Combating Antibiotic Resistance Through Development of a Novel Antimicrobial Delivery Vehicle

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COMBATING ANTIBIOTIC RESISTANCE THROUGH DEVELOPMENT OF A NOVEL ANTIMICROBIAL DELIVERY VEHICLE

by

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Thesis submitted in partial fulfillment of the requirements for the degree of

DEPARTMENTAL HONORS

in

Biological Engineering in the Department of Biological Engineering

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Combating Antibiotic Resistance Through Development of a Novel Antimicrobial Delivery Vehicle

Alex Hatch, Alyssa Calder, Cody Gunnell, Aaron Winder

Senior Design Final Report/Honors Senior Thesis
Project Summary:

A thermoreversible gel for antimicrobial agent delivery would provide a novel platform suitable for medical applications from persistent infections arising from localized biofilms. Thermoreversible gels could be used to coat medical implants, to inject for subdermal treatments, or to implement in topical applications. FDA approved Pluronics® form thermoreversible gels and certain Pluronics® display inherent varying degrees of antimicrobial activity. Pluronics® have been shown to affect efflux pumps, and reduce ATP levels within the bacterial cell. In combination with antibiotics and other pharmaceuticals, Pluronics® have been used to sequester drugs and act as a delivery vehicle. With advances in nanomedicine, nanoparticles offer alternatives to traditional antibiotics. Silver nanoparticles (Ag NPs) have been shown to affect enzymes, bind to DNA, and induce structural changes in bacterial membranes. These properties make Ag NPs a good candidate for overcoming antibiotic resistant bacteria. We designed a thermoreversible gel combining Pluronic® F-127 and Ag NPs that can be applied as a liquid to conform to the site, followed by gelation and sustained release of antimicrobials or other therapeutics.

Aims:

- Select thermoreversible polymer based on cost, application, and ease of implementation.
- Synthesize Ag NPs of a consistent shape and dimension.
- Build on the work of Wang (2007) by synthesizing Ag NPs using a thermoreversible solution ready for immediate use.
- Create thermoreversible, pharmaceutical formulation containing combinations of Pluronic® F-127 and Ag NPs.
- Analyze the antibiotic effects of combining Pluronic® F-127 and Ag NPs in a single formulation.
- Characterize physical properties of the thermoreversible gel and Ag NPs.

Background:

A worldwide increase of nosocomial infections affects thousands of people each year. Individuals admitted to or treated in hospitals often have a weakened immune response that predisposes them to infection. Not only is an acquired infection an undesirable consequence of being treated in a hospital, but the problem is complicated by an increasing number of infections from bacteria exhibiting antibiotic resistance, the result of selective pressures in the hospital environment. Addressing this development has proven to be a challenge to public health officials and hospital administrators.

The introduction of antibiotics and development of new formulations of antibiotics has been a focus in medicine since the early 1900s. Antibiotics have three principle targets. The first is the machinery involved in cell wall biosynthesis. This is accomplished using Beta-Lactam drugs like penicillins and cephalosporins and also the non-beta-lactam drug, vancomycin. The second antibiotic target includes factors involved in bacterial protein synthesis. These antibiotics take advantage of non-eukaryotic characteristics of ribosomal subunits and other factors involved with initiation, elongation, and termination in bacterial protein synthesis. Drugs effective in this class include macrolides, tetracyclines, and aminoglycosides. The third principle target of
antibiotics is bacterial DNA replication and repair. Antibiotics in this class inhibit enzymes such as topoisomerases and DNA gyrase and include the fluoroquinolones. A final general classification of antibiotics describes a drug's effectiveness as broad spectrum--affecting both gram positive and gram negative bacteria--and narrow spectrum, affecting only one or the other. Effective antibiotics target characteristics associated with bacteria but not employed by the host organism (Walsh 2000).

Antibiotic resistance is the result of bacterial characteristics that nullify the action of the drug on the target. One type of antibiotic resistance involves preventing the antibiotic from reaching its target in sufficient concentration. The cell accomplishes this in part by having a protective coating like a peptidoglycan layer and or a lipid polysaccharide layer. Gram negative bacteria possess both an inner and outer plasma membrane that reduces drug diffusion rates. Additionally, planktonic bacteria (free floating) are more susceptible to drug transport than bacteria that colonize and form biofilms. The complex matrix associated with biofilms typically contains an exopolysaccharide layer, significantly reduces drug diffusion and the ability of the drug to reach its target. An additional challenge with biofilms is that a certain number of bacteria are dormant and not susceptible to antibiotics that target metabolically active bacteria.

Another type of resistance results when both gram negative and gram positive bacteria produce membrane proteins that act as efflux pumps, exporting toxic materials outside of the cell. Efflux pumps can exhibit a low degree of specificity allowing the expulsion of multiple antibiotics, and in some resistance mechanisms these pumps are overproduced. The effectiveness of the efflux pumps is increased by the ability of the bacteria to slow diffusion rates of drugs as discussed above (Nikaido 1998). Two common and problematic, opportunistic pathogens have developed resistance that incorporates this paradigm. *Pseudomonas aeruginosa* has a low susceptibility to antibiotic treatment due to the action of multidrug efflux pumps and low permeability of the antibiotics across bacterial cellular envelopes (Poole 2004).

Another means of antibiotic resistance involves the modification of the antibiotic. This is a prevalent method of resistance in beta-lactam antibiotics. The beta-lactam structure functions by transferring an acyl group to the active site of bacterial enzymes involved in the synthesis of cell wall components (Walsh 2000). Resistant bacteria enzymatically hydrolyze the beta-lactam structure rendering the drug ineffective. Methicillin resistant *Staphylococcus aureus* (MRSA) is characterized by the ability to modify this class of antibiotics. MRSA is one of the major antibiotic resistant bacteria affecting hospitals and care centers and was reported to be the cause of 63% of the total staph infections in the US in 2004 (Pray 2008).

Current methods employed to counter antibiotic resistance focus on: modifying the active portion or substituents of the active portion of antibiotics, developing new synthetic antibiotics, identifying the genetic causes of antibiotic resistance and suppressing the expression of those genes, and educating health providers and their patients about correct therapeutic doses and rotation of antibiotic treatment in a hospital setting (Walsh 2000).

The present project focused on the development of an engineered antimicrobial formulation capable of targeting multiple cell functions and countering understood antibiotic
resistance mechanisms. While most antibiotics target one mode of cellular action, a new field is looking at general antimicrobial agents, such as silver, that target multiple cellular pathways. Silver has long been known as an antimicrobial agent; although the mechanism of action is not well understood. It is thought that silver ions interact with bacterial enzymes and deactivate them, bind to DNA, or induce structural changes in the cell membrane (Morones 2005 and Lok 2007). Because silver ions target a broad range of processes, bacterial organisms are less likely to develop resistance to silver. Silver ions are useful in the treatment of topical bacterial infections in humans because silver is nontoxic to humans in minute concentrations (Pal 2007).

Even though silver ions are effective antimicrobial agents the properties of nanoparticles provide an interesting alternative. The field of nanotechnology is increasingly gaining more interest and attention. In 2005 the total global investment in nanotechnology was $10 billion and is estimated to reach $1 trillion by 2015 (Roco 2005). Ag NPs are being used in various areas such as textiles, pharmaceutics, paint and cosmetics due to their antimicrobial properties (Navarro 2008). A true nanoparticle must have at least one dimension measuring smaller than 100 nm (Navarro 2008). The unique properties of nanoparticles are due to their small size creating a high surface area to volume ratio. High specific surface area means more atoms are on the exterior of nanoparticles (Navarro 2008). Indeed, the antimicrobial effects of silver increases as particle size decreases (Pal 2007). Ag NPs are made by two methods, using laser ablation or the reduction of AgNO$_3$ by a variety of compounds (Mafune 2000 and Dadosh 2009). Each method causes Ag NPs to come in a variety of shapes such as spherical, cuboidal, rod-shaped, and triangular. Ayyad (2010) formed Ag NP’s in the presence of agar biopolymers and found that using a biopolymer matrix gives greater control over size, dispersion and shape of the particles. The shape and size of Ag NPs is determined using TEM imaging among other diagnostic methods. Dadosh (2009) found that reduction of AgNO$_3$ with tannic acid produced spherical nanoparticles with a diameter between 18-30 nm. Wang (2004) used the Pluronic$®$ F-127 to create rod-shaped nanoparticles. It has also been shown that fungi can reduce silver nitrate to create spherical nanoparticles with a diameter between 60-80 nm (Birla 2008). It has been found that the shape of the Ag NPs will determine the extent of toxicity. Truncated triangular Ag NPs display the strongest antimicrobial properties followed by spherical and rod-shaped (Pal 2007). This could be due to the fact that the points of the triangles are able to puncture cells or they are more readily release silver ions. Triangular shape can be achieved by synthesizing Ag NP’s in the presence of UV or light radiation, aromatic solvents, or heat. (Deivaraj 2005, Callegari 2003, Jia 2006)

Research has been done determining the minimal inhibitory concentration (MIC) of Ag NPs for a variety of organisms. The MIC for $E. coli$ is around 20 mg/L with 12 nm spherical nanoparticles (Sondi 2004) whereas the MIC for $P. aeruginosa$ is 25 mg/L with 21 nm icosahedral nanoparticles (Morones 2005). Using only Ag NPs as an antimicrobial agent would require a fairly large amount of the expensive Ag metal. Combining Ag NPs with other compounds could limit the amount of silver actually needed and make medical treatments more economically feasible. Simple zone of inhibition tests have been done combining Ag NPs and antibiotics. In these tests Ag NPs did increase the zone of inhibition for ampicillin, gentamycin, kanamycin, streptomycin, and vancomycin against $P. aeruginosa$, $E. coli$, and $S. aureus$ (Birla 2008). Ag NPs have also been sequestered in hydrogel networks for controlled release and coated with amphiphilic molecules such as amidated polyethyleneimine (PEI) which do not
release the nanoparticles but still maintain the antibacterial effect (Aymonier 2002, Ma 2007, Chudasama 2010). Amphiphilic molecules have also been used as a capping agent to stabilize Ag NPs and prevent aggregation (Shervani 2008).

Thermoreversible gels are a valuable tool in drug delivery. These gels have low critical solution temperatures (LCST) that allow them to have a phase transition from liquid to gel at temperatures ranging from 0-50 °C. The liquid phase allows for easy loading of a drug and the gel phase provides a stable delivery vehicle. Many thermoreversible gels are triblock copolymers. Triblock copolymers are amphiphilic molecules that spontaneously form micelles with a hydrophobic core in aqueous environments. This core allows sequestration of hydrophobic drugs in an aqueous environment (Shah 2003). This is known as the critical micelle concentration (CMC). The properties of PEG-PLGA-PEG, PCL-PEG-PCL, and PEO-PPO-PEO (Pluronic®) triblock copolymers will be introduced and compared to determine the best delivery vehicle for topical antimicrobial applications.

The triblock copolymer poly-(ethylene glycol-b-(DL-lactic acid-co-glycolic acid)-b-ethylene glycol) (PEG-PLGA-PEG) has thermoreversible gelation properties, Figure 1. PEG-PLGA-PEG is not commercially available, but is synthesized by ring opening polymerization of DL-lactide and glycolide initiated by monomethoxy poly (ethylene glycol) creating PEG-PLGA. Adding hexamethylene diisocyanate to the diblock copolymer results in the formation of PEG-PLGA-PEG. This triblock copolymer will also spontaneously form micelles above certain concentrations with the hydrophilic PEG portion on the outside of the micelle in aqueous solutions (Jeong 1999). This polymer (33 % w/w) is liquid at room temperature (viscosity of 10 cp), but has a phase transition to a solid at body temperature (viscosity around 400 cp). PEG-PLGA-PEG will maintain a 3-D shape in the gel form even when shaken (Jeong 1999). This polymer can be naturally degraded by the body, but maintains the gel form for over 1 month subdermally in rats (Jeong 2000). PEG-PLGA-PEG is not erodible by dilution and is beneficial as a long term drug delivery vehicle (Jeong, Aug 1999). A drug can be sequestered in the polymer by simply adding it to an aqueous solution of the polymer. No organic solvents or surgical procedures are required to sequester the drug in the gel matrix (Jeong 1999).

\[
\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_n[\text{(COCH}_2\text{O})_m\text{(COCH}_2\text{H}_3\text{O})_n]\text{OCNH}(\text{CH}_2)_6\text{NHC}(\text{OCH}_3\text{H}_2\text{CO})_n[\text{(OCH}_2\text{CO})_m\text{(OCH}_2\text{H}_3\text{O})_n]\text{OCCH}_3
\]

![Figure 1. Structure of PEG-PLGA-PEG triblock copolymer (Jeong 1999)](image)

Poly ethylene glycol (PEG) and poly (ε-caprolactone) (PCL) have been widely used in the biomedical field. They are both well-known, FDA-approved, biodegradable and biocompatible materials (Liu 2007), Figure 2. PCL-PEG-PCL is a triblock copolymer that is not commercially available and must therefore be synthesized in lab.
Figure 2. Synthesis scheme and structure of PCL-PEG-PCL triblock copolymer (Liu 2007).

PCL-PEG-PCL block copolymers are prepared using ring-opening polymerization of ε-caprolactone initiated by low-molecular-weight PEG (Mₙ= 400-2000). The triblock components are combined with Sn(Oct)₂ in a three-necked vessel under a dry nitrogen atmosphere. The mixture is then dissolved using toxic dichloromethane, precipitated in excess cold petroleum ether, and then filtrated. A concentration of 15-30% or more of PCL-PEG-PCL is needed in solution in order to gel. Depending on ratio of PCL and PEG the gel transition temperature is between 10-30° C (Gong 2009).

PCL-PEG-PCL was shown to be effective as a drug delivery vehicle in vitro and in vivo as described by Gong et al. In vitro testing was performed using the hydrophobic small-molecule drug VB₁₂, the hydrophilic small-molecule drug, honokiol, and the macromolecular, hydrophilic protein, BSA. In vitro testing showed sustained drug release from a 30% w/w PCL-PEG-PCL gel in PBS buffer for periods over 24 hours with the hydrophilic drug, and 14 days with the hydrophobic drug, and the highest release rate of the protein between 24 and 48 hours. Lidocaine release and effectiveness was tested in vivo in rats, showing effective drug release and effect 4 times as long as lidocaine alone. This gel was also found to be stable in vitro in PBS with shaking for more than 9 weeks at 37° C. Another amphiphilic molecule is the commercially available surfactant known as Pluronic® (Loyd 1994). In general, polyoxamers (Pluronics®) are composed of white, waxy, free-flowing granules that are practically odorless and tasteless (Loyd 1994). Pluronics® are tri-block copolymers consisting of a hydrophobic Polyoxypropylene (PPO) portion and a hydrophilic Polyoxyethylene (PEO) portion arranged in a PEO-PPO-PEO fashion with varying weight proportions of PEO/PPO (Housley 2009).

Pluronic® have been used to sequester several drugs including antibiotics, anti-inflammatory drugs, and cancer treatments. In addition to being a delivery vehicle, Pluronic® have inherent antimicrobial activity (Marsh 2002). Researchers have reported that Pluronics® increase the cytotoxicity of anthracyclines in multiple drug resistant (MDR) cancer cells, inhibit drug efflux transporters in MDR cells, affect the ability of MDR cells to sequester drugs in cytoplasmic vesicles, and induce a reduction in ATP levels selectively in MDR cells (Kabanova 2003). Additionally, several antibiotics coupled with various Pluronics® formulations showed synergistic effects on various microbes. Furthermore, the presence of Pluronics® enables a sustained release of the drug, rendering them effective for extended periods of time. (Veyries 1999, Jagannath 1999) Additionally, reports indicate that exposure of mammalian cells to Pluronics® has negligible effects on the cells and some Pluronics® have been approved for use in humans by the FDA (Kabanova 2003, Faulkner 1997). Pluronics® have a two year shelf life in
The fact that Pluronics® exist in varying sizes and PEO/PPO proportions confers unique properties and determines the class and name of each Pluronic®. From our literature review involving numerous Pluronics® in existence, Pluronic® F-127 and F-68 have been studied and used in medical applications. Pluronic® F-68 has been previously subjected to numerous experimental and clinical studies using mice (Faulkner 1997). This surfactant is not biodegradable and is rapidly excreted from the body after intravenous administration (Faulkner 1997). Pluronic® F-68 and F-127 are commercially available as a solid flake, are soluble in water, and are both approved by the FDA (BASF). We have chosen to use F-127 in our experiment due to the abundance of literature on this Pluronic® as well as procedures and methods on how to synthesize Ag NPs using Pluronic® F-127. Also, in order to form a gel using F-68 the concentration in solution must be at least 60% (w/w) whereas F-127 only requires 20% (w/w) thus making it more economical (Schmolka 1972). A greater molecular weight and higher percentage of PEO in a Pluronic® decreases their cytotoxicity in the body. F-68 and F-127 each have the same percentage of PEO, however, F-68 has a molecular weight of approximately 8,400 Daltons and F-127 is about 12,600 Daltons (Schmolka 1972).

Pluronic® F-127 (Poloxamer 407) (Figure 3) is a nonionic surfactant that has a good solubilizing capacity, low toxicity, and is, therefore, considered a good medium for drug delivery systems. F-127 polymers are produced by condensation of ethylene oxide and propylene oxide (Lundsted 1972.). F-127 is more soluble in cold water than hot as a result of increased solvation and hydrogen bonding at lower temperatures (Gilbert 1986). F-127 in concentrations of 20 to 30% w/w in aqueous solution shows the characteristics of reverse thermal gelation (Miyazaki 1984). These poloxamers are liquid at refrigerated temperatures (4-5˚C), but gel upon warming to room temperature (20°C). This gelation is reversible upon cooling (Jorgensen 1997). The unique thermoreversible and drug release characteristics of F-127 render it an attractive candidate for pharmaceutical drug delivery through different routes of administration. Barichello (1999) found that F-127 gels made at a concentration of 20% released insulin better than 30% F-127 gels. It was also found that gels made at pH 7.0 had a better release rate of insulin than gels at pH 5.5 and 4.0 (Barichello 1999).

Figure 3. Chemical structure of F-127 (Guzman 1994); a = 0.35, b = 0.3

One study used Pluronic® CRL-1072 with antimicrobial agents to modulate surface proteins of Mycobacterium avium and increased antibiotic uptake (Jagannath 1999). Conveniently, Pluronics® are also commonly used as a reducing and capping agent in the synthesis of Ag NPs and are able to stabilize the resulting silver colloid due to their amphiphilic properties (Chudasama 2010, Shervani 2008, Abdullin 2009). After stabilizing Ag NPs with F-68, Ma (2009) introduced the Pluronics® into a hydrogel as a guest molecule. They showed that the silver impregnated hybrid gel demonstrated good catalytic activity in the reduction of
methylen blue, indicating that Ag NP coated with Pluronics® retain their potency. The dissolution of F-127 in human body conditions has also been studied. Liu (2006) found that the gel dissolution rate of 20% F-127 in simulated arterial flow conditions was 0.14 ± 0.007 g/cm²/h.

In both PEG-PLGA-PEG and PCL-PEG-PCL the integrity of the gel can be maintained for weeks after a subdermal injection, whereas Pluronics® only last for a few hours (Gong, 2008). Degradation of Pluronics® occurs by surface degradation while PEG-PLGA-PEG degrades by chemical means (Jeong Sept 1999). PEG-PLGA-PEG can maintain a rigid 3D shape, but this feature would be undesirable in topical applications. The LCST of PEG-PLGA-PEG is very close to body temperature (37°C) so the triblock copolymer would not be in the gel state on the surface of the skin. Pluronics®, on the other hand, are in a gel state at room temperatures (~20°C). Both PEG-PLGA-PEG and PCL-PEG-PCL are not commercially available. They would need to be synthesized by various processes. After the synthesis is complete the structure would need to be verified by extensive methods such as NMR which are costly and time consuming. Pluronics® are commercially available and small samples (~250 g) can be requested for research purposes.

An understanding of properties that confer antibiotic characteristics and an understanding of current resistance mechanisms increases our ability to formulate and design increasingly effective antibiotics. For example, the antibiotic resistance effect of multi drug efflux pumps may be limited by creating a more hydrophilic drug, by limiting the energy available for efflux pump activity, or by increasing the efficiency of delivery of the antibiotics across the membrane. Pluronics® exhibit the potential to meet all of these requirements. The ability of bacteria to enzymatically modify the active portion of drugs may be decreased by the development or identification of materials possessing antibiotic properties. The introduction of effective new antimicrobial formulations is beneficial simply by the fact that it provides new treatment options and increases the ability of physicians to rotate treatments. As shown, Ag NPs overcome several resistance mechanisms simultaneously and their combination with a Pluronic® delivery vehicle may provide a valuable treatment alternative.

**Significance and innovation:**

The project aims to establish a novel method for synthesizing silver nanoparticles with Pluronics® F-127 and simultaneously sequester them in a Pluronics® thermoreversible gel. Ag NPs are less likely to be overcome by antibiotic resistance. The thermoreversible properties of F-127 allow the Ag NPs to be sequestered in a liquid phase and gel at a defined temperature.

**Approach:**

Methods
1) Culturing *P. aeruginosa*
   a. Reviving Freeze Dried Cultures-
      i. Decrimp culture tube or break serum vial
      ii. Resuspend the cell pellet in 0.5 mL of sterile water or liquid LB media
      iii. Spread 100 µL of culture on a LB plate using aseptic technique. Using the same spreader, spread an additional plate
      iv. Incubate plates at 37°C sealed with parafilm resting upright
v. Select colony and inoculate a liquid culture
vi. Create glycerol freezer stock (750 µL of culture, 750 µL of 30 % glycerol). Store at -80˚C

b. Antimicrobial Susceptibility Testing
   i. Take 100 µL of freezer stock and suspend in 1 mL of LB
   ii. Spread 100 µL of suspension on LB agar plate
   iii. Drop cold gel solution (8 µL) from a pipette directly onto plate as well as the bottom and top of a 0.6 cm diameter Whatman qualitative fast filter paper without disturbing the plate
   iv. Incubate plate for 24 hours at 37˚C
   v. Measure the zone of inhibition (diameter)

2) Thermoreversible Gel
   a. Make thermoreversible gel using F-127
      i. For 20% solution: Weigh 20 g F-127, add slowly to 80 ml of water at 5-10˚C in 250 ml beaker while stirring with a magnetic stirring bar. Leave container in refrigerator (4˚C) overnight or place in ice bath and mix slowly until F-127 has completely dissolved (usually 4 hours). Once F-127 has dissolved, bring it back to room temperature. (Schmolka 1972)
      ii. For 30% solution: Weigh 30 g F-127, add slowly to 70 ml of water at 5-10˚C in 250 ml beaker while stirring with a magnetic stirring bar. Leave container in refrigerator (4˚C) overnight or place in ice bath and mix slowly until F-127 has completely dissolved (usually 4 hours). Once F-127 has dissolved, bring it back to room temperature. (Schmolka 1972)

   b. Characterize antimicrobial and physical properties
      i. Test gel on Instron to determine elasticity and ultimate tensile strength
         1. An aliquot of 5.0 g of F-127 gel was added to plastic 50 mm diameter Petri dishes and equilibrated to room temperature (20˚C). The gels were approximately 3 mm tall when solidified in the Petri plates. The elastic modulus of the gels was measured using an Instron 5542 instrument and Bluehill software version 2.21. A plastic 25.5 mm diameter lexan cylindrical head was used in the compression test. Data was recorded from the point the head touched the surface of the gel. The cylinder compressed the gel at a rate of 1.0 mm/min and until a final depth of 1.5 mm.
      ii. Gelation Temperature/LCST
         1. To test the LCST, 5 mL samples of the gels were added to a 15 mL tube. Gels transitioned to the solid phase upon warming. Tubes were inverted using a clamp stand in a cold room (4˚C) and allowed to equilibrate to the new temperature. When gels reached LCST they liquefied falling from the tube. Temperature was measured using an IR temperature gun RYOBI TV4V and recorded.

3) Ag NPs
   a. There are several possible protocols for synthesis of Ag NPs. Protocols listed are in order of intended use.
i. **Using F-127**: A 0.05 M AgNO₃ aqueous solution, instead of water, was used to form a 50% F-127 gel by adding 1 gram of Pluronic® to each ml of AgNO₃. The resulting mixture forms a thick, yellow paste. Mixing was facilitated by centrifugation at 3000 rpm with a radius of 10 cm equally 1008 g’s. Samples were allowed to sit for variable time periods. The mixture was brought down to 20% and 30% solutions by adding dH₂O and further mixed in various exposures to UV light. (Wang et al 2004)

ii. **Tannic Acid**: 20 mL of 6.8 mM tri-sodium citrate with (1.8-23.5 uM) tannic acid was heated to 60 C and added with vigorous stirring to 80 mL of 0.74 mM AgNO₃ preheated to 60 C. The mixture was kept at 60 C for 3 min, or until color turned yellow, boiled for 20 min, cooled down to room temperature and stored in a dark bottle at 4 C. (Dadosh 2009)

b. Characterize Ag NPs
   i. UV-Vis scan on a Thermo Labsystems Multiskan Spectrum Spectrophotometer with Multiskan Spectrum 1.0 software was used to check for the absorbance peak at 410 nm.
   ii. Image Ag NPs to determine shape and size using AFM (Digital Instruments Bioscope Atomic Force Microscope, Model #BS3-N2, Nanoscope software version F.31R1)

4) Characterize thermoreversible gel with Ag NPs
   a. Alterations to gel properties
      i. Gelation temperature
      ii. Elasticity and ultimate tensile strength
   b. Antimicrobial activity of Gel + Ag NPs

**Materials**

<table>
<thead>
<tr>
<th>Glassware/Labware/General Supplies:</th>
<th>Diagnostic Instruments:</th>
<th>Specific Products:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carboid</td>
<td>UV-vis spectrophotometer</td>
<td>F-127</td>
</tr>
<tr>
<td>Glassware</td>
<td>Cuvettes</td>
<td>Deionized Water</td>
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<tr>
<td>250 mL beakers</td>
<td>AFM</td>
<td>Ethanol</td>
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<td>50 mL Centrifuge tubes</td>
<td>λ = 254 nm</td>
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<td>Microcentrifuge tubes (1.5 mL)</td>
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<td>Citric Acid</td>
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<tr>
<td>Biohazard Bags</td>
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<td><em>Pseudomonas aeruginosa</em> ATCC #27853</td>
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</tbody>
</table>
Results

Spectrophotometry

Figure 4. UV-Visible spectrum of Ag NPs synthesized using the tannic acid protocol.

Figure 5. UV-Visible spectrum of Ag NPs synthesized using 30% F-127 with silver nitrate and varying levels of UV exposure.
Figure 6. Control solutions with and without exposure to UV.

Atomic Force Microscopy

Figure 7. 30% solution of F-127 with AgNO₃ exposed to UV for 1 hour.
Figure 8. 30% solution of F-127 with AgNO₃ exposed to UV for 6 hours.

Table 1. Effect of UV exposure on Ag NP size as measured using AFM.

<table>
<thead>
<tr>
<th>UV Exposure (Hrs)</th>
<th>Diameter (nm)</th>
<th>St. Dev</th>
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<td>1</td>
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<tr>
<td>8</td>
<td>17.58</td>
<td>10.46</td>
<td>17</td>
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</table>
Figure 9. LCST of Pluronic® and nanoparticle preparations. NS is a non sterile sample; ST has been autoclaved and is sterile.

Figure 10. Triplicate of Instron compressive stress strain curve of 20% non-sterile F-127. Average elastic modulus is: 0.233 MPa.
**Figure 11.** Triplicate Instron compressive stress strain curve of 20% sterile F-127. Average elastic modulus is: 1.16 MPa.

**Figure 12.** Triplicate of Instron compressive stress strain curve of 30% NS F-127. Average elastic modulus is: 2.21 MPa.

**Figure 13.** Several Instron compressive stress strain curve of 20% F-127 with Ag NPs.
Zone of Inhibition

**Figure 14.** Zone of inhibition images using Pluronic® and Ag NPs with confluent lawns of *P. aeruginosa*. (A) 30 µg/mL Tetracycline (B) 20% NS F-127 (C) 20% ST F-127 (D) 30% F-127 (E) 20% F-127 with 7.5 hours UV exposure Ag NPs (F) 30% F-127 with 8 hours UV exposure Ag NPs.

**Table 2.** Average diameter of the zone of inhibition tests from Figure 14.

<table>
<thead>
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<th>Treatment</th>
<th>Average Diameter (cm)</th>
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<td>30 µg/ml Tetracycline</td>
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<tr>
<td>20% NS F-127</td>
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<tr>
<td>20% ST F-127</td>
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<tr>
<td>30% NS F-127</td>
<td>1.00</td>
</tr>
<tr>
<td>20% NS F-127 + Ag NPs</td>
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</tr>
<tr>
<td>30% NS F-127 + Ag NPs</td>
<td>1.23</td>
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**Discussion:**

Literature outlining methods for creating Ag NPs using Pluronic® suggested using 75% (w/w) F-127 to synthesize Ag NPs (Wang 2004). Because of the difficulty of creating an even dispersion of F-127 and AgNO₃ at such a high concentration, attempts at synthesis using a lower
concentration (50% w/w) of Pluronic® were made. UV-vis scans of the resulting gels were
compared to control solutions of Ag NPs synthesized using the Tannic acid method, Figure 4,
silver nitrate, and F-127 only. The Tannic acid method yielded a peak centered around 400-420
nm, which agrees with results in the literature (Callegari 2003). However, no such peak was
observed with the Pluronic® method, though a large precipitate was present in the bottom of the
tube suggesting that some reaction was taking place. Further literature review claimed that UV
radiation can be useful in altering the size and shape of the particles and alter the UV-vis
absorbance readings (Deivaraj 2005). Subsequent samples were prepared and exposed to UV
radiation (λ=254 nm, 13 mW/cm²) for variable time periods. It was observed that the
characteristic absorbance band (400-500 nm) appeared and became more prominent with
increasing UV exposure, Figure 5. These results were also compared to UV treated silver nitrate
and Pluronic®, Figure 6. To attempt to establish the cause for this absorbance increase, samples
were imaged using AFM.

AFM images of the silver nanoparticles obtained by the method above showed a
dispersion of nanoparticle sizes, Figures 7 and 8. A parameter of interest was the effect of UV
exposure on the particle shape and size. As previously stated, the absorbance of samples around
410 nm grew as the UV radiation increased, this suggested that there may be some evolution in
size, shape or concentration that caused this change. However, no distinct trend in any was
observed in the AFM images as measured by nanoparticle height over different UV exposure
times, Table 1. It should be noted that time constraints only allowed for a limited number of
fields to be scanned and the volumes taken for AFM imaging were very small and may not be
representative of the remainder of the sample since it cannot be definitively said that the sample
was well mixed due to the viscosity and thermoreversible properties. More comprehensive
classification of particle sizes, shapes and concentrations in the sample should therefore be
attempted before any conclusions as to the effect of UV can be made.

A possible explanation for the increase in absorbance around 400 nm without a
perceivable evolution in Ag NP size or shape may be an encapsulation of the Ag NP’s by
Pluronic® polymer adhesion. It is feasible that UV radiation may disrupt this adhesion and reveal
the actual absorbance signature. Another possible explanation for the increased absorbance could
be an alteration of the Ag NP oxidation state by the UV radiation.

Two 50 mL solutions of 20% F-127 were prepared. One sample was autoclaved at 120°C
for 30 minutes. Beakers were sealed with aluminum foil and parafilm and placed in the
refrigerator at 4°C. The pH of the sterile (ST) and non-sterile (NS) solutions of F-127 were
measured using pH strips. The pH of the NS sample was ~5.5 while the pH of the ST sample was
~5.8. The pH of the 30% Ag NP 8 hour solution was ~4. The pH of the 20% 7.5 hour solution
was ~4. The pH of the 0 hour Ag NP sample was ~4. The samples with Ag NPs have a lower pH
than the Pluronic® alone. This could be due to various factors including UV exposure time or
presence of Ag NPs.

The Kibron, Inc. µTrough S tensiometer instrument was used to test the surface tension
of F-127. Unfortunately the wells on the instrument were so thin that the cold F-127 began to
warm too quickly and transitioned to the gel phase before any significant data could be obtained.
Unless the tensiometer could be utilized in a cold room, the surface tension of F-127 will not used to characterize the gel.

To determine the LCST, all tubes started at a temperature of 20° C and four replicates were completed for NS and ST 20% F-127 gels 30% F-127 and for each Ag NP preparation. The LCST was measured using an infrared temperature sensor. Results can be found in Figure 9. The LCST for the 20% F-127 solutions is higher compared to the 30% F-127, and the presence of nanoparticles affect the LCST. A two-sided statistical analysis was run with α=0.1 and we observed that there was a statistically significant difference between 20% F-127 and 20% F-127 with Ag NPs. Also, there was a statistical significance between 30% F-127 and 30% F-127 with Ag NPs. This suggests that Ag NPs change the LCST.

An interesting phenomenon was observed with the NS gels. The gels would be solid in the container but after the compression test turned liquid and required days to re-solidify. This trend was not observed in the ST gels. During the autoclave process perhaps the polymers in the ST gels were cross-linked and became more stable and solid. Figure 10 shows the stress vs. strain curve of NS 20% F-127 gels. One sample liquefied very quickly and provided different results from our other samples. The average elastic modulus was 0.233 MPa. Figure 11 shows the stress vs. strain curve of ST 20% F-127 gels. The average elastic modulus was 1.16 MPa. The ST gels could withstand greater forces when compared to the NS gels as evident in the elastic modulus. A 50 mL 30% F-127 solution was prepared per the above protocol. Figure 12 shows the stress vs. strain curve for the 30% solution. The average elastic modulus was 2.21 MPa suggesting that higher concentration of Pluronics® increase the elastic modulus. These data were compared to gels impregnated with Ag NPs, Figure 13. A two-sided statistical analysis was run at the α=0.1 level and there was not a statistically significant difference between 20% F-127 and 20% F-127 with Ag NPs. There was, however, a statistical significance between 20% F-127 and 30% F-127. This suggests that a higher concentration of F-127 results in an increase in elastic modulus.

A lyophilized strain of *Pseudomonas aeruginosa* was obtained from ATCC #27853, resuspended in LB media, and spread on LB media agar plates. After one day’s growth, two different colony morphologies were present on the plates. The two colony types were streaked for isolation on new plates and colonies were selected for PCR and sequencing. A universal primer set specific for the 16S ribosomal region was used for PCR and sequencing. The sequence obtained showed 97% homology to various *P. aeruginosa* strains contained in the NCBI genome database. Freezer stock solutions of the sequence verified *P. aeruginosa* colony were created containing 15% glycerol.

The antimicrobial affect of synthesized Ag NPs was measured using zone of inhibition tests. When treated with a 30 μg/mL concentration of tetracycline, which was shown to inhibit growth of other pseudomonads, no visible zone of clearing was observed. Pluronics® alone slightly reduced growth around the filter paper but no zones of complete clearing were observed. The Pluronics® appear to form a non-fouling layer limiting attachment of the microbes to the surface of the LB plate. Pluronics® with Ag NPs proved most effective at inhibiting growth (Figure 14, Table 2). Multiple zone of inhibition tests were conducted using various UV exposure times as well as 20% and 30% solutions of F-127. A two-sided statistical analysis was
run at the $\alpha=0.1$ level and we observed a significant difference between 20% F-127 and 20% F-127 with Ag NPs. We also observed statistical significance between 30% F-127 and 30% F-127 with Ag NPs. This suggests that the F-127 with Ag NPs significantly improve the antimicrobial properties of the gel.

**Timetable:**

<table>
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<tr>
<th>Date:</th>
<th>Task:</th>
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<tr>
<td>September 2010</td>
<td>Turn in Senior Design proposal</td>
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<tr>
<td>September 2010</td>
<td>Test gel preparation methods</td>
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<tr>
<td>September 2010</td>
<td>Determine if frozen <em>P. aeruginosa</em> stocks are viable</td>
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<tr>
<td>October 15, 2010</td>
<td>Turn in URCO proposal</td>
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<td>October 2010</td>
<td>Make F-127 gels</td>
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<tr>
<td>November 2010</td>
<td>Test gel properties</td>
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<td>November 2010</td>
<td>Make Ag NPs with known procedure</td>
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<tr>
<td>December 2010</td>
<td>Determine antimicrobial properties of gel</td>
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<tr>
<td>December 2010</td>
<td>Synthesize Ag NPs using F-127 gel</td>
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<td>December 2010</td>
<td>Submit abstract to national research conference</td>
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<td>December 7, 2010</td>
<td>Turn in interim report</td>
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<tr>
<td>January 2010</td>
<td>Have prepared stocks of gel and Ag NPs</td>
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<td>January 2010</td>
<td>Preliminary Ag NP characterization</td>
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<tr>
<td>February 2011</td>
<td>Continue characterizing Ag NPs and gel</td>
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<tr>
<td>March 2011</td>
<td>Test properties of gel and Ag NPs (LCST, antimicrobial activity, etc.)</td>
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<tr>
<td>April 2011</td>
<td>Analyze data, write final paper, and prepare for final presentation.</td>
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<tr>
<td>April 15, 2011</td>
<td>Turn in URCO final report</td>
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<tr>
<td>May 2011</td>
<td>Present final project</td>
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<tr>
<td>May 2011</td>
<td>Graduation</td>
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**Communication of Results:**

We presented our findings at the IBE Regional Conference at Utah State University in October 2010, Research on Capitol Hill at the Utah State Capitol in January 2011, and Utah Conference on Undergraduate Research at Weber State University in February 2011.

In addition we provided weekly summaries of our progress to Dr. Taylor and Dr. Britt. We also provided the interim written report of our progress up to December 2010. By the end of April 2011 we will have presented our project to the faculty in Biological Engineering and its advisory board.

**Benefit of Project:**

The introduction of antibiotics with novel, and improved modes of action is valuable to many fields including human and animal health care. As far as we know, research has been done analyzing the effects of combining Pluronics® with antibiotics and antibiotics with Ag NPs with desirable antibiotic effects. But to date, no research has analyzed the effects of combining Pluronics® with Ag NPs or combining Pluronics® with Ag NPs and existing antibiotics. Additionally, the development of a thermoreversible formulation would allow for implementation as a topical solution for presurgical preparation or wound/burn treatment, an injectable sub dermal treatment, or into the pretreatment of prosthetic devices implanted into a
living system. The broad antibiotic effects of Ag NPs and Pluronics® are such that the
development of resistance to these materials by bacteria is less likely. These broad effects would
also likely counter existing and problematic multidrug resistant bacteria that plague hospitals and
care centers. The development of such a product would prove extremely valuable at present and
contribute to our existing body of knowledge for further advancement.

Future Studies:

Future studies could be used to test the gel with Ag NP formulation using mammalian
cells to test for toxicity. Zone of inhibition tests using antibiotic resistance strains of bacteria
would be beneficial to see the effects of the gel formulation. Determine concentration of silver
ions present in the Ag NP with F-127 solution using ICP-MS. It would be interesting to view the
Pluronic® after exposure to UV using NMR could be used to see if UV exposure changes the
physical properties of F-127.

Knowledge gained regarding implementation and management of projects:

We gained an understanding about the importance of getting funding early. At the
beginning of this project we had to wait several weeks for our funding and therefore could not
begin any of our experiments. We also learned the importance of dividing tasks. Each of us had
specific roles and tasks that made the work flow more easily. Along with this division of tasks it
was important to meet weekly as a group to discuss progress and concerns. For those weeks that
we lacked funding, it gave us the opportunity to perform a thorough literature review. This
helped us to understand our protocols better before starting any lab work. Lastly, we observed
that living organisms are unpredictable and results are never what you expect.

Description of Personnel:

Alex Hatch: During his undergraduate studies, Alex has been involved in research projects
involving bioplastics. Alex plans to attend medical school after his undergraduate studies and for
this project has researched nosocomial infections and modes of antibiotic resistance. Alex also
compiled the aims of our project as well as the benefits. Alex brings a level of expertise in
bacterial culturing and aseptic techniques. Alex focused his efforts in the project to the synthesis
and characterization of Ag NPs.

Alyssa Calder: Alyssa has been involved in the laboratory of Dr. Anne Anderson and Dr. David
Britt working with *P. putida* and *P. chloraphis*. Alyssa also brings experience in bacteria
culturing. For this project Alyssa gathered information and graphical materials related to
Pluronics® as well as finding methods of characterizing nanoparticles. Alyssa outlined plans to
communicate results of our project, organized our references, and brings a level of expertise in
Ag NPs and Pluronics®. Alyssa focused on creating the thermoreversible gel and characterized
its properties as described above. Alyssa was also involved with zone of inhibition testing.

Aaron Winder: During his undergraduate studies Aaron has worked in the tissue engineering
laboratory of Dr. Soonjo Kwon involving breast cancer research. For this project Aaron found
information relating to antibiotic resistance and modes of antibiotic resistance. Provides expertise
in computer programs and formulated the significance and innovation of our design project. Aaron also worked on the synthesis and characterization of the Ag NPs for this project.

Cody Gunnell: During Cody’s undergraduate studies he volunteered in the tissue engineering laboratory of Dr. Soonjo Kwon researching carbon nanotubes and their effects on stem cell differentiation. For this project, Cody gathered information specific to Pluronic® F-127 as well as general information about Pluronics® and micelle formation. Wrote the description of personnel and provides expertise in cell culture. Cody also worked on creating the thermoreversible gel and characterizing its properties. Cody was also involved in zone of inhibition testing as well as running statistical analysis of our results.

We plan on utilizing the expertise of Dr. David Britt, PhD for his knowledge involving surfactants, biomaterials, and gels.

References:


