. 2006, McCullen et al. 2007]. Current nanofibrous scaffold systems that utilize Ag-nps have only done so through the incorporation of Ag nitrate into the polymer solutions, subsequently subjected to an annealing process [Jeon et al. 2008, Liu et al. 2010a].

**Silver Nanoparticle Exposure**

The ever-increasing use of Ag-nps will likely lead to their accumulation in the air, water, soil, and within organisms. Therefore, exposure to Ag-nps may occur unintentionally in the environment, or through the intentional use of Ag-np incorporated products.
Currently, The Project for Emerging Nanotechnologies database indicates that the concentration of Ag-nps within the hundreds of consumer products ranging from dietary supplements to cosmetics is on the order of <6ppm to 10,000ppm [www.nanotechproject.org]. For example, socks that contain Ag-nps in an effort to prevent foot odor were found to release up to 100% of the incorporated Ag-nps during washings [Benn and Westerhoff 2008]. These released Ag-nps have the potential to harm helpful bacteria in wastewater treatment facilities or endanger aquatic organisms in lakes and streams, and hand washings increase the risks associated with human interactions.

There currently exists little regulation regarding the manufacture of Ag-nps due to the extensive, historically safe use of Ag. However, ingestion of Ag can cause argyria, the benign condition characterized by the bluish-graying of the skin that occurs through its preferential deposition in the basal lamina of soft tissues such as the skin, liver and spleen [Fung and Bowen 1996], blood vessels, gastrointestinal tract, liver, and kidney [Danscher 1980]. Although argyria is most commonly reported clinically after excessive Ag ingestion, Ag deposition has been seen after Ag sulfadiazine treatment of burned skin [Lee and Lee 1994; Marshall 1979; Temple and Farooqi 1985]. In response to argyria, but not to Ag toxicity, the National Institute for Occupational Safety and Health set a daily exposure limit for all forms of Ag at 0.01 mg/m³, and the Environmental Protection Agency established the oral reference dose at 0.005mg/kg/day [EPA 1996; NIOSH 2003]. Additionally, the only applicable regulation, aimed more at the sale of Ag-nps-containing products, falls under the Environmental Protection Agency’s [EPA] Federal Insecticide, Fungicide, and Rodenticide Act, but only if a product explicitly states that it contains a substance, in this case Ag-nps,
that is intended to disinfect, sanitize, reduce, or mitigate growth or development of microbiological organism.

Ag-nps may be introduced into the gastrointestinal tract after being shed from coatings on food-handling machinery or directly from Ag-nps sprayed onto produce for the prevention of food-spoiling bacteria [Silver 2003]. The homeopathic ingestion of Ag-nps has led to various in vivo studies; generally, exposure to Ag and Ag-nps over time lead to distribution in various tissues without causing toxicity. Drinking water containing Ag administered orally to rats for 1 to 2 weeks resulted in Ag distribution to the musculus soleus, cerebellum, spleen, duodenum, and myocardial muscle [Pelkonen et al. 2003]. Repeated oral exposure of 60nm Ag-nps to rats over 28 days revealed a dose-dependent accumulation of Ag in the liver [Kim et al. 2008b]. Chronic symptoms from prolonged intake of low doses of Ag salts are fatty degeneration of the liver and kidneys as well as changes in blood cells [Wijnhoven et al. 2009]. Intravenous injection in rats with 4, 10, 20, and 40mg/kg of Ag found that the 40mg/kg dose caused a significant increase in liver enzymes while blood serum had an increase in ROS; TEM showed particle deposition in the liver and kidney at the 40mg/kg dose with a dose >20mg/kg considered toxic for rats [Tiwari et al. 2011].

The respiratory system is another major port of entry for Ag-nps. When inhaled, Ag-nps may be deposited in any region of the respiratory system, be taken into cells, enter the bloodstream, and circulate to potentially sensitive target organs. Inhalation of dusts or fumes containing Ag occurs primarily in occupational settings, such as industrial plants involved in Ag chemical manufacturing, jewelry manufacturing, Ag reclamation, and production of tableware. Due to the small diameter of Ag-nps, Brownian diffusion results in deep penetration of Ag-nps into the lungs and diffusion to the high lung surface area presented in
the alveolar region. Several 28-day and 90-day inhalation studies with rats have demonstrated that inhalation of Ag-nps of approximately 15nm resulted in slight increases in neutral mucins, with some hepatic necrosis and Ag-nps accumulation in the lung and liver [Wijnhoven et al. 2009].

However, the majority of all products incorporated with Ag-nps come into direct contact with skin; skin exposure to Ag-nps is greatest to the skin due to their increasing inclusion into textiles, burn wound creams, catheters, keyboards, ATM buttons, and children’s toys [www.nanotechproject.org]. Although Ag compounds have long been used in wound dressings, the incorporation of Ag-nps into wound dressings is recent. The release of Ag ions from the Ag-nps in wound dressings is generally triggered by wound fluids and tissue exudates. While it has been deemed that 10-40ppm is required for appropriate antimicrobial action, the Ag content of dressings currently available varies from <0.1mg/cm² to more than 1mg/cm², with the amount of Ag ions released ranging from <1ppm to >70ppm [Wijnhoven et al. 2009]. However, absorption of Ag ions through intact skin is low (<1ppm) since much of the free ion is precipitated as Ag sulfide in the superficial layers of the skin.

Skin

Structure and Function

Skin is the largest organ of the body and is a complex, multilayered primary sense organ that performs numerous important roles. It provides a protective layer against mechanical, chemical, and microbiological insults and it performs crucial roles in thermoregulation, water regulation, ultraviolet radiation protection, and vitamin D metabolism. For many of these reasons, the skin provides a large route of exposure to Ag-
nps; in order to evaluate the multiple interactions between Ag-nps and the skin, it is important to first understand the structure of skin.

The skin consists of two distinct layers: the epidermis and the dermis, as outlined in Figure 2. The outermost layer, the epidermis, is derived from ectoderm and is capable of regeneration. Wounds involving only the epidermis heal by regeneration of epidermal cells not only from the wound’s periphery but also from skin adnexal structures, including hair follicles, sebaceous glands, and sweat glands. Given the ability of the epidermis to regenerate, pure epidermal wounds heal without scarring [Paletta et al. 2006]. The dermis is below the basement membrane layer of the epidermis, is derived from mesoderm, and contains connective tissues cells, nerves, and blood vessels [Monteiro-Riviere 2008].
Figure 2.2: Schematic depicting mammalian skin. Left side of schematic depicts animal skin; right side of the schematic depicts human skin. Image from: Monteiro-Riviere NA. 1991. Comparative Anatomy, Physiology, and Biochemistry of Mammalian Skin. Dermal and Ocular Toxicology: Fundamentals and Methods (Ed. DW Hobson). CRC Press, Inc. Chapter 1, pp. 3-71.

**Epidermis**

The epidermis can be further divided into five layers, or strata: stratum corneum (SC), stratum lucidum, stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB). The primary cell of the epidermis, the keratinocyte, forms the five strata, representing its progressive differentiation, a process known as cornification. Tight intercellular connections
within the epidermis form the basis of its physical integrity and lamellar granules, containing sterols, polar lipids and hydrolytic enzymes, released into the intercellular space give the epidermis its impermeable quality, and its low pH inhibits bacterial growth [Paletta et al. 2006].

The SC is the outermost layer and its thickness is variable depending on the location on the body [Monteiro-Riviere et al. 1990]. The SC can be further classified into the outermost stratum dysjunctum and the inner stratum compactum. The SC is comprised of nonviable, terminally differentiated cells called corneocytes that lack nuclei and cytoplasmic organelles. They are packed together ten cells deep and are filled with keratin filaments that provide a protective barrier from the environment. The corneocytes are polyhedral and approximately 30μm in diameter and 0.5μm in thickness. Keratin filaments in the corneocytes constitute a reinforcement network and provide resistance to mechanical stress. These 8-10nm diameter keratin intermediate filaments are bundled together in the cytoplasm along with desmosomes and hemidesmosomes at the cell membrane [Eichner et al. 1986]. The cells are bound by cornified envelopes containing loricin, involucrin, filagrin, and cystatin A to stabilize the corneocytes. Desmosomes in the SC connect individual corneocytes and the corneosomes degradation induces desquamation. The major lipid components in the SC layer are ceramides, cholesterol and free fatty acids. The structure of intercellular lipids is composed of broad lamellar sheets oriented parallel to the skin surface [Gray et al. 1975, Monteiro-Riviere et al. 2001, Uchida and Hamanaka 2006]. The SC barrier can be conceptualized as a two compartment system, the “brick and mortar model”, where keratin filament based protein rich corneocytes are “bricks” and the extracellular lipid
matrix is “mortar” [Elia et al. 1983]. The indirect path of intercellular spaces between corneocytes accounts for the barrier properties of the skin.

There are also three other non-keratinocyte cell populations within the epidermis; melanocytes, Langerhans cells, and Merkel cells do not participate in the process of keratinization [Smack et al. 1994]. Melanocytes reside in the basal layer of the epidermis; they produce and secrete melanin pigment protects the skin from ultraviolet light and is found in the basement membrane and are capable transferring melanin to surrounding keratinocytes. Langerhans cells are dendritic cells that are specialized for defense, and account for 2% of the total epidermal cell population. They originate from the bone marrow and migrate to the skin; these cells play a key role in allograft rejection through the recognition of foreign substances and presentation of antigens to lymphocytes in order to stimulate an immune response. By TEM, Langerhans cells may be identified by their granules within a rod-shaped organelle [Breathnach 1988]. Merkel cells reside in the palms and soles, nail beds, and oral and genital epithelium and are specialized neuroendocrine cells that produce nerve growth factor receptors and function as mechanoreceptors [Monteiro-Riviere 1991]. Merkel cells are joined to SB and adjacent keratinocytes by desmosomes. They cannot be seen directly by light microscopy but staining can help locate them in the epidermal layers [Moll et al. 1984; Moll 1994].

The next epidermal layer, the SG, normally contains two to three layers of granular cells parallel to skin surface. The cells in SG are comprised of keratohyalin granules with dense staining regions and irregular shape that contain several proteins including profilaggrin, loricrin, cysteine-rich proteins and keratin intermediate filaments. These granules move toward and fuse with the cell membrane, releasing their components into the
intracellular spaces. The SS is located under the SG and consists of several layers of irregularly shaped polyhedral cells. Tonofilaments in the cytoplasm and melanosomes between cells are found in the cytoplasm of cells in the SS layer. The SB layer contains mitotically active columnar or cuboidal keratinocytes in a single layer, attached to the adjacent cells in SS or to neighboring basal cells by desmosomes and to the underlying basement membrane by hemidesmosomes [Elias and Feingold 2006]. The SB cells function as stem cells to continuously produce keratinocytes or act to anchor the epidermis [Monteiro-Riviere 2006; Monteiro-Riviere et al. 2008].

Dermis, Hypodermis and Others

The deeper layer of the skin, the dermis, can give mechanical protection to the body, provide nutrients via blood in the vessel, and regulate body temperature by control of blood flow and sweating. It is between 15 to 40 times thicker than the epidermis, and is derived from mesoderm and the connective tissue provides elasticity and tensile strength. Structurally, the dermis is a complex network of cellular and acellular components. Collagen, elastic, and reticular fibers are the main components of dense connective tissue that anchors the dermis to muscle or bone. Collagen is the major acellular structural component and constitutes approximately 70% of the skin’s dry weight. Type I and type III collagens represent 80% and 15% of the total dermal collagen, respectively. Elastic fibers comprise 2% of the dry weight of skin and play a crucial role in wound contraction after a loss of skin integrity. Finally, glycosaminoglycans (GAGs) are the third major acellular component of the dermis. The principle GAGs in the dermis are heparin and heparin sulfate; their major functions include binding water and cationic molecules, serving as cofactors for multiple
enzyme pathways, and participating in cell adhesion and basement membrane formation [Cuono 1988]. In addition, two important GAGs, chondroitin sulfate and dermatan sulfate, have been implicated in endothelial proliferation during wound healing [Faham et al. 1996].

Capillaries, nerves, and lymphatics are also located in the dermis. The major cell types of the dermis are the fibroblasts, which is responsible for both the synthesis and the degradation of dermal proteins. In addition, the dermis contains a small number of hematopoietic derived cells including macrophages, mast cells, lymphocytes and eosinophils. Other cell types found in the dermis are associated with the vascular, lymphatic and nervous systems and the epidermal appendages such as plasma cells, fat cells and extravasated leukocytes.

The mature dermis may be divided into two main layers: the superficial papillary layer and the deeper reticular layer. The papillary dermis is directly beneath the basement membrane and contains blood vessels, lymphatic vessels, and nerve processes. The reticular dermis is located under the papillary layer and is thicker than the superficial layer and is characterized by type I collagen, coarser elastic fibers with very few cells and is continuous with the papillary layer. The hypodermis is underneath the dermis and anchors to muscles and bones via loose connective tissue of collagen and elastic fibers. The hypodermis serves as a reserve energy supply and allows its mobility on underlying structures. It is composed of connective tissue with a loose arrangement of collagen and elastic fibers. Hair follicles, sebaceous glands, and sweat glands are skin appendages. Hairs are keratinized structures derived from hair follicles and epidermal invaginations into the dermis and may extend into the hypodermis. Hair follicles represent a route for skin penetration of nanoparticles and may contribute to an increase in transdermal absorption [Monteiro-Riviere 1998].
Therefore, the skin represents a large area for potential Ag-nps penetration and consequent systemic absorption. Overall, there are three ways for chemical penetration or absorption through skin to occur: transcellular, intercellular, and appendageal pathways (through hair follicles or sweat glands). The flux and diffusion of a compound across the SC varies depending on nanoparticle size, morphology, surface structure, and polarity.

**Skin Biomechanics**

Generally, skin may be considered a viscoelastic material since it exhibits a nonlinear stress-strain relationship, a hysteresis loop in cyclic loading and unloading, stress relaxation at constant strain, and creep at constant load [Dunn and Silver 1983]. At rest, skin in naturally under tension in vivo, as evidenced by the very large contraction of excised skin of magnitudes up to 200%. The stress-strain behavior of skin is composed of generally three phases: (1) up to strains of about 0.3 the collagen network offers little resistance to deformation (high deformation (strain) for a low force (stress), ‘toe’ region), (2) between strains of 0.3 and 0.6 the collagen fibrils begin to deform and a linear response is seen, and (3) cross-linked collagen begins to yield and finally fail [Silver et al. 2001]. In region (1) characteristic of naturally occurring stresses resulting from flexion and extension at joints is seen. In region (1), during initial deformation, the random network of collagen fibers is being straightened out in the direction of the applied stress. At the beginning of region (2) some of the fibers have straightened out completely and as deformation progresses additional fibers are recruited into the load-carrying role until in region (3) all of the possible fibers have been recruited and no further deformation is possible so that any additional load results in the failure of the fibers and network. Due to this process, skin becomes stiffer with
increasing strain rate because with increased stretch the collagen fibers orient along the
direction of stretch, thereby increasing its resistance to the applied stretch.

The tight junctions, desmosomes, and hemidesmosomes that attach the epidermal
cells provide the epithelial layer with the mechanical strength needed for it to withstand
various stresses placed upon it. However, the mechanical effect of the epidermis is
negligible when it is much thinner than the dermis, but significant on the palms and soles of
the feet where the epidermis is relatively thick [Harkness 1971]. Unfortunately, the values
for the mechanical properties of the skin can vary drastically (by a factor of 3000), depending
on the model proposed, type of species, orientation of the skin specimen tested, location on
the body, and the stress applied. Generally, the mechanical strength of the dermis is derived
from the connective tissue that runs in all directions, which imparts it with a tensile strength
ranging from 3.4 to 68.9MPa, ultimate load ranged from 20 to 250N, ultimate strain ranging
from 35 to 115%, modulus of elasticity values range from 0.02MPa to 150MPa, time
constants are typically of the order 10ms [Doubal and Klemera 2006, Ankerson et al. 1999,

**Models in Dermatological Research**

In skin research, human epidermal keratinocytes (HEK) are used as in vitro cell
models for testing the cutaneous toxicity of chemicals, drugs, and nanoparticles because they
are the primary cell type in the epidermis and also produce inflammatory factors. The most
commonly used method to assess the in vitro toxicity of Ag-nps is the cell viability test. In
general, the percentage of dead cells is directly proportional to the toxicity of Ag-nps. Cell
viability assays are typically chemical based and depend on differential inclusion, exclusion,
or conversion of a dye or dye precursor which can only be enzymatically converted to detectable dye in living cells but not dead cells. In this case, the toxicity can be evaluated by quantitating colorimetric or fluorescent dyes and include MTT, MTS, 96AQ and aqueous One (96AQ), alamarBlue, lactate dehydrogenase (LDH), live/dead, and neutral red assays. Since nanoparticles are capable of adsorbing chemicals onto their surface, they may interact and interfere with the ability of the assay dye to react appropriately, and produce false results [Monteiro-Riviere et al. 2009]. Ag-nps above 1ppm of various sizes and surface conditions were shown to interfere with MTT, 96AQ, and alamarBlue viability assays, with alamarBlue having the least interaction. Therefore, prior to initiating a study and selecting a particular assay as a viability biomarker, it is important to investigate any potential interactions between the nanoparticle and assay dye.

However, in vitro skin cell culture cannot substitute for in vivo skin due to its lack of biological complexity. In this instance, porcine skin has possesses many similarities with human skin when compared across various anatomical regions, the structure of its epidermal and dermal layers, hair density and follicle arrangement, and chemical composition [Monteiro-Riviere and Stromberg 1985; Monteiro-Riviere 1986]. For ex-vivo tissue models, flow-through diffusion cell systems with porcine or human skin may be used to investigate cutaneous penetration and absorption of chemicals and nanoparticles [Bronaugh and Stewart 1985, Monteiro-Riviere and Riviere 2005]. This model can be used in absorption, dermatopharmacokinetic and dermatotoxicology studies by evaluating the amount of penetrated chemical or nanoparticle in the venous effluent [Riviere et al. 1995; Monteiro-Riviere and Inman 1997].
Biological Interactions of Silver Nanoparticles

Surface Adsorption

When Ag-nps are introduced into a fluid environment such as blood, plasma or interstitial fluid, they become coated with proteins that consequently undergo conformational changes. These changes expose new epitopes and may alter their function and/or avidity, potentially decreasing the nutrients available to cells. These adsorbed proteins may also promote their attachment onto cell surfaces; for example complement and immunoglobulin adsorption increases nanoparticle opsonization and promotes receptor-mediated phagocytosis. As Ag-nps approach the cell surface, they may bind to released cellular biomolecules in suspension or on the cell membrane, to surface ligands, hydrophobic or charged regions on the cell surface, or proteins, effectively interfering with cellular function. Additionally, multiple Ag-nps might form rafts that could bind to and block the functioning of surface proteins.

Risk Assessment

With the advent of nanotechnology, the potential unintended consequences to human health and the environment that might accompany nanomaterials should be considered. There is a growing body of literature that details various degrees of adverse biological effects induced by nanoparticles at cellular, subcellular and molecular scales [Berry et al. 2003, Berry et al. 2004a, Berry et al. 2004b; Brunner et al. 2006, Fortner et al. 2005, Lin et al. 2006, Tabata et al. 1997, Thill et al. 2006, Tokuyama et al. 1993]. Due to their increased reactivity, nanoparticles are more biologically active than their bulk constituents. They are also capable of gaining access to locations not accessible to their larger particle counterparts.
Given that many biological processes occur at the nanoscale, there are many opportunities for these ultrafine particles to interfere with normal biological functions.

Although Ag-nps may be synthesized as zero-valent species their stabilization is achieved, regardless of actual intent, by the deposition of surface species during fabrication and vehicle exposure. These surface species could originate from: 1) residues from the manufacturing process or exposure to ambient gases; 2) industrial chemicals and stabilizers used to prepare dispersions; or 3) organic and inorganic constituents of biologically relevant buffers used in preparing laboratory stock solutions. Any of these surface species may contribute directly to Ag-nps toxicity and is dependent on the adsorbed species.

Therefore, critical risk assessment of a nanomaterial should investigate their potential human and environmental toxicological risks throughout its life cycle, from raw nanomaterial through commercialization and end of product life, where possible exposure may arise during material fabrication, handling, usage, and waste disposal. The toxicological risk associated with nanomaterials are critically influenced by their physicochemical properties such as size, surface area, zeta potential, surface chemistry, and chemical composition. Therefore, the physicochemical characterization of nanoparticles is an important first step in order to correctly assess their potential exposure routes, toxicity, and related risk. This characterization should be conducted both before and after their application, particularly following their dispersion into their study medium.

**Toxicity**

In its metallic form Ag is inert and exerts no toxicological activity; instead, toxicity may be derived from the dissolution of toxic Ag ions from the surface of Ag-nps in the
presence of water or tissue fluids. The driving force for Ag ion dissolution depends on many factors, such as surface area and curvature, roughness, nanoparticle aggregation, functionalization, and solubility within a given environment as well as the concentration gradient between the particle surface and the bulk solution phase. A nanoparticle with higher solubility such as ZnO may be more cytotoxic than one with low solubility, such as TiO$_2$ [Brunner et al. 2006]. Unlike other heavy metals, the speciation of Ag does not seem to play a large part of its toxicity; while Ag$^+$ is highly reactive, the toxicity of Ag$^{2+}$ or Ag$^{3+}$ is unlikely due to the transient nature of these forms of Ag, particularly in an aqueous environment. Agglomeration of multiple Ag-nps, as well as the protracted release of ions from an immobile Ag-np, increases the potential toxicological impact of Ag-nps.

The dissolution of Ag ions from the surface of Ag-nps would increase the incidence of cellular entry of Ag ions, either directly through the use of non-specific phosphate/sulphate anionic transporters ion transporters or using specific metal ion binding proteins [Nel et al. 2009]. Ag ions may bind to specific serum proteins to form metal-protein complexes, or to the electron-donor groups of biological molecules containing sulphur, oxygen, and nitrogen; the proteins that bind most strongly to carbon nanotubes, iron oxide particles, liposomes and polymeric nanoparticles are albumin, immunoglobulins, complement, fibrinogen, apolipoproteins, ferritin, and transferrins [Sun et al. 1999, Torti and Torti 1994, Qian et al. 2002]. Adsorbed proteins and organic substances increase the dissolution rates of Ag-nps through at least two mechanisms: aqueous complexation whereby aqueous species complex free ions released from Ag-nps surface, and ligand-enhanced dissolution whereby adsorbed natural organic material and organic acids extract surface metal atoms from Ag-nps surfaces. In the acidic phagosome and endosome, the particles are
exposed to a series of oxidative mechanisms designed to destroy the foreign body, which leads to the generation of metal ions and free radicals [Galle et al. 1992, Lundborg et al. 1992]. Ultimately, the accumulation of Ag ions within lysosomes and endosomes causes swelling and rupture, leading to further particle deposition in the cytoplasm.

Presently, there is no consensus on the cytotoxicity of Ag-nps; however, the majority of publications do show reduced cell viability following Ag-np exposure. The variability of the cytotoxicity is linked to several characteristics, such as the synthesis method and purity of the Ag-nps, the concentration of released Ag ions from the nanoparticle, and the physicochemical properties of the nanoparticles such as size, shape, and surface functionalization. In vitro cell studies have demonstrated size-dependent Ag-np cytotoxicity and the generation of ROS to various Ag-nps in a variety of human cell lines such as fibrosarcoma, skin carcinoma, lung fibroblast, glioblastoma, hepatoma, alveolar, and keratinocyte [Wijnhoven et al. 2009, Samberg et al. 2010, Christensen et al. 2010, Hussain et al. 2005, Braydich-Stolle et al. 2005, Ahamed et al. 2008, Schrand et al. 2008]. Toxicity of Ag-nps may be attributed to multiple effects: the binding of single and multiple Ag-nps to the surface of cells, the interactions of Ag-nps after uptake into a cell, and the resulting degradation of Ag-nps inside cells. Within the cell, Ag-nps ions may undergo rapid reduction, which would generate reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS are known to be involved in protein oxidation, leading to their degradation, lipid peroxidation, DNA damage, and apoptosis. Free radicals can react with DNA to induce damage to purine and pyrimidine bases as well as to the deoxyribose backbone [Dizdaroglu et al. 2002].
Inflammation

Inflammation is a skin response to allergens, irritants, UV light and mechanical perturbation. When skin comes into contact with irritants or other stimuli, skin inflammation begins with the secretion of cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α. IL-1 and TNF-α released from epidermal cells results in the expression of other cytokines (IL-6 and IL-8) to activate differentiation and proliferation of leukocytes. Inflammation also induces vasodilatation and cellular infiltrate, resulting in an increase in epidermopoiesis and thickening of skin [Barker et al. 1991; Nickoloff 1993]. The inflammatory factors can be divided as follows: inflammatory cytokines: IL-1 and TNF-α; chemotactic cytokines: IL-8, IL-10; growth promoting cytokines: IL-6, IL-7, IL-15, GM-CSF, TGF-α and immunomodulatory cytokines: IL-10, IL-12, IL-18. Here only IL-1, TNF-α, IL-6 and IL-8 will be reviewed in detail due to their important role in skin inflammation.

IL-1α/β and TNF-α are primary cytokines that initiate and activate a number of effector molecules independently to trigger cutaneous inflammation. The IL-1 family is mainly composed of α and β forms which reside in cytoplasm. The precursor IL-1α is found on the surface of monocytes and B lymphocytes, IL-1β is produced by monocytes, macrophages, Langerhans cells, and dendritic cells, and keratinocytes in normal skin constitutively produce precursor and active IL-1α and inactive pro-IL-1β [Mizutani et al. 1991]. Due to this, the epidermis is a reservoir for IL-1α which is released when the body is injured, and the release of IL-1α activates keratinocytes to produce more IL-1α, as well as increase the expression of IL-6, IL-8 GMCSF, intracellular adhesion molecules, and memory T cells [Kupper and Grove 1995, Groves et al. 1991]. The increased IL-6 expression stimulates keratinocytes and fibroblasts proliferation, down regulates Langerhans cell E-
cadherin expression, which induces the cells to migrate from the epidermis to lymph nodes where they encounter the naïve T lymphocytes and present antigen on the surface of Langerhans cells. Alternatively, IL-1β needs to be cleaved by interleukin-1β converting enzyme to its active form [Mosley et al. 1987, Black et al. 1988]. Keratinocytes are the main producers of TNF-α in skin in response to stimuli such as UV light [Kolde et al. 1992, Kock et al. 1990]. TNF-α activates T cells and Langerhans cells and regulates fibroblast collagen synthesis. IL-1β and TNF-α together can augment Langerhans cell migration to lymph nodes. In addition, there is some storage of TNF-α in mast cells as constitutive expression to initiate the quick response to irritants, allergens, and sunburns [Gordon and Galli 1990].

Interleukin-8 (IL-8) is a neutrophil chemotactic polypeptide that can stimulate the mast cell histamine release, induce leukocytes and keratinocyte chemotaxis and proliferation [Larsen et al. 1989; White et al. 1989, Nickoloff 1993, Matsushima et al. 1988]. IL-8 can be produced by different types of cells present in the skin, stimulated by IL-1α and TNF-α in dermal fibroblasts and by IL-1α only in keratinocytes [Larsen et al. 1989].

Some nanomaterials have been shown to cause an increase in IL-1 and TNF-α levels, which may directly induce keratinocyte proliferation and initiate keratinocyte response to other signaling molecules. Fourteen-nanometer carbon black nanoparticles enhanced the expression of IL-1β in lung [Inoue et al. 2006]. IL-1β and TNF-α were released when HEK were exposed to QD 565 or 655 coated with carboxylic acid [Ryman-Rasmussen et al. 2007], but was not detectable with functionalized SWCNT and fullerene-based amino acid nanoparticles [Zhang et al. 2007, Rouse et al. 2006]. It is unknown whether long-term exposure to nanomaterials on the skin surface can cause over proliferation of the epidermis and possibly carcinogenesis, and there are no long-term studies that have been conducted to
date. By definition as an inflammatory marker of skin, IL-8 is a suitable and standard marker for nanomaterial inflammation. IL-8 is found to be one of the early biomarkers produced by HEK when exposed to some aromatic and aliphatic hydrocarbon components of jet fuels, multi-walled carbon nanotubes, fullerene-based amino acid, single-walled carbon nanotubes, and QD led to increases in the expression of proinflammatory IL-8 by HEK [Monteiro-Riviere et al. 2005, Rouse et al. 2006, Zhang et al. 2007, Ryman-Rasmussen et al. 2007, Allen et al. 2000, Allen et al. 2001a, Chou et al. 2003, Yang et al. 2006].
3. Evaluation of Silver Nanoparticle Toxicity in vivo Skin and in vitro Keratinocytes

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Abstract

Products utilizing the antimicrobial properties of silver nanoparticles (Ag-nps) may be found in health and consumer products that routinely contact skin. The study was designed to assess the potential cytotoxicity of Ag-nps in human epidermal keratinocytes (HEK), and their inflammatory and penetrating potential into porcine skin in vivo. Eight different Ag-nps were used in this study. HEK viability was assessed by MTT, alamarBlue (aB), and CellTiter 96Aqueous One (96AQ); release of pro-inflammatory mediators IL-1β, IL-6, IL-8, IL-10, and TNF-α were measured. Skin was dosed topically for 14 consecutive days. The effect of the unwashed Ag-nps on HEK viability after 24 h exposure indicated a significant dose-dependent decrease (p<0.05) at 0.34µg/ml with aB and 96AQ and at 1.7µg/ml with MTT. However, both the washed Ag-nps and carbon-coated Ag-nps showed no significant decrease in viability at any concentration assessed by any of the three assays. For each of the unwashed Ag-nps a significant increase (p<0.05) in concentration IL-1β, IL-6, IL-8, and TNF-α was noted. Localization of all Ag-nps was depicted in cytoplasmic vacuoles of HEK. Macroscopic observations showed no gross irritation in porcine skin, while microscopic and ultrastructural observations showed areas of focal inflammation and localization of Ag-nps on the surface and in the upper stratum corneum layers of the skin. This study provides a better understanding Ag-nps safety in vitro as well as in vivo and a basis for occupational and risk assessment. Ag-nps are non-toxic when dosed in washed Ag-nps solutions or carbon-coated.
Introduction

Historically, Ag compounds have been used in numerous fields to prevent microbial growth. Like many non-essential heavy metals Ag is a natural biocide, but compared to titanium, zinc, and copper, Ag-nps show the highest antimicrobial efficacy against bacteria, viruses and other eukaryotic microorganisms [Gong et al. 2007]. The Phoenicians coated milk bottles with Ag to inhibit bacterial growth, doctors have administered drops of Ag nitrate solutions to newborn babies to prevent neonatal conjunctivitis [Crede 1881], and Ag sulfadiazine creams have long been considered the standard of care for the prevention of widespread bacterial growth on burn patient’s denuded skin [Moyer et al. 1967]. Both dietary supplements and homemade varieties of Ag colloids have been sold for decades as a “cure-all” for diseases such as tuberculosis, syphilis, scarlet fever, shingles, herpes, pneumonia, and arthritis [NCCAM 2006]. Furthermore, advances in nanotechnology have facilitated the increase of Ag-containing merchandise available to the public, making Ag the most used nanomaterial of all manufacturer-identified products in the world [www.nanotechproject.org]. Products such as room deodorizing sprays, acne creams, clothing that prevents body odor, baby wipes, and pacifiers all exploit the natural antimicrobial activity of Ag [www.nanotechproject.org]. A study investigating the release of Ag-nps from commercially available sock fabric showed that socks could contain up to 1360µg Ag/g-sock and could release as much as 1.3µg/ml of silver into distilled water [Benn and Westerhoff 2008].

Ingestion of Ag can cause argyria, the benign condition characterized by the bluish-graying of the skin that occurs through its preferential deposition in the basal lamina of soft tissues such as the skin, liver and spleen [Fung and Bowen 1996], blood vessels,
gastrointestinal tract, liver, and kidney [Danscher 1980]. Although argyria is most commonly reported clinically after excessive Ag ingestion, silver deposition has been seen after Ag sulfadiazine treatment of burned skin [Lee and Lee 1994; Marshall 1979; Temple and Farooqi 1985]. In response to argyria, but not to Ag toxicity, the National Institute for Occupational Safety and Health set a daily exposure limit for all forms of Ag at 0.01 mg/m³, and the Environmental Protection Agency established the oral reference dose at 0.005mg/kg/day [EPA 1996, NIOSH 2003].

Studies indicating Ag toxicity exist from as early as 1983, Rungby and Danscher showed that intraperitoneal administered Ag salts can accumulate in neurons and in protoplasmic glial cells of the brain and spinal cord. *In vitro* cell line studies have shown decreased mitochondrial function after exposure to Ag-nps in murine neuroblastoma cells [Schrand et al. 2008], hepatic cells [Hussain et al. 2005], germ-line stem cells [Braydich-Stolle et al. 2005], human skin carcinoma cells [Arora et al. 2008], and HEK and fibroblasts [Burd et al. 2007]. Although *in vivo* studies have not been performed with Ag-nps, polyvinylpyrrolidone-stabilized Ag-nps with a mean size of 25nm were shown to penetrate into the upper layers of the epidermis in excised human skin in static diffusion cells [Larese et al. 2009]. The ability for other nanomaterials such as quantum dots (QD) and fullerenes [Rouse et al. 2007, Ryman-Rasmussen et al. 2006, Zhang and Monteiro-Riviere 2008], as well as zinc oxide [Cross et al. 2007; Gamer et al. 2006] to penetrate into the stratum corneum has been shown and warrants examination of the ability for Ag-nps to penetrate the skin.
The objectives of this study were to determine the optimal viability assay for use with Ag-nps in order to assess their toxicity to skin cells, their inflammatory potential and capacity to penetrate skin.

**Materials and Methods**

**Ag-nps**

To encompass the variety of manufactured Ag-nps on the market, eight different Ag-nps were used in this study with different sizes and surface conditions, the properties of which are summarized in Table 1. All Ag-nps used in this study were supplied by nanoComposix (San Diego, CA, USA) and consisted of the following: commercially-used unwashed and not coated Ag-nps suspended in deionized water with diameters of 20nm, 50nm and 80nm (“unwashed”), washed and not coated Ag-nps suspended in deionized water with diameters of 20nm, 50nm and 80nm (“washed”), and commercially-used dried carbon-coated Ag-nps with diameters of 25nm and 35nm (“carbon-coated”). The sizes of each type of Ag-nps was determined by the manufacturer and confirmed in this study by DLS and TEM.
Table 3.1 - Physicochemical Properties of Ag-nps

<table>
<thead>
<tr>
<th>Description</th>
<th>MDD (nm)</th>
<th>DLS diameter (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>TEM diameter (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Supplied Concentration (mg/ml)</th>
<th>Particle Concentration (particles/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Zeta&lt;sup&gt;b&lt;/sup&gt; Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwashed, colloid</td>
<td>20</td>
<td>30.8±0.6</td>
<td>22.4±2.6</td>
<td>0.20</td>
<td>2.41E+12</td>
<td>-29.7</td>
</tr>
<tr>
<td>Unwashed, colloid</td>
<td>50</td>
<td>47.7±0.5</td>
<td>49.4±6.2</td>
<td>0.20</td>
<td>4.44E+11</td>
<td>-27.8</td>
</tr>
<tr>
<td>Unwashed, colloid</td>
<td>80</td>
<td>75.5±1.0</td>
<td>79.2±8.0</td>
<td>0.20</td>
<td>7.09E+10</td>
<td>-33.2</td>
</tr>
<tr>
<td>Washed, colloid</td>
<td>20</td>
<td>25.5±0.4</td>
<td>21.4±3.1</td>
<td>2.86</td>
<td>1.89E+14</td>
<td>-46.0</td>
</tr>
<tr>
<td>Washed, colloid</td>
<td>50</td>
<td>43.7±1.1</td>
<td>50.0±5.9</td>
<td>3.45</td>
<td>5.01E+12</td>
<td>-44.3</td>
</tr>
<tr>
<td>Washed, colloid</td>
<td>80</td>
<td>79.9±28.0</td>
<td>77.0±6.0</td>
<td>2.79</td>
<td>1.07E+12</td>
<td>-43.7</td>
</tr>
<tr>
<td>Carbon-coated, powder</td>
<td>25</td>
<td>149.0±89</td>
<td>27.2±10.3</td>
<td>N/A</td>
<td>N/A</td>
<td>-24.0</td>
</tr>
<tr>
<td>Carbon-coated, powder</td>
<td>35</td>
<td>167.0±110</td>
<td>37.0±11.6</td>
<td>N/A</td>
<td>N/A</td>
<td>-29.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are expressed as Mean±SD, <sup>b</sup>Zeta Potential in deionized water.

MDD, Manufacturer-Designated diameter; DLS, Dynamic light scattering; TEM, transmission electron microscopy.

Both the unwashed and washed Ag-nps were synthesized by ammonium hydroxide catalyzed growth of Ag onto 5nm gold (Au) seed particles. Concentration of the particles was achieved via tangential flow filtration (TFF). The unwashed Ag-nps solution contained approximately 5.55mg/ml of formaldehyde solvent and methanol byproduct from their formation. These unwashed Ag-nps were then ultra centrifuged to obtain the solution supernatant for toxicity testing (“as synthesized” supernatant). The Ag-nps were then serially washed with 20 volume equivalents of 2mM phosphate buffer (pH 7.5) and the 5, 10, 15 and 20 washing permeates were collected. The washing permeates were found to have
20-50ppb of dissolved silver content. The colloidal Ag-nps were stored at 4°C in the dark. The carbon-coated Ag-nps, synthesized by pulsed plasma reactor and coated with polyaromatic graphitic carbon, were supplied as a powder and stored at room temperature.

**Cell Culture**

Cryopreserved primary neonatal human epidermal keratinocytes (HEK; Lonza, Walkersville, MD) were grown in keratinocyte growth medium-2 (KGM-2; Lonza, Walkersville, MD) in cell culture flasks (75cm²; 1,000,000 cells) to approximately 80% confluency in a 37°C humidified 5% CO₂ incubator. The cells were passed into clear or black 96-well microplates (12,500cell/well; 200μl) in which the peripheral wells contained only KGM-2 to prevent the evaporation of treatment medium. Between 18-24 h later, after reaching approximately 80% confluency, the HEK were exposed to either KGM-2 (control) or serial dilutions of each Ag-nps for the following experiments.

**Evaluation of Best Viability Assay**

A nanoparticle and a nanoparticle/cell control was run in parallel with each viability assay as described by Monteiro-Riviere et al. (2009) to assess the interactions between the viability assays and the Ag-nps (See Supplemental Material).

**Ag-nps treatment of HEKs**

An initial dose response study was conducted to assess the concentrations of Ag-nps that could affect HEK after 24 h exposure. The majority of the colloidal Ag-nps tested were supplied in both low volume and concentration, which limited the highest HEK dosing
concentration to 1.7 µg/ml. Combined with KGM-2 medium, a 1.7 µg/ml solution of the Ag-nps was serially diluted (1:5) to provide concentrations ranging from 1.7 to 0.000544 µg/ml. The effect of the Ag-nps on the HEK viability was assessed by three different toxicity assays: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), alamarBlue (aB), and CellTiter 96 AQueous (96 AQ) assays.

Dosing concentrations of the washed and carbon-coated Ag-nps were tested up to 42.5 µg/ml but did not show a response. Additionally, to differentiate the potential cytotoxicity between the particles and the contaminants present in the colloidal solution, HEK were also treated with the “as synthesized” supernatant and the 5, 10, 15 and 20 washing permeate for 24 h at concentrations ranging from 1.7 µg/ml to 0.068 µg/ml.

**Cytokine Release**

For the concentrations of Ag-nps that showed toxicity, cytokine analysis was conducted to determine their pro-inflammatory potential by assessing the release of interleukin (IL)-8, IL-6, tumor necrosis factor-alpha (TNF)-α, IL-10, and IL-1β; the procedure is described in Supplemental Material.

**In Vivo Porcine Skin Exposure**

The effects of the unwashed Ag-nps were compared to the washed Ag-nps in vivo. Assuming the two smallest Ag-nps could penetrate, the comparison was limited to the 20 and 50 nm washed and unwashed samples. Ag-nps solutions ranging from 34.0 to 0.34 µg/ml were dosed on the back skin of the pigs (dosing information in Supplemental Material). Skin was evaluated for erythema and edema according to the Draize system (Erythema: 0, no
change; 1, very slight change; 2, pale red in defined area; 3, definite red in well-defined area;
4, crimson red. Edema: 0, no change; 1, very slight change; 2, slight change with edges
barely defined; 3, moderate change, with area raised 1 mm; 4, severe change, with area raised
more than 1 mm and extending beyond the exposure area) (Draize et al. 1944). All animals
were treated humanely and with regard for alleviation of suffering.

Microscopic Observations

To assess morphological alterations during the in vivo study, tissue samples were
harvested after the pigs were euthanized and processed routinely for light microscopy (see
Supplemental Material). Approximately 1 cm sections were evaluated for intercellular and
intracellular epidermal edema, dermal edema, and inflammation using the following scoring
system: 0, no change; slight, inflammation on less than half the sample; moderate,
inflammation on half the sample; severe, inflammation on greater than half the sample.

Ultrastructural Observations

Particle size analysis was conducted with both DLS and TEM to confirm the
manufacturer-identified diameters and surface characterization (for details see Supplemental
Material). To localize Ag-nps uptake in vitro, HEK were grown to approximately 70%
confluency in cell culture flasks (25 cm²) and treated for 24 h with each Ag particle at
1.7 µg/ml in KGM-2. The cells and skin samples were processed routinely for TEM (for
details see Supplemental Material). All TEM samples were observed on an FEI/Philips EM
208S TEM operating at an accelerating voltage of 80 kV. Additionally, unstained samples
were analyzed by EDS with a Hitachi HF2000 FE TEM equipped with an Oxford Instruments INCA EDS.

**Statistical Analysis**

The mean values for HEK percent viability (normalized by viability) and cytokine concentration for each treatment were calculated, and the significant differences ($p<0.05$) determined by the PROC GLM Procedure (SAS 9.1 for Windows; SAS Institute, Cary, NC). When significant differences were found, multiple comparisons were performed with the Tukey’s Studentized Range HSD test at $p<0.05$ level of significance. The Dunnett’s t-test was performed to determine the significance at $p<0.5$ of differences between control and treatment group. Data are expressed as the means ± Standard Error of the Mean (SEM) of two plates (n=6/plate).

**Results**

**Evaluation of Best Viability Assay**

Table 2 summarizes the control studies for the three viability assays by listing the absorbance or fluorescence value for each Ag-nps that significantly shows an interaction between assay and Ag-nps. For MTT, at 1.7µg/ml both the 25nm (Figure 1A) and 35nm (Figure 1B) carbon-coated Ag-nps nanoparticle/cell controls show a statistically significant increase in absorbance after exposure of Ag-nps to cell-reacted assay dye. The nanoparticle controls showed a statistically significant increase in absorbance at 1.7µg/ml, shown in Figure 1C with the 20nm unwashed Ag-nps for 96AQ and MTT but not for aB.
Table 3.2- Control studies summary of Ag-nps concentration that significantly shows an interaction between assay and Ag-nps

<table>
<thead>
<tr>
<th>Description</th>
<th>No Cell Control</th>
<th>Cell Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT</td>
<td>aB</td>
</tr>
<tr>
<td>20nm Unwashed</td>
<td>1.7</td>
<td>--</td>
</tr>
<tr>
<td>50nm Unwashed</td>
<td>0.34</td>
<td>--</td>
</tr>
<tr>
<td>80nm Unwashed</td>
<td>1.7</td>
<td>--</td>
</tr>
<tr>
<td>20nm Washed</td>
<td>--</td>
<td>0.34</td>
</tr>
<tr>
<td>50nm Washed</td>
<td>--</td>
<td>1.7</td>
</tr>
<tr>
<td>80nm Washed</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>25nm Carbon-coated</td>
<td>1.7</td>
<td>--</td>
</tr>
<tr>
<td>35nm Carbon-coated</td>
<td>1.7</td>
<td>--</td>
</tr>
</tbody>
</table>

aNo cell control value at which a significant increase in absorbance or fluorescence was observed (p< 0.05), bCell control value at which a significant increase in absorbance was observed before and after Ag-nps (p< 0.05), cSignificant decrease was observed for this data set. Data are expressed as micrograms of Ag-nps per milliliter of media.

Figure 3.1- Evaluation of viability assays, and HEK viability after exposure to Ag-nps. (A) 25nm carbon-coated Ag-nps MTT nanoparticle/cell control; (B) 35nm carbon-coated Ag-nps MTT nanoparticle/cell control; (C) Nanoparticle controls of 20nm unwashed Ag-nps; (D)
HEK viability after 24h exposure to various Ag-nps as assessed by aB. *p<0.05, nanoparticle/cell controls compared for each concentration before and after Ag-nps, nanoparticle control run with multiple comparisons between concentrations, each Ag-nps assessed independently. Different letters denote a significant difference. \(^{\Lambda}\)p<0.05, 20nm unwashed; \(^{\alpha}\)p<0.05, 50nm unwashed; \(^{\Lambda}\)p<0.05, 80nm unwashed; \(^{\alpha}\)p<0.05, “As synthesized” supernatant; multiple comparisons between concentrations, each Ag-nps assessed independently. Different letters denote a significant difference.

All other nanoparticle control data can be found in the supplemental material section. No significant change was seen in the fluorescence values for aB for the 20nm, 50nm or 80nm unwashed Ag-nps (See Supplemental Material, Figure 1A). The unwashed 50nm and 80nm Ag-nps caused a significant increase in absorbance values for MTT and 96AQ but not for aB (See Supplemental Material, Figures 1B, 1C). A significant increase in the fluorescence value for aB was seen at 0.034µg/ml for both 20nm and 50nm washed Ag-nps and at 1.7 for 80nm washed Ag-nps (See Supplemental Material, Figure 2A). A significant change in absorbance value for 96AQ was seen at 1.7µg/ml for each of the 20nm, 50nm, and 80nm washed Ag-nps (See Supplemental Material, Figure 2B). The 80nm washed Ag-nps caused a significant increase in absorbance value for MTT at 1.7µg/ml (See Supplemental Material, Figure 2C). A significant increase in fluorescence value for aB was seen at 1.7µg/ml for 35nm carbon-coated Ag-nps (See Supplemental Material, Figure 3A). A significant increase in absorbance value was seen for 96AQ at 1.7µg/ml for 25nm carbon-coated and at 0.034µg/ml for 35nm carbon-coated (See Supplemental Material, Figure 3B).
A significant increase in absorbance value was seen at 1.7µg/ml for 25nm and 35nm carbon-coated Ag-nps (See Supplemental Material, Figure 3C).

**Ag-nps Treatment of HEK**

Generally, for concentrations ranging from 0.000544 to 1.7µg/ml and exposure for 24 h the unwashed Ag-nps resulted in a dose-dependent viability decrease with all three assays, whereas the washed and carbon-coated Ag-nps did not show a decrease in viability for any assay.

As seen in Figure 1D, the 20nm and 50nm unwashed Ag-nps caused a significant decrease in viability at 0.34µg/ml for both aB and 96AQ and at 1.7µg/ml for MTT. The 80nm unwashed Ag-nps caused a significant decrease in viability at 0.34µg/ml for all three assays. The “as synthesized” supernatant showed a significant decrease in viability at 0.34µg/ml for MTT and aB assays and at 1.7µg/ml for 96AQ. Toxicity was not present for any of the supernatant obtained from the 5, 10, 15 or 20 washing permeates. Exposure of HEK to washed 20nm, 50nm, and 80nm Ag-nps and the carbon-coated Ag-nps ranging in concentration from 0.000544 to 1.7µg/ml for 24 h showed no significant decrease in viability with any assay (See Supplemental Material, Figures 4, 5, 6).

**Cytokine Release**

There was a significant increase above the limit of detection in IL-1β (Figure 2A), IL-6 (Figure 2B), IL-8 (Figure 2C) and TNF-α (Figure 2D) from HEK exposed to 0.34µg/ml unwashed 20, 50, and 80nm Ag-nps for 24 h. The lowest limit of detection for each cytokine
was IL-1β, 0.8pg/ml; IL-6, 1.1pg/ml; IL-8, 0.5pg/ml; TNF-α, 3.0pg/ml. The values for IL-10 were below the detectable limit of the assay (0.9pg/ml).

Figure 3.2- Cytokine release by HEK exposed to Ag-nps. (A) IL-1β; (B) IL-6; (C) IL-8; (D) TNF-α. *p<0.05, multiple comparisons between concentrations, each Ag-nps assessed independently. Different letters denote a significant difference.

**Macroscopic Observations**

No gross erythema or edema based on the Draize system was noted in any of the treated sites during the entire 14 day study *in vivo*. Treated sites were grey in appearance representing residual Ag-nps on the surface of the skin.

**Microscopic Observations**

Morphological observations of untreated porcine skin exhibited normal epidermis and dermis (Figure 3A). Skin dosed daily with Ag-nps for 14 days exhibited a concentration-
dependent response, regardless of particle size or if washed or unwashed. Treatment with the lowest dosing concentration of 0.34µg/ml of 20nm washed Ag-nps typically showed a slight intracellular and intercellular epidermal edema (Figure 3B), with 3.4µg/ml of the 20nm washed Ag-nps showed moderate focal intracellular and intercellular epidermal edema, and focal epidermal and dermal inflammation (Figure 3C). Treatment at the highest concentration of 34µg/ml of the 20nm washed Ag-nps showed severe intracellular and intercellular epidermal edema with severe focal dermal inflammation (spongiosis), epidermal hyperplasia and parakeratosis. An increase in the extension of the rete pegs into the superficial papillary layer of the dermis was noted (Figure 3D).
Figure 3.3- LM images of porcine skin treated with Ag-nps. (A) control; (B) 20nm washed Ag-nps, 0.34µg/ml (C) 20nm washed Ag-nps, 3.4µg/ml (D) 20nm washed, 34µg/ml. Bars=60µm. Large arrows point to intracellular epidermal edema, smaller arrows point to focal areas of intercellular epidermal edema, arrowheads point to perivascular inflammation. SC, stratum corneum; E, epidermis; D, dermis; RP, rete peg. H&E.

Results of the 20nm unwashed Ag-nps (See Supplemental Material, Figure 7) were similar to the 20nm washed Ag-nps. At 0.34µg/ml, the 20nm unwashed Ag-nps showed slight intracellular epidermal edema. The 3.4µg/ml of the 20nm unwashed Ag-nps showed
moderate intracellular and intercellular epidermal edema. The 34µg/ml of the 20nm unwashed Ag-nps showed severe intracellular and intercellular epidermal edema with focal areas of intraepidermal infiltrates and superficial papillary dermal inflammation.

*Ultrastructural Observations of HEK Exposed to Ag-nps*

Ultrastructural observations of the 20nm washed and 25nm carbon-coated Ag-nps are depicted in Figure 4A, B (all other Ag-nps, see Supplemental Material, Figure 8).
Figure 3.4- TEM images of Ag-nps, HEK and porcine skin exposed to Ag-nps. (A) 20nm unwashed Ag-nps, bar=100nm; (B) 25nm carbon-coated Ag-nps, bar=100nm; (C) Control HEK, bar=1μm; (D) 80nm washed Ag-nps, bar=1μm; (E) Control porcine skin, bar=500nm; (F) 34μg/ml of 50nm washed Ag-nps in stratum corneum of porcine skin, bar=500nm. Arrows point to Ag-nps. SC, stratum corneum; N, nucleus; unstained sections.
The HEK controls appeared normal with a prominent nucleus, nucleolus and mitochondria (Figure 4C). HEK dosed with Ag-nps of all sizes and surface conditions depicted Ag-nps localization within membrane-bound cytoplasmic vacuoles; a representative image of internalized Ag-nps can be seen in Figure 4D (all other Ag-nps electron micrographs, see Supplemental Material, Figure 9). EDS analysis of HEK dosed with the 20nm washed Ag-nps confirmed the presence of Ag in the vacuoles, copper from the grid and Au from the particle seed was present (See Supplemental Material, Figure 10).

The control skin consisted of a normal compact stratum corneum with approximately 20-30 layers attached by desmosomes (Figure 4E). Skin dosed daily for 14 days depicted all Ag-nps localization within or on top of the stratum corneum. TEM images depicts skin dosed with 34µg/ml of the 50nm washed Ag-nps within the superficial layers of the stratum corneum (Figure 4F), and 20nm washed Ag-nps on top of the stratum corneum (see Supplemental Material, Figure 9H). EDS analysis conducted in the areas detected Ag, osmium from the post fixation process, and copper from the grid (See Supplemental Material, Figure, Figure 10).

**Conclusions**

Ag-nps have been integrated into hundreds of products that affect the daily lives of millions of people in many countries. Their main usage is for disinfection in wound care and in products such as odor-reducing clothing, acne creams and face masks. The majority of these products come into direct contact with skin, the largest organ of the human body, and could serve as a potential route for nanoparticle penetration. Therefore, the relationship of
Ag-nps in skin needs to be investigated with particular focus on their irritation potential, toxicity, and penetration into skin and skin cells. This study evaluated the cytotoxic potential of Ag-nps of varying size and surface conditions in HEK, their penetrating capacity into porcine skin after topical repetitive daily dosing for two weeks, and the localization of the Ag-nps within HEK and porcine skin.

The use of several viability assays is important to determine the optimal assay to assess Ag-nps toxicity; therefore, mortality of HEK after Ag-nps exposure was evaluated with three different assays that use colorimetric or fluorescent dyes as markers to determine cell viability by assessing cell metabolism.

Nanomaterials, such as single-walled carbon nanotubes [Zhang et al. 2007], carbon black [Monteiro-Riviere and Inman 2006], fullerenes and quantum dots [Monteiro-Riviere et al. 2009], are capable of interfering with viability assay dye and dye products through the adsorption of cell medium constituents and cytokines. The potential interactions between assays and Ag-nps were assessed by the nanoparticle and nanoparticle/cell controls which showed an increase in absorbance and fluorescence values at the highest concentration. The increase in absorbance and fluorescence values could cause the toxicity of the Ag-nps in HEK to be underestimated. Additionally, the nanoparticle/cell control showed that both the 25nm and 35nm carbon-coated Ag-nps interfered with the MTT assay at the 1.7µg/ml concentration due to the increase in absorbance values following incubation of the reduced formazan product with the Ag-nps (Figure 1A, B). Overall, all assays were affected by the Ag-nps, and based on its fluorescence values aB may be the best viability assay to use when conducting experiments with Ag-nps.
MTT, aB, and 96AQ viability assays did not show toxicity for the 25nm and 35nm carbon-coated Ag-nps as well as the 20nm, 50nm and 80nm washed Ag-nps. All three assays also showed that the 20nm, 50nm and 80nm unwashed Ag-nps contributed to a decrease in HEK viability at 24 h after exposure to the 0.34-1.7µg/ml concentrations, but did not show a size-dependent decrease in viability. However, the difference in toxicity between the unwashed and washed Ag-nps is inferred to be due to the presence of contaminants in the unwashed solution such as formaldehyde which has shown to have cytotoxic effects on cell culture [Ku and Billings 1984]. These residual contaminants were removed by the washing step 5, as indicated by the lack of cell death after exposure to any of the 5, 10, 15, and 20 washing supernatant. This study also showed that Ag-nps of different sizes, surface conditions, and synthesis method are all internalized into membrane-bound vacuoles in HEK, without a decrease in viability after 24 h.

*In vitro* cell line studies have shown that 25µg/ml of 25nm Ag-nps in murine neuroblastoma cells decreases mitochondrial function and causes the production of reactive oxygen species that could potentially contribute to neurodegenerative diseases [Schrand et al. 2008]. A significant decrease in mitochondrial function has been shown in hepatic cells after single exposures of 15nm and 100nm Ag-nps at concentrations ranging from 5-50µg/ml [Hussain et al. 2005], in germ-line stem cells at a concentration of 10µg/ml for 15nm Ag-nps [Braydich-Stolle et al. 2005], and in HEK and fibroblasts after exposure to Ag-nps extracted from commercially available Ag-based wound dressings with Ag content ranging from 13-934µg/cm² at approximately 15µg/ml [Burd et al. 2007]. Interactions between Ag-nps ranging in size from 7-20nm and human skin carcinoma cells showed a decrease in mitochondrial function and the onset of apoptosis at concentrations of 0.78µg/ml and
1.56µg/ml respectively [Arora et al. 2008]. The 0.34-1.7µg/ml toxic concentrations of the 20, 50 and 80nm unwashed Ag-nps are slightly more sensitive compared to the *in vitro* toxicity studies conducted by others in different cell lines, although it is important to consider such factors as agglomeration, surface conditions, size, cell lines, and interactions with the assay dye products when comparing across studies.

Keratinocytes produce proinflammatory cytokines such as IL-8, IL-6, TNF-α, and IL-1β that serve as mediators for inflammatory and immunologic reactions in skin exposed to irritants [Allen et al. 2000, Allen et al. 2001a, Allen et al. 2001b, Barker et al. 1991, Corsini and Galli 2000, Grone 2002, Monteiro-Riviere et al. 2003, Nickoloff et al. 1991]. Although different toxicants may elicit different responses in HEK, studies in our laboratory have shown cytokine release by HEK in response to jet fuel exposure [Allen et al. 2000, 2001a,b; Chou et al. 2003; Monteiro-Riviere et al. 2003], multi-walled carbon nanotubes [Monteiro-Riviere et al. 2005], 6-aminohexanoic acid-functionalized single-walled carbon nanotubes [Zhang et al. 2007], fullerenes (Rouse et al. 2006), and QD [Ryman-Rasmussen et al. 2006, Zhang et al. 2008]. The inflammatory potential of the Ag-nps was confirmed by the increase in IL-1β, IL-6, IL-8, and TNF-α detected in the media from HEK cell culture exposed to each of the unwashed Ag-nps at a concentration of 0.34µg/ml.

Nanomaterials are also capable of being internalized into cells and penetrating through skin; QD-621 have the ability to penetrate into the intercellular lipid layers of the stratum corneum of porcine skin [Zhang et al. 2008], QD-565 and QD-655 with diverse physiochemical properties have been shown to penetrate into the dermis of abraded skin [Zhang and Monteiro-Riviere 2008], derivatized fullerenes localized within the intercellular space of the stratum granulosum layer of flexed excised porcine skin [Rouse et al. 2007].
Topical application of zinc oxide 26–30nm in a sunscreen formulation on *in vitro* human skin localized nanoparticles in the upper stratum corneum with minimal penetration [Cross et al. 2007], and microfine zinc oxide with a mean size of 80 nm and agglomerates of titanium dioxide less than 160nm were not shown to penetrate the porcine stratum corneum layer of *in vitro* static diffusion cells [Gamer et al. 2006].

Porcine skin is an excellent model for human penetration was because of its comparable thickness and absorption rates to human skin [Bronaugh et al. 1982, Monteiro-Riviere and Riviere 1996, Reifenrath et al. 1984]. It was surprising that after 14 consecutive days of topical dosing, the Ag-nps did not cause any macroscopic irritation, although the grey appearance of the skin due to the deposition of Ag-nps may have masked any subtle signs of erythema. When viewed microscopically, focal inflammation and edema increased with an increase in Ag-nps concentration. The highest concentration consistently caused epidermal hyperplasia with elongated extension of rete pegs down into the dermis which is typical of a chronic irritation reaction as reported with jet fuels [Monteiro-Riviere et al. 2001, Muhammad et al. 2005]. TEM depicted the localization of Ag-nps only in the superficial layers of the stratum corneum, which was similar to results found in a static cell diffusion study [Larese et al. 2009], and suggests ionic flux into the epidermis could attribute to focal inflammation. Many Ag-nps that were not bound to the skin were washed away during both the light and electron microscopy processing techniques, yet their location is confirmed with other nanoparticles that were not shown to penetrate into the deeper epidermis [Cross et al. 2007, Zhang and Monteiro-Riviere 2008].

In summary, this study indicates that toxicity of Ag-nps in HEK is influenced by the residual contaminants in the Ag-nps solutions, and that the Ag-nps themselves may not be
responsible for an increase in cell mortality. Complete characterization of not only the nanoparticles but also the vehicle is important in order to distinguish between Ag-nps and contaminant toxicity. Additionally, this study showed that 20, 50 and 80nm washed and unwashed Ag-nps as well as 25nm and 35nm carbon-coated Ag-nps interfered and/or reacted with MTT, 96AQ and aB viability assays and that aB may be the best viability assay due to its lower interference with these Ag-nps. Since Ag-nps of several types have been shown to enter cells and remain on the skin, the possibility of Ag-nps entry into the body through damaged or abraded skin is important to consider, particularly since many Ag-containing products specialize in wound care. With the ability for Ag-nps to enter HEK, their degradation within the cell may create reactive oxygen species (ROS) that would be damaging to cell machinery and DNA [Arora et al. 2008]. Given that focal inflammation, specifically intracellular and intercellular epidermal edema, was seen after 14 days of topical application of Ag-nps to skin, that inflammation had occurred. Longer studies over several weeks with Ag-nps in different vehicles should be, potentially with the addition of compromised skin. Overall, this study provides knowledge on the Ag-nps toxicity and penetration \textit{in vitro} and \textit{in vivo} over 14 days as well as provides a basis for occupational and risk assessment.

\textbf{Supplemental Material}

\textbf{Methods}

\textit{Evaluation of the Optimal Viability Assay}

One control was to assess nonspecific interactions between the Ag-nps and the assay dye without the use of cells (“nanoparticle control”). In this case, Ag-nps dosing solutions
equal to those used for viability tests were placed into collagen-coated 96-well plates devoid of cells and incubated for 24 h. The plates were then centrifuged at 1000rpm for 5 min and the media was replaced with the assay solution and read according to normal assay protocol as described above. Any change in absorbance values was reported and compared to control (KGM-2 only, no Ag-nps). The second control was to assess the specific interactions between the Ag-nps and the metabolized dye (“nanoparticle/cell control”). In this case, two plates were involved: in one 96-well plate, Ag-nps dosing solutions equal to those used for viability tests were placed into collagen-coated wells devoid of cells; in the other 96-well plate, cells were plated and grown. After incubating each plate for 24 h, the cell-containing plate was assayed according to normal viability protocol and read as described above. Immediately after reading, the particle-containing plate was spun down, the medium removed, and the assay solution from each well of the cell-containing plate was transferred into the corresponding well of the particle-containing plate. After incubation for 3 h, the plate was read and the difference in absorbance (before and after Ag-nps) was recorded.

**MTT**

The cell culture medium was removed from each treatment well and 200µl of 0.5mg/ml MTT (Sigma-Aldrich, St. Louis, MO) in KGM-2 warmed to 37°C was added to Ag-nps and vehicle-treated wells. The plates were incubated for 3 h, the MTT medium was removed, and HEKs rinsed with 200µl of Hank’s balanced salt solution (HBSS) for 2 min. The HBSS was removed, 100µl of 70% isopropanol was added to each well, and the plates were agitated for 25 minutes to extract the dye from the cells. Absorbance, directly proportional to cell viability, was determined at 550 nm in a Multiskan RC plate reader.
(Labsystems, Helsinki, Finland) equipped with Ascent software (version 2.6). The absorbance values were normalized by the controls and expressed as percent viability.

**aB**

One hundred microliters of cell culture medium was removed from each well and 10µl of aB (Molecular Probes, Invitrogen, Eugene, OR) solution was added to the medium. The plates were incubated for 3 hours, the fluorescence was quantitated (top read) on a Spectra Max Gemini EM spectrophotometer with an excitation wavelength of 545 nm and an emission wavelength of 590 nm. The fluorescence values were normalized by the controls and expressed as percent viability.

**96 AQ**

One hundred microliters of cell culture medium was removed from each well and 20µl of 96 AQ (Promega, Madison, WI) was added to the medium. The plates were incubated for 3 hours and then read at 450nm in a Multiskan RC microplate reader (Lab Systems, Helsinki, Finland) equipped with Ascent software (version 2.6). The absorbance values were normalized by the controls and expressed as percent viability.

**Cytokine Release**

The medium from each treatment set of the dosed cells was removed, pooled into a microfuge tube, and quickly frozen to -80°C until assayed. On the day of the assay, samples were thawed and 50µl of each sample added (in triplicate) to a filter plate with beads coupled to the cytokine antibodies (Bio-Plex Cytokine Assay, Bio-Rad Laboratories, Hercules, CA),
and the assay was performed according to the manufacturer’s instructions. Samples were assayed on a Bio-Plex System (Luminex x MAP Technology) equipped with Bio-Plex software (version 4.0). The samples were quantified by linear regression to logarithmic standard curves.

**In vivo Porcine Skin Exposure**

A 34µg/ml stock solution of each sample was prepared in deionized water and serially diluted (1:10) to provide Ag-nps solutions with concentrations ranging from 34.0 to 0.34µg/ml. Two female weanling pigs (Sus scrofa) weighing 20-30kg were housed in an AALAC accredited facility on elevated floors and were provided water and 15% protein pig and sow pellets ad libitum. The procurement, care, and use of animals were in accordance with the regulations and terms of the federal Animal Welfare Act and North Carolina State University’s Institutional Animal Care and Use Committee guidelines. Approximately 24 h before the topical application of the Ag-nps, the pigs were sedated with an injection of telazol-ketamine-xylazine (TKX) and the excess hair on the dorsum carefully clipped. On the first day of the experiment each pig was sedated with TKX and placed in a sling. Fourteen sites, 7 on each side of the midline, were randomly assigned on the back of each pig. All sites were topically dosed with 500µl of the appropriate Ag-nps solution or control, allowed to air dry and then occluded with a Hilltop® chamber inset with a cotton pad (19mm inside diameter, 284mm² surface area; Hill Top Research Inc., Miamiville, OH, USA). The chambers were secured with non-irritating Medipore® (3M, St. Paul, MN) tape. Lastly, a body stocking was placed over the entire dorsum of each pig to further secure the chambers. Any change in the skin was recorded and photographed. After this, the pigs were redosed,
the chambers were replaced and secured as described above and the dosing regiment was repeated for 14 days. At the conclusion of the 14 day study, a final Draize score was taken, all dosing sites were photographed, and the pigs were euthanized with 100mg/kg Euthasol® (Delmarva Laboratories, Inc., Midlothian, VA). The dosing sites were biopsied and placed in 10% NBF for light microscopy (LM) and in Trump’s fixative for TEM. In addition, samples were also frozen in liquid nitrogen and stored at -80°C.

**Ultrastructural Observations**

Samples were prepared by either diluting the washed and unwashed Ag-nps with deionized water, or suspending the carbon-coated Ag-nps in deionized water and sonicating for 10 minutes. Primary diameter was then determined using a Zetasizer (Malvern Instruments Ltd.) at 25°C. Additionally, to visualize particle morphology and uniformity of size, the samples were prepared by placing a drop of homogenous suspension of each Ag-nps (colloid; dry Ag-nps suspended in deionized water) onto a formvar-coated copper mesh grid and allowed to air dry. The samples were observed with an FEI/Philips EM 208S transmission electron microscope operating at an accelerating voltage of 80 kV.

HEK treated with Ag-nps were harvested with trypsin, rinsed in HBSS, and fixed for at least 24 h in Trump’s fixative at 4°C. The cells were rinsed in 0.1M phosphate buffer (pH 7.2), pelleted in a microfuge tube, resuspended, and quickly pelleted in 3% molten agar. Agar-embedded samples were post fixed in 1% osmium tetroxide (Polysciences, Inc., Warrington, PA) in 0.1M phosphate buffer for one hour at 4°C, washed with deionized water, dehydrated through an ascending ethanol concentration, cleared in acetone, infiltrated and embedded with Spurr resin, and polymerized at 70°C overnight. Cells were not stained to
allow for better visualization of the Ag-nps and to ensure the absence of stain artifacts resulting from lead citrate and uranyl acetate.

Skin treated with Ag-nps was harvested, trimmed into approximately 1mm² pieces and immersed in Trump’s fixative at 4°C. The sections were then dehydrated and embedded in Spurr resin as described above. The samples were sectioned on a diamond knife and placed onto formvar-coated copper mesh grids for added stability and to help prevent both rolling of the edges and separation of the stratum corneum from the epidermis.

Results

Figure 3.5- Nanoparticle control of unwashed Ag-nps. (A) aB; (B) 96AQ; (C) MTT.

^p<0.05, 20nm unwashed; ^p<0.05, 50nm unwashed; ^p<0.05, 80nm unwashed, multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.
Figure 3.6- Nanoparticle control of washed Ag-nps. (A) aB; (B) 96AQ; (C) MTT. \(^{\Lambda}p<0.05, 20\text{nm washed}; ^{a}p<0.05, 50\text{nm washed}; ^{\Lambda}p<0.05, 80\text{nm washed}, \) multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.

Figure 3.7- Nanoparticle control of carbon-coated Ag-nps. (A) aB; (B) 96AQ; (C) MTT. \(^{\Lambda}p<0.05, 25\text{nm carbon-coated}; ^{a}p<0.05, 35\text{nm carbon-coated}, \) multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.
Figure 3.8- Viability of HEK exposed to unwashed Ag-nps and “as synthesized” supernatant. (A) 96AQ; (B) MTT. \( ^{\text{A}}p<0.05, \) 20nm unwashed; \( ^{\text{a}}p<0.05, \) 50nm unwashed; \( ^{\text{A}}p<0.05, \) 80nm unwashed; \( ^{\text{a}}p<0.05, \) “As synthesized” supernatant; multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.

Figure 3.9- Viability of HEK exposed to washed Ag-nps. (A) aB; (B) 96AQ; (C) MTT. \( ^{\text{A}}p<0.05, \) 20nm washed; \( ^{\text{a}}p<0.05, \) 50nm washed; \( ^{\text{A}}p<0.05, \) 80nm washed; multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.
Figure 3.10- Viability of HEK exposed to carbon-coated Ag-nps. (A) aB; (B) 96AQ; (C) MTT. $^a$p$<0.05$, 25nm carbon-coated; $^b$p$<0.05$, 35nm carbon-coated; multiple comparisons between concentrations. Each Ag-nps assessed independently. Different letters denote a significant difference.
Figure 3.11- LM images of porcine skin treated with Ag-nps. (A) 20nm unwashed, 0.34µg/ml; (B) 20nm unwashed, 3.4µg/ml; (C) 20nm unwashed, 34µg/ml. Bars=60µm. Large arrows point to intracellular epidermal edema, small arrows point to focal areas of intercellular epidermal edema. SC, stratum corneum; E, epidermis; D, dermis. H&E.
Figure 3.12- TEM images of Ag-nps. (A) 50nm unwashed; (B) 80nm unwashed; (C) 20nm washed; (D) 50nm washed; (E) 80nm washed; (F) 35nm carbon-coated. Bars=100nm.
Figure 3.13- TEM images of HEK and porcine skin exposed to Ag-nps. (A) 20nm unwashed; (B) 50nm unwashed; (C) 80nm unwashed; (D) 20nm washed; (E) 50nm washed; (F) 25nm carbon-coated; (G) 35nm carbon-coated; (H) porcine skin and 34µg/ml of 20nm washed. HEK bars=1µm; Porcine skin bar=100nm. Arrows point to Ag-nps. N, nucleus; unstained sections.
Figure 3.14- EDX spectra of HEK and porcine skin exposed to Ag-nps. (A) HEK dosed with 20nm washed; (B) porcine skin dosed with 20nm washed. Arrows point to Ag peaks. Au from Ag-nps core; Copper from grid; Osmium from tissue post fixation.
4. The Use of Electron Energy-Loss Spectroscopy to Analyze Oxygen Acquisition on Silver Nanoparticles Following Human Cell Internalization

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Abstract

Hundreds of consumer products have taken advantage of the increased innate antimicrobial activity of silver (Ag) at the nanoscale. The majority of consumer products that incorporate silver nanoparticles (Ag-nps), such as textiles, baby wipes and acne creams come into direct contact with skin. Human epidermal keratinocytes (HEK), the main cell of the skin, were exposed for 24h to 20nm Ag-nps. To evaluate any potential surface changes of the Ag-nps at the biological interface, transmission electron microscopy electron energy loss spectroscopy (TEM-EELS) was used. Compared to the initial purely metallic Ag-nps, the presence of oxygen was detected on the surface of Ag-nps internalized into HEK, and the amount of oxidation was quantified. TEM-EELS revealed the production of up to 8 oxide monolayers on the surface of Ag-nps as a result of cellular uptake.

Introduction

As a transition metal, silver nanoparticles (Ag-nps) possess unique electrical and optical properties which make them candidates for targeted drug delivery systems and biosensors. Additionally, the controlled dissolution of Ag ions from the surface of Ag-nps is a desirable property for indiscriminate antimicrobial activity. Advances in nanotechnology have facilitated the incorporation of Ag-nps into consumer and healthcare products, resulting in a rapid increase in human exposure [www.nanotechproject.org].
The majority of Ag-nps in consumer products may have direct contact with skin, the largest organ of the human body, and could serve as a potential route of exposure. Some nanomaterials have been shown to penetrate intact skin; quantum dots (QD) of diverse sizes and physicochemical properties have demonstrated intercellular penetration through the lipid bilayers of the stratum corneum layer of skin [Ryman-Rasmussen et al. 2006, Zhang et al. 2008], very small derivatized fullerenes have penetrated to the deeper dermis layer of skin subjected to cyclic flexion [Rouse et al. 2007], and Ag-nps penetrated intact and damaged in vitro human skin in static cells but with low flux [Larese et al. 2009], whereas Ag-nps did not penetrate in vivo porcine skin following 14 days of repetitive dosing [Samberg et al. 2010]. Minimal penetration was observed for UVB damaged skin with TiO₂ nanoparticles [Monteiro-Riviere et al. 2011], and for abraded rat skin with QD [Zhang and Monteiro-Riviere 2008].

Once past the outermost stratum corneum layer of the skin, nanomaterials have the potential to interact with skin cells (keratinocytes) which are the primary cell type of the epidermal layer of the skin. Various nanomaterials such as aluminum nanoparticles with surface aluminum oxide [Monteiro-Riviere et al. 2010], derivatized fullerenes [Rouse et al. 2006], single-walled carbon nanotubes [Zhang et al. 2007], multi-walled carbon nanotubes [Monteiro-Riviere et al. 2005], and various QD [Zhang et al. 2008, Ryman-Rasmussen et al. 2007, Ryman-Rasmussen et al. 2007] have been identified within the cytoplasmic vacuoles of HEK. Furthermore, Ag-nps have been internalized into different cell lines such as human epidermal keratinocytes (HEK) and fibroblasts [Samberg et al. 2010, Burd et al. 2007], murine neuroblastoma cells [Schrand et al. 2008], rat hepatic cells [Hussain et al. 2005],
human germ-line stem cells [Barydich-Stolle et al. 2005], and human skin carcinoma cells [Arora et al. 2008].

Transmission electron microscopy (TEM) is paramount for the visualization of nanoparticles that lack a fluorescent label, and is particularly useful for localization of metallic nanoparticles within biological samples. Additionally, analytical methods such as energy dispersive X-ray (EDX) or electron energy-loss spectroscopy (EELS) are useful for both characterization and corroboration of the identity of nanoparticles. The advantage of using EELS over EDX lies in its higher resolution and ability to identify the atomic composition, chemical bonding, valence and conduction band electronic properties, and surface properties of a specimen. When EELS is performed on a biological specimen, the chemical elements of a compound localized within a cellular or intracellular compartment at the ultrastructural level may be studied.

The use of ultrathin specimens is desired to minimize plural scattering contributions to the spectrum, which requires t/λ < 0.5, where t is specimen thickness and λ is the inelastic mean free path of electrons within the sample which is 100 nm for Ag [Iakoubovskii and Mitsuishi 2008]. Biological specimens are rarely analyzed by EELS primarily due to difficulty of preparing such thin samples and ultrathin section instability under the electron beam. Elemental microanalysis in biological specimens has shown calcium in various tissues [Bordat et al. 1998, Pezzati and Grohovaz 1998], iron oxide nanoparticles in rat lymph nodes [Bordat et al. 2000], copper accumulation in human liver [Jonas et al. 2001], phosphorous in various cellular compartments [Ottensmeyer and Andrew 1980], ultrafine inhaled TiO₂ particles in rat lung tissue [Kapp et al. 2007], and radiolytic products in frozen samples [Aronova et al. 2001].
Though Ag-nps are utilized in hundreds of products for their antimicrobial activity, the exact process of Ag-nps dissolution and their subsequent change in oxidative state within a cellular compartment has yet to be investigated. Here, we report on the use of EELS to analyze the production of an oxide shell on Ag-nps following cellular internalization into cytoplasmic vacuoles of human skin cells.

**Experimental Methods**

**Ag-nps**

The 20 nm spherical Ag-nps were obtained from NanoComposix (San Diego, CA, USA). According to the manufacturer, the Ag-nps were synthesized by ammonium hydroxide catalyzed growth of Ag onto 5 nm gold seed particles with concentration achieved via tangential flow filtration. Prior to arrival, they were serially washed with 20 volume equivalents of 2 mM phosphate buffer (pH 7.5) and suspended in deionized (DI) water at a concentration of 2.86 mg/ml. The Ag-nps were stored at 4 °C in the dark.

**Culture of HEK**

Cryopreserved primary neonatal human epidermal keratinocytes (HEK; Lonza, Walkersville, MD) were seeded in keratinocyte growth medium-2 (KGM-2; Lonza, Walkersville, MD) in cell culture flasks (75 cm²; 1,000,000 cells) and grown to approximately 80% confluency in a 37°C humidified 5% CO2 incubator. The cells were passed into two smaller T-25 cell culture flasks (25 cm²; 120,000 cells), grown to approximately 80% confluency and then exposed for 24 h to either KGM-2 alone (control) or a solution of 17 µg/ml Ag-nps made fresh in KGM-2.
TEM processing of HEK incubated with Ag-nps

HEK treated with the 20 nm Ag-nps were harvested from their T-25 flasks with trypsin, rinsed in HBSS, and fixed for at least 24 h in Trump’s fixative at 4 °C. Cells were rinsed in 0.1M phosphate buffer (pH 7.2), pelleted in a microfuge tube, resuspended, and quickly pelleted in 3% molten agar. Samples were post fixed in 1% osmium tetroxide (Polysciences, Inc., Warrington, PA) in 0.1 M phosphate buffer for 1 h at 4 °C, washed with DI water, dehydrated through an ascending ethanol series, cleared in acetone, infiltrated and embedded with Spurr’s resin, and polymerized at 70 °C overnight. Samples were sectioned with a diamond knife to a thickness of 20-30 nm and placed onto a clean 200-mesh copper grid with a lacey carbon support film. The grids were not post-stained with lead citrate and uranyl acetate to allow for better visualization of the Ag-nps, to ensure the absence of stain artifacts, and to ensure a lack of interference during analysis. Immediately after sectioning, each grid was placed on a clean glass slide and degassed on top of an inverted halogen lamp (approximately 50 °C) for no less than 10min to ensure maximum section stability.

TEM processing of Ag-nps

For initial analysis of Ag-nps, approximately 10 µl of 20 nm Ag-nps suspended in DI water at 17 µg/ml was dropped onto a copper mesh grid with a lacey carbon support film in a pumped down glove box, and allowed to dry. The grid was secured into the grid holder within the glove box, and quickly transferred into the TEM to minimize oxygen contamination. Alternatively, to mimic cell culture conditions and atmospheric oxygen exposure during section cutting and transfer, approximately 100 µl of 20 nm Ag-nps were spun down at 12,500 rpm for 3 min to form a pellet. The water was removed and the Ag-nps
were embedded into molten agar by quick suspension and pelleting, and then processed routinely as described above for HEK.

**TEM-EELS Analysis of Samples**

For initial analysis of native Ag-nps, Z-contrast images and intensity profiles were obtained from native Ag-nps dropped onto a lacey carbon support film was obtained on a JEOL 2010F scanning/transmission electron microscope (S/TEM) operating at 200 kV using a HAADF detector (n=8). Alternatively, individual particles within small agglomerates of resin-embedded Ag-nps and HEK internalized Ag-nps were analyzed immediately after sectioning by EELS on a VG HB501 UX high resolution dedicated STEM operating at 100 kV (n=4). The STEM was equipped with a third order Nion spherical aberration corrector, a high-angle annular dark-field (HAADF) detector, and a Gatan Enfina EELS system which utilizes parallel detection energy loss spectrometers. For each EELS acquisition a convergence semi-angle of 25 mrad was used, the EELS collection semi-angle was 12 mrad, the energy resolution as defined by the full width at half maximum of the zero loss peak (ZLP) was 0.6 eV, and a dispersion of 0.3 eV/channel (Advanced Microscopy Laboratory; Oak Ridge National Laboratory).

Individual Ag-nps internalized into HEK were visualized by TEM within cytoplasmic vacuoles and were imaged using Z-contrast, brightfield and darkfield imaging (n=4). The low-loss data was used to calculate the thickness of the sections. TEM-EELS analysis quantified the elemental distribution of Ag, O, C, and N. Spectrum imaging was used to obtain EELS and Z-contrast images simultaneously every 0.5 nm with an exposure time of 2.5s/pixel. All the elemental quantification including pixel analysis of a spectrum image was
done using the program Quantifit [Duscher 2010]. Background subtraction of the core-loss spectra was done using a power law fit, where the Quantifit program fit the background subtracted spectrum in two regions - the pre-edge background region and the tail of the ionization edge. The C-K edge has an expected onset at 284 eV, and the obtained EELS spectra were fit by excluding 5 eV pre-edge and 40 eV post-edge. The O-K edge has an onset at 532 eV and the obtained EELS spectra were fit by excluding 2 eV pre-edge and 15 eV post-edge from the spectrum. The M_{4,5} edge of Ag has a delayed onset at 367 eV, and the obtained EELS spectra was fit by excluding 10 eV pre-edge and 140 eV post-edge. This was necessary in order to get the correct onset of O-K edge. The Hartree-Slater model was used to compute the cross-sections of constituent elements. The atomic percentage of each element was then calculated by a ratio method that uses the product of two ratios: the ratio of the cross-sections, and the ratio of the background subtracted integrated core-loss peak intensities [Duscher 2010].

**Results and Discussion**

Transmission electron micrographs depicted native 20 nm Ag-nps to be highly uniform with spherical shape and a relatively narrow size distribution of 21.4 nm ±3.1nm (Figure 1A). Chemically-sensitive HAADF transmission electron micrographs of native Ag-nps revealed a core-shell structure due to the Au seed used to synthesize the Ag-nps (Figure 1B). These particles were only briefly exposed to atmospheric oxygen during grid transfer, but there were no abrupt intensity changes (as verified in the intensity profile of Figure 1C) in the shell of the Ag-nps, suggesting that the Ag was unary and that an oxide layer had not formed. In Figure 1B, the separation between the planes is ~2.3 Å which corresponds to the Ag {111}.
planes. Since Ag is a fifth-period element, the EELS spectrum displays a delayed maximum due to the “centrifugal potential.” While the Ag-M edge is supposed to start at 367 eV, it becomes apparent only at about 397 eV [Fredrickx et al. 2004]. Congruent with the work of others, this centrifugal effect was clearly seen for small agglomerates of Ag-nps, and Ag was positively identified by the Ag-M\(_{4,5}\) edges in the obtained spectra (Figure 1D) [Fredrickx et al. 2004, Egerton 1996, Seiler et al. 1991, Oleshko et al. 1995]. Elemental quantification of the EELS spectra using the Quantifit routine of native Ag-nps embedded in Spurr’s resin showed that the control Ag-nps contained only Ag (80-100 at. %) from the particles and C (0-20 at. %) from the surrounding resin (n=8). Based on the combination of EELS data, the HAADF image, and intensity profile it may be concluded that native Ag-nps are not initially oxidized, and that the resin embedding routine, grid sectioning, and grid transferring process does not contribute any oxygen to the particles.
In order to quantify the amount of oxygen present on Ag-nps within cellular vacuoles, HEK were incubated for 24 h with a sublethal 17 µg/ml concentration of 20 nm Ag-nps solution. TEM images of untreated HEK appeared normal with a prominent nucleus, nucleolus, and mitochondria (Figure 2A). TEM images of HEK exposed to 17 µg/ml of 20 nm Ag-nps for 24 h depicted small agglomerates of Ag-nps localized within membrane-bound cytoplasmic vacuoles (Figure 2B; inset; higher magnification depicting Ag-nps within
a vacuole). Internalized Ag-nps were observed with HAADF along a <220> direction (Figure 2C).

To quantify the amount of oxygen present on Ag-nps within HEK, EELS spectra were acquired from Ag-nps agglomerates within vacuoles, as well as in areas devoid of Ag-nps. Elemental quantification of the EELS spectra revealed that HEK were composed of C (93 at. %), O (4.5 at. %), and N (2.5 at. %) (n=4); analysis of Ag-nps localized within cellular vacuoles contained Ag (80-100 at. %), O (0-20 at. %), and C (0-10 at. %) (n=4). Based on the diameter of the Ag-np being analyzed, the separation between planes and the thickness of section, simple geometry was used to subtract out the atomic percentage of each element in the surrounding cell and calculate the total number of atomic oxide monolayers present on the surface of the particle. For each of the four samples, 6 to 8 monolayers of oxygen was detected; the large number of oxide monolayers was indicative that the particle degraded in vitro.
Figure 2: Transmission electron micrograph of untreated HEK, Bar = 2 µm; B) Transmission electron micrograph of HEK incubated with the sublethal concentration of 17 µg/ml of 20 nm Ag-nps depicting internalized Ag-nps, Bar=2 µm, Arrows indicate agglomerates of internalized Ag-nps, arrowhead indicates Ag-nps depicted in inset, Bar=100 nm; C) High-angle annular dark-field (HAADF) transmission electron micrograph of an internalized Ag-np, Bar=2 nm; D) EELS spectrum for internalized Ag-nps.
Conclusion

In conclusion, HEK incubated with a sublethal 17 µg/ml concentration of 20 nm Ag-nps for 24 h resulted in the production of up to 8 atomic oxide monolayers on the surface of Ag-nps found within cytoplasmic vacuoles. The acquisition of oxide layers is likely due to cellular reactive oxygen species and exposure to low pH through the fusion of cytoplasmic vacuoles containing Ag-nps with lysosomes. The consequent release of Ag ions into the cell has been cited result in interference with cellular processes through anionic site binding on critical biomolecules, and ultimately cell death [Burd et al. 2007, Schrand et al. 2008, Hussain et al. 2005, Braydich-Stolle et al. 2005, Arora et al. 2008, Park et al. 2010, Ahamed et al. 2008].

Acknowledgement

This research was supported by the United States Air Force Office of Scientific Research (USAFOSR) grant number FA 9550-08-1-0182 and NIH grant number RO1 ES016138. Dr. Aaron C. Johnston-Peck acknowledges salary support from a GAANN fellowship. The authors thank Drs. Roger Narayan and Ravi Aggarwal for helpful discussions on TEM-EELS.
5. Antibacterial efficacy of silver nanoparticles of different sizes, surface conditions and synthesis methods

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Abstract

Silver nanoparticles (Ag-nps) are used as a natural biocide to prevent undesired bacterial growth in clothing and cosmetics. The objective of this study was to assess the antibacterial efficacy of Ag-nps of different sizes, surface conditions, and synthesis methods against *Escherichia coli*, Ag-resistant *Escherichia coli*, *Staphylococcus aureus*, Methicillin-Resistant *Staphylococcus aureus* (MRSA), and *Salmonella* sp. Ag-nps samples were synthesized by: base reduction with unmodified surfaces and used as synthesized (“unwashed”; 20nm, 50nm, 80nm) or after 20 phosphate buffer washes (“washed”; 20nm, 50nm, 80nm), or synthesized by laser ablation with carbon stabilized surfaces (“carbon-coated”; 25nm, 35nm). Unwashed Ag-nps were toxic to all bacterial strains at concentrations between 3.0-8.0µg/ml. The washed Ag-nps and carbon-coated Ag-nps were toxic to all bacterial strains except Ag-resistant *Escherichia coli* at concentrations between 64.0-1024.0µg/ml. Ag-resistant *Escherichia coli* died only when treated with unwashed Ag-nps or its supernatant, both of which contained formaldehyde.

Introduction

Silver (Ag) is a natural biocide and compared to titanium, zinc, and copper, Ag nanoparticles (Ag-nps) have shown the greatest antimicrobial efficacy against bacteria, viruses and other eukaryotic microorganisms [Gong et al. 2007]. Ag sulfadiazine is the
standard care for the prevention of widespread bacterial growth on the skin of burn patients [Moyer et al. 1965]. Nanomaterials have specific physicochemical characteristics that may differ from their bulk form due to their increased surface area to volume ratio that results in heightened reactivity [Fubini et al. 2007]. The development of new synthesis methods may result in high yield concentrations and stable dispersions of Ag-nps, thereby increasing antibacterial applications of Ag-nps, which is currently the most common used nanomaterial of all engineered products in the world [www.nanotechproject.org].

Recent investigations have shown similar effects for low concentrations of Ag-nps and Ag ions having effective biocidal concentrations in the nanomolar and micromolar ranges (Lok et al. 2006; Pal et al. 2007). Ag-nps have been shown to inactivate bacteria and inhibit cellular functions such as growth, permeability, regulation of enzymatic activity, and respiratory processes hypothesized by a preferential attachment to the phosphate and sulfur groups of the cell membrane [Baker et al. 2005, Li et al. 2010, Lok et al. 2006, Morones et al. 2005, Sondi and Salopek-Sondi 2004]. Studies have related various physicochemical property of Ag-nps to their antibacterial effect: concentration [Pal et al. 2007, Sondi and Salopek-Sondi 2004], bacterial type [Kim et al. 2007], bacterial strain [Ruparelia et al. 2008]) Ag-nps structure [Kim et al. 2007, Pal et al. 2007], Ag-nps size [Baker et al. 2005, Morones et al. 2005, Panáček et al. 2006, Sondi and Salopek-Sondi 2004], and addition of surfactants or polymers [Dror-Ehre et al. 2009].

Despite the rapidly increasing number of silver-containing products, there exist uncertainties regarding the use of Ag-nps relating to human health effects. These uncertainties relate directly to factors that influence the potential efficacy of an antimicrobial compound such as the pharmacokinetic properties of absorption, distribution metabolism,
elimination, and protein binding, or the pharmacodynamics or drug effects on the bacteria such as inhibition of growth (bacteriostatic), killing kinetics of the organism (bactericidal), and post-antibiotic effects [Wanger 2007].

Based on the results of a previous study in our lab that showed washing and carbon-coating of Ag-nps was crucial for the removal of toxicity to human epidermal keratinocytes (HEK) [Samberg et al. 2010], the objective of this study was to further evaluate whether a decrease in eukaryotic toxicity coincided with a decrease in antibacterial efficacy of Ag-nps that varied in size, surface condition, and synthesis method against Escherichia coli, Ag-resistant Escherichia coli, Staphylococcus aureus, methicillin-resistant Staphylococcus aureus (MRSA), and Salmonella sp.

Methods

Reagents

Eight different Ag-nps that varied in size, surface condition and synthesis method were studied with their properties summarized in Table 1. All Ag-nps were obtained from nanoComposix (San Diego, CA, USA) and consisted of the following: commercially-used unwashed Ag-nps with unmodified surface suspended in deionized (DI) water with diameters of 20nm, 50nm and 80nm (“unwashed”), washed Ag-nps with unmodified surface suspended in DI water with diameters of 20nm, 50nm and 80nm (“washed”), and commercially-used dried Ag-nps with carbon-coated surface with diameters of 25nm and 35nm (“carbon-coated”).
Table 5.1- Physicochemical Properties of Ag-nps

<table>
<thead>
<tr>
<th>Dispersal Conditions</th>
<th>MDD (nm)</th>
<th>DLS diameter (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DLS diameter (nm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>TEM diameter (nm)</th>
<th>Supplied Conc. (mg/ml)</th>
<th>Particle Conc. (particles/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Zeta Potential&lt;sup&gt;c&lt;/sup&gt; (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwashed, Colloidal</td>
<td>20</td>
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<td>579.1±16.1</td>
<td>22.4±2.6</td>
<td>0.20</td>
<td>2.41E+12</td>
<td>-29.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>47.7±0.5</td>
<td>674.6±8.6</td>
<td>49.4±6.2</td>
<td>0.20</td>
<td>4.44E+11</td>
<td>-27.8</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>75.5±1.0</td>
<td>529.2±4.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>79.2±8.0</td>
<td>0.20</td>
<td>7.09E+10</td>
<td>-33.2</td>
</tr>
<tr>
<td>Washed, Colloidal</td>
<td>20</td>
<td>25.5±0.4</td>
<td>707.6±40.1</td>
<td>21.4±3.1</td>
<td>2.86</td>
<td>1.89E+14</td>
<td>-46.0</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>43.7±1.1</td>
<td>775.2±50.9</td>
<td>50.0±5.9</td>
<td>3.45</td>
<td>5.01E+12</td>
<td>-44.3</td>
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<tr>
<td></td>
<td>80</td>
<td>79.9±28.0</td>
<td>645.9±37.5</td>
<td>77.0±6.0</td>
<td>2.79</td>
<td>1.07E+12</td>
<td>-43.7</td>
</tr>
<tr>
<td>Carbon-coated, Powder</td>
<td>25</td>
<td>149.0±89</td>
<td>501.5±24.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>27.2±10.3</td>
<td>N/A</td>
<td>N/A</td>
<td>-24.0</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>167.0±110</td>
<td>689.4±79.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>37.0±11.6</td>
<td>N/A</td>
<td>N/A</td>
<td>-29.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are expressed as Mean±SD; in DI water, <sup>b</sup>in MH media <sup>c</sup>Zeta Potential in DI water. <sup>d</sup>Polydispersion affects quality of value. MDD, Manufacturer-Designated diameter; DLS, Dynamic light scattering; TEM, transmission electron microscopy.

Both the unwashed and washed Ag-nps were synthesized by ammonium hydroxide catalyzed reduction growth of Ag onto 5nm gold (Au) seed particles. A major reducing agent for the formation of Ag-nps was formaldehyde and its concentration in the unwashed solutions was analyzed by high performance liquid chromatography (HPLC) with UV detection; samples of the unwashed Ag-nps were diluted, derivatized with 2,4-dinitrophenylhydrazine to confirm the presence of 5.55mg/ml formaldehyde [Samberg et al.
Concentration of the particles was achieved via tangential flow filtration. The unwashed Ag-nps were removed by ultracentrifugation (L5 Beckman, Ti-70.1 rotor, 30 min at 109,000 x g) to obtain the supernatant for toxicity testing (“supernatant”). The washed Ag-nps were achieved by serially washing the unwashed Ag-nps with 20 volume equivalents of 2mM phosphate buffer (pH 7.5). Analysis with inductively coupled plasma optical emission spectrometry (ICP-OES) showed that all solutions contained ≤60ppb dissolved silver content and that over 99% of the formaldehyde contaminant was removed by the 5th washing volume equivalent. All colloidal Ag-nps were stored at 4°C in the dark. According to the manufacturer, the carbon-coated Ag-nps were synthesized by pulsed plasma reactor and coated with polyaromatic graphitic carbon and were supplied as a powder and stored at room temperature.

Silver nitrate (AgNO₃; 99.9%, Sigma-Aldrich, St. Louis, MO) was used without further purification as a source of Ag⁺ ions. Prior to each usage, a 5mg/ml formaldehyde solution was made fresh from paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in DI water at 60°C and cleared with 1 drop of 1N sodium hydroxide.

**Bacterial Strains**

Cation-adjusted Mueller-Hinton (MH) broth and agar (Difco Laboratories, Detroit, MI) was used as the bacterial cultivating medium for *Escherichia coli* J53, Ag-resistant *Escherichia coli* J53(pMG101), *Staphylococcus aureus* (ATCC 25213), methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC 43300), and *Salmonella* sp. (ATCC 35664). Isolated bacterial colonies were grown overnight at 37°C from frozen samples on an agar plate (with 100µg/ml ampicillin for Ag-resistant *Escherichia coli* J53(pMG101)).
bacterial colony was suspended in phosphate buffered saline (PBS) to a 0.5 McFarland ($10^5$ CFU/ml). Microplates were incubated at 37ºC and shaken at 200 rpm for 24h. To ensure quality control and for seeding accuracy, bacteria were diluted in PBS at $10^3$, $10^4$, $10^5$, and $10^6$ and plated overnight.

**Susceptibility of Bacteria to Ag-nps**

The broth microdilution minimum inhibitory concentration (MIC) test was conducted to measure the in vitro activity of Ag-nps against each bacterial isolate. A sterile round-bottom plastic 96-well plate containing 100µl of serially 1:2 diluted concentrations of Ag-nps was inoculated with 100µl of 5-8x$10^5$ CFU/ml of each bacterial isolate ($n=6$ wells/treatment) [Wikler et al. 2003]. Each of the Ag-nps samples were tested at ten serially diluted concentrations starting at the highest dosing concentration that their supplied concentrations would allow. Therefore, the washed and carbon-coated Ag-nps, as well as the formaldehyde and Ag ion solutions were tested from 512 to 0.5µg/ml, while the unwashed Ag-nps and supernatant was tested from 32 to 0.125µg/ml due to their lower starting concentration; this lower starting concentration was not shown to limit the results of the study.

After the microplates were incubated for 24h at 37ºC, the lowest concentration showing no visible growth was recorded as the MIC. After 24h of incubation with Ag-nps, 10µl of the suspension from all of the clear wells was dropped onto a MH agar plate and incubated for 24 hr at 37ºC. The minimum bactericidal concentration (MBC) was determined by the concentration that failed to yield growth.
Ultrastructural Observations

Particle size was determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM) to confirm the manufacturer-identified diameters and surface characterization. Unwashed and washed Ag-nps were suspended at the highest dosing concentration in DI water and MH broth, and the carbon-coated Ag-nps were sonicated in DI water for 10min and then suspended in DI water and MH media. Immediately after dispersion, the Ag-nps were placed in a disposable cuvette and DLS measurements carried out on a Zetasizer Nano-ZS (Malvern Instruments, Inc., Worcestershire, UK). The initial DLS readings were performed at the standard characterization temperature of 25 °C. Each measurement was repeated five times, with 10-20 runs as optimized by the instrument. Data was culled based on the correlogram, size quality report, and expert advice rendered by the Dispersion Technology Software (5.03).

Additionally, TEM was utilized to characterize the structure, shape and size uniformity of each Ag-nps. Samples were prepared by placing a drop of homogeneous suspension of each Ag-nps at the highest dosing concentration in both DI water onto a formvar-coated copper mesh grid and air dried. Bacterial interactions with Ag-nps were investigated using the 20nm washed Ag-nps and a representative gram-positive (Staphylococcus aureus) and gram-negative (Escherichia coli J53) strain. Incubated in a shaker (200rpm and 37°C), the bacteria were grown to mid-exponential growth phase within 2 hours (confirmed by optical density) in 12ml of MH media, and dosed for one hour at the sublethal concentration of 10µg/ml of the 20nm washed Ag-nps. Samples were centrifuged at 14 500rpm for 3min, the media aspirated and the pellet was resuspended in 1ml Trump’s fixative for 24h. Cells were rinsed in 0.1M phosphate buffer (pH 7.2), pelleted in a
microcentrifuge tube, resuspended, and quickly pelleted in 3% molten agar. Agar-embedded samples were post-fixed with 1% osmium tetroxide (Polysciences, Inc., Warrington, PA) in 0.1M phosphate buffer for one hour, washed twice with DI water, dehydrated through ascending ethanol concentrations, cleared in acetone, infiltrated and embedded in Spurr’s resin, and polymerized overnight at 70°C. Samples were sectioned with a diamond knife and placed onto copper mesh grids. Images were taken prior to post-staining to show the location of Ag-nps relative to bacteria, and post-stained with lead citrate and uranyl acetate in order to visualize cell morphology and membrane integrity. The osmolarity of the solutions were confirmed as 0.1M with a micro-osmette (Precision Systems, Natick, MA, USA).

All TEM samples were examined on an FEI/Philips EM 208S TEM operating at an accelerating voltage of 80 kV.

**Statistical Analysis**

The mean values for bacterial MIC and MBC for each Ag-nps treatment were calculated, and significant differences \((p<0.05)\) were determined by the PROC GLM Procedure (SAS 9.1 for Windows; SAS Institute, Cary, NC). Data are expressed as the means ± standard error of the mean (SEM) of 6 replicates.

For the sake of statistical analysis and graphical representation, when bacteria persisted to grow at the highest dosing concentration, the value of 1024µg/ml was used. In these circumstances, a qualifier (†) can be noted above its value in Tables 2 and 3.
Results

Characterization of Ag-nps

Each of the washed and unwashed Ag-nps was spherical in shape with a relatively narrow size distribution, formed stable dispersions as evidenced by higher negative zeta potentials; the carbon-coated Ag-nps were spherical in shape, with a larger size distribution, and formed slightly agglomerated dispersions supported by their lower negative zeta potentials which (Figure 1, Table 1). DLS measurements showed multiple peaks for MH media alone, indicative of protein presence. Agglomeration of Ag-nps occurred after incorporation into MH which is consistent with previously reported nanomaterials shown to tightly interact with proteins to promote agglomeration [Monteiro-Riviere et al. 2009, Monteiro-Riviere et al. 2010, Stone and Kinloch 2007]. TEM micrographs in Figure 1a-h demonstrate the symmetrically spherical shape and uniformity of size distribution for all Ag-nps. Although the carbon coating is not visible by TEM, 25nm and 35nm carbon-coated Ag-nps shown in Figures 1g and 1h have slightly less defined boundaries.
Susceptibility of Bacteria to Ag-nps

Minimum inhibition concentrations (MIC; Table 2) and minimum bactericidal concentration (MBC; Table 3) were calculated for the Ag-nps, AgNO₃, supernatant, and formaldehyde solutions on each bacterial strain (n=6). In certain cases (reflected by † symbol) the bacteria grew even at the highest concentration, so the next highest concentration (1024.0µg/ml) was used strictly for statistical analysis); this is particularly true for
Escherichia coli J53(pMG101) where neither an MIC nor an MBC was obtainable for silver nitrate even when tested at the highest possible concentration of 16 987µg/ml.

Table 5.2- Values of Minimum Inhibitory Concentrations (MIC)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>MDD (nm)</th>
<th>Escherichia coli J53</th>
<th>Escherichia coli J53pMG101</th>
<th>Salmonella</th>
<th>Staphylococcus aureus</th>
<th>MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(µg/ml)(^a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Unwashed, Colloidal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>3.7±0.3</td>
<td>4.0±0.0</td>
<td>3.0±0.4</td>
<td>4.0±0.0</td>
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</tr>
<tr>
<td>50</td>
<td>6.0±0.9</td>
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<td>4.0±0.0</td>
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<td>4.0±0.0</td>
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<tr>
<td>80</td>
<td>4.0±0.0</td>
<td>4.0±0.0</td>
<td>4.0±0.0</td>
<td>3.0±0.5</td>
<td>4.0±0.0</td>
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<tr>
<td><strong>Supernatant</strong></td>
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<td>4.0±0.0</td>
<td>3.0±0.4</td>
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<td>3.0±0.4</td>
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<tr>
<td><strong>Washed, Colloidal</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>64.0±0.0</td>
<td>1024.0±0.0</td>
<td>96.0±14.3</td>
<td>192.0±28.6</td>
<td>256.0±0.0</td>
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</tr>
<tr>
<td>50</td>
<td>192.0±28.6</td>
<td>1024.0±0.0†</td>
<td>192.0±28.6</td>
<td>256.0±0.0</td>
<td>384.0±57.2</td>
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</tr>
<tr>
<td>80</td>
<td>384.0±57.2</td>
<td>1024.0±0.0†</td>
<td>640.0±171.7†</td>
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<td><strong>Carbon-coated, Powder</strong></td>
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<td></td>
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<tr>
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<td>298.7±42.0</td>
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<tr>
<td>35</td>
<td>384.0±57.2</td>
<td>1024.0±0.0†</td>
<td>384.0±57.2</td>
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<tr>
<td><strong>Silver Nitrate</strong></td>
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<td><strong>Formaldehyde</strong></td>
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<td>32.0±0.0</td>
<td>48.0±7.2</td>
<td>64.0±0.0</td>
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</table>

MDD, Manufacturer-designated diameter; MRSA, Methicillin-resistant Staphylococcus aureus. "Data are expressed as Mean±SEM (n=6), µg/ml. † denotes that an MIC could not be obtained since the bacteria grew even at the highest treatment concentration. (Escherichia coli J53pMG101, Salmonella, Staphylococcus aureus and MRSA)
Table 5.3- Values of Minimum Bactericidal Concentrations (MBC)

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>MDD (nm)</th>
<th>Escherichia coli J53</th>
<th>Escherichia coli J53pMG101</th>
<th>Salmonella</th>
<th>Staphylococcus aureus</th>
<th>MRSA</th>
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<tr>
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<td>(µg/ml)</td>
<td>(µg/ml)</td>
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MDD, Manufacturer-Designated diameter; MRSA, Methicillin-resistant *Staphylococcus aureus*. *Data are expressed as Mean±SEM (n=6), values in µg/ml. † denotes that an MBC could not be obtained since the bacteria grew even at the highest treatment concentration.

The 20nm, 50nm, and 80nm unwashed Ag-nps had an MIC value ranging from 3.0-8.0µg/ml, and an MBC value between 6.0-14.7µg/ml for each of the bacterial strains. Similarly, the supernatant had an MIC between 2.0-4.0µg/ml and an MBC value between
3.7-11.0μg/ml. The 20nm, 50nm and 80nm washed Ag-nps had MIC values ranging from 64.0-1024μg/ml for all bacterial strains and MBC values ranging from 85.3-1024μg/ml. The 25nm and 35nm carbon-coated Ag-nps had MIC values ranging from 256.0-1024μg/ml for all bacterial strains and MBC values ranging from 384.0-1024μg/ml. The AgNO₃ had an MIC of 1.7-1024μg/ml and an MBC between 4.0-1024μg/ml. The formaldehyde MIC value ranged from 32.0-64.0μg/ml and the MBC value was 128.0μg/ml for each bacterial strain. As expected, the negative control of MH broth alone showed no antibacterial activity indicating a lack of contamination.

A representative microplate is shown in Figure 2 that compares the differences in MIC values between *Escherichia coli* J53 (rows B, C, and D) and *Escherichia coli* J53(pMG101) (rows E, F, and G) after treatment with 20nm washed Ag-nps (rows B and E), silver nitrate (rows C and F) and supernatant (rows D and G) samples. The plate clearly shows that while *Escherichia coli* J53 is susceptible to each of the treatments, the only treatment that affects *Escherichia coli* J53(pMG101) is the supernatant, indicating that something other than Ag is present in the solution in a sufficient quantity.
Figure 5.2- Representative microplate of MIC assay. *Escherichia coli* J53 (rows B, C, and D) and *Escherichia coli* J53pMG101 (rows E, F, and G) treated with 20nm washed Ag-nps (rows B and E), silver nitrate (rows C and F), and supernatant (rows D and G) samples. Wells with a cloudy appearance and a bacterial ‘button’ denote growth. The first well showing no growth is denoted the MIC. The MIC values for this plate are: Row B, 64µg/ml; Row C, 8µg/ml; Row D, 4µg/ml; Row E, exceeds testing; Row F, exceeds testing; Row G, 4µg/ml. Rows A and H as well as columns 1 and 12 are filled with MH medium to help prevent evaporation in the inner wells and as a quality control to ensure a lack of contaminated medium.

**Ultrastructural Observations**

Transmission electron micrographs of control and treated bacteria are depicted in Figure 3. Only TEM images depicting bacterial strains treated with 10µg/ml of 20nm washed Ag-nps are illustrated. TEM images of bacterial strains treated with unwashed Ag-nps were omitted due to the overwhelming effects of formaldehyde, and TEM images of
bacterial strains treated with carbon-coated Ag-nps were omitted due to the lack of effects seen even at the highest concentration testable. While untreated *Escherichia coli J53* displayed characteristic bacilli shape (Figure 3a), *Escherichia coli J53* treated with 10µg/ml of 20nm washed Ag-nps was depicted with condensed cytoplasm (Figure 3b), and ruptured cells (Figure 3c). *Escherichia coli J53*(pMG101) appeared normal and similar to controls after exposure to Ag-nps (Figures 3d and e). Control *Staphylococcus aureus* displayed the characteristic cocci shape (Figure 3f), while *Staphylococcus aureus* exposed to Ag-nps displayed membrane integrity loss (Figure 3g) and ruptured cells (Figure 3h). *Escherichia coli J53* (Figure 3c) and *Staphylococcus aureus* (Figure 3h) treated with Ag-nps depicted whole bacteria that ruptured with Ag-nps agglomerates near the degenerate cells.
Figure 5.3- TEM images of bacteria exposed to 10µg/ml of 20nm washed Ag-nps. (a) control *Escherichia coli* J53; (b) and (c) treated *Escherichia coli* J53; (d) control *Escherichia coli* J53pMG101; (e) treated *Escherichia coli* J53pMG101; (f) control *Staphylococcus aureus*; (g) and (h) treated *Staphylococcus aureus*. Bar=100nm. Arrows point to agglomerated Ag-nps.

Energy dispersive X-ray (EDX) spectrum for bacteria samples dosed with 10µg/ml of 20nm washed Ag-nps confirmed the presence of Ag-nps. Figure 4 shows the spectrum from analysis of the *Escherichia coli* J53 (Figure 4a) and *Staphylococcus aureus* (Figure 4b) samples. The arrows point to Ag peaks, Au from Ag-nps core, copper from grid and osmium from cell post fixation are also present in the spectra.
Discussion

The antimicrobial properties of Ag-nps are of significant value for consumer products, food processing, packaging and storage, textiles, medical applications such as wound care products and implantable medical devices. Accordingly, Ag-nps have been integrated into hundreds of products that affect the daily lives of millions of people in many countries with their main usage focusing on disinfection in wound care and infection prevention [www.nanotechproject.org]. Our previous study evaluated the toxicity of Ag-nps in human epidermal keratinocytes (HEK) and showed that washing or carbon-coating Ag-nps prevented toxicity in skin cells [Samberg et al. 2010].

Ag may exist in four different oxidation states: Ag$^0$ (metallic silver), Ag$^{+1}$, Ag$^{+2}$, and Ag$^{+3}$. The first two states are the most abundant, while the latter two are unstable in aquatic environments [Wijnhoven et al. 2009]. Although Ag$^0$ shows little to no reactivity, Ag$^{+1}$ has a strong binding affinity for thiol and disulfide groups, and preferentially binds to anionic sites on teichoic acid and peptidoglycan present in gram-positive bacterial cell walls, and to the
phosphoryl groups of lipopolysaccharide present in gram-negative bacterial cell walls [Collins and Stotzky 1989]. The toxicity of Ag-nps may be explained by several mechanisms: 1) excessive binding of Ag$^{+1}$ and Ag-nps could prevent the uptake of essential nutrients to the cell, ultimately leading to cell death, 2) Ag$^{+1}$ entry into the cell by competitive binding with essential heavy metals such as Ca$^{2+}$, Mg$^{2+}$ and Mn$^{2+}$, or 3) their transport and irreversible accumulation in the cell could occur by complexation with ligands or substrates. Lastly, Ag$^{+1}$ could inhibit respiration, or bind and condense DNA once inside [Holt and Bard 2005]. Ultimately, the biocidal activity of Ag ions is likely caused by a synergistic effect between the binding of Ag ions to the cell wall, their uptake and subsequent accumulation in the cell, and their interference with critical biomolecules within the cell. Therefore, it can be concluded from these theories that the steady release of Ag$^{+1}$ from the degradation of Ag-nps is a critical function of Ag-nps that should be considered prior to synthesis.

The effect of Ag-nps size on the antibacterial activity has been shown to increase the reactivity with decreasing particle size, and may relate that the inactivation effect increases with increasing number of Ag-nps that can be attached to a bacterium [Dror-Ehre et al. 2009, Lok et al. 2006, Morones et al. 2005, Panáček et al. 2006]. In both the aqueous and MH media dispersions, each of the unwashed and washed Ag-nps exhibited long-term stability, whereas the carbon-coated Ag-nps formed larger agglomerates immediately. This increased agglomeration of carbon-coated Ag-nps could also have contributed to their decreased antibacterial efficacy compared to the washed Ag-nps. In the MH medium, a gradual increase in hydrodynamic diameter occurred over time and resulted in the formation of large agglomerates according to DLS. The tendency for nanomaterials to agglomerate in cell
culture media has been previously reported in our lab for aluminum nanoparticles under cell culture conditions over time and with changes in temperature [Monteiro-Riviere et al. 2009]. Although a linear relationship between the size of the washed Ag-nps and their corresponding MIC value appears for nearly each bacteria tested, it is unlikely to be an actual trend due to the nonlinear relationship between Ag-nps size in water and in MH media. Another observation was that all Ag-nps tested precipitated at the bottom of the wells after 24h, most likely due to protein and dead cell binding.

In this study, the surface condition of the Ag-nps was directly related to the synthesis method. There are several methods to synthesize Ag-nps; one of the most common is by direct reduction of a precursor followed by a stabilizer such as formaldehyde, while common physical techniques include milling, metal condensation, laser ablation, electrolysis, and metal-organic chemical vapor deposition [Cioffi et al. 2009]. The base catalyzed reduction method resulted in Ag-nps with unmodified surfaces, whereas the laser ablation method resulted in a carbon modified surface. Therefore, both the surface condition and synthesis method was shown to affect the antibacterial efficacy of Ag-nps. It can be noted from Table 2 that unmodified Ag-nps synthesized by the base catalyzed reduction method have greater antibacterial activity than carbon modified Ag-nps synthesized by carbon-stabilized laser ablation method.

To date, most antibacterial studies involving Ag-nps have evaluated their efficacy using disk or cup diffusion techniques, likely due to its simplicity and cost effectiveness [Cho et al. 2005, Kim et al. 2007, Morones et al. 2005, Pal et al. 2007]. However, during preliminary testing, we observed that Ag-nps first soaked and then dried into cotton disks failed to diffuse outward, killing only the bacteria directly under the disk. This may be
explained by the fact that high molecular weight compounds will not diffuse well in agar and are therefore difficult or inappropriate to test by the diffusion method [Wanger 2007].

This study showed that washed or carbon-coated Ag-nps have an MIC ranging between 64-512µg/ml. The majority of studies by others yielded MIC values under 10µg/ml which closely corresponds to our results for control Ag ions or unwashed Ag-nps [Choi et al. 2008, Li et al. 2010, Kvitek et al. 2008]. A few studies by others reported slightly higher MIC values between 40-180µg/ml [Morones et al. 2005, Ruparelia et al. 2008, Sondi and Salopek-Sondi 2004], and it is interesting to note that these studies used either washed Ag-nps or Ag-nps in a carbon matrix. The toxicity of the unwashed and supernatant solutions, and general lack thereof in the washed solutions, may be attributed to the 5.55mg/ml of formaldehyde. The toxic concentration range for the unwashed Ag-nps (3.0-8.0µg/ml) contained approximately 77.0-220.0µg/ml formaldehyde which corresponds closely to the range of MIC values for freshly synthesized formaldehyde (32.0-64.0µg/ml). Strong differences in MIC values for Ag-nps have been noted between differing bacterial strains [Kvitek et al. 2008] and additional differences may be accounted for by variations in Ag-nps size or initial bacterial concentration.

In vitro toxicity studies of Ag-nps in skin, liver and stem cell lines have shown that Ag-nps readily enter cells and cause cellular damage through the generation of reactive oxygen species [Braydich-Stolle et al. 2005, Hussain et al. 2005, Samberg et al. 2010]. Additionally, it has been implied by various authors that Ag-nps are capable of attaching to the bacterial cell membrane as well as entry into cells, though many authors do not use EDX and TEM to confirm the actual penetration of Ag into whole bacteria [Dror-Ehre et al. 2009, Pal et al. 2007, Smetana et al. 2008, Sondi and Salopek-Sondi 2004]. Some investigators
have reported that only Ag-nps with a diameter less than 10nm were capable of entering *Escherichia coli* and *Pseudomonas aeruginosa* [Morones et al. 2005], while others have shown that 80nm Ag-nps can accumulate within *Pseudomonas aeruginosa* after the addition of the chloramphenicol [Xu et al. 2004]. However, we did not observe Ag-nps in our bacteria but showed ruptured and damaged bacteria with Ag-nps agglomerates nearby.

Plasmid pMG101 is an Ag-resistance plasmid containing nine genes that also confers resistance to mercury, tellurite and several antibiotics [Silver 2003]. The plasmid encodes a periplasmic Ag$^{+1}$-binding protein and two parallel membrane Ag$^{+1}$ efflux pumps. Axiomatically, as bacterial contact with Ag increases, the number of Ag-resistant bacteria will correspondingly increase. Accordingly, Ag-resistant bacteria have been reported in Ag-saturated environments such as in hospital burn wards, polluted soil around Ag mines, and water catchment associated with photographic film production [Silver 2003]. One particular outbreak at Massachusetts General Hospital resulted in the death of several patients and required the closing of the burn ward [Silver 2003]. Our observation of Ag-resistant *Escherichia coli* J53(pMG101) morbidity in the presence of each of the unwashed Ag-nps samples suggests that residual contaminants and not Ag is responsible for killing this strain.

It is crucial to perform several characterization methods on nanomaterials to confirm the manufacturer’s specifications, as well as to perform chemical analysis to detect the presence of contaminants. Otherwise, it is difficult to conclude with absolute certainty that an antibacterial effect is due solely to Ag. This study showed that Ag-nps of the exact same size and synthesis method can yield vastly different MIC values simply by washing. The unwashed and washed Ag-nps were identical except for the contents of their surrounding solutions; the use of both unwashed and washed Ag-nps in this study aided in differentiating
the effects between Ag-nps and those solutions. It is important to note that although the washed Ag-nps solutions was crucial for the identification of the actual antimicrobial efficacy of the Ag-nps themselves, and not those of their surrounding solutions, it is the unwashed Ag-nps that are sold commercially. It was additionally found that carbon-coating was shown to virtually eliminate toxicity of Ag-nps. While no significant size-dependent toxicity was noted, it may be concluded that Ag-nps synthesized by carbon-stabilized laser ablation produce a less effective antibacterial agent, compared to Ag-nps synthesized by the base catalyzed reduction method. As expected, a decrease in eukaryotic toxicity through washing or carbon-coating of Ag-nps similarly decreases the antibacterial efficacy of Ag-nps. Additionally, the use of this Ag-resistant *Escherichia coli* strain proved to be a valuable tool for the identification in difference between Ag and contaminant toxicity.

**Acknowledgement**

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The authors would like to thank Dr. Anne Summers of the University of Georgia for donation of *Escherichia coli* J53 and *Escherichia coli* J53(pMG101), Megan Fauls of North Carolina State University for donation of Methicillin-Resistant *Staphylococcus aureus* and *Salmonella* sp., Mitsu Suyemoto of North Carolina State University for technical assistance and Dr. Steven Oldenburg of NanoComposix for provision of Ag-nps.
6. Incorporation of Silver Nanoparticles into Degradable poly(L-lactide-co-epsilon-caprolactone) Copolymer Scaffold for Skin Regeneration

Manuscript in preparation for submission.

Abstract

Silver nanoparticles (Ag-nps) are used as a natural biocide to prevent undesired bacterial growth in clothing and cosmetics. The objective of the study was to impart antibacterial properties through the incorporation of Ag-nps to electrospun degradable 50:50 poly(L-lactide-co-epsilon-caprolactone) (PLCL) scaffolds for skin tissue engineering applications. The biocompatibility of the scaffold containing Ag-nps was evaluated with human epidermal keratinocytes (HEK); cell viability and proliferation were evaluated using Live/Dead and alamarBlue viability assays following 7 days and 14 days of cell culture on the scaffolds. No significant decreases in cell viability were noted at either time point and cell proliferation increased 120% by 7 days and 200% by 14 days on both control and Ag-nps incorporated scaffolds. After 14 days, scanning electron microscopy revealed a confluent layer of HEK on the surface of the scaffolds, and fluorescent microscopy confirmed cell migration into the scaffold interior. Uniaxial tensile tests revealed a slight decrease in the modulus of elasticity following Ag-nps incorporation compared to the control, while cellular attachment significantly increased the modulus of elasticity. Therefore, these findings suggest that Ag-nps incorporated scaffolds are biocompatible and suitable for skin tissue engineering graft scaffolds.
Introduction

The skin, the largest organ of the body, performs numerous vital functions, including fluid homeostasis, thermoregulation, immunologic functions, neurosensory functions, and metabolic functions such as vitamin D synthesis. The skin also provides primary protection against infection by acting as a physical barrier, and when this barrier is damaged pathogens have a direct route to infiltrate the body, possibly resulting in infection. Annually, over 2 million wounds in the United States alone require advanced care; the major etiologies of these complex wounds include venous, diabetic and pressure ulcers, burns, amputations and trauma [Garfein 2009]. Dermatomed, meshed skin grafts, and micrografting of autologous donor skin is efficient for closing large wounds, but results in the creation of additional partial thickness wounds that also need to heal. Alternatively, biological membranes such as cadaver skin, pig skin, and neonatal skin substitutes provide good temporary coverage, but are hindered by limited supply and immunological complications [Burd and Chiu 2005]. Despite great advances in skin wound care, the resulting severe scarring and deformity continues to be one of the greatest challenges facing patients with skin lesions.

The field of tissue engineering and regenerative medicine with its mission to “develop biological substitutes that restore, maintain, or improve tissue function” has been revolutionary in terms of attempting to resolve these shortcomings [Langer and Vacanti 1993]. Skin tissue regeneration may be achieved through the use of a degradable synthetic membrane on which a patient’s cells may be seeded. In contrast to conventional grafting, in which autologous donor skin expansion is limited to approximately 1:4 by meshing and micrografting procedures, the rapid growth of patient-derived cells in vitro can provide in excess of 60 times the area of the initial biopsy. In this method, cells isolated from biopsies
as small as 4 square inches can be expanded to cover catastrophic burns covering over 80% of the body in about two months from the time of injury [Boyce et al. 2006]. The requirements for suitable tissue engineering scaffolds are demanding and extensive. The scaffold should be biocompatible to facilitate host cell adhesion and cell growth, be mechanically strong, capable of being fabricated into a desired shape, have a suitable degradation rate to meet the requirements of new tissue growth, produce nontoxic degradation products, and be easily handled and processed during manufacture [Tateishi et al. 2002]. More specific requirements are necessary on a per tissue basis; for example, skin replacements are required to be bacteriostatic, semi-permeable to water, and cosmetically acceptable. Skin scaffolds should also be elastic enough to withstand cyclic mechanical strains without any significant permanent deformation or creep [Chung et al. 2010, Jeong et al. 2004, Jeong et al. 2005, Kim et al. 1999, Kim and Mooney 2000].

In the past, tissue scaffold have been fabricated from synthetic products using nylon (Biobrane; Smith & Nephew) [Whitaker et al. 2008], silicone (Myskin; CellTran) [Moustafa et al. 2004], and polyurethane (Tegaderm; 3M) [Phan et al. 2005], or from acellular matrices sourced from human tissue (AlloDerm, GraftJacket, and Repliform; LifeCell) [Horch et al. 2005]. More recently, polymers such as poly(L-lactide) (PLA), poly(Glycolic Acid) (PGA), and poly(ɛ-caprolactone) (PCL), have been used to fabricate successful tissue engineered scaffolds for various soft tissues [Kwon et al. 2001, Athanasiou et al. 1996, de Groot et al. 1997, den Dunnen 1995, Rodriguez et al. 1999]. Hydrolyzable and biocompatible copolymers of PLA and PCL (poly(l-lactide-co-epsilon-caprolactone); PLCL) have also been fabricated into scaffolds by various methods, including extrusion, particulate leaching [Jeong et al. 2004 Jeong et al. 2005] melt spinning [Chung et al. 2010], and electrospinning [Kwon
et al. 2005, Mo et al. 2004, Inoguchi et al. 2006]. In particular, the 50:50 PLCL scaffold has high elastomeric properties with breaking strains in excess of 100% [Jeong et al. 2004, Jeong et al. 2005, Kwon et al. 2005, Hiljanen-Vainio et al. 2005, Chung et al. 2010]. Compared to other bioresorbable polymers such as PGA and PLA, this PLCL copolymer has a slow rate of degradation, with in vivo studies reporting 81% mass retained after 15 weeks of implantation [Jeong et al. 2004].

Scaffolds may be fabricated using an array of different techniques such as particle leaching, fiber bonding, phase separation, and rapid prototyping; however, electrospun nanofibrous polymer scaffolds are increasingly being favored since they provide a three-dimensional structure similar to the natural environment and are easily tailored to provide the necessary requirements such as mechanical properties, surface topography and chemistry, and in vivo degradation rate [Yang et al. 2001, Pham et al. 2006]. Electrospinning is a method for producing nonwoven fibers with diameters in the range of submicrometers down to nanometers. In this process, a continuous filament is drawn from a polymer solution by high electrostatic forces. As the charged jet of polymer solution is ejected the solution evaporates and the attenuated polymer is deposited as ultrafine fibers on a grounded conductive collector [Kwon et al. 2005, Inoguchi et al. 2006, McCullen et al. 2007, Chung et al. 2010]. This enables the fabrication of a structure resembling the extracellular matrix with a high surface area to volume ratio.

Infection risks are a danger in any surgical technique, and tissue engineered constructs risk contamination during the in vitro culture step and also during implantation. To address this unresolved problem, silver nanoparticles (Ag-nps) have been incorporated into several different medical devices due to their ability to exert antibacterial effects against
a both Gram-positive and Gram-negative bacteria, as well as multidrug-resistant strains [Samberg et al. 2011, Nair and Laurencin 2007, Lok et al. 2006, Kim et al. 2007, Pal et al. 2007, Shahverdi et al. 2007, Panacek et al. 2006]. A few Ag-nps electrospun scaffolds have been developed; however, they have taken the approach of loading AgNO₃ into the polymer solution and then exposing the scaffold to an annealing process to reduce the precursor into Ag-nps [Jeon et al. 2008, Liu et al. 2010a]. While this process negates complications involving particle agglomeration upon introduction into the polymer solvent, the reduction process can produce a large range in particle sizes and particle attachment location.

The objective of this study was to characterize and assess the biocompatibility of an electrospun scaffold of PLCL nanofibers incorporating antibacterial 20nm Ag-nps. Since this type of resorbable scaffold has potential applications in the regeneration of skin tissue, this study included an evaluation of the mechanical properties, attachment and proliferation of human epidermal keratinocytes (HEK), the level of antimicrobial activity promoted by the slow release of the silver from the degrading polymer matrix.

**Materials and Methods**

*Synthesis and characterization of Ag-nps*

20nm Ag-nps (1.00mg/ml) were obtained from NanoComposix (San Diego, CA, USA). The Ag-nps were synthesized by ammonium hydroxide catalyzed growth of Ag onto 5nm gold seed particles while in the presence of citric acid. According to the manufacturer, following synthesis the Ag-nps were concentrated via tangential flow filtration, serially washed and suspended in deionized (DI) water and 2mM citrate buffer. Upon arrival, the Ag-nps were stored at 4°C in the dark.
Particle size and surface characterization was determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM) to confirm the manufacturer-identified diameters and surface characterization. Ag-nps were suspended at the highest dosing concentration of 100µg/ml in deionized (DI) water, Mueller Hinton bacterial broth, and KGM-2 cell culture medium. Immediately after dispersion, the Ag-nps were placed in a disposable cuvette and DLS measurements were carried out on a Zetasizer Nano-ZS (Malvern Instruments, Inc., Worcestershire, UK). The initial DLS readings were performed at the standard characterization temperature of 25 °C. Each measurement was repeated five times, with 10-20 runs per measurement; data was culled based on the correlogram and size quality report rendered by the Dispersion Technology Software (5.03). Additionally, TEM was utilized to characterize the structure, shape and size uniformity of the Ag-nps. Specimens were prepared by placing a drop of homogeneous suspension of Ag-nps at the highest dosing concentration in DI water onto formvar-coated copper mesh grids and air dried. The grids were then examined on an FEI/Philips EM 208S TEM operating at an accelerating voltage of 80 kV. Using ImagePro software, Ag-np diameters were measured on 100 randomly selected particles from each individual TEM image using the magnification recorded during TEM viewing.

*Poly(lactide-co-epsilon-caprolactone) (PLCL) Copolymer*

The PLCL copolymer was created using L-lactide (100 mmol), ε-caprolactone (100mmol), and 1,6-hexanediol (0.5mmol), which were polymerized at 150°C for 24h, using stannous octoate (1mmol) as a catalyst. After the reaction, the product was dissolved in chloroform and precipitated in methanol, filtered and dried under vacuum. The molar ratio
of the two synthetic monomers (PLA and PCL) in the PLCL copolymer was 50:50). The average molecular weight of the copolymer was \( \text{MW} = 350,000 \). The bulk copolymer was stored in sealed plastic bags in a vacuum desiccator.

**Solution Preparation**

The solvent used for dissolving the polymer was 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma-Aldrich, USA). Based on a previous study performed in our lab [Chung et al. 2010], the optimal electrospinning polymer concentration of 11% (w/v) was prepared. Homogeneous solutions were obtained by slow agitation with a magnetic stirrer at 300rpm for 3h. The polymer was readily dissolved at room temperature, and the solution remained stable during storage at room temperature for up to 7d prior to electrospinning. Control polymer solutions were prepared without Ag-nps, and were observed to be clear in appearance.

It was noted that direct addition of Ag-nps into the polymer solution caused particle agglomeration; in order to overcome this problem, prior to Ag-nps addition into the polymer solution, the desired quantity of Ag-nps were first spun down at 16000g to form a soft pellet, the water was removed, and the pellet was resuspended into HFIP. The resuspended Ag-nps sample was then added to a concentrated polymer solution in order to yield the optimal 11% (w/v) polymer concentration. In this experiment, the Ag-nps sample was added to the polymer solution at a concentration of 0.5mg Ag-nps (0.5ml of 1 mg/ml Ag-nps stock solution, spun down, and water removed) to give a total polymer volume of 15ml. Using inductively-coupled plasma mass spectroscopy (ICP-MS), the resulting scaffolds were shown
to contain $0.11 \pm 0.01$ mg Ag/g scaffold. All scaffolds were sterilized by ethylene oxide prior to use.

*Electrospinning*

The custom designed electrospinning apparatus consisted of a high-voltage power supply (Gamma High Voltage Research, Inc), an infusion pump (New Era Pump System, Inc), a 15ml plastic syringe, a stainless steel blunt-ended needle (20 gauge) and a 15cm diameter metal collector. The syringe was mounted horizontally on the infusion pump and the sample solution was fed at a constant rate through the syringe to the needle tip. The distance between the needle tip and the collector was maintained at 15 cm. The applied voltage to the needle tip was 7.5-10kV and the flow rate of the solution was 1.0 ml/hr. Control PLCL polymer films were electrospun without Ag-nps.

*Fiber Characterization*

In order to determine the morphology and diameter of the filaments, the scaffolds were viewed using TEM and scanning electron microscopy (SEM). SEM images were acquired from a JEOL JSM 6360LV SEM using an accelerating voltage of 5 kV. Specimens were mounted on aluminum stubs using conductive carbon tape. They were then sputter coated with gold/palladium using a Hummer™ 6.2 Sputter Coating System (Anatech, CA, USA) to obtain a conductive coating about 100 Å thick. SEM images of the control and Ag-nps PLCL webs were used to measure the mean fiber diameters. Using ImagePro software, fiber diameters were measured on 100 randomly selected fibers from each individual SEM image using the magnification recorded during SEM viewing.
TEM was utilized to confirm the incorporation of Ag-nps within the fibers; copper mesh grids were mounted on the electrospinning collector and the polymer was allowed to spin onto their surface. Grids were then examined on an FEI/Philips EM 208S TEM operating at an accelerating voltage of 80 kV.

**Biocompatibility Analysis**

Cryopreserved primary neonatal human epidermal keratinocytes (HEK; Lonza, Walkersville, MD) were grown in keratinocyte growth medium-2 (KGM-2; Lonza, Walkersville, MD) in cell culture flasks (75cm²; 1,000,000 cells) to approximately 80% confluency in a 37°C humidified 5% CO₂ incubator. Cells were then passed into cell culture plates for biocompatibility testing.

For initial toxicity testing of the Ag-nps, HEK were passed into black 96-well microplates (12,500 cells/well; 200µl) in which the peripheral wells contained only KGM-2 to prevent the evaporation of treatment medium. Between 18-24 h later, after reaching approximately 80% confluency, the HEK were exposed for 24h to either KGM-2 (control) or serial dilutions of Ag-nps starting at 100µg/ml. The viability of the HEK was assessed using alamarBlue (aB) viability assay; aB viability assay was used since it was determined to be the best viability assay for examining the toxicity of Ag-nps [Samberg et al. 2010]. The dosing medium was aspirated and replaced with aB solution (10% aB in KGM-2), incubated for 3h, and the fluorescence read on a Spectra Max Gemini EM spectrophotometer with an excitation wavelength of 545nm and an emission wavelength of 590nm. The fluorescent values of treated cells were normalized to the control cells, and the data expressed as percentage viability.
Concurrently, to assess the various interactions of Ag-nps with aB, both a nanoparticle control and a cell control was performed as described previously [Monteiro-Rivere et al. 2009, Samberg et al. 2010]. The nanoparticle control assay was used to assess nonspecific interactions between the Ag-nps and the assay dye without the use of cells. For this assay, Ag-nps were suspended in centrifuge tubes containing aB medium (10% aB in KGM-2) at the same dosing concentrations as the toxicity test and incubated for 3h. The aB medium was transferred to a black plate and fluorescence read at intervals between 565nm and 640nm with an 545/590nm excitation/emission wavelength. In addition, the cell control assay was conducted to measure how the Ag-nps affected the assay after the dye reacted with the viable cells. For this assay, confluent HEK were treated with only KGM-2 media and viability assayed after 24h. Plates containing the Ag-nps concentrations (without cells) were incubated in parallel. Once the viability of the normal HEK had been assayed, the reacted dye was transferred to the parallel Ag-nps plate and incubated for 3h. Any change in the fluorescence values following incubation with the Ag-nps indicated the effect of the Ag-nps on the reacted dye. Only one plate (n=6 replicates per plate) was run per control assay.

For biocompatibility testing of the scaffolds, control and Ag-nps PLCL webs were first soaked in KGM-2 medium overnight prior to cell seeding to facilitate protein adsorption and cell attachment onto the fiber surface. Cells were seeded onto the control and Ag-incorporated scaffolds in 6-well plates (50,000 cells/well; 2ml), and the medium was refreshed every other day. After 7d and 14d, the alamarBlue assay was used to determine cell proliferation within the scaffolding, compared to cells grown as a monolayer in an identical area. Live/Dead viability assay was used to determine the viability of HEK within
the scaffolding and visualize cell attachment, as determined by the percentage of cells that fluoresced green.

Cell attachment and proliferation within the scaffold was also observed using SEM. Images were acquired from a JEOL JSM-6360LV SEM using an accelerating voltage of 5 kV. After cell incubation at 7d and 14d, scaffolds were fixed with Trump’s fixative for 24h. They were then rinsed with 0.1M phosphate buffer (pH 7.2), followed by deionized water, and then dehydrated by processing through an ascending series of aqueous ethanol solutions. The scaffolds were then submersed in hexamethyldisilazane overnight, sputter coated as described above, and then observed by SEM.

**Degradation**

Papain enzyme was used to simulate an accelerated in vivo enzymatic environment. The papain enzyme solution was activated prior to the start of the degradation study by adding 0.01M EDTA solution with 0.05M of cysteine, papain from papaya latex (Sigma Aldrich, USA), 0.01M ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, USA), and 0.05M L-cysteine hydrochloride monohydrate (Sigma Aldrich, USA). The activated solution was prepared based on the mole ratio of 1: 2.5: 12.5 between enzyme, EDTA, and cysteine respectively. In order to maintain the activity of the enzyme during the entire degradation period, the enzyme solutions were replenished with activated enzymes after every 72 hours. Scaffolds were subjected to the degradation solution for 7d at 37 °C. The mass of dry scaffolds was measured before and after degradation treatment.
Antibacterial Efficacy

Cation-adjusted Mueller-Hinton (MH) broth and agar (Difco Laboratories, Detroit, MI) was used as the bacterial cultivating medium for *Escherichia coli* J53, Ag-resistant *Escherichia coli* J53(pMG101), *Staphylococcus aureus* (ATCC 25213), methicillin-resistant *Staphylococcus aureus* (MRSA; ATCC 43300), and *Salmonella* sp. (ATCC 35664). Isolated bacterial colonies were grown overnight at 37°C from frozen samples on an agar plate (with 100µg/ml ampicillin for Ag-resistant *Escherichia coli* J53(pMG101)).

The broth microdilution minimum inhibitory concentration (MIC) test was conducted to measure the in vitro activity of Ag-nps against each bacterial isolate [Wikler et al. 2009]. Briefly, a sterile round-bottom plastic 96-well plate was loaded with 100µl of serially 1:2 diluted concentrations of Ag-nps. Ag-nps samples were tested at ten serially diluted concentrations starting at the highest dosing concentration that their supplied concentrations would allow (1.00mg/ml). Silver nitrate (AgNO₃; 99.9%, Sigma-Aldrich, St. Louis, MO) was used as the positive control source of Ag⁺ ions.

For each bacterial strain, a bacterial colony was suspended into phosphate buffered saline (PBS) to 0.5McFarland (10⁸ CFU/ml), diluted 1:20 into MH broth, and added into the treatment wells at 100µl of 5-8x10⁵ CFU/ml (n=8 wells/treatment). To ensure quality control and for seeding accuracy, inoculating bacteria were diluted in PBS at 10³, 10⁴, 10⁵, and 10⁶ and plated overnight. Microplates were incubated at 37°C and shaken at 200rpm for 24h. After the microplates were incubated for 24h at 37°C, the lowest concentration showing no visible growth was recorded as the MIC. To determine the MBC, 10µl from all of the clear wells (MIC and higher concentrations) was dropped onto a MH agar plate and incubated for 24hr at 37°C. The minimum bactericidal concentration (MBC) was determined by the
concentration that failed to yield growth. Additionally, *Escherichia coli* J53(pMG101) exposed to 16,987 µg/ml AgNO₃ was not growth inhibited, which confirmed that the strain was Ag resistant.

The broth microdilution MIC test was also used to evaluate the antibacterial efficacy of released Ag-nps from the scaffolds over time. In addition to collecting KGM-2 collected every other day during the 14d cell growth, the medium was also collected every other day for 14d from the Ag-nps PLCL scaffolds exposed to KGM-2 without the presence of cells, as well as the papain enzyme solution to simulate accelerated in vivo conditions. The concentration of Ag in these solutions, as well as the concentration of Ag remaining in the scaffold at the end of testing was evaluated using ICP-MS.

*Tensile Testing*

The mechanical properties of the PLCL scaffolds was assessed via uniaxial tensile testing to failure at a cross-head speed of 0.5mm/sec, during which force and displacement data as well as videos were recorded, as well as video recording of the gauge length over time (n=4 for each group). All specimens were prepared by punching into dog-bone shaped specimens using a die with a gauge length of 20mm and 5mm. Acellular scaffolds were tested “as is,” while 14d cell growth scaffolds and degraded scaffolds were transferred directly from the cell culture plates to a board where they were lightly blotted, punched, and tested. Specimens were mounted between pneumatic grips with a 250g load cell mounted under the bottom fixed grip. Acellular and 14d cell growth control scaffolds were tested using an ElectroForce (Bose, Minnesota, USA) mechanical system, but malfunction of the system required all other specimens to be tested using a Mini-Bionix II mechanical testing
machine (MTS, Minnesota, USA). The load cell and all other test conditions were maintained. The modulus of elasticity was calculated from the slope of the linear portion of the standard stress-strain curves. The strain was calculated from the recorded videos using ImagePro software to track the movement of dots drawn onto the scaffold gauge length.

**Statistical Analysis**

The mean values for HEK percent viability (normalized to control cells grown as a monolayer), proliferation, and MIC/MBC values were calculated, and the significant differences between the means \( (p<0.05) \) were determined by the PROC GLM Procedure (SAS 9.1 for Windows; SAS Institute, Cary, NC). When significant differences were found, multiple comparisons were performed using Tukey’s Studentized Range High Standard Deviation test at \( p < 0.05 \) level of significance. The data were expressed as the mean ± standard error of the mean (SEM) for \( n=6 \).

**Results**

**Toxicity**

As the concentration of the 20nm Ag-nps increased there was no significant change in percent viability of HEK following 24h exposure until 50µg/ml was reached. At this concentration, only 63% viability remained whereas at 100µg/ml there was nearly a complete loss of viability (Figure 1). The nanoparticle control showed that Ag-nps caused a significant \( (p<0.05) \) increase in aB fluorescence values above control for all Ag-nps concentrations at the relevant 590nm emission wavelength (Figure 2), indicative of particle interaction with aB viability assay. The cell control showed that Ag-nps further affected reacted assay dye; the
20nm Ag-nps caused a significant (p<0.05) increase in fluorescence values at the relevant 590nm emission wavelength starting at 50µg/ml (Figure 3).

Figure 6.1: HEK viability after 24h exposure to 20nm Ag-nps.
*, **, *** denotes statistical significance (p < 0.05), multiple comparisons between concentrations.

Figure 6.2: Nanoparticle control assay for 20nm Ag-nps, for concentrations ranging from 0.39 to 100µg/ml.
Figure 6.3: Cell control assay for 20nm Ag-nps, for concentrations ranging from 0.39 to 100µg/ml.

Fiber and Ag-nps Morphology

The physicochemical properties of the 20nm Ag-nps can be found in Table 1, which lists their physical and hydrodynamic diameters according to TEM and DLS, respectively, as well as the supplied Ag-nps solution concentration, particle concentration, and zeta potential. As is typical for nanomaterials, the hydrodynamic diameter of the 20nm Ag-nps measured by DLS was found to be slightly larger (27.4nm) than the physical diameter measured by TEM (19.2nm). The significantly larger hydrodynamic diameter is most likely attributable to the formation of small, loosely bound Ag-nps dimers. Agglomeration of Ag-nps occurred after incorporation into MH broth (659.3±30.7) and KGM-2 medium (2721.5±178.3) which is consistent with the previously reported behavior of nanomaterials which are known to physically interact with proteins and promote agglomeration [Stone and Kinloch 2007, Monteiro-Riviere et al. 2009, 2010, Samberg et al. 2011]. The zeta potential of a colloid suspension characterizes its stability and ability to resist aggregation, with greater zeta
potentials having greater stability. The 20nm Ag-nps suspension had a zeta-potential of -50eV which indicates good stability due most likely to the stabilizing effect of the citrate buffer. According to TEM, the 20nm Ag-nps samples were found to be spherical in shape and highly uniform in size both before and after incorporation into the PLCL fibers (Figure 4A-B).

Table 6.1: Physicochemical Properties of Ag-nps

<table>
<thead>
<tr>
<th>MDD</th>
<th>DLS diameter (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DLS diameter (nm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>DLS diameter (nm)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>TEM diameter (nm)</th>
<th>Supplied Conc. (mg/ml)</th>
<th>Particle Conc. (particles/ml)</th>
<th>Zeta&lt;sup&gt;g&lt;/sup&gt; (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>27.4±0.2</td>
<td>2721.5±178.3</td>
<td>659.3±30.7</td>
<td>19.2±2.2</td>
<td>1.00</td>
<td>2.6x10&lt;sup&gt;13&lt;/sup&gt;</td>
<td>-49.8±0.5</td>
</tr>
</tbody>
</table>

Data are expressed as mean ±standard error of the mean; <sup>a</sup> DI water, <sup>b</sup> KGM-2 medium, <sup>c</sup> MH broth; MDD, manufacturer-designated diameter; DLS, dynamic light scattering; TEM, transmission electron microscopy.

By visual observation, control PLCL scaffold appeared white, whereas Ag-nps PLCL scaffold appeared grey. The morphology of the obtained PLCL fibers was observed using SEM. Figure 4C-F shows selected SEM images of control and Ag-nps PLCL webs after HEK cell culture for 7d and 14d. Smooth, connected fibers are observed in Figures 4C and 4D as well as fiber agglomeration, indicative of incomplete solvent evaporation. No significant difference in average fiber diameter (p<0.05) was noted between the control and the Ag-nps sample; the average diameter for the control PLCL web was 2.25µm±0.18µm, while the average fiber diameter for the Ag-nps PLCL web was 2.70µm±0.19µm.
Figure 6.4: Transmission and scanning electron micrographs of control and Ag-nps PLCL scaffolds A) 20nm Ag-nps, Bar=50nm; B) Ag-nps within PLCL nanofiber, Bar=0.5µm; C) Control PLCL scaffold with 7d cell growth, Bar=10µm; D) Ag-nps PLCL scaffold with 7d
Biocompatibility

SEM images depicted the attachment and proliferation of HEK on both control and Ag-nps PLCL samples throughout their 7d and 14d time period of cell culture, exhibiting characteristic HEK morphology (Figures 4C-F). Following 7d of incubation, individual HEK were easily observed on both the control and Ag-nps PLCL webs (Figure 4C and 4D, respectively). After 14d, the HEK were able to form confluent monolayers on both the control and Ag-nps PLCL webs (Figures 4E and 4F, respectively).

This proliferation was confirmed using the αβ assay; HEK proliferation was observed to increase to approximately 120% by 7d and over 200% by 14d on both control and Ag-nps PLCL scaffolds, compared to cells grown in a monolayer (Table 2). Additionally, cell viability was evaluated using the Live/Dead viability assay following 7d and 14d of cell growth. No significant decreases in cell viability were noted at either time point for the control or Ag-nps PLCL scaffold (Figure 5). Some cells in each image appear out of focus, indicative of cell growth into the scaffold interior. To analyze whether the cells in the scaffolds remained viable, a Live/Dead cell viability assay was used. As shown in Figure 5A-5D, >99% of the cells were viable 7d and 14d on both control and Ag-nps PLCL scaffolds after the initial seeding, as determined by the percentage of cells that fluoresced green.
Table 6.2: HEK proliferation on PLCL scaffolds

<table>
<thead>
<tr>
<th>Scaffold Type</th>
<th>Percent Viability Compared to Monolayer Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time point</td>
</tr>
<tr>
<td></td>
<td>7d</td>
</tr>
<tr>
<td>Control</td>
<td>124.5±0.5</td>
</tr>
<tr>
<td>Ag-nps</td>
<td>112.6±0.4</td>
</tr>
</tbody>
</table>

All data is expressed as mean±standard error of the mean of percent viability compared to cells grown as a monolayer in a well.

Figure 6.5: Fluorescent images of HEK on control and Ag-nps PLCL scaffolds after 7d and 14d stained with Live/Dead viability assay. A) Control PLCL scaffold with 7d cell growth;
B) Ag-nps PLCL scaffold with 7d cell growth; C) Control PLCL scaffold with 14d cell growth; D) Ag-nps PLCL scaffold with 14d cell growth. Live cells stain green, dead cells stain red, 10X magnification.

**Mechanical Properties**

The mechanical properties of the PLCL scaffold were altered by the addition of Ag-nps, by the exposure to 14d of cell culture, as well as by 7d of in vitro enzymatic degradation (Table 3). Uniaxial tensile tests of the PLCL scaffolds revealed a decrease in the modulus of elasticity (E) values with the addition of Ag-nps (1.14MPa) compared to the PLCL control (1.78MPa). On the other hand, the elastic modulus increased significantly for both the Ag-nps PLCL scaffold (1.66MPa) and the PLCL control scaffold (1.96MPa) during 14d of the cell culture compared to their acellular counterparts. As expected, in vitro enzymatic degradation of the scaffolds was associated with a decrease in the modulus of elasticity for both the Ag-nps PLCL scaffold (0.94MPa) and the PLCL control scaffold (1.45MPa).

Table 6.3: Uniaxial tensile test mechanical properties of PLCL scaffolds

<table>
<thead>
<tr>
<th>Scaffold</th>
<th>Treatment</th>
<th>E (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Acellular</td>
<td>1.78±0.2</td>
</tr>
<tr>
<td></td>
<td>14d Cell Growth</td>
<td>1.96±0.0</td>
</tr>
<tr>
<td></td>
<td>7d Degraded</td>
<td>1.45±0.1</td>
</tr>
<tr>
<td>Ag-nps</td>
<td>Acellular</td>
<td>1.14±0.2</td>
</tr>
<tr>
<td></td>
<td>14d Cell Growth</td>
<td>1.66±0.1</td>
</tr>
<tr>
<td></td>
<td>7d Degraded</td>
<td>0.94±0.1</td>
</tr>
</tbody>
</table>

All data is expressed as mean ±standard error of the mean.
Antibacterial Efficacy

The 20nm Ag-nps alone were shown to inhibit the growth of *S. aureus*, MRSA, *Salmonella* sp., and *E. coli* J53. The MIC and MBC values for each strain were found to be 32.0 and 32.0µg/ml for the Gram-positive strains, respectively, whereas the MIC and MBC was 64.0 and 64.0-85.3µg/ml for the Gram-negative strains, respectively (Table 4). The MIC and MBC for the positive control of Ag ions (AgNO₃) was 2.0 and 4.0µg/ml for the Gram-positive strains, respectively, and 2.0 and 2.0µg/ml for the Gram-negative strains, respectively (Table 4). As expected, the 20nm Ag-nps did not inhibit the growth of the Ag-resistant *E. coli* J53(pMG101) strain at any concentration tested, which confirms the noncytotoxic behavior of the Ag-nps colloidal suspension.

Table 6.4: Minimal Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC).

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>AgNO₃</th>
<th>20nm Ag-nps</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (µg/ml)</td>
<td>MBC (µg/ml)</td>
<td>MIC (µg/ml)</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>2</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td>MRSA</td>
<td>2</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>2</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td><em>E. coli</em> J53</td>
<td>2</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td><em>E. coli</em> J53(pMG101)</td>
<td>512†</td>
<td>512†</td>
<td>512†</td>
</tr>
</tbody>
</table>

*The data are expressed as means. All standard errors of the means were zero.
†*Escherichia coli* J53pMG101 grew at all concentrations, so the highest testable value 512 was reported.

Exposure to in vitro enzymatic degradation was performed to mimic accelerated in vivo degradation, and allow for the release of antimicrobial Ag-nps and Ag ions. Degradation for 7d caused a loss in mass of 40.9% for the control PLCL scaffold, compared
to 37.6% for the Ag-nps PLCL scaffolds. ICP-MS analysis showed that the concentration of Ag content in the Ag-nps PLCL scaffolds before and after cell growth or degradation treatment remained constant (0.1mg Ag / g scaffold). However, the amount of Ag-nps released from the PLCL scaffold into the KGM-2 medium or the enzymatic degradation solution was insufficient to impart antibacterial efficacy against any of the bacterial strains included in this study.

**Discussion**

In recent years, interest has grown for the utilization of Ag-nps rather than traditional antibiotics due to their broad antimicrobial activity against both Gram-negative and Gram-positive bacteria, including multidrug-resistant strains [Samberg et al. 2011, Nair and Laurencin 2007, Lok et al. 2006, Kim et al. 2007, Pal et al. 2007, Shahverdi et al. 2007, Panacek et al. 2006]. The application of Ag-nps in tissue engineered scaffolds for skin regeneration is pivotal in the reduction of infection that is rampant particularly in burn wounds. Many successful and promising attempts have been made to incorporate Ag-nps into biomaterials for continuous release to impart antibacterial activity including nanoparticulate Ag bone cement [Alt et al. 2004], titanium implants coated with a silver-hydroxyapatite layer [Chen et al. 2006], silver-incorporated bioglass system for dental applications [Balamurugan et al. 2008], and Ag-nps incorporated into polyurethane foam water filters [Jain and Pradeep 2005]. Attempts have also been made to investigate the feasibility of the incorporation of Ag-nps into polymer scaffolds for various antimicrobial applications [Furno et al. 2004, Hong et al. 2006].
However, before the scaffold is used for *in vitro* tissue engineering, it is essential to identify an optimum concentration of released Ag-nps that would show antimicrobial activity without having any cytotoxic effect on the cells of the targeted tissue. Nanomaterials, such as carbon black [Monteiro-Riviere and Inman 2006], aluminum nanoparticles [Monteiro-Riviere et al. 2010], and Ag-nps [Samberg et al. 2010] are capable of interfering with viability assay dye and dye products through the adsorption of cell medium constituents and cytokines. The potential interactions between assays and Ag-nps were assessed by the nanoparticle and cell controls, and showed an increase in fluorescence values. Based on this data, it can be inferred that Ag-nps interact with the assay dye to mimic the presence of metabolically active cells. Therefore, the increase in fluorescence values may cause the level of toxicity of the Ag-nps to HEK to be underestimated. Additionally, it has been reported that Ag-nps of various sizes can cause toxicity to various human cell lines in vitro, and it was therefore important to limit the concentration of the Ag-nps incorporated into PLCL scaffolds in this study [Liu et al. 2010b, Ahamed et al. 2008, Park et al. 2010].

The present study investigated the incorporation of 20nm Ag-nps into electrospun PLCL scaffolds to both foster the growth of HEK, and to ideally impart some level of antimicrobial activity through the protracted release of Ag-nps into the culture medium. This was achieved through the fabrication of PLCL scaffolds that incorporated Ag-nps, and was based upon the previously established electrospinning parameters developed in our laboratory [Chung et al. 2010]. It was confirmed by TEM that the 20nm Ag-nps were well incorporated within the fibers. While HEK exposed to the 20nm Ag-nps alone caused a significant decrease in viability, their incorporation into the PLCL scaffold did not result in a decrease in cell viability. In fact, the HEK flourished on and within the scaffolds after 7d
and 14d of culture. The observations of cell behavior on the nanofiber scaffolds confirmed that throughout this period of cell culture the HEK proliferated well on the Ag-nps containing nanofibers as monolayer cultures, as well as migrating through the thickness of the scaffold. The antibacterial results for the 20nm Ag-nps alone are congruent with our previous results for 20nm Ag-nps stabilized in a phosphate buffer [Samberg et al. 2011]. In this study, the amount of Ag-nps incorporated into the scaffold was based upon overestimates of the polymer surface area and polymer degradation rate, which unfortunately led to an insufficient amount of Ag-nps release. The modulus of elasticity of 1.14MPa for the Ag-nps containing PLCL scaffold compares well to that found by an electrospun PLCL scaffold of similar dimensions (0.8Mpa) [Kwon et al. 2005].

The present study sought to use electrospinning to fabricate a novel antibacterial skin scaffolding material composed of PLCL degradable polymer incorporating with Ag-nps. The potential use of the electrospun Ag-nps PLCL scaffold for skin regeneration was evaluated in vitro with human skin cells, keratinocytes, using models for cell attachment and proliferation, antibacterial efficacy, and biomaterial mechanical properties. In conclusion, PLCL is a suitable choice for soft tissue scaffolds since it is biocompatible, facilitates rapid attachment and growth of cells, and has a low modulus of elasticity similar to that of human skin. These results showed that although the release of Ag content was insufficient to inhibit bacterial growth, the scaffold supported the robust attachment and proliferation of keratinocytes throughout the scaffold, while maintaining sufficient mechanical properties to warrant skin implantation. To maximize the benefit of Ag-nps in terms of both biocompatibility and antimicrobial activity, future studies will be undertaken out to optimize their content and distribution within PLCL scaffolds.
Acknowledgements

The authors would like to acknowledge Katharina Sippel for help with the initial scaffold fabrication, and Dr. Steven Oldenburg of NanoComposix (San Diego, CA, USA) for the donation of the 20nm Ag-nps. This research was partially supported by the National Institutes of Health (NIH) RO1 ES016138.
7. Silver Nanoparticles Do Not Influence Stem Cell Differentiation But Cause Minimal Toxicity.


Abstract

The aim of this study was to evaluate the toxicity and cellular uptake of both undifferentiated and differentiated human adipose derived stem cells (hASC) exposed to silver nanoparticles (Ag-nps), and to assess their effect on hASC differentiation. hASC were exposed to 10nm or 20nm Ag-nps at concentrations of 0.1, 1.0, 10.0, 50.0 and 100.0µg/ml either before or after differentiation down the adipogenic or osteogenic pathways. Exposure of hASC to either 10nm or 20nm Ag-nps resulted in no significant cytotoxicity to hASC, and minimal dose-dependent toxicity to adipogenic and osteogenic cells at 10µg/ml. Each of the hASC, adipogenic and osteogenic cells showed cellular uptake of both 10 and 20nm Ag-nps, without causing significant ultrastructural alterations. Exposure to 10nm or 20nm Ag-nps did not influence the differentiation of the cells, and at antimicrobial concentrations of Ag-nps resulted in a minimal decrease in viability. The biocompatibility of Ag-nps with both undifferentiated and differentiated hASC establishes their suitability for incorporation into tissue engineered graft scaffolds, for the prevention of bacterial contamination upon implantation.
Introduction

Over 300,000 incidences of surgical site infection occur annually in the United States, arising in approximately 2% - 3% of patients who have undergone surgery. Although rates vary widely according to the type of surgery, 33% of infections occur from orthopedic surgeries resulting in increased treatment intensity, prolonged length of stay, higher costs, and greater risk for readmission or death [CDC 2011]. The most common bacterial strain responsible for surgical infection is *Staphylococcus aureus*, and the only method employed currently to avoid infection is through strict adherence to sterile surgical conditions and through the administration of prophylactic antibiotics [AAOS 2010]. However, drug resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *enterococci* (VRE) are still problematic [AAOS 2010].

Due to the enhanced antibacterial activity of Ag at the nanoscale, Ag-nps are the most used nanomaterial and the most valuable in the medical and personal hygiene fields through their incorporation into hundreds of consumer products [www.nanotechproject.org]. Previous studies have demonstrated that Ag-nps exert antimicrobial effects on a wide spectrum of bacteria such as *Escherichia coli*, *Enterococcus faecium*, *Vibrio cholera*, *Staphylococcus aureus*, and even MRSA [Samberg et al. 2011, Morones et al. 2005, Sondi and Salopek-Sondi 2004]. Since Ag compounds have been used for centuries with a lack of toxicity or carcinogenicity to the immune, cardiovascular, nervous, or reproductive systems, the use of Ag-nps in products has largely been unregulated [Silver 2011]. Presently, there is no consensus on the cytotoxicity of Ag-nps; however, the majority of publications do show reduced cell viability and increased reactive oxygen species generation following Ag-np exposure [Wijnhoven et al. 2009].
An application for Ag-nps is their incorporation into implantable materials such as prostheses and skin or bone grafts. In recent years, tissue engineering of patient-specific bone grafts have been used to replace damaged or defective tissues, and have become a viable alternative to autografts and allografts [Vergroesen et al. 2011]. Advances in human stem cell research demonstrate that these cells have unique properties that are important for the development of engineered tissue constructs, including high proliferation rates and self-renewal capacity, maintenance of the unspecialized state, and specialized differentiation under given conditions. Human mesenchymal stem cells (hMSC) isolated from either adult bone-marrow or adipose tissue possess a high capacity for self-replication and have the potential to differentiate into osteogenic, chondrogenic, adipogenic, and tenogenic lineages when placed in an appropriate environment [Zhao and Ma 2005, Zuk et al. 2002].

The interaction and metabolism of Ag-nps with cultured osteoblasts grown from hMSC should be minimal for their incorporation into a scaffold to be successful. There are a number of studies that showed that various mesoporous silica and magnetic nanoparticles used for cell tracking have no apparent deleterious effects on the morphology, cell proliferation, cell viability, and differentiation efficiency of stem cells [Chung et al. 2007, Huang et al. 2008, Lewin et al. 2000]. However, Ag-nps and Ag ions have been reported to attach to cell membranes, and interact with cellular molecules such as DNA, bacterial cell wall components, or sulphhydril groups of metabolic enzymes [Samberg et al. 2011, Morones et al. 2005, Sondi and Salopek-Sondi 2004]. Therefore, bacterial replication, membrane permeability, and different metabolic pathways may be interrupted. The objective of this study was to evaluate the toxicity and cellular uptake of both undifferentiated and differentiated human adipose derived stem cells (hASC) exposed to Ag-nps, and to assess
their potential effect on hASC differentiation. Understanding the interactions between Ag-nps and undifferentiated hASC is important to determine whether the antimicrobial activity of Ag-nps is detrimental to the differentiation of hASC down the adipogenic or osteogenic pathways for tissue engineering scaffold systems.

**Materials & Methods**

*Ag-nps*

Two solutions of spherical Ag-nps were obtained from NanoComposix (San Diego, CA), each at Ag-nps concentration of 1.00mg/ml with diameters of 10nm and 20nm. Ag-nps were synthesized by ammonium hydroxide catalyzed growth of Ag onto 5nm gold seed particles. Concentration of the particles was achieved via tangential flow filtration. The Ag-nps were then serially washed with 20 volume equivalents of phosphate buffer (PB), resuspended in stabilizing citrate buffered deionized (DI) water, and stored at 4°C in the dark.

Particle size was determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM) to confirm the manufacturer-identified diameters. To measure the hydrodynamic diameters and the zeta potential, samples of Ag-nps were suspended at the highest dosing concentration in DI water, immediately placed in a disposable cuvette and analyzed using a Zetasizer Nano-ZS (Malvern Instruments, Inc., Worcestershire, UK). The DLS readings were performed at the standard characterization temperature of 25 °C. Each measurement was repeated five times, with 10-20 runs as optimized by the instrument. Data was culled based on the correlogram, size quality report, and expert advice rendered by the Dispersion Technology Software (5.03). Additionally, TEM was utilized to characterize Ag-
np structure, shape and size uniformity. Samples were prepared by placing a drop of homogeneous suspension of each Ag-np solution at the highest dosing concentration in DI water onto a formvar-coated copper grid and air dried. Samples were examined on an FEI/Philips EM 208S TEM operating at an accelerating voltage of 80 kV.

**hASC Isolation and Expansion**

All protocols involving human tissue were approved by the Institutional Review Boards of the University of North Carolina at Chapel Hill (UNC-CH) and North Carolina State University. Excess adipose tissue from elective plastic surgery procedures was obtained with donor consent from 5 premenopausal female donors of Caucasian, Native American or other ethnicities that ranged from 24-37 years of age. The hASC were isolated from the adipose tissue by enzymatic digestion using 0.075% collagenase type I, and characterized as described [Bernacki et al. 2008, Wall et al. 2007]. Cells were seeded into T-75 flasks, and grown to approximately 70-80% confluency in a 5% CO₂ incubator at 37°C for expansion and culture. Cells were harvested using trypsin which increased their passage number incrementally (passage-0, passage-1, etc.). At passage-1, hASC from each of the 5 donors were pooled into a T-75 flask (20,000 cells/donor; 100,000 cells total), expanded and frozen in liquid nitrogen until needed.

Pooled passage 3 cells were seeded into T-75 cell culture flasks at 100,000 cells/flask, cultured in complete growth medium (minimum essential medium eagle, alpha-modified supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 100units/ml penicillin and 100µg/ml streptomycin), and maintained at 37°C in a humidified atmosphere of 5% CO₂. Once hASC reached 80% confluency, after 6d, cultures were washed twice with
phosphate buffered saline (PBS) and cells were retrieved using trypsin. At this point, hASC were plated into 12 well plates (5,000 cells/well) to assess viability, into T-25 flasks (50,000 cell/flask) to assess cellular uptake, and into 6 well plates (50,000 cells/well) to assess any Ag-nps effects on differentiation. Differentiation of cells was achieved via hASC culture in 1) osteogenic medium (complete growth medium supplemented with 50µM ascorbic acid, 0.1µM dexamethasone, and 10mM β-glycerophosphate), or in 2) adipogenic medium (complete growth medium supplemented with 1µM dexamethasone, 5µg/mL insulin, 100µM indomethacin, and 500µM isobutylmethylxanthine), or in 3) complete growth medium to maintain the cells in their progenitor cell state.

Cell Viability

To determine the effect of Ag-nps on both undifferentiated and differentiated hASC viability, undifferentiated hASC were grown to 100% confluency in complete growth medium for 4d. To determine the baseline viability, hASC were first differentiated down the osteogenic and adipogenic pathways or maintained in their proliferative state for 14d and then exposed for 24h to concentrations of 0, 0.1, 1.0, 10.0, 50.0 and 100.0µg/ml of either 10nm or 20nm Ag-nps. As a comparison to examine any effect internalized Ag-nps have on hASC, cells were first exposed for 24h to Ag-nps and then differentiated down the osteogenic or adipogenic pathways (or maintained in their proliferative state) for 14d. At the conclusion of each of these alternatives, the viability of the hASC was assessed with alamarBlue viability assay; this assay was chosen based upon its dye having minimal interactions with Ag-nps [Samberg et al. 2010]. Briefly, a 10% alamarBlue solution was added to each well (Molecular Probes, Invitrogen, Eugene, OR), incubated for 3h and
fluorescence quantitated on a Spectra Max Gemini EM spectrophotometer at an excitation wavelength of 545nm and an emission wavelength of 590nm. The fluorescence values were normalized to controls and expressed as percent viability.

**Cellular Uptake**

To view potential cellular uptake of Ag-nps into hASC, undifferentiated hASC were grown to 100% confluency in complete growth medium for 5d. To determine baseline uptake data, hASC were first differentiated down the osteogenic or adipogenic pathways or maintained in their proliferative state for 14d and then exposed for 24h to concentrations of 0, 0.1, 1.0, 10.0, 50.0 and 100.0µg/ml of either 10nm or 20nm Ag-nps. As a comparison to examine any morphological and ultrastructural effect internalized Ag-nps have on hASC, cells were first exposed for 24h to Ag-nps and then differentiated down the osteogenic or adipogenic pathways (or maintained in their proliferative state) for 14d. At the conclusion of each of these alternatives, cells were harvested with trypsin, rinsed in Hank’s balanced salt solution, and fixed for 24h in Trump’s fixative at 4°C. Cells were rinsed in 0.1M PB (pH 7.2), pelleted in a microfuge tube, resuspended, and quickly pelleted in 3% molten agar. Samples were post fixed in 1% osmium tetroxide in 0.1M PB for one hour at 4°C, washed with DI water, dehydrated through an ascending ethanol concentration, cleared in acetone, infiltrated and embedded with Spurr’s resin, and polymerized at 70°C overnight. Cells were not stained to allow for better visualization of the Ag-nps and to ensure the absence of stain precipitate artifacts. TEM analysis was performed to determine if any morphological or ultrastructural changes occurred between untreated cells and cells exposed to Ag-nps either
prior to or following differentiation, as well as any abnormalities in intracellular lipid accumulation within adipogenic cells or extracellular calcium accretion by osteogenic cells.

**Effects on Differentiation**

To assess any effects of Ag-nps on hASC differentiation, undifferentiated hASC were grown to 100% confluency in complete growth medium for 5d. Then, hASC were exposed for 24h to either 10nm or 20nm Ag-nps at concentrations of 0, 0.1, 1.0, 10.0, 50.0 and 100.0µg/ml, made fresh in complete growth medium. After 24h the medium was removed, and cells were differentiated down the osteogenic or adipogenic pathways or maintained in their proliferative state for 14d. Osteogenic differentiation was determined with Alizarin Red S staining for extracellular calcium accretion, and adipogenic differentiation was determined with Oil Red O staining for intercellular lipid accumulation. To assess differences in staining between control and treatment wells, digital light microscopy images were obtained from each well (n=6 wells per treatment), and the pixel area per image (3 images/well) covered by the stained calcium or lipid was determined by color-based image analysis using Adobe Photoshop.

**Statistical Analysis**

The mean values for hASC percent viability (normalized to control) and pixel area per image for each treatment were calculated, and the significant differences ($p<0.05$) determined by the PROC GLM Procedure (SAS 9.1 for Windows; SAS Institute, Cary, NC). When significant differences were found, multiple comparisons were performed with the Tukey’s Studentized Range HSD test at $p<0.05$ level of significance. The Dunnett’s t-test
was performed to determine the significance at p<0.5 of differences between control and treatment group. Data were expressed as the mean ± Standard Error of the Mean (SEM) of two plates (n=6 wells per treatment) and three images per treatment.

Results

Ag-nps Characterization

The physicochemical properties of the Ag-nps can be found in Table 1, which lists the physical and hydrodynamic diameters of the Ag-nps according to TEM and DLS, respectively, as well as the supplied Ag-nps solution concentration, particle concentration, and zeta potential. As is typical for nanomaterials, the hydrodynamic diameter of the 10nm and 20nm Ag-nps measured by DLS was found to be slightly larger (21.7 and 27.4 nm, respectively) than the physical diameter measured by TEM (7.2 and 19.2nm, respectively). The significantly larger hydrodynamic diameter of the 10nm Ag-nps is most likely attributable to the formation of small, loosely bound Ag-nps dimers. The zeta potential of a colloid characterizes its stability and ability to resist aggregation, with greater zeta potentials having greater stability. Both the 10nm and 20nm Ag-nps had zeta-potentials between -40 and -50eV which indicated good stability. The stability and lack of aggregated clusters is largely to the addition of stabilizing citrate buffer. According to TEM, both 10nm and 20nm Ag-nps were found to be spherical in shape and highly uniform in size (Figure 1A-B).
Table 7.1- Physicochemical Properties of Ag-nps

<table>
<thead>
<tr>
<th>MDD</th>
<th>DLS diameter (nm)</th>
<th>TEM diameter (nm)</th>
<th>Supplied Concentration (mg/ml)</th>
<th>Particle Concentration (particles/ml)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>21.7±0.3</td>
<td>7.2±1.2</td>
<td>1.00</td>
<td>4.8×10^{14}</td>
<td>-40.6±0.4</td>
</tr>
<tr>
<td>20</td>
<td>27.4±0.2</td>
<td>19.2±2.2</td>
<td>1.00</td>
<td>2.6×10^{13}</td>
<td>-49.8±0.5</td>
</tr>
</tbody>
</table>

aData are expressed as Mean±SD; in DI water. MDD, Manufacturer-Designated diameter; DLS, Dynamic light scattering; TEM, transmission electron microscopy.

Cell Viability

When undifferentiated hASC were exposed to 10nm and 20nm Ag-nps for 24h, and then differentiated for 14d, no significant decrease in viability was observed for the undifferentiated hASC. A concentration-dependent toxicity was observed for both
osteogenic and adipogenic cells starting at an Ag-nps concentration of 10µg/ml (Figure 2A-B). Similarly, the addition of either 10nm or 20nm Ag-nps following hASC differentiation resulted in no significant decrease in viability of undifferentiated hASC at any concentration, while concentration dependent toxicity was observed for 10nm Ag-nps at 100µg/ml for osteogenic cells and at 10µg/ml for adipogenic cells (Figure 2C), and for 20nm Ag-nps at 50µg/ml for osteogenic cells and at 100µg/ml for adipogenic cells (Figure 2D). It should be noted that although significant decreases in viability were observed, the maximal loss of viability was only 30%.

Figure 7.2- AlamarBlue viability of undifferentiated hASC treated with either 10nm or 20nm Ag-nps and then differentiated for 14d (A and B); and hASC differentiated for 14d and then treated with 10nm or 20nm Ag-nps (C and D). *†p<0.05 significant differences in viability were assessed by multiple comparisons between concentration.
**Cellular Uptake**

Exposure of 10nm and 20nm Ag-nps to differentiated hASC served as the control for the evaluation of 10nm and 20nm Ag-nps exposure to undifferentiated hASC. Any morphological and ultrastructural changes in cells, as well as changes in intracellular lipid accumulation in adipogenic cells and extracellular calcium accretion in osteogenic cells, were compared to untreated cells. It was observed that for each treatment group, approximately 50% of all cells contained Ag-nps within their cytoplasmic vacuoles, and an increase in treatment concentration resulted in an increase in Ag-nps agglomerates within the vacuoles. Transmission electron micrographs of cells exposed to 10µg/ml are shown as a representative sample (Figures 3-5).

![TEM images](image)

Figure 7.3- TEM images of hASC exposed to either 10nm or 20nm Ag-nps at 10µg/ml. A) hASC; B) hASC treated with 10nm Ag-nps; C) higher magnification of vacuole containing Ag-nps; D) higher magnification of vacuole containing Ag-nps; E) higher magnification of vacuole containing Ag-nps.
Ag-nps from B; D) hASC treated with 20nm Ag-nps; E) higher magnification of vacuoles containing Ag-nps from D. Bars= 2µm (A, B, D); Bars= 200nm (C, E). Arrows denote agglomerates of Ag-nps.

Neither 10nm nor 20nm Ag-nps exposure to either undifferentiated or differentiated hASC resulted in any morphological abnormalities to cells compared to controls. Control hASC were slightly elongated and displayed indented nuclei, a prominent nucleolus, and small intracytoplasmic vacuoles along the periphery of the nucleus (Figure 3A). Small agglomerates of 10nm Ag-nps were observed at low magnification within cytoplasmic vacuoles of hASC (Figure 3B), and high magnification of these vacuoles confirmed the presence of Ag-nps through the highly uniform shape and size of the Ag-nps (Figure 3C). Similarly, 20nm Ag-nps were seen in small agglomerates within vacuoles along the periphery of the nucleus (Figure 3D). High magnification confirmed numerous Ag-nps within these vacuoles (Figure 3E).
Figure 7.4- Transmission electron micrographs of adipogenic cells exposed to either 10nm or 20nm Ag-nps at 10µg/ml.  A) Adipogenic cell showing spherical lipid accumulation, B) adipogenic cell treated with 10nm Ag-nps, C) higher magnification of the vacuole containing Ag-nps from B, D) adipogenic cell treated with 20nm Ag-nps, E) higher magnification of the vacuole containing Ag-nps from D.  Bars= 2µm (A, B, D); Bars= 200nm (C, E). Arrows denote agglomerates of Ag-nps.

Both 10nm and 20nm Ag-nps exposure to hASC prior to, or following, adipogenic differentiation did not result in any morphological or ultrastructural changes to cells compared to controls. Control adipogenic cells exhibited a more globular morphology with extensive lipid droplet accumulation within the cell (Figure 4A). Small agglomerates of 10nm Ag-nps were observed at low magnification within cytoplasmic vacuoles of unstained
adipogenic cells, and Ag-nps were not observed within the lipid droplets (Figure 4B). High magnification of these vacuoles confirmed the presence of Ag-nps through the highly uniform shape and size of the Ag-nps (Figure 4C). Similarly, 20nm Ag-nps were seen in small agglomerates within cytoplasmic vacuoles (Figure 4D), and high magnification again confirmed Ag-nps within these vacuoles (Figure 4E).

Figure 7.5- Transmission electron micrographs of osteogenic cells exposed to either 10nm or 20nm Ag-nps at 10µg/ml. A) Osteogenic cell showing extracellular calcium accretion (dark dots), B) higher magnification of accreted calcium, C) osteogenic cell treated with 10nm Ag-nps, D) higher magnification of the vacuole containing Ag-nps from C, E) adipogenic cell treated with 20nm Ag-nps, F) higher magnification of the vacuole containing Ag-nps from E. Bar=5µm (A); Bars=2µm (B, C, E); Bars= 200nm (D, F). Arrows denote agglomerates of Ag-nps.
Both 10nm and 20nm Ag-nps exposure to hASC prior to, or following, osteogenic differentiation did not result in any morphological changes to the cells compared to controls. Control osteogenic cells were more fusiform (Figure 5A), with extensive extracellular calcium accretion (Figure 5B). Numerous small agglomerates of 10nm Ag-nps were observed at low magnification within cytoplasmic vacuoles of the osteogenic cells (Figure 5C), and high magnification of these vacuoles confirmed the presence of Ag-nps through the highly uniform shape and size of the Ag-nps (Figure 5D). Similarly, 20nm Ag-nps were seen in small agglomerates within vacuoles (Figure 5E), and high magnification confirmed Ag-nps within these vacuoles (Figure 5F).

**Effects on Differentiation**

Exposure of undifferentiated hASC to either 10nm or 20nm Ag-nps for 24h at any concentration did not influence hASC differentiation. Light micrographs of cells exposed to 10µg/ml are shown as a representative sample (Figures 6-8).
Figure 7.6- Light micrographs of undifferentiated hASC exposed to either 10nm or 20nm Ag-nps at 10µg/ml showing no stain for either calcium accretion with Alizarin Red S (A-C) or for lipid accumulation with Oil Red O (D-F). hASC (A, D), exposed to 10nm Ag-nps (B, E), or 20nm Ag-nps (C, F). Bars=0.1mm

Undifferentiated hASC were stained for calcium accretion with Alizarin Red S (Figure 6A-C), and lipid accumulation with Oil Red O (Figure 6D-F). Since the hASC were maintained in complete growth medium, control cells stained for neither calcium (Figure 6A) nor lipid (Figure 6D). There was no significant occurrence of either lipid or calcium following exposure to 10nm Ag-nps at any of the concentrations as evidenced by a lack of calcium and lipid staining (Figure 6B, E). These results were consistent for 20nm Ag-nps (Figure 6C, F).
Figure 7.7- Light micrographs of adipogenic cells exposed to either 10nm or 20nm Ag-nps at 10µg/ml showing no stain for calcium accretion with Alizarin Red S (A-C) and strong staining for lipid accumulation with Oil Red O (cherry red spots; D-F). Control cells (A, D), exposed to 10nm Ag-nps (B, E), or 20nm Ag-nps (C, F). Bars=0.1mm

Adipogenic cells were stained for calcium accretion with Alizarin Red S (Figure 7A-C), and lipid accumulation with Oil Red O (Figure 7D-F). Control adipogenic cells did not stain for calcium (Figure 7A) and stained strongly for lipid (Figure 7D). There was no significant change in lipid accumulation or calcium accretion following exposure to 10nm Ag-nps at any concentration as evidenced by the continued lack of calcium staining (Figure 7B) and continued strong lipid staining (Figure 7E). Similarly, exposure to 20nm Ag-nps at any concentration did not influence the differentiation down the adipogenic pathway, as
evidenced by the continued lack of calcium staining (Figure 7C), and continued strong lipid staining (Figure 7F).

Figure 7.8- Light micrographs of osteogenic cells exposed to either 10nm or 20nm Ag-nps at 10µg/ml exhibiting strong staining for calcium accretion with Alizarin Red S (red spots; A-C) but none for lipid accumulation with Oil Red O (D-F). Control cells (A, D), exposed to 10nm Ag-nps (B, E), or 20nm Ag-nps (C, F). Bars=0.1mm

Osteogenic cells were stained for calcium accretion with Alizarin Red S (Figure 8A-C), and lipid accumulation with Oil Red O (Figure 8D-F). Control osteogenic cells stained strongly for calcium (Figure 8A) and did not stain for lipid (Figure 8D). There was no significant change in lipid accumulation or calcium accretion following exposure to 10nm Ag-nps at any concentration as evidenced by the continued strong calcium staining (Figure
8B) and continued lack of lipid staining (Figure 8E). Similarly, exposure to 20nm Ag-nps at any concentration did not influence the differentiation down the osteogenic pathway, as evidenced by the continued strong calcium staining (Figure 8C), and continued lack of lipid staining (Figure 8F).

**Discussion**

Bone grafts are the second most transplanted material, second only to blood transfusions [Giannoudis et al. 2005]. Currently, conventional treatment methods for bone replacement are limited to autografts (grafts derived from the patient), and allografts (grafts derived from a donor). While autografts are the most desirable option due to the significantly reduced immune reaction, patients possess a relative lack of supply. The use of cadaverous allografts provides a larger tissue supply but may cause immunological reactions. Alternatively, hASC are capable of providing patient-specific cells for tissue regeneration therapies due to their high proliferation rates and self-renewal capacity, maintenance of the unspecialized state, migration capability, and specialized differentiation into various cell types such as bone, cartilage, fat, and muscle. This differentiation may be achieved through chemical induction; but, differentiation may also be influenced by the material properties of the tissue engineered scaffolds onto which they are seeded.

Every year approximately 300,000 patients who have undergone surgery suffer from surgical site infection [CDC 2011]. Studies investigating the effects of surgical site infection have estimated that an infection increases patient stay by an additional 5 days at a cost of nearly $18,000 per patient, bringing the yearly cost of surgical site infection patient care to over ten billion dollars [CDC 2011]. So, while the development of tissue engineering of
patient-specific bone grafts have become a viable alternative to autografts and allografts, the implantation of these tissue grafts would still suffer from the high rate of surgical site infection. To overcome this, once an optimal scaffolding material is achieved for each tissue type, antimicrobial activity to prevent infection could be achieved through the incorporation of Ag-nps. The indiscriminate activity of Ag-nps and the released ions would provide broad-spectrum antimicrobial activity to scaffolding, and decreasing the rate of incidence of infection while improving the success rate and ultimate feasibility of tissue engineered grafts. Additionally, this would help to decrease the widespread use of prophylactic antibiotics that has increased the occurrence of bacterial resistance to typical antibiotics such as methicillin. Therefore, the interaction and metabolism of Ag-nps with hASC should be minimal for their incorporation into a scaffold to be successful. However, the biocompatibility of Ag-nps with hASC is a prerequisite to their incorporation into scaffolding. Therefore, this study validated the use of Ag-nps incorporation into a tissue engineered scaffolding by assessing the toxicity, cellular uptake, and effect on hASC differentiation following exposure to either 10nm or 20nm Ag-nps at concentrations of 0.1, 1.0, 10.0, 50.0 and 100.0µg/ml both prior to, and following, hASC differentiation down their adipogenic and osteogenic pathways.

Fortunately, there are a number of studies that have shown that internalized nanoparticles have no apparent deleterious effects on the morphology, cell proliferation, cell viability, and differentiation efficiency of mesenchymal stem cells [Chung et al. 2007, Huang et al. 2008]. Studies involving easily functionalized polystyrene and silica nanoparticles and derivatized magnetic nanoparticles used for biomedical imaging, drug delivery, and gene therapy have shown that their internalization into hASC does not illicit cytotoxicity [Chung et al. 2007, Huang et al. 2008, Lewin et al. 2000, Lorenz et al. 2006]. Multi-walled carbon
nanotubes incorporated into tissue engineered scaffolding were not shown to affect the viability or proliferation of hASC [McCullen et al. 2007]. Additionally, nanoparticles composed of osteogenic factors have shown remarkable improvements in mesenchymal stem cell adhesion, attachment, and differentiation on tissue engineered bone scaffolds [Lock and Liu 2011].

The results of this study were similar to our previous research which showed that washed 20, 50 and 80nm Ag-nps did not cause a decrease in viability to human epidermal keratinocytes [Samberg et al. 2010]. This study determined that metallic nanoparticles such as Ag-nps are capable of interfering with viability assay dye and dye products through the adsorption of cell medium constituents and cytokines, which is a common effect and has been reported for several other viability assays and nanomaterials [Monteiro-Riviere and Inman 2006, Monteiro-Riviere et al. 2009, Zhang et al. 2007]. Based upon these findings, alamarBlue was chosen for this study due to its limited interaction with Ag-nps. These same Ag-nps have already been evaluated for their antibacterial efficacy in a previous study, and were found to inhibit the growth both gram-positive and gram-negative bacterial strains such as Escherichia coli, Staphylococcus aureus, Methicillin-Resistant Staphylococcus aureus and Salmonella beginning at approximately 64µg/ml. In the same study, AgNO₃ was used as a positive control for Ag ions and was shown to inhibit the strains at 1.7µg/ml, indicative that the 64µg/ml inhibitory concentration of Ag-nps released at least an equivalent 1.7µg/ml of Ag⁺. Therefore, the concentrations used in the present study were chosen to encompass the bacterial inhibitory concentration [Samberg et al. 2011].

There is general agreement that dissolved Ag ions are responsible for the biological action that is especially pronounced against microorganisms, and this ability of Ag-nps to
slowly release these ions from their surface has made them an attractive antimicrobial additive to products. The rate of dissolution is obviously a complicated process that is dependent upon many factors including Ag-nps synthesis method, storage length before use, temperature, light exposure, surface functionalization, and cell culture conditions [Kittler et al. 2010]. The biological activity of freshly prepared and aged Ag-nps is remarkably different due to the different amounts of released ions. For example, in a study that utilized hASC as reporter cells to analyze the effect on cell viability and morphology from the release of Ag ions over time from 50±20nm citrate-stabilized Ag-nps, it was found that 25µg/ml of 3-day old Ag-nps were enough to cause a decrease in viability to 70%, compared to a complete loss of viability for Ag-nps first aged in water for 1 to 6 months [Kittler et al. 2010]. Similarly, 8nm Ag-nps in DI water were shown to release less than 0.1µg/ml of Ag ions after 1d of incubation and up to 2µg/ml after 120d of incubation, while dissolution rates decreased with the addition of citrate-stabilization or in the presence of organic matter [Liu and Hurt 2010]. Additionally, the investigators showed that their release rates were congruent with commercial Ag-nps stabilized with gelatin and gum Arabic, similar to previous reports [Liu and Hurt 2010, Braydich-Stolle et al. 2005].

Axiomatically, the lethal concentration of Ag-nps will be higher than that of Ag⁺ due to the limited release rate of Ag⁺ from the surface of the particles. The lethal concentration of Ag⁺ to human cell lines such as germ-line stem cells, dermal fibroblasts, and leukocytes is generally below 5µg/ml, so it is curious that many papers report toxicity of Ag-nps at the same concentration of reported toxicity of Ag⁺ alone [Liu and Hurt 2010, Braydich-Stolle et al. 2005, Hidalgo and nguez 1998, Jansson and Harms-Ringdahl 1993, Park et al. 2010]. For example, 15nm Ag-nps synthesized in a pulsed-plasma reactor have also caused
toxicity in germ-line stem cells at 10µg/ml [Braydich-Stolle et al. 2005]. Controversy concerning the concentration of Ag-nps toxicity is most likely due to the Ag-np synthesis method, whether or not the Ag-nps are washed, and their length of storage prior to use. Ag-nps solutions may contain residual reducing agent contaminants that are known toxicants, a finding confirmed in this lab where the presence of formaldehyde in freshly synthesized Ag-nps solutions was found to be responsible for the toxicity in skin cells compared to thoroughly washed Ag-nps [Samberg et al. 2010]. The citrate stabilization of the 10nm and 20nm Ag-nps used in this study may explain their higher lethal concentration, which is congruent with the rare studies that explicitly state that when particles are washed they result in higher lethal concentrations compared to studies that do not mention washing, generally greater than 25µg/ml [Samberg et al. 2010, Kittler et al. 2010, Ahamed et al. 2008, Liu et al. 2010b].

The results of this study depicted that exposure of 10nm and 20nm Ag-nps either prior to differentiation, or following 14d of hASC differentiation, resulted in no significant cytotoxicity to hASC, and minimal dose-dependent toxicity to adipogenic and osteogenic cells at 10µg/ml and greater. Drawing on the results of the previous bacterial study, it is reasonable to conclude that at least 1.7µg/ml was released from the Ag-nps at the highest concentration of 100µg/ml and resulted in increased cell toxicity. In each of the analyses where the hASC were first exposed to Ag-nps and then differentiated, residual Ag-nps remained attached to the cells throughout the 14d of differentiation, which was confirmed by TEM. This is particularly relevant; since degradable tissue engineered scaffolds containing Ag-nps would shed these nanoparticles and their ions over the course of the scaffold lifetime to impart their antimicrobial activity, and would therefore interact with the seeded progenitor
cells over the course of their differentiation. Each of the progenitor, adipogenic and osteogenic cells showed cellular uptake of both 10nm and 20nm Ag-nps, without eliciting and abnormal morphological or ultrastructural changes. Lastly, exposure to 10nm and 20nm Ag-nps for 24h did not influence the intended differentiation of the undifferentiated hASC, adipogenic, or osteogenic cells and at the antibacterial concentration of 64µg/ml, the Ag-nps showed minimal toxicity.

Conclusions & Future Perspective

This study showed that exposure of hASC to 10nm and 20nm Ag-nps either prior to differentiation, or following 14d of differentiation, resulted in no significant cytotoxicity to undifferentiated hASC. Minimal dose-dependent toxicity was observed for adipogenic and osteogenic cells at concentrations of 10µg/ml and greater. The cellular uptake of Ag-nps into each of the hASC, osteocytes and adipocytes did not alter their ultrastructural morphology, or influence hASC differentiation. Exposure of 10nm and 20nm Ag-nps at concentrations up to 100µg/ml did not influence hASC differentiation. Although the cell quantity limited this study to the investigation of Ag-nps effect on cell viability, uptake and differentiation, these results reasonably conclude that either 10nm or 20nm Ag-nps are suitable to incorporate into tissue engineered scaffolds that utilize hASC.

Further studies may be conducted to ensure the biocompatibility of the Ag-nps with hASC, including assays for hASC immunophenotype preservation or DNA stability, cytokine release, or the effects of multiple Ag-nps exposures during differentiation. These results are promising for future studies where Ag-nps will be incorporated into tissue engineered scaffolds. Previously evaluated scaffolding for bone graft therapies comprised of collagen,
collagen coated poly(ε-caprolactone), and poly(L-lactic acid)/tricalcium phosphate nanofibers has been shown to increase cell-mediated calcium accretion [Sumanasinghe et al. 2006, Haslauer et al. 2011, McCullen et al. 2009], and would be a suitable model to evaluate Ag-nps incorporation. The addition of antimicrobial functionality, namely through the incorporation of Ag-nps into a tissue engineered graft scaffold would be pivotal in preventing bacterial growth and thereby minimizing infections, as well as patient and hospital costs.

Executive Summary

- Exposure of 10nm and 20nm Ag-nps either prior to differentiation, or following 14d of hASC differentiation, resulted in no significant cytotoxicity to hASC. Minimal dose-dependent toxicity was observed for adipogenic and osteogenic cells at concentrations of 10µg/ml and greater.
- Exposure of 10nm and 20nm Ag-nps either prior to differentiation, or following 14d of hASC differentiation, resulted in cellular uptake into each of the progenitor, adipogenic and osteogenic cells, without eliciting ultrastructural alterations.
- Exposure of 10nm and 20nm Ag-nps for 24h did not influence the intended differentiation of the cells.
- Ag-nps are suitable for tissue engineered scaffolding incorporation at the antibacterial concentration of 64µg/ml.

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8. Conclusions and Future Recommendations

Conclusions

In addressing the question of Ag-nps safety, this series of studies has been successful in evaluating the toxicity and inflammatory potential of Ag-nps of various sizes, surface conditions, and synthesis methods. This research helps to provide a better understanding of the biological interactions between Ag-nps and human cells, specifically HEK and hASC, and the risks associated with Ag-np exposure. Due to their minimal toxicity, lack of effect on intended cell differentiation, proven antibacterial efficacy, and minimal effect on scaffold mechanical properties, this report strongly suggests their use in tissue engineered scaffolds. These studies have clearly provided an interdisciplinary approach and made a significant contribution to research in the fields of toxicology and tissue engineering.

Future Recommendations

The study described in Chapter 7 investigated the fabrication and testing of an Ag-nps incorporated electrospun skin scaffold for skin tissue engineering applications. As the scaffold failed to release sufficient Ag-nps to produce an antibacterial effect, future research should investigate the fabrication of a scaffold with a greater concentration of Ag-nps in order to achieve antibacterial efficacy. Also combining this electrospun layer with another electrospun scaffold seeded with dermal fibroblasts may be the next step to achieve a multi-layer composite scaffold structure so as to mimic the hierarchical configuration of the skin.

The study described in Chapter 8 investigated the feasibility of using antibacterial Ag-nps in a tissue engineered scaffold that utilizes adipose derived stem cells. The low level of toxicity and lack of effect to hASC differentiation reasonably concluded that either 10nm
or 20nm Ag-nps are suitable for incorporation into tissue engineered scaffolds that utilize hASC. Further studies could be conducted to ensure the biocompatibility of the Ag-nps with hASC, including assays for hASC immunophenotype preservation or DNA stability, cytokine release, or the effects of multiple Ag-nps exposures during differentiation. Additionally, the logical next step is their incorporation into a suitable scaffold, optimized for the intended growth and differentiation of hASC.
9. References


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