

# Improving wound healing by breaking down biofilm formation and reducing nosocomial infections

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## SUMMARY

A retrospective study that was published concluded that hospital admissions for adult patients in the United States suffering from skin infections is increasing by significant amounts. In a 10-year period in the early 2000's, infections due to *S. aureus* increased by 123%, and this number is increasing as time goes on [1]. The purpose of this experiment was to use hyaluronic acid, silver nanoparticles, and a bacteriophage cocktail to create a hydrogel. Unlike traditional hydrogels, which only aid in the protection and healing of wounds, this hydrogel promoted wound healing by increasing cell proliferation while simultaneously disrupting biofilm formation and breaking down *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are two strains of bacteria that attribute to nosocomial infections and are increasing in antibiotic resistance. The biofilm formation forms a barrier that gives the bacteria the power to resist antibiotics and give a pathway to create a source of systemic chronic infections [2]. If hyaluronic acid is used in conjunction with colloidal silver and species-specific bacteriophages, we predicted that cell proliferation in an in vitro wound healing model would increase. We tested our bacteriophage-containing hydrogel on human fibroblast cells and on *S. aureus* and *P. aeruginosa* cultures to investigate its potential wound healing and antibacterial properties. In support of our hypothesis, we found that cell proliferation increased. Simultaneously, the bacteriophage cocktail and colloidal silver decreased biofilm growth in both *S. aureus* and *P. aeruginosa* while not affecting cell viability. This finding is important because it offers a cost-effective, non-invasive approach to improving wound healing without the use of antibiotics, which is important in a world where antibiotic resistance is becoming an increasingly prevalent problem.

## INTRODUCTION

*Pseudomonas aeruginosa* is a gram-negative bacterium. Gram-negative bacteria are typically more resistant to antibiotics than gram-positive bacteria due to the fact that they have a unique outer membrane able to prevent antibiotics from penetrating the cells. Its natural habitat typically includes moist locations. *Pseudomonas aeruginosa* is considered an opportunistic human pathogen. Not only

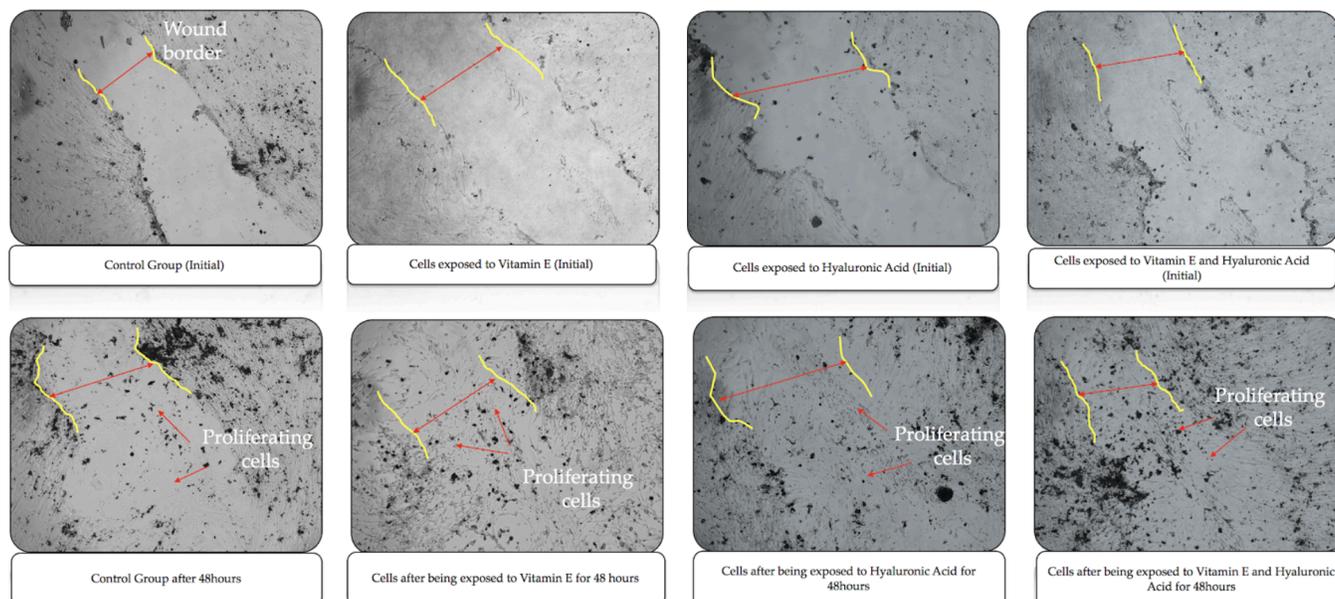
can it cause both acute and chronic infections in patients that are immunocompromised or being treated for severe burns and injuries, but it is also both invasive and toxigenic [3]. These bacteria are able to survive in temperatures up to 41°C (105.8°F), can be transferred through environmental conditions, and frequently cause life-threatening infections in patients that have cystic fibrosis. [4].

However, drug-resistant gram-negative infections, caused by bacteria such as *Pseudomonas aeruginosa*, have emerged as major concerns in hospitals, nursing homes, and other healthcare settings. *P. aeruginosa* is a major cause of nosocomial infections which affect more than 2 million patients every year and account for around 90,000 deaths annually [5]. In some cases, the bacteria are able to enter the body through urinary and intravenous catheters, ventilators, or wounds and can lead to pneumonia and infections of the bloodstream, bones, joints, and urinary tract. These types of infections disproportionately affect the very ill and the elderly and are often difficult to treat [6].

Unlike *Pseudomonas aeruginosa*, *Staphylococcus aureus* is a gram-positive bacteria strain. Similar to *Pseudomonas aeruginosa*, it is a common cause of systemic infections, particularly if the cutaneous barrier is disrupted [7]. *Staphylococcus aureus* causes many types of infections, including superficial skin lesions, boils, styes, localized abscesses, and furunculosis. It is a dangerous infection due to three main virulence factors: surface proteins that promote colonization of the host, factors that inhibit phagocytosis, and the production of toxins that damage host tissues and cause disease symptoms [8].

Hydrogels are polymer networks composed of 90% water in a gel base. They create a moist healing environment that promotes granulation and epithelialization, which is the generation of new connective tissue and blood vessels that eventually leads to the closing of the wound [9]. Additionally, the hydrogel's high-water content cools the wound, producing pain relief that can last up to 6 hours [10]. Hydrogels also decrease patient discomfort when changing burn dressings because they do not adhere to the wound surface [11].

Bacteriophages are viruses that live within bacteria and self-replicate, eventually destroying the bacterial cell. They cannot reproduce alone and they require the bacterial cell as a host to reproduce. The receptor structure on bacteriophages is so specific that a phage can only attack bacteria that has a cell surface that exactly "matches," therefore they are species-



**Figure 1: Hyaluronic acid and vitamin E enhance cell proliferation.** The cell proliferation assay showed an increase in proliferation in the cells treated with hyaluronic acid, vitamin E, and both hyaluronic acid and vitamin E compared to untreated cells. Forty-eight hours post treatment, there is a significant difference in proliferation between the treatment groups and the control group. In the pictures of the control group, the borders of the wound can still be seen while in the pictures of the treatment group the borders of the wound have mostly given way to the proliferating cells.

specific. As bacteria evolves, bacteriophages also evolve so that they can increase in numbers and propagate over time. Due to their specificity, bacteriophages are an effective use for medicine because they can destroy antibiotic-resistant strains of bacteria. Literature has shown that treatments using bacteriophages began as early as 1921 [12].

As an element, silver is effective against more than 650 pathogens and has a broad spectrum of activity [13]. As such, it has a high potential for solving the problem of multidrug resistance, which is observed in several bacterial strains. When used as a nanoparticle, silver's antimicrobial properties are enhanced; this is because silver particles have a larger surface area allowing for the bacteria to be exposed to more silver, though the antibiotic mechanism is currently not understood [14]. This allows silver to be used in a wider range of applications, including wound dressings and surgical devices [15]. It has the ability to inhibit both enzymatic systems in the bacteria and alter the synthesis of DNA. Multiple studies have demonstrated silver nanoparticles' effectiveness at preventing bacterial growth and development [16].

Hyaluronic acid is the main component of the extracellular matrix and is considered one of the key factors in the tissue regeneration process. It is present through all steps of the wound healing process as a factor that actively modulates tissue regeneration [17]. Many studies investigating hyaluronic acid have demonstrated its ability to modulate inflammation, cellular migration and angiogenesis [17]. Adding excess hyaluronic acid to a wound speeds up the recovery process of the wound and is considered a safe and effective approach to treat wounds [18].

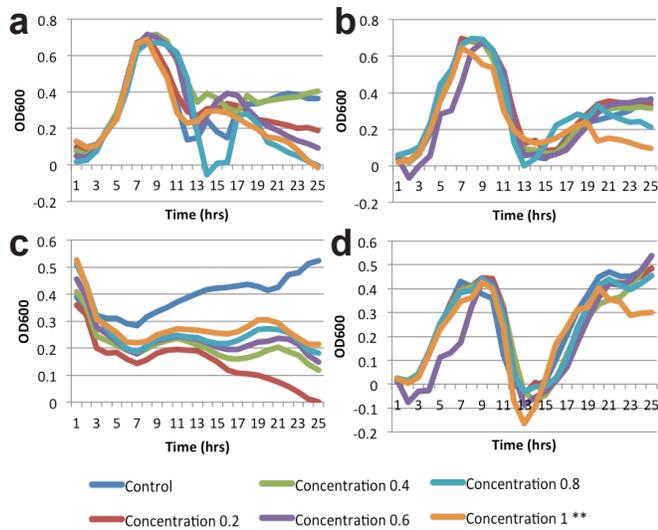
Vitamin E has been shown to regulate cellular signaling

and gene expression and influence wound healing [19]. It defends cellular membranes from reactive oxygen species by activating various signal transduction pathways and can be classified as an antioxidant. It also modulates the expression of connective tissue growth factor, thereby protecting wounds from infections such as *Staphylococcus aureus* infections and *Pseudomonas aeruginosa* infections [20]. Thus, we predicted that using hyaluronic acid and Vitamin E in conjunction with colloidal silver and species-specific bacteriophages would increase cell proliferation in an in vitro wound healing model.

The purpose of this experiment is not only to use hyaluronic acid, Vitamin E, silver nanoparticles, and a species-specific bacteriophage cocktail to create a hydrogel, but to test its beneficial wound-healing capabilities and antibiotic properties. Unlike traditional hydrogels, which only aid in the protection and healing of wounds, this hydrogel is not only able to protect and heal wounds, but it would be able to heal wounds and burns by increasing cell proliferation while simultaneously disrupting biofilm formation and breaking down harmful bacteria strains that cause systemic nosocomial infections, therefore providing a more efficient wound-healing mechanism.

## RESULTS

In the cell proliferation assay, the cells exposed to Vitamin E, hyaluronic acid, and the combination of Vitamin E and hyaluronic acid all showed an increase in proliferation of the human dermal fibroblasts over the control group, almost reaching confluency in a two-day period. Simultaneously, the assay run to test for a decrease in bacterial growth and cell viability yielded results that showed silver nanoparticles are



**Figure 2: The effects of silver nanoparticles on bacteria growth.** (A) There was a significant decrease in growth of *Staphylococcus aureus* when exposed to an 80% concentration of a 0.02 mg/mL stock solution of 40 nm silver nanoparticles ( $p < 0.05$ ) and an 0.02 mg/mL stock solution of silver nanoparticles ( $p < 0.05$ ). (B) There was a significant decrease in growth of *Staphylococcus aureus* when exposed to 0.02 mg/mL stock solution of 40nm silver nanoparticles. A one-tailed T-Test was performed to test for statistical significance and the only p-values less than 0.05 was the p-value for the 100% concentration. (C) There was a significant decrease in growth of *Pseudomonas aeruginosa* when exposed to all concentrations of 20nm silver nanoparticles ( $p < 0.05$ ). (D) There was a significant decrease in growth of *Pseudomonas aeruginosa* when exposed to 40nm silver nanoparticles. ( $p < 0.05$  for 100% concentration,  $p > 0.05$  for all others).

able to decrease the growth of both *P. aeruginosa* and *S. aureus* by significant amounts without a significant effect on the viability of the human dermal fibroblasts.

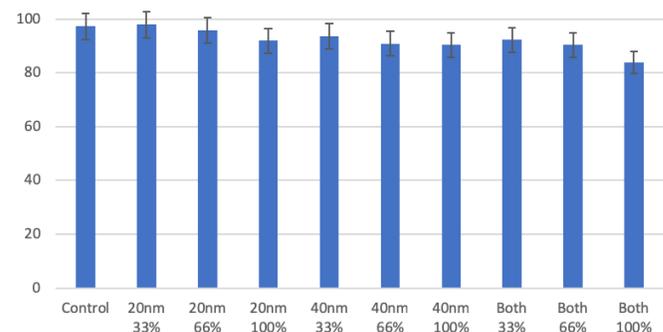
In the cell proliferation assay (Figure 1), a clear difference could be seen between cell proliferation into the wound of the control group and cell proliferation into the wound of experimental groups, even within 24 hours of treatment. By 48 hours of treatment, the three experimental groups had almost reached confluency while the control group still showed the outline of the scratch. Not only this, but the control group still had many clear spaces and showed minimal cell movement. The cells exposed to both hyaluronic acid and Vitamin E had the most proliferation, but individually the cells exposed to vitamin E exhibited more proliferation than the cells exposed to hyaluronic acid. There were three trials per experimental group and each group showed similar results.

Due to some literature suggesting that 20nm silver nanoparticles were small enough to enter human cells and cause harm [21], we used both 20nm and 40nm silver nanoparticles. When we ran an OD600 assay using the silver nanoparticles, the results showed that there were concentrations in each experimental group that were statistically significant in reducing the amount of bacteria present (Figure 2). We performed a one tailed T-Test to test for statistical significance and there was a comparison between

a normalized initial concentration and then the corrected final concentration of bacteria present to determine which group was statistically significant in reducing the amount of bacterial growth.

The cell viability assay showed that both the 20nm and 40nm silver nanoparticles did not affect cell viability by a statistically significant amount in any of the groups (Figure 3;  $p = 0.0622$  for the lowest percent viability, all else were larger, one-tailed T-test). The lowest percent viability was the treatment with undiluted 0.02 mg/mL silver nanoparticles of both 20nm and 40nm silver nanoparticles together, which still had a viability above 80%. This is important because it suggests that while the silver nanoparticles are able to break down bacteria, they do not display short term negative effects on the body. In the parts of the experiment where silver nanoparticles are tested solely on bacteria, each trial had one or more concentrations that reduced the bacterial population by a statistically significant amount (Figure 2). In the cell viability assay there were three trials per experimental group and each group showed similar results. A one tailed T-Test was performed to compare the differences in the viability of the experimental groups to the control groups.

After isolation, purification, and propagation of the bacteriophages were complete, 7 bacteriophages for *P. aeruginosa* and 5 bacteriophages for *S. aureus* were isolated from the samples collected from the Broward Sewer Treatment Plant. This constituted a bacteriophage cocktail of 12 individual bacteriophages that was specific to *S. aureus* and *P. aeruginosa*. When an OD600 assay was run using the bacteriophage cocktail, all concentrations significantly reduced the number of bacteria present in the solution ( $p = 0.0191$ , one-tailed T-test) and did not have an effect on the cells ( $p = 0.117$ ; Figure 3). Ultimately, the results of these experiments support our hypothesis that the use of hyaluronic acid and vitamin E in conjunction with colloidal silver and species-specific bacteriophages increases cell proliferation in an in vitro wound healing model while decreasing bacterial growth.



**Figure 3: Silver nanoparticles do not affect HDF viability.** The results of the cell viability assay show that both the 20nm and 40nm silver nanoparticles do not have a statistically significant effect on the viability of the human dermal fibroblasts ( $p > 0.05$ ).

## DISCUSSION

### Application

Each year in the United States, over 2 million people develop an antibiotic resistant infection and roughly 23,000 people die due to infections from antibiotic resistant strains of varying types of bacteria [22]; the findings of this project have implications in ultimately reducing that number. The results offer a cost-effective, non-invasive approach to improving wound healing without the use of antibiotics, therefore not contributing to antibiotic resistance.

By using silver nanoparticles and species-specific bacteriophages (**Figure 2A, 2B, 2C, 2D**), the hydrogel created in this project targets strains of bacteria that have the potential to adapt to antibiotics without affecting the viability of the cells, increasing wound-healing efficiency. Moreover, the use of bacteriophages ensures that the hydrogel does not exacerbate antibiotic resistance, providing solvency to a problem that has increasingly plagued the medical community. In essence, our experiment demonstrated that both the silver nanoparticles and the species-specific bacteriophage cocktail are able to significantly reduce the number of bacteria present, thereby having implications in decreasing hospitalized patients' risk of infection while simultaneously circumventing the issue of antibiotic resistance.

The scratch assay (**Figure 1**), which used both hyaluronic acid and vitamin E, showed that both substances were able to decrease the time for new cells to proliferate into a wound after a confluent layer of cells has been disrupted. Therefore, hyaluronic acid and vitamin E reduce the healing time and could potentially decrease the chances of infection in hospitalized patients, thereby enhancing hospital efficiency. In addition, by simply using a hydrogel, the discomfort of the patient can be decreased for hours at a time.

Another benefit of our approach is that the hydrogel can be customized to benefit the patient or the hospital. If there is a certain antibiotic resistant strain of bacteria present in the hospital, bacteriophages for that specific strain can be isolated from local sewer treatment plants or even the patients and added to hydrogel for a more targeted and specific treatment.

In summary, the results of this experiment provide conclusive support of a more efficient mechanism for treating patients by decreasing the time of hospital stay, undermining antibiotic resistance, and diminishing the presence of nosocomial infections. This multifaceted approach targets multiple problems that the medical community faces and makes for a more successful healthcare system.

### Limitations

Originally, we planned on gene-sequencing the bacteriophages to show genotypic variation between bacteriophages. Due to the lack of availability of primers to sequence the bacteriophages collected in the experiment, we were not able to gene-sequence the phages.

Another limitation is that the experiment was performed in a 12-well plate using a single cell type as a model for humans,

a much more complex recipient. Due to this, the effects of the project were limited and cannot predict how the hydrogel would react when exposed to multiple layers of skin as well as blood and inflammation. While prior research and other experiments have suggested that such limitations would not negatively impact the implications of this experiment [citations needed for this prior research], the lack of human trials leaves room for uncertainty. Small molecular differences may seem minor, but these minor molecular differences could cause a cascade effect in an in vivo system.

Due to the absence of biokinetics, it can only be hypothesized that the hydrogels' success in an in vitro model will translate to success in an in vivo system. Therefore, this in vitro wound healing model can simply lay the groundwork for further research where it will eventually be tested in an in vivo system.

### Future Research

In the future, we would like to test the hydrogel on a 3D burn model created using collagen, fibrin, human foreskin fibroblasts, and human epithelial keratinocytes due to its success in increasing proliferation on human dermal fibroblasts. Such an experiment would provide insight into how hyaluronic acid and vitamin E would affect proliferation in multiple cell types.

Also, we would like to test the hydrogel on tissue samples from various organisms. This will allow us to test the hydrogel on models most similar to the human skin. In addition, we would like to experiment with multiple injury models to determine how effective the hydrogel is at increasing the proliferation of multiple cell types in a wound affecting multiple layers of the skin.

We would also like to expand the experiment beyond the scope of the two bacterial strains used here and create a more all-encompassing bacteriophage cocktail. Such a treatment would allow for a more systemic, multi-faceted approach to wound care by addressing a widespread variety of bacterial infections that plague hospitals on a global scale.

## MATERIALS & METHODS

### Plating Cell Culture

The external portion of a vial of Human Dermal Fibroblasts: HDF, adult (Sigma-Aldrich 106-05A) was decontaminated using 70% alcohol in a sterile biological safety cabinet and the cells were resuspended in the vial by gently pipetting the cells 5 times with a 2 mL pipette. The cell suspension was pipetted from the vial into a T-75 flask containing 15 mL of fibroblast growth medium (Sigma-Aldrich 116-500) and placed in a 37°C, 5% CO<sub>2</sub> humidified incubator. The fibroblast growth medium was changed every other day until the cells covered roughly 60% of the plate and were split onto new plates when the cells reached 80% confluency. [23]

### Isolation of Phages from Environmental Samples

10 mL of nutrient broth (20 g nutrient broth

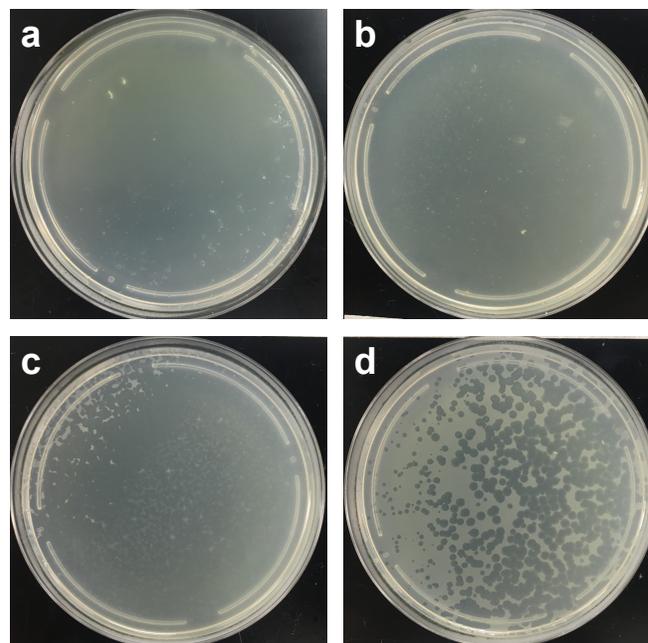
(ThermoScientific)) was inoculated with a single colony of *Pseudomonas aeruginosa* and incubated overnight at 37°C. The next morning, 0.5 mL of 10x nutrient broth (20 g nutrient broth (ThermoScientific), 50 mM CaCl<sub>2</sub>, 50 mM MgCl<sub>2</sub>, 100 mL dH<sub>2</sub>O) was added aseptically to a sterile glass bottle along with 4.5 mL of 0.2micron filter-sterilized sewage samples, obtained and given to us by the North Regional Water and Wastewater Treatment Plant, and mixed well. The broth was inoculated with 1 mL of the overnight culture and incubated overnight at 37°C. The next day, nutrient agar plates were labeled to distinguish between the control. *P. aeruginosa*, and *S. aureus*. The required number of agar overlays (2 g nutrient broth (ThermoScientific), 10 mL 50 nM CaCl<sub>2</sub>, 10 mL 50 nM MgCl<sub>2</sub>, 80 mL H<sub>2</sub>O, 0.2 g molecular-grade agarose) were prepared and cooled to 45°C. Once cooled, 200 µL of overnight culture was added to an overlay. It was swirled gently and poured over the surface of the appropriately labelled nutrient agar plate. The plates were then incubated at 37°C for 6h. For experimental samples, 1 mL of the overnight sewage–bacteria culture was centrifuged at 13,000 rpm for 5 min. 100 µL was spotted onto the center of a set overlay and the remaining supernatant was stored at 4°C in a sterile Eppendorf. If phages are present in the sample, a zone of clearing or individual plaques will appear in the center of the plate (Figures 4 and 5). [24]

#### Propagation of Phages from Environmental Samples

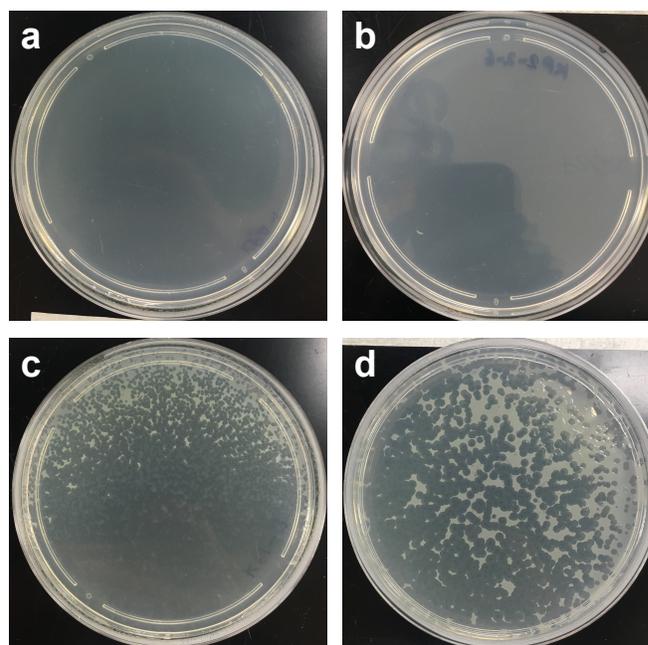
10 mL of nutrient broth were inoculated with a single colony of the host strain and incubated overnight at 37°C. Using the supernatant from the “Isolation of Phages from Environmental Samples” protocol, a dilution series of the sample in sterile nutrient broth was prepared from 1:10 to 1:107. The required number of overlays were prepared and cooled to 45°C. Once cooled, 200 µL of overnight culture was aseptically added to an overlay along with 20 µL of the diluted phage sample. The overlay was gently swirled and poured over the surface of the appropriately labelled nutrient agar plate. The plate was gently pushed back and forth so that the overlay completely covered the surface of the agar plate. The plate was transferred to the 37°C incubator and incubated for 6h with the plate lid facing upward. If phages are present in the sample, individual plaques will be seen in the lower dilutions (Figures 4 and 5). The higher dilutions will likely be completely lysed. [24]

#### Purification of Phages from Environmental Samples

Plates from the “Propagation of Phages from Environmental Samples” protocol were inspected to assess plaque distribution. From the plates with well-separated individual plaques, a sterile inoculation loop was used to carefully scoop off a single plaque into 1 mL sterile nutrient broth in a 1.5 mL Eppendorf tube. The sample was vortexed and stored at 4°C for at least 2h. From the previously prepared plaque suspension, a dilution series was created where in tube 1, 20 microliters of supernatant were added to 180



**Figure 4. Photographs of *P. aeruginosa* Bacteriophages.** Photos of (A) 10<sup>-1</sup>, (B) 10<sup>-2</sup>, (C) 10<sup>-3</sup>, and (D) 10<sup>-4</sup> dilutions of the bacteriophage cocktail used to treat *P. aeruginosa*. Unlike the dilutions performed in the isolation, propagation, and purification stage in the procedures, these images show serial dilutions of the bacteriophage cocktail that were tested in the OD600 assay. Note that the 10<sup>-1</sup> dilution has no bacteria, but as the dilutions progress, more bacteria can be seen (the bacteria appears as cloudy while the clearings are the areas phages destroyed the bacteria.)



**Figure 5. Photographs of *S. aureus* Bacteriophages.** Photos of (A) 10<sup>-1</sup>, (B) 10<sup>-2</sup>, (C) 10<sup>-3</sup>, and (D) 10<sup>-4</sup> dilutions of the bacteriophage cocktail used to treat *P. aeruginosa*. These images show serial dilutions of the bacteriophage cocktail that were tested in the OD600 assay. Note that the 10<sup>-1</sup> dilution has no bacteria, but as the dilutions progress, more bacteria can be seen (the bacteria appears as cloudy while the clearings are the areas phages destroyed the bacteria.)

microliters of nutrient broth and then in tube 2, 20 microliters of tube 1 were added to 180 microliters of nutrient broth, until you got to tube 10. For each plaque type, the plaque picking process was completed three times until a pure phage sample was created, this is when all phages on the plate are the same size/shape and visually display the same structure. [24]

### Cell Viability

Human dermal fibroblasts were taken out of T-75 flasks, and equal amounts of cell suspension were then plated in a 24 well plate. After letting the cells reach confluency, human dermal fibroblast growth medium was supplemented with varying amounts of 20nm, 40nm, and a combination of both 20 and 40nm silver nanoparticles made with the fibroblast growth medium [25]. After a 24 hour period of incubation with the silver nanoparticles, the cells were counted.

### Cell Counting

The T-75 flasks from the "Cell Viability" protocol were gently swirled to ensure that the cells were evenly distributed. Before the cells had a chance to settle, 0.5 mL of cell suspension were removed using a 5 mL sterile pipette and placed in an Eppendorf tube. 100  $\mu$ L of cells were pipetted into a new Eppendorf tube and 400  $\mu$ L 0.4% Trypan Blue (final concentration 0.32%) was added to the solution. Using a pipette, 100  $\mu$ L of Trypan Blue-treated cell suspension were removed from the Eppendorf tube and placed in the hemocytometer. A microscope with a 10X objective was used to focus on the gridlines of the hemocytometer. Using a counter, the live, unstained cells were counted in one set of 16 squares. The hemocytometer was then moved to the next set of 16 corner squares and this process was repeated until all 4 sets of 16 corners were counted. The average cell count from each of the sets of 16 corner squares were multiplied by 10,000, and then multiplied by 5 to correct for the 1:5 dilution from the Trypan Blue addition. The final calculation was the number of viable cells/mL in the original cell suspension. To calculate viability, the live and dead cell counts were added to obtain a total cell count. Then, the live cell count was divided by the total cell count to calculate the percentage of viable cells [26].

### Cell Proliferation Assay

Equal amounts of cells were transferred from T-75 flasks to each of the wells in a 24-well plate. After letting the cells reach confluency, a scratch assay was performed by taking a sterile pipette tip and dragging it across the center of the well, stimulating a wound [27]. Varying concentrations of a 1% stock solution of hyaluronic acid, varying concentrations of a 36mg/ml stock solution of vitamin E, and a combination of the two were added to the wells. Over a two-day period, proliferation into the wound was recorded and images were taken.

### OD600 Assay

The OD600 method was performed automatically in a high throughput manner using a microtiter plate reader over a 24-hour period. 1/10 dilutions of both *P. aeruginosa* and *S. aureus* were taken from active cultures that were stored at 4°C and a 12-well multichannel pipette was used to pipette equal amounts of bacteria into the wells of a 96-well plate. The control was set aside as row A in the 96-well plate, and various concentrations of a stock solution of 0.02 mg/mL silver nanoparticles, of both 20nm and 40nm solutions, were made. These solutions were then pipetted into the rows and placed into the spectrophotometer that was set to 37°C. The program was set to run for 24 hours and take readings every hour [28].

### Statistics

To test for significance in decrease in the experimental groups compared to the control group for the OD600 assay, the Cell Viability Assay, and the Cell Counting Assay, a 1 Tailed T-Test was performed comparing each experimental group to the control group.

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