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# Controlling bacterial persister cells with low level electric currents

Henry Lars Peterson

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# **Controlling bacterial persister cells with low level electric currents**

A Capstone Project Submitted in Partial Fulfillment of the  
Requirements of the Renée Crown University Honors Program at  
Syracuse University

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and Renée Crown University Honors  
May 2013

Honors Capstone Project in Bioengineering

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Date: 1 May 2013

## **Abstract**

Bacterial persister cells present a growing concern as they inherit the ability to tolerate high concentrations of antibiotics and repopulate after an antibiotic treatment leading to chronic diseases. *Pseudomonas aeruginosa* causes many human infections including skin infections and those associated with burn injuries, and implanted medical devices, and are associated with Cystic Fibrosis. Recently, the Ren Lab developed a novel approach to eliminate persister cells of *P. aeruginosa*, including those in biofilms, using low level electric currents. To evaluate the safety of this method and to better understand how the underlying elements, this study focused on the cytotoxicity of treatments with low level DCs to different human cell lines, effects on *P. aeruginosa* in co-culture with human cells, and protein expression of *P. aeruginosa* in response to DC treatments. Four human cell lines, Lung Cancer 5803, Lung Cancer 5908, Breast Cancer 231, and Fibroblast cells, were tested for cytotoxicity during DC treatment. Treatment with  $70 \mu\text{A}/\text{cm}^2$  DC with  $4.0 \mu\text{g}/\text{mL}$  tobramycin led to a two-log killing of *P. aeruginosa* and 90%+ survival of mammalian cells. Consistently, the proteomic revealed the stress response in *P. aeruginosa* was induced by DC treatment. These findings provide new insight in bacterial control with DCs which will help further development of this technology.

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## 1. Introduction

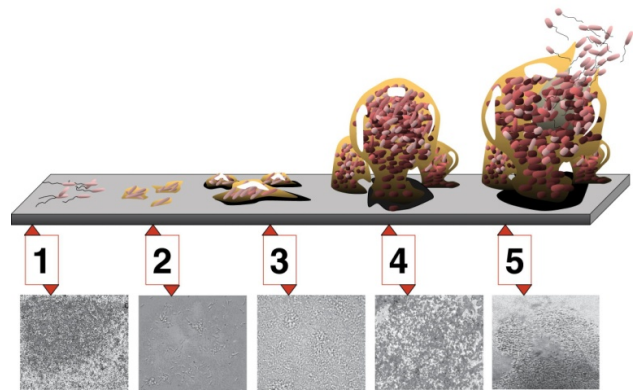
There is an ever increasing concern over the rapid development and spread of bacteria that are resistant to multiple antibiotics. Such bacteria present a major threat to of public health and economy. Bacteria can grow both as individual cells (planktonic) and in multi-cellular communities attached to surfaces, known as biofilms. The formation of

biofilms provides an ideal condition for the development of multi-antibiotic resistant strains of bacteria. Biofilms are commonly found on

infected tissues and implanted medical devices causing chronic infections in human

bodies with high mortality and morbidity. For some bacterial species, biofilm formation is critical for survival in hostile environments because the cells benefit from the protection of a hydrated matrix of protein and polysaccharide. As exemplified in Figure 1, biofilm formation starts with individual cells irreversibly attached to a surface followed by microcolony formation, maturation, and dispersion. These microcolonies are organized with functional heterogeneity to allow the transfer of nutrients and metabolic wastes[8].

Most antibiotics are unable to penetrate the exopolysaccharide (EPS) matrix to reach individual cells because the polymeric substances that construct the biofilm



**Figure 1:** The Five Stages of *Pseudomonas aeruginosa* growth on biofilms: Stage 1, early attachment; Stage 2, irreversible attachment; Stage 3, primary maturation; Stage 4, secondary maturation; Stage 5, dispersion [3]

matrix slowing down diffusion. Further evidence points out that the cells in the inner layers of biofilms receive less nutrients and are not susceptible to antibiotics in their nutrient deficient starving state [1,2,5].

Another mechanism of antibiotic tolerance is persister formation. Persister cells do not die when antibiotics are administered allowing them to remain in the body until an antibiotic regime has concluded. At this point, persister cells are able to return to normal cells and grow rapidly causing chronic infections that can lead to extended hospital stays and potentially death depending on the physical condition and immunity of the patient [9].

The Gram-negative bacterium, *Pseudomonas aeruginosa*, can be found in both natural environments and disease conditions. It is highly versatile because of its ability to grow under many different conditions and to elevate a variety of stress responses. Because it is a Gram-negative bacterium, *P. aeruginosa* has the presence of an outer membrane made up of some lipopolysaccharides and proteins that is not present in Gram-positive bacteria. This outer membrane is important for the study of this bacterium as it helps with the resistance to antibiotics by halting the diffusion of antibiotic into the cell. *P. aeruginosa* is an opportunistic pathogen that infects patients with cystic fibrosis and in persons with compromised immune systems (i.e. HIV/AIDS). For patients with cystic fibrosis, *P. aeruginosa* presents a serious problem because 80% of the population gets infected by this bacterium [4]. As discussed by Davies JC, *P. aeruginosa* is the main pathogen found in lungs of cystic fibrosis patients. It is thought that the accumulation of mucus in the airways leads to the breakdown of antibacterial

peptides as well as increased bacterial receptors on the membranes of eukaryotic cells, and a decreased ability to fight against infection due to the lack in essential molecules such as nitric oxide and glutathione [15].

Like many other pathogenic bacteria, *P. aeruginosa* can form biofilms and persister cells, which are tolerant to most, if not all, antibiotics. Recently, the Ren Lab developed a novel approach to eliminate persister cells of *P. aeruginosa*, including those in biofilms, using low level electric currents. Research using the bioelectric effect originated in the 1950s and has continuously progressed to include a multitude of areas of research, but research with its advantages when used against biofilm formation started only in the 1990s.

A phenomenon named, bioelectric effect, was reported by Costerton and co-workers that low level electric fields produced by electric currents can enhance the efficacy of the antibiotics against bacterial biofilms. Research has shown the amount of antibiotic required to control biofilm cells decreases in the presence of electric current. Alternating current (AC) and direct current (DC) have shown to be effective. This is especially important to fight biofilm infections because it allows the doses of antibiotics to be reduced to clinically and physiologically acceptable values. Normally, antibiotics alone are not effective against biofilms due to their high tolerance, up to 1000 times higher than planktonic cells.

The use of the bioelectric effect has proven to be promising thus far. The use of DC current alone has shown to 1 log reduction in CFU/mL. This phenomenon has been tested and proven for *P. aeruginosa*, *Staphylococcus epidermis*, *Staphylococcus gordonii*, *Candida albicans*, and *Klebsiella pneumonia* with



tobramycin, gentamicin, and cycloheximide [6, 7, 14]. The exact mechanism by which the bioelectric effect enables for a large increase in bacterial killing, however, is not well understood.

Recently, the Ren Lab reported that low level DCs can also sensitize persister cells to antibiotics. This study focuses on the killing of planktonic bacteria cells (PAO1) by the efficacy of combining low level electrical currents with the antibiotic tobramycin. Further, the study examines the potential cytotoxic effects of low level electrical currents on representative mammalian cells to determine if this method can be safely integrated into medical applications. Finally, the last part of the study aims to gain a better understanding of how extracellular protein expression changes during electrical treatment of PAO1. By separating cellular proteins and comparing the protein expression profiles of the control and treated samples, we expect to obtain new insight into persister control by low-level DCs. Proteomics allows quantitative comparison of proteins expression under different conditions. It was hypothesized that EC treatment can induce and repressed different sets of proteins in *P. aeruginosa* cells. Thus, by comparing a control sample (no EC treatment) with an experimental sample (EC treatment), the physiological changes in cells can be investigated at the protein level.

Great importance is also put on the need to study the cytotoxicity of the EC treatment on human cells. Although the EC treatment proves to be effective in the killing of persister cells, it is necessary to prove that there are no significant effects of EC treatment on human cells. In this study, we characterized the viability of LC 5908, LC 5803, and BC 231 with treatment of  $70 \mu\text{A}/\text{cm}^2$  and 84

$\mu\text{A}/\text{cm}^2$ . We further tested the killing of *P. aeruginosa* PAO1 in co-cultures with tobramycin and 500  $\mu\text{A}$  DC. To my best knowledge, this is the first study to investigate the cytotoxicity of low level DCs on LC 5908 and LC 5803 cells, to test the effects of DCs on *P. aeruginosa* in co-culture with and to compare protein expression profiles of *P. aeruginosa* in response to DCs.

## 2. Materials and Methods

### 2.1 DC treatment of human cells in a planktonic suspension

Two experimental groups were studied to determine the effects of an applied electrical field on varying cultures of mammalian cells. An electrochemical cell (Figure 3) was created by putting two stainless steel 304 (SS 304) electrodes inside a cuvette. An Ag/AgCl reference electrode was used to maintain a steady current load. Two current densities were used to test the effects of DC on cell

viability,  $70 \mu\text{A}/\text{cm}^2$  ( $500\mu\text{A}$ ) and  $84 \mu\text{A}/\text{cm}^2$  ( $600\mu\text{A}$ ). The cell lines tested were Lung Cancer 5908, Lung Cancer 5803, and Breast Cancer 231 (obtained from Dr. Jing An at SUNY Upstate Medical University). Initially fiber blast cells were also included in the cells, but issues with cell proliferation led to their exclusion from the study. All cell lines were grown in T-flasks, each containing 25mL the phosphate dense Roswell Park Memorial Institute medium supplemented with 10% Fetal Bovine Serum (FBS) and

1% Glutamine-Penicillin-Streptomycin (GPS) for 7-8 days. The cells are cultured with 5%  $\text{CO}_2$  at  $37^\circ\text{C}$  and the medium was replenished every 4 days. To harvest cells, the medium was removed using a pipet and TE buffer containing 0.25%

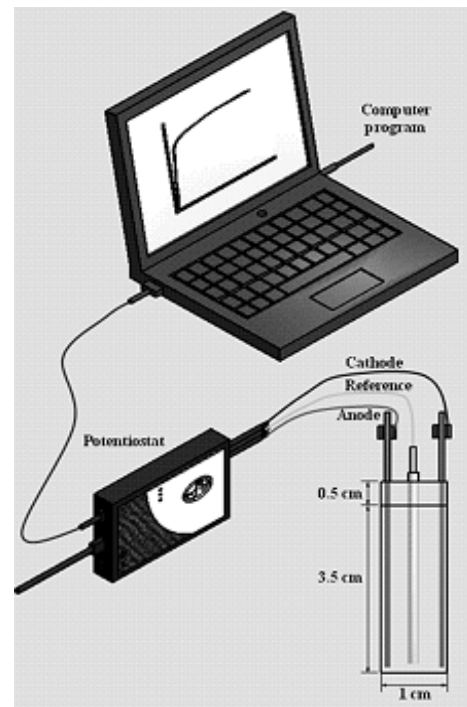


Image 2: Display of how the bioelectric effect is set up. Cells are suspended in the cuvette shown here

Trypsin and 0.53mM EDTA added and let sit for 15 minutes to break apart proteins in the extracellular matrix. The cells were re-suspended in 10mL RPMI were transferred to a conical tube and centrifuged at 1000 rpm for three minutes. The suspension was discarded and 0.85% NaCl solution was added to the solution to re-suspend the cells for testing. For each test, 3 mL of the mixture was put into a sterile cuvette and the electrochemical cell was assembled using the two SS 304 coupons and an Ag/AgCl reference electrode. The flow of electricity was set and monitored by the AfterMath Scientific Data Organizer Software (Pine Research Instrumentation). The program was set to run at the desired current for sixty minutes. At time zero, twenty minutes, forty minutes, and sixty minutes, 40  $\mu$ L of sample was extracted and dyed with 10  $\mu$ L of Trypan blue. Ten  $\mu$ L of the mixture was put onto a hemocytometer. A hemocytometer is a special slide used to count cells in four different grid quadrants. The cell membrane color number and color were recorded to determine the viability at 20 minute intervals during the one-hour treatment.

## ***2.2 Effect of DC on the viability of human cells infected with PA01***

Mammalian cells were collected after incubation for 7-8 days and collected with TE buffer and centrifuged at 1000 rpm for 3 minutes as described above. Cells were then re-suspended in 0.85% NaCl. Concurrently, *P. aeruginosa* PA01 cells were collected from a 25 mL overnight culture by taking 10mL of the culture and adding it to a 25 mL conical tube. The PA01 were prepared from a 25mL overnight culture in Luria-Bertani (LB) medium containing 10 g/L yeast extract, and 10 g/l NaCl [16]. The tube was centrifuged for 10 minutes at 1000rpm. The

supernatant was discarded and the cells were re-suspended in equal amounts of 0.85% NaCl, mixed using a vortex, then centrifuged for 10 minutes at 1000 rpm. This step was then repeated one more time. Using a cuvette with 3 mL 0.85% NaCl buffer as a reference, the  $OD_{600}$  of the 3mL of the PA01 suspension was adjusted to reach  $OD_{600}=0.6$  for co-culture experiment. OD or optical density is a measure of the amount of light absorbed by a suspension. In this case the suspension contains PA01 and 0.85% NaCl was used as a baseline such that measuring the  $OD_{600}$  of PA01 does not take into the account the presence of the buffer. Once the  $OD_{600}$  was measured, the sample was diluted such that an  $OD_{600}=0.6$  was added to the human cells. Upon infection, a current density of  $70\mu A/cm^2$  was applied for 1 hour using SS 304 electrodes and an Ag/AgCl reference electrode to create an electrochemical cell (Figure 3). The Trypan Blue excision method was used to determine the cell viability of human cells during the one-hour treatment. Also at twenty minute intervals the number of viable PA01 cells was quantified by counting colony forming unit (CFU). CFU is a technique to approximate the number of bacterial cells in a sample by diluting and plotting bacterial cells on agar plates (1.5% agar). The process uses 96 well plates with the addition of 180 $\mu$ L of 0.85% NaCl into an area of 5x7 wells for each sample. During testing, 20 $\mu$ L of the solution was put into the 5 wells in the first row. By pipetting 20 $\mu$ L of this mixture, after mixing, to the second row, the concentration of bacteria is effectively diluted by a factor of ten. Continual dilutions allow accurate count of bacterial cells.

## 2.3 Proteomics

### *Step 1: Protein sample preparation*

To obtain quality 2DE images it is important to know the optimal level of sonication to break the cells. To do this, *P. aeruginosa* samples were extracted and sonicated at the following settings of power and time: 50% 1:30 min, 50% 2:00 min, 50% 3:00 min, 75% 1:30 min, 75% 2:00 min, 75% 3:00 min, 100% 1:30 min, 100% 2:00 min, and 100% 3:00 min. For this test, protein samples were extracted from the pellet and supernatant to better understand how the amount of proteins varied with increased sonication. A following test was conducted with 100% sonication at intervals of 1:00 min, 2:00 min, 3:00 min, 4:00 min, and 5:00 min with the addition of 200  $\mu$ L of lysis buffer. For information for extraction techniques refer to Step 1 in part 2.4. Note: for the first test no lysis buffer was used and the sonication information was not available at the time of this experiment.

### *Step 2: 1D gel preparation and running*

To make 1D gels, the following recipe is used to make two gels (12% acrylamide):

Distilled water	5.1 mL
0.5M Tris-HCl, pH 6.8	2.5 mL
10% (w/v) SDS stock	100 $\mu$ L
Acrylamide/ Bis (40% stock)	975 $\mu$ L
10% ammonium persulfate	50 $\mu$ L
Temed	10 $\mu$ L

Once the gel solution is made it is poured into a cassette then a comb is placed on top to make the wells for running the proteins. To run the mixture a running buffer is used. Running buffer is made with glycine (115.2g), Tris (24g), SDS (4g), and dH<sub>2</sub>O (qs to 4L). The cassettes run until the bands reach the bottom of the gels

When loading the individual wells with proteins, each sample is died with bromophenol blue before addition.

### *Step 3: 1D gel imaging*

Gels are imaged on the Molecular Imager Gel Doc XR+ System with Image Lab Software using a 1D gel imaging protocol.

## ***2.4 Variable protein expression mapping due to electrochemical treatment***

### *Step 1: Protein sample preparation*

A control and experimental sample of PA01 were prepared from a 25mL overnight culture in Luria-Bertani (LB) medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/l NaCl. Overnight cultures were shaken at 200 rpm and incubated at 37 °C. Samples were prepared by centrifuging samples at 8,000 rpm at 4°C, 10 min, and washed with Solution W (KCl [3mM] 22.5mg, KH<sub>2</sub>PO<sub>4</sub> 1.5mM] 20.5mg, NaCl [68mM] 400mg, Na H<sub>2</sub>PO<sub>4</sub> [9mM] 108mg, and dH<sub>2</sub>O 100mL). The process was repeated three times. Samples are mixed by pipetting (avoid vortexing). The amount of lysis buffer needed is calculated such that the final protein concentration is between 5-7 mg/mL by the following:

$$1 \text{ OD} = 1\text{mg/mL of cell mass}$$

$$\text{Protein} = 55\% \text{ of cell mass}$$

Ex: 5mL culture with OD=0.6 will have

$$5\text{mL} \times 0.6 \times 1\text{mg/mL} \times 55\% = 1.65\text{mg protein}$$

The samples are then frozen over dry ice then thawed with running tap water and a vortex is used to mix for ten seconds. The sonicator is used on the 100% pulse setting (10 seconds on, 10 seconds off) for two, two and a half minute intervals with the tubes suspended in ice cold water. Samples are then centrifuged at 13.2k rpm and 4°C for a minimum of 45 minutes, but preferentially 60+ minutes. The supernatant is then transferred to a new microcentrifuge tube.

*Step 2: Rehydration*

The rehydration mixture follows for each sample:

Protein sample	42 $\mu\text{L}$
Soln A	60 $\mu\text{L}$
Rehydration buffer	298 $\mu\text{L}$

The mixture is then pipetted into a channel of an Immobile Strip Tray plate. After an Immobile DryStrip is placed gel side down, the protective peel is taken off and it is cut to fit the tray. Ensure optimal absorption of protein by pushing out any air bubbles under the strip. The addition of ~1.5mL of mineral oil on top of the strip prevents the strip from drying. The strips must remain overnight to incubate.

*Step 3: 1-D: IsoElectric Focusing (IEF)*

First electrode wicks are placed on top of the wires of the acidic and basic ends of the Multiphor plate to enable the transfer of electricity from the salts to the gel strip without direct contact. Immobile DryStrips (Bio-Rad) are then transferred after letting excess mineral oil drip off and placed gel side up. Addition of ~2mL



is used to cover the strips. Check that the tray is in proper orientation before running the program on the IEF machine. The program should be set as:

500V, 2 min, rapid

3500V, 24 hr, rapid

50 $\mu$ A/gel

Note: ensure to put into the IEF machine program the number of gels so it reaches set voltages.

#### *Step 4: Gel Preparation*

A large Büchner flask or sidearm flask is used to mix:

	Tris	dH <sub>2</sub> O	PDA	40% acrylamide soln	0% APS	TEMED
1 gel:	22mL	35.82mL	0.2645g	24.78mL	100 $\mu$ L	45 $\mu$ L
2 gels:	44mL	71.64mL	0.528g	49.56mL	165 $\mu$ L	75 $\mu$ L

APS solution is made by diluting 0.05g in 500 $\mu$ L dH<sub>2</sub>O (for two samples, save the extra 150 $\mu$ L for each sample for later).

The contents must be added from left to right. Before the addition of 40% acrylamide solution, the mixture is mixed well. Once the 40% acrylamide solution is added, the solution is de-gased for 10 minutes under a hood with a vacuum and a rubber stopper to reduce the presence of volatile acrylamide monomers. The addition of 10% APS and TEMED are added in a circular mixture with gentle swirling to initiate polymerization. The solution is poured into the top of the gel cassettes leaving about 2cm of space from the top. One to two mL of saturated sec-Butanol is added to top of the gel to create an even gel matrix. The gel should sit for at least 2 hours to polymerize. Excess APS and some TEMED are used to polymerize excess solution.

*Step 5: 2-D Polyacrylamide Gel Electrophoresis (PAGE)*

Upon the completion of 1-D IEF, the machine is unplugged and the tray is drained of the mineral oil. The gel strips are then transferred onto the Immobile Tray gel side up adding ~3mL of E1 onto each strip and placing it onto a rocker for fifteen minutes. After draining E1 from the tray, the strips are washed off with dI H<sub>2</sub>O, then ~3mL of E2 is added to each strip and it is put on the rocker for five minutes. E2 is then drained from the tray. E1 and E2 are made from the same base solution, Solution C, which follows this recipe:

Urea [6M]	9g
Glycerol	6mL
SDS [2%]	0.5g
0.05M Tris pH6.8	11.75mL

To make E1, 0.12 g DTT [2%] is added to 3 mL of solution C. To make E2, 0.15g of Iodoacetamide [2.5%] and a few grains of bromophenol blue are added to 3mL of solution C.

Before putting the strips into the glass cassettes, dI H<sub>2</sub>O is added to the top of the gel. Strips have to be trimmed in order to properly fit into the cassette by snipping a portion of the strip from either end. The strip is now softly pushed into contact with the 2-D gel ensuring the acidic end is on the left side of the gel. To push the gel in, use a piece of filter paper.

The cassettes are then loaded into the Protean cartridges and ~3L of running buffer is added ensuring the space between the cassettes is full before pouring the remaining buffer into the tank. Ensure the liquid buffer is not leaking as leaking

will stop the flow of electricity. Running buffer is made with glycine (115.2g), Tris (24g), SDS (4g), and dH<sub>2</sub>O (qs to 4L). The machine was 250 mA. During the first fifteen minutes, it is checked that there is no leaking, the dye front starts going down, and bubbles are rising. Dye fronts and amp are checked periodically until the gel front reaches the bottom of the 2D gel and the machine is turned off. It is normal if the current drops over time.

*Step 6: Gel retrieval, pre-imaging, and imaging*

Gels are unloaded and put into trays containing dI H<sub>2</sub>O by carefully using the spacers to separate the pieces of glass containing the gel. The gels are then eased into the water and orbited for five minutes at 42 rpm. The water is then dumped out and a fixation solution (10% methanol and 7% acetic acid) is added and the gels are put back onto the orbiter for up to one hour. At this point the fixation solution is put into a hazardous waste container and Sypro Ruby stain (Bio-Rad) is added. The trays set on the orbit machine overnight covered in aluminum foil to prevent ultraviolet radiation from degrading the stain. Finally, a 24 hour destaining period is recommended with two solution changes using the fixation solution before the sample is ready to image. For imaging, the Molecular Imager Gel Doc XR+ System with Image Lab Software was used with a Sypro Ruby stain protocol.

*Step 7: Protein extraction*

Using a UV box, gels are imaged so that selected plugs can be extracted from the gel for analysis. Plugs are chosen by comparison between two different gels. For this experiment, eight protein gels were chosen. Protein plugs are extracted from

the gels using a One Touch Plus Spotpicker with a 1.5 mm diameter (The Gel Company) and with disposable tips with floating filter for PDM 1.5 One Touch Plus sp (The Gel Company). Once the spots are extracted they were put into microcentrifuge tubes.

*Step 8: In-Gel Digestion and Extraction*

First the gel pieces are washed by adding 100  $\mu\text{L}$  dI  $\text{H}_2\text{O}$  and letting sit for 5 minutes before discarding the  $\text{H}_2\text{O}$ . Next a 100  $\mu\text{L}$  (50:50) mixture of 100 mM Ammoniumbicarbonate (Sigma) and acetonitrile were added and let sit for 10 minutes before discarding the mixture. After adding 50  $\mu\text{L}$  of acetonitrile and incubating for 5 minutes at 56 °C. The liquid was discarded and the gel pieces were opaque and shrunken. Gel pieces were then allowed to dry in a sealed dessicator for 10 minutes.

The next step is for reduction and alkylation of the protein samples. This step serves break disulfide bonds. First, 20  $\mu\text{L}$  of 10 mM D,L 1,4 Dithiothreitol (Acros Organics) in 10 mM Ammoniumbicarbonate is added to each sample and incubated for 45 minutes at 56 °C. Microcentrifuge tubes are removed and allowed to cool to room temperature before adding 20  $\mu\text{L}$  of 55 mM iodoacetamide (Acros Organics) in 100 mM Ammoniumbicarbonate and incubated in darkness for one hour. Note: for the 2 steps in reduction and alkylation make 100 mM Ammoniumbicarbonate solutions then add the D,L 1,4 Dithiothreitol and iodoacetamide in their respective molalities (i.e. for step add 10 mM D,L 1,4 Dithiothreitol to 20  $\mu\text{L}$  of 100 mM Ammoniumbicarbonate). The washing process was then repeated.

Next, the proteins are enzymatically cut into peptide chains using trypsin. A trypsin solution is made on ice using 10 ng/ $\mu$ L trypsin in 4 °C 50 mM Ammoniumbicarbonate with 10% acetonitrile. Each protein is rehydrating using 15  $\mu$ L of the trypsin solution and incubated for thirty minutes on ice. Subsequently, 10  $\mu$ L of 50 mM Ammoniumbicarbonate is added to each protein then put into a 30 °C water bath overnight. The microcentrifuge tubes are wrapped in Parafilm (Pechiney Plastic Packaging Company) to ensure that no water enters.

Lastly, peptides are collected for sequencing by stopping the enzymatic reactions occurring with the addition of formic acid (Acros Organics) to the final concentration of 1.0%. The supernatant was removed and saved. Afterward, the addition of 30  $\mu$ L of 50% acetonitrile with 5% formic acid to each protein was allowed to sit for 45 minutes before sonicating at 100% for five minutes. The supernatant was removed and saved with the previous supernatant. The previous step was repeated again to ensure maximum collection. Lastly, the addition of 30  $\mu$ L of 90% acetonitrile with 5% formic acid to each protein was allowed to sit for 5 minutes. The supernatant was removed and added to the other supernatants of the respective proteins. The supernatants were dried in a dessicator. Samples were sent to Cornell's Proteomics and Mass Spectrometry Facility. During initial drying process two protein plugs were lost due to the variation in air pressure.

### ***2.5 Statistical Analysis***

All the data was obtained and compared to multiple results and expressed as averages +/- standard deviations. One-way ANOVA at a significance of  $p < 0.05$

was performed using Microsoft Excel's Data Analysis package. In addition, some tests were analyzed using the two-tail 95% confidence t-test.

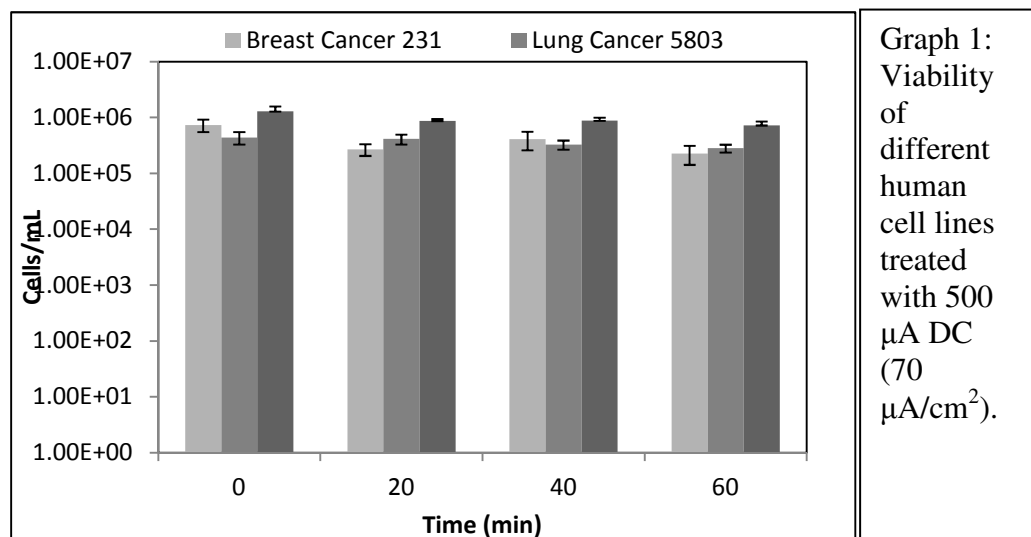
### 3. Results

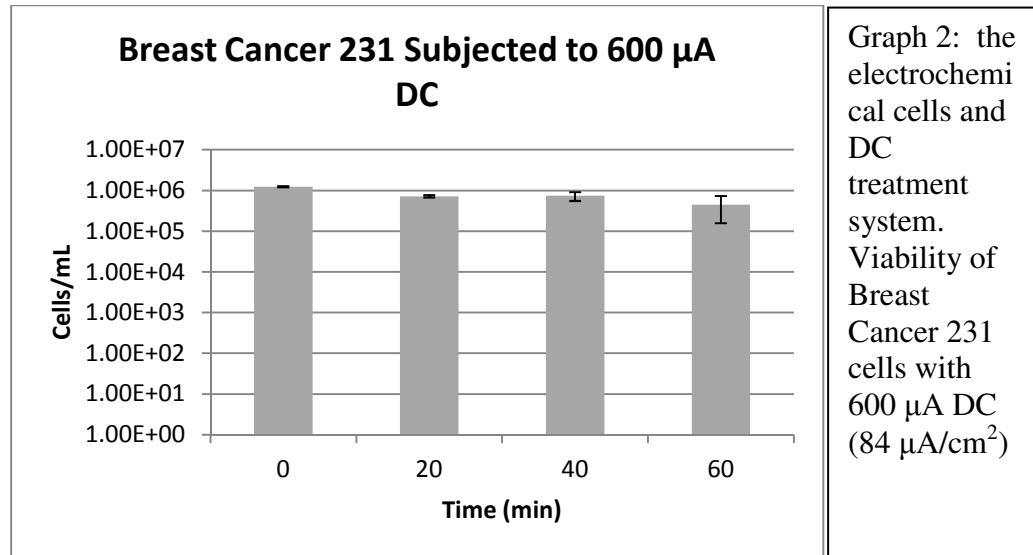
#### 3.1 DC treatment of human cells in a planktonic suspension

In order to understand the cytotoxicity of DC treatment, three different human cell lines were tested in an aqueous solution for one hour with current levels of 500 $\mu$ A or 600 $\mu$ A (or 70 - 84  $\mu$ A/cm<sup>2</sup> DC). The three cell lines used were Lung Cancer 5908, Lung Cancer 5803, and Breast Cancer 231. Figure 4 shows the results of this test. For cell line BC 231, cell viability was only decreased by 7.19% with 1h treatment at 70  $\mu$ A/cm<sup>2</sup> DC. for 500 $\mu$ A. For cell line LC 5803, cell viability decreased by 7.83% during with 1h treatment at 70  $\mu$ A/cm<sup>2</sup> DC. For cell line LC 5908, cell viability decreased by 9.24% during with 1h treatment at 70  $\mu$ A/cm<sup>2</sup> DC. Overall, the results showed that there is no major toxicity to human cells with DC treatment at the same DC levels the was found effective against *P.*

*aeruginosa*.

The testing with 600 $\mu$ A DC on mammalian cells showed levels of cytotoxicity too high to consider for co-culture tests. This is due to a 64% reduction in cell count during one hour of treatment.





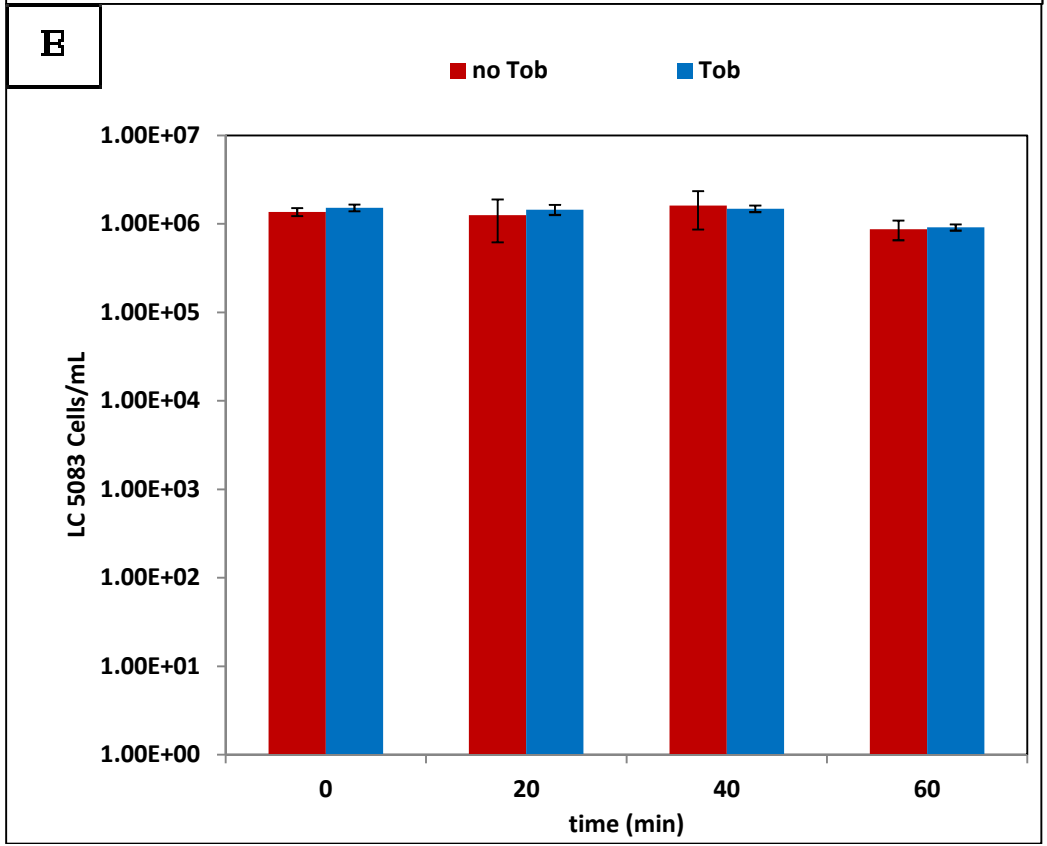
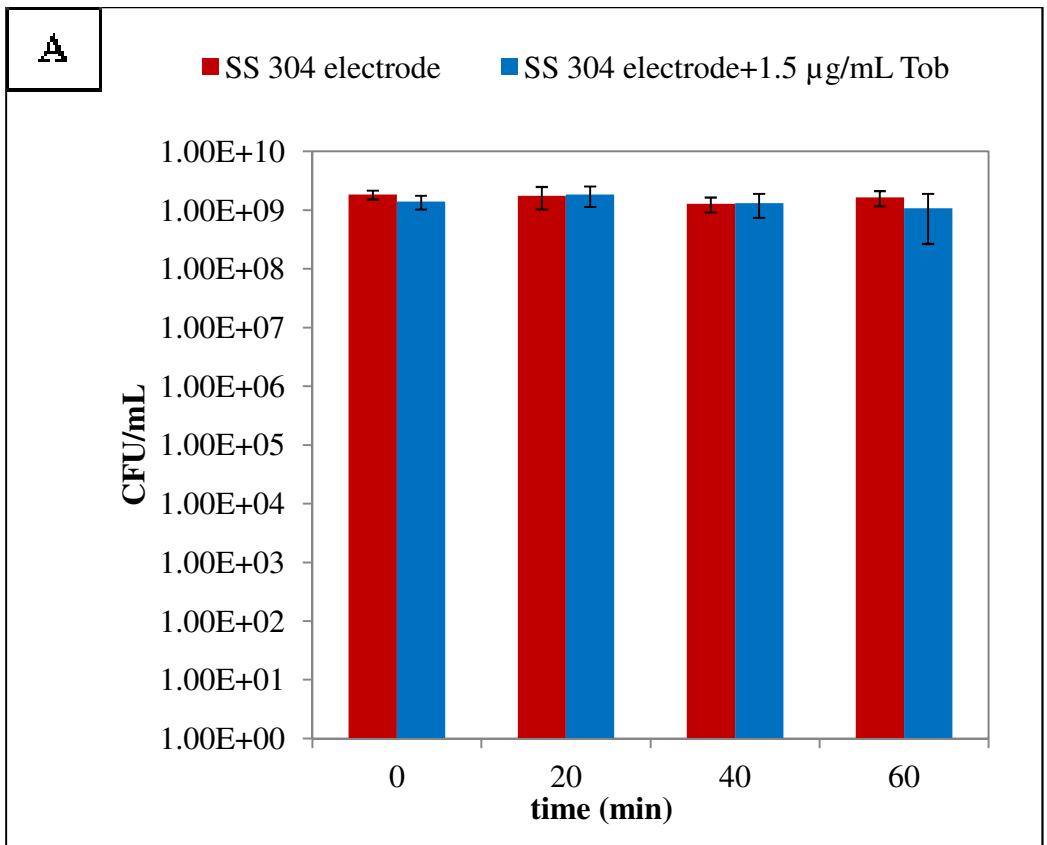
### 3.2 Effect of DC on the viability of human cells infected with PA01

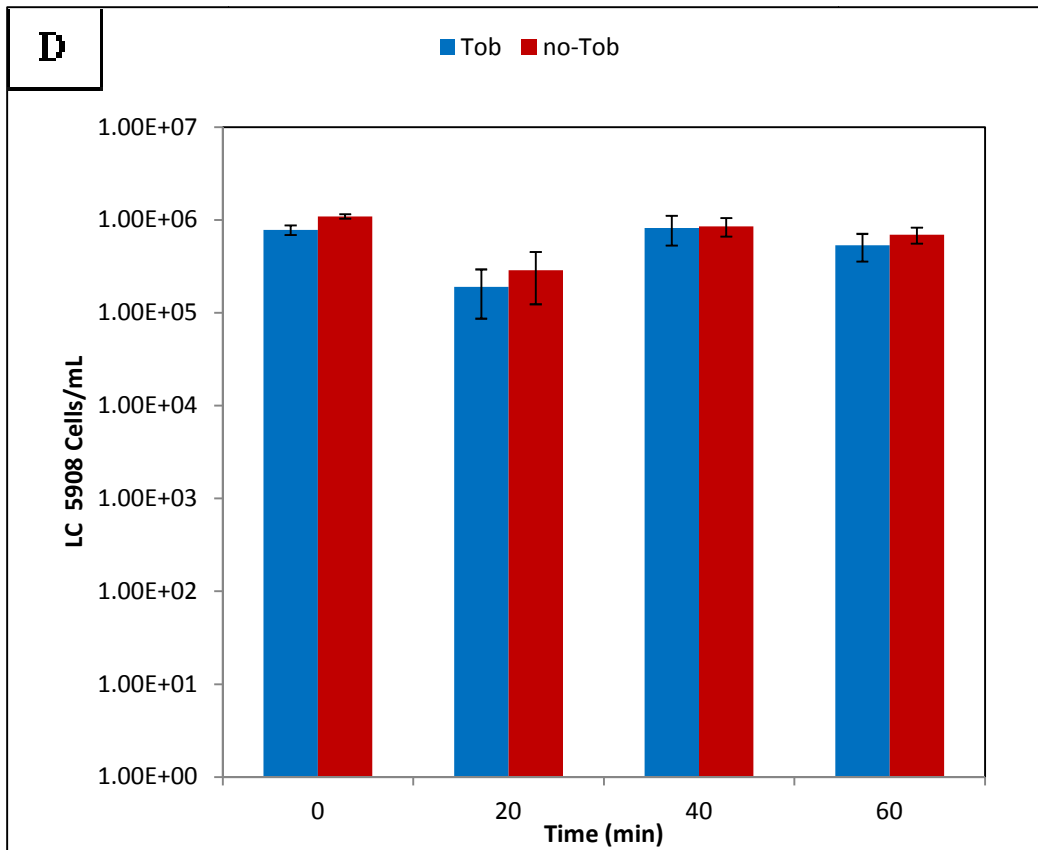
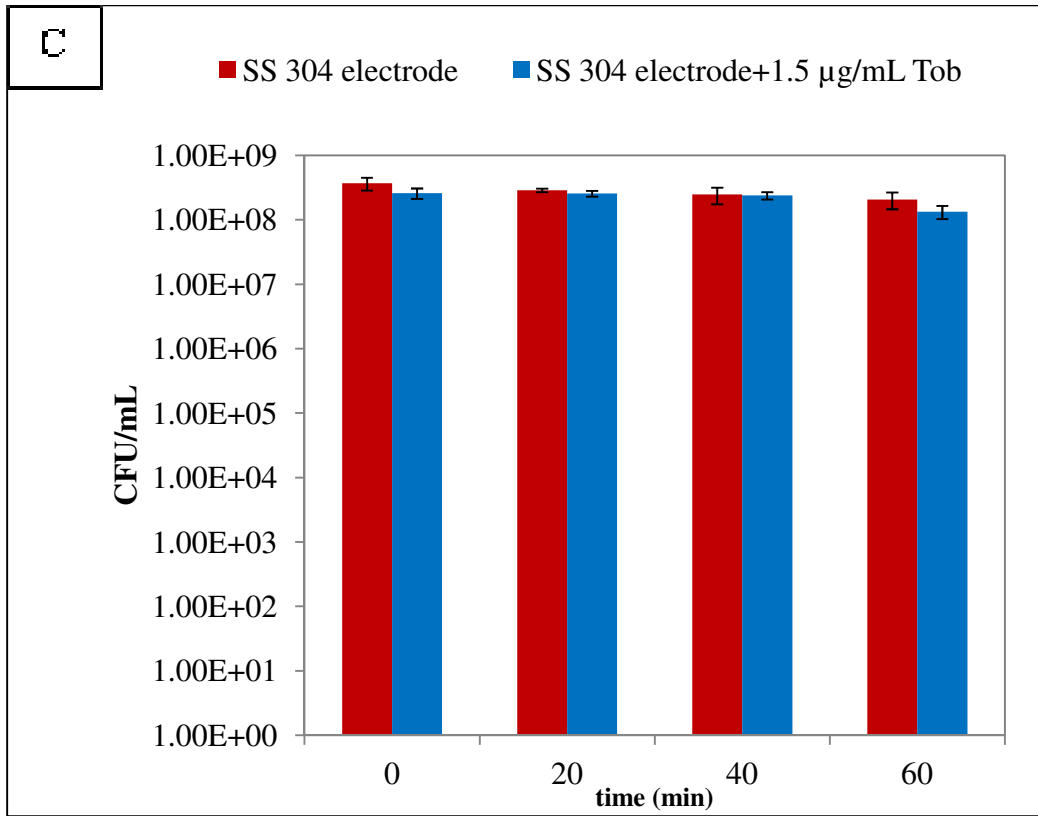
To determine if we can selectively kill *P. aeruginosa* in co-cultures, the viability of human cells infected with PA01 under electric currents and in the presence of the antibiotic Tobramycin was tested.

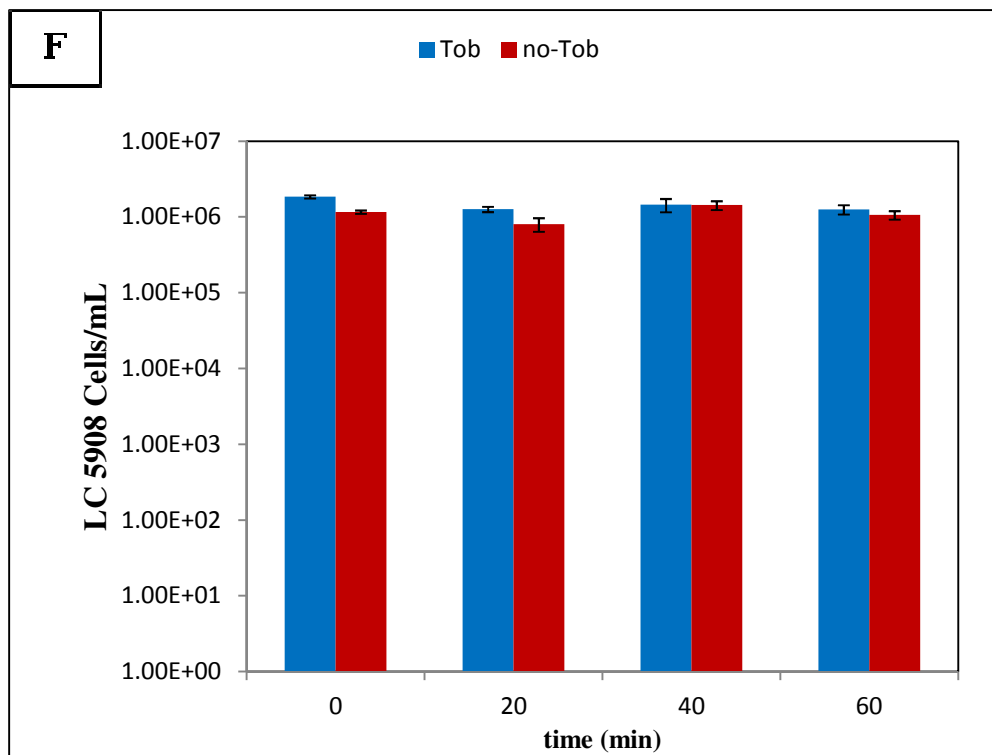
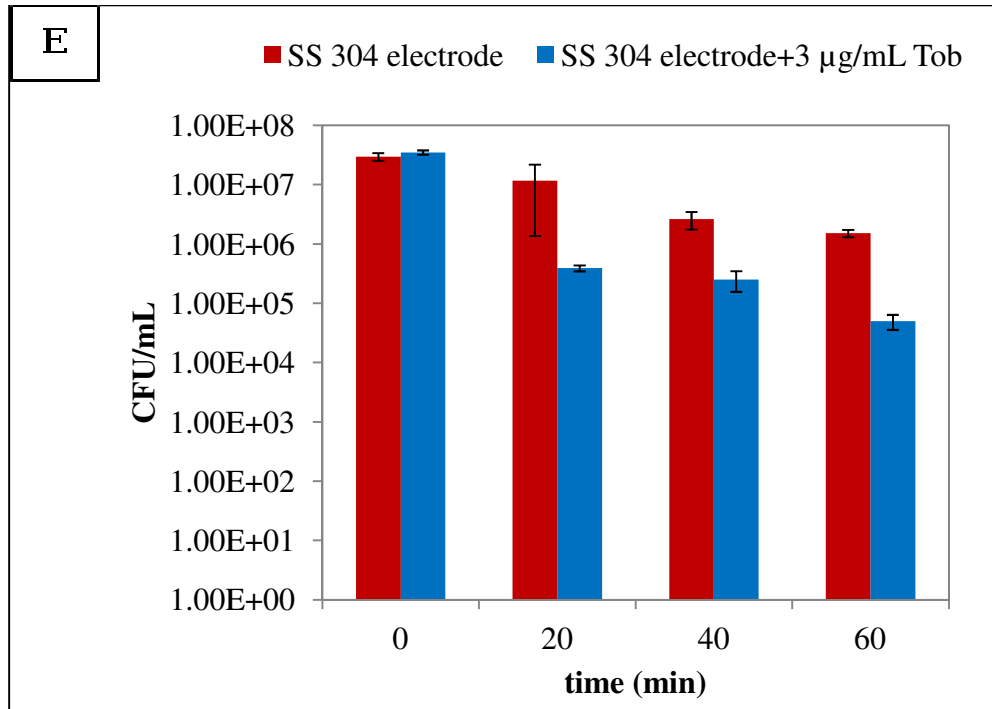
The first test was designed to determine the effectiveness of 1.5  $\mu$ g/mL of tobramycin with DC treatment on mammalian cells infected with PA01. The cell lines LC 5908 and LC 5803 were tested with 1.5  $\mu$ g/mL tobramycin. The two tests showed less than 1 log killing of bacteria after one hour of DC treatment. Thus the use of 1.5  $\mu$ g/mL tobramycin was not sufficient to obtain significant killing of PA01. This led to a test using 3.0  $\mu$ g/mL to see if killing would be achieved with increased tobramycin concentration. As for the mammalian cell longevity, neither tobramycin concentration showed significant effects on cell death. To demonstrate the viability of human cells infected with PA01, tests were set up with cell line LC 5908 and PA01 and treated with 3  $\mu$ g/mL tobramycin for one hour of 500  $\mu$ A DC treatment. After one hour treatment LC 5908 showed a



5% and 12% decrease in viability for the test with and without Tobramycin. More than two logs of killing was achieved for bacteria in the presence of tobramycin compared to 1 log killing of bacteria in the absence of tobramycin. The two tests showed that with the use 3.0  $\mu\text{g}/\text{mL}$  there was over two-log killing of PAO1. In contrast, there was less than one log killing with 1.5  $\mu\text{g}/\text{mL}$  tobramycin. Interestingly, the tests run as a control for the 3.0  $\mu\text{g}/\text{mL}$  (exclusion of antibiotic) showed 1 log killing, but the test run as a control for the 1.5  $\mu\text{g}/\text{mL}$  showed no killing. Overall, synergy was observed between 70  $\mu\text{A}/\text{cm}^2$  and 3  $\mu\text{g}/\text{mL}$  in killing *P. aeruginosa* PAO1 in co-cultures with Lung Cancer 5908 cells. This condition was found to be safe to human cells.







Graph 3: A-F show the results of DC treatment on human cells infected with PA01. A,C,E show bacteria population during treatment. B,D,F show mammalian cell population during treatment. Tests compared concentrations of tobramycin.

### ***3.3 Determination of appropriate sonication protocol for 2DE analysis***

To better understanding appropriate time and power levels for sonication, different levels of sonication were tested. The following images show the two different tests that were run to gain a better understanding of how different levels of sonication affected the degree of proteins harvested. The first test (Image 4) showed that the amount of sonication at its maximum of three minutes was still not enough to obtain results from the supernatant where the proteins should be found. The images show that as sonication time increase and intensity increased there were no effects on the amount of protein bands and their respective densities. This meant that there was a necessity to run the test with the lysis buffer to help break down the cells before sonication.

Due to this, the next test was designed to use 100% sonication at one minute time intervals between one and five minutes with 200  $\mu$ L of lysis buffer. The results showed that sonication of three, four, and five minutes led to similar results once lysis buffer was used, whereas, sonication of less than two minutes resulted in less protein densities in the 1D gels (Image 5). As a conclusion of this experiment, parameters for the protocol for the variable protein expression mapping due to electrochemical treatment experiment were set and results were improved.

Finally in Image 6, results for 2D PAGE show how results improve dramatically. The first image lacks a lot of proteins spots and those that are present are not well spread out. The second image shows an abundance of protein spots well spread Out on the gel.

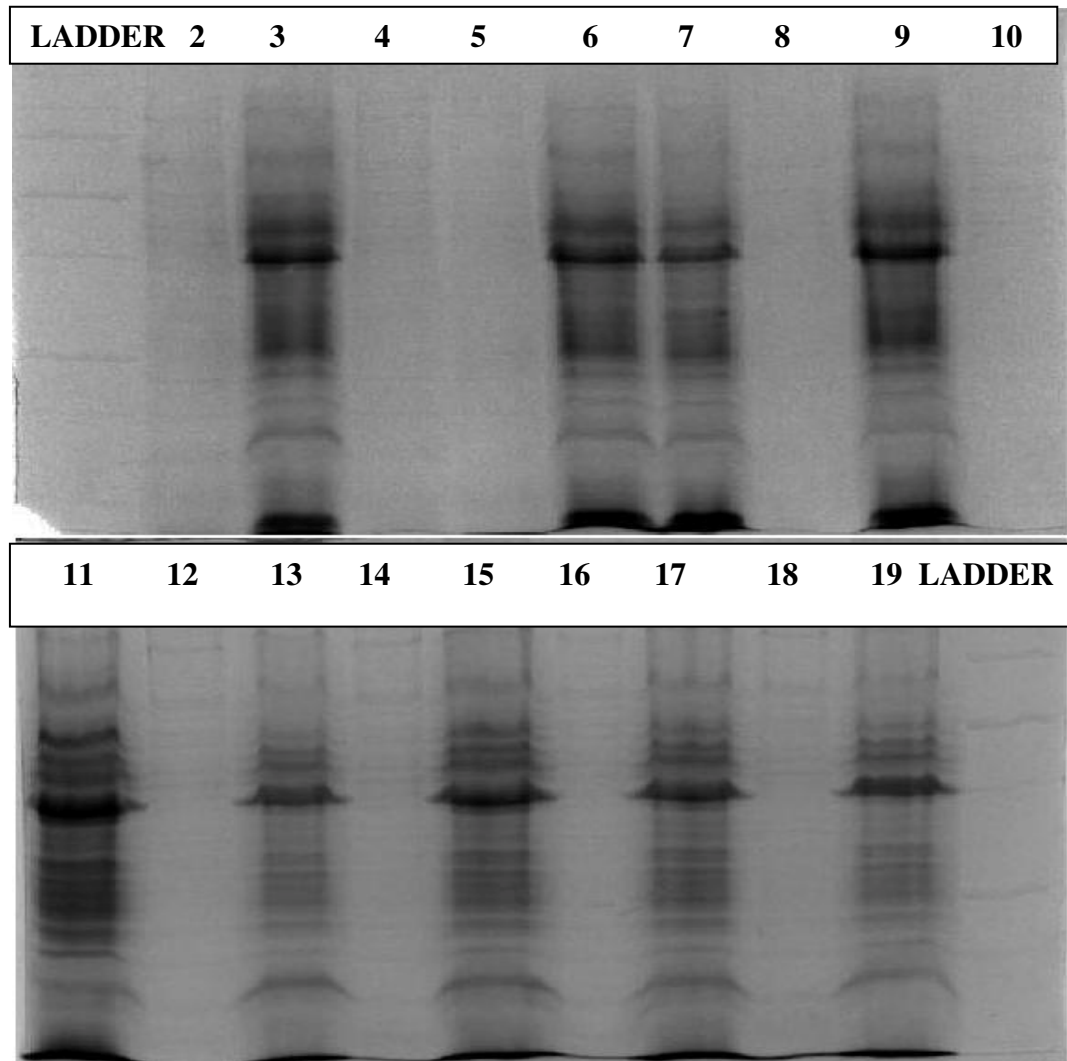


Image 4: The test results show how varying time and intensity of sonication affect the amount of protein present in the pellet and supernatant. From left to right the test shows: (1) Ladder, (2) Supernatant: 75% 2:00 min, (3) Pellet: 75% 1:30 min, (4) Supernatant: 75% 1:30 min, (5) Supernatant: 50% 3:00 min, (6) Pellet: 50% 3:00 min, (7) Pellet: 50% 2:00 min, (8) Supernatant: 50% 2:00 min, (9) Pellet: 50% 1:30 min, (10) Supernatant: 50% 1:30 min, (11) Pellet: 75% 2:00 min, (12) Supernatant: 75% 3:00 min, (13) Pellet: 75% 3:00 min, (14) Supernatant: 100% 1:30 min, (15) Pellet: 100% 1:30 min, (16) Supernatant: 100% 2:00 min, (17) Pellet: 100% 2:00 min, (18) Supernatant: 100% 3:00 min, (19) Pellet: 100% 3:00 min, and (20) Ladder.

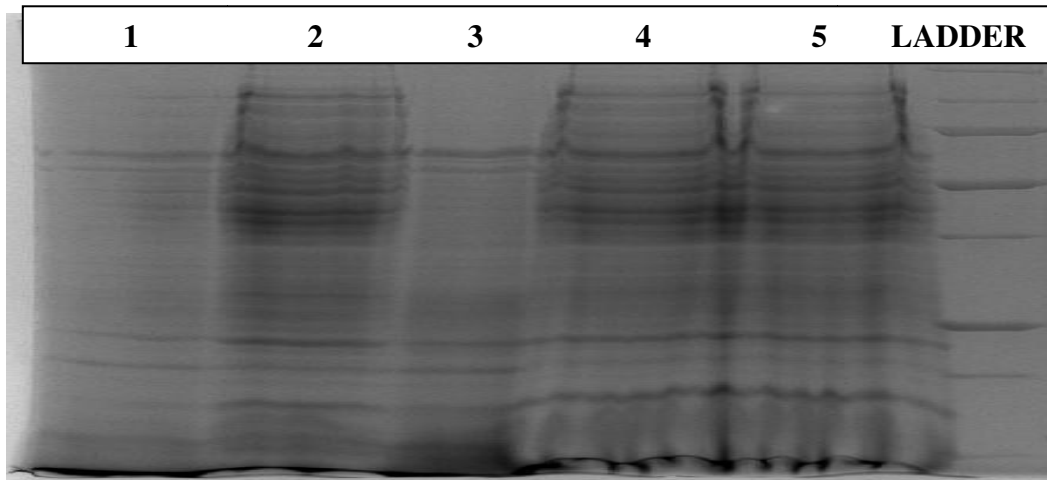


Image 5: The test results show how varying time at 100% sonication with lysis buffer affects the presence of proteins in the supernatant. Times from right to left are (1) 1 minute, (2) 3 minutes, (3) 2 minutes, (4) 4 minutes, and (5) 5 minutes.

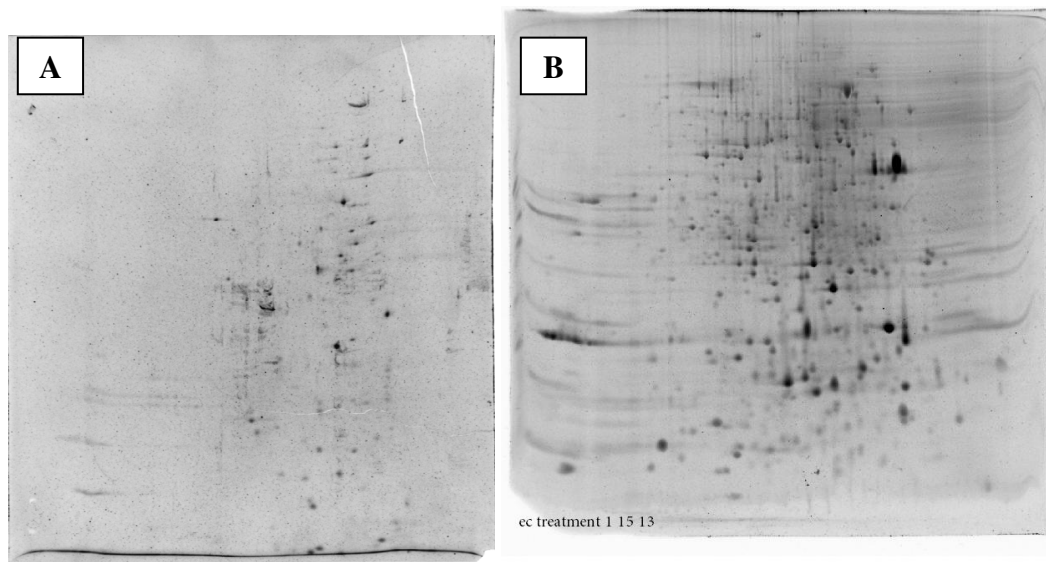


Image 6: The protein gels show the 2DE gels of *P. aeruginosa* before (A) and after (B) optimizing the protocol.

### 3.4 Differential protein expression *P. aeruginosa* in response to DC treatment

In order to better understand the mechanism of bacteria killing by DC, protein expression of *P. aeruginosa* PAO1 was analyzed using the 2D SDS-PAGE.

Because the protocol used was designed for *Escherichia coli*, eight tests were

done using the technique to ensure that proper and accurate results were achieved. Further, 1D gel tests were run to examine how varying degrees of intensity at different time intervals of sonication affected the yield and separation of proteins. Our results showed that sonication for five minutes at 100% power gave best results. By comparing 2DE images of the control and DC treated samples of several proteins, up- or down- regulated by DC were identified. Six represented proteins were sampled from the gels. Four of them were processed with in-gel digestion with trypsin and sent to the proteomic facility at Cornell University for protein identification by sequencing. Protein spot 1 and 3 were found up-regulated and extracted from the treated bacteria. Protein spot 1 was identified to be a 50s ribosomal protein L25 which assists in protein formation, stops peptidyl hydrolysis, and supports peptide bond formation. Protein spot 3 was found to be a stress protein, which is expressed in consistent anaerobic conditions due to energy starvation. Protein spots 4 and 5 were found down-regulated and extracted from the control bacteria. Protein spot 4 was found to be outer membrane porin F, which allows for passive diffusion of glucose, nutrients, ions, and water. Protein spot 5 was found to be DsbA, which is a soluble thiol:disulfide oxidoreductase periplasmic protein (responsible for forming disulfide bonds in proteins) and necessary for type III secretion system. Type III secretion system is made of a protein appendage used to detect eukaryotic cells and influence protein expression for bacterial infection (proteins are directly secreted into bacteria).



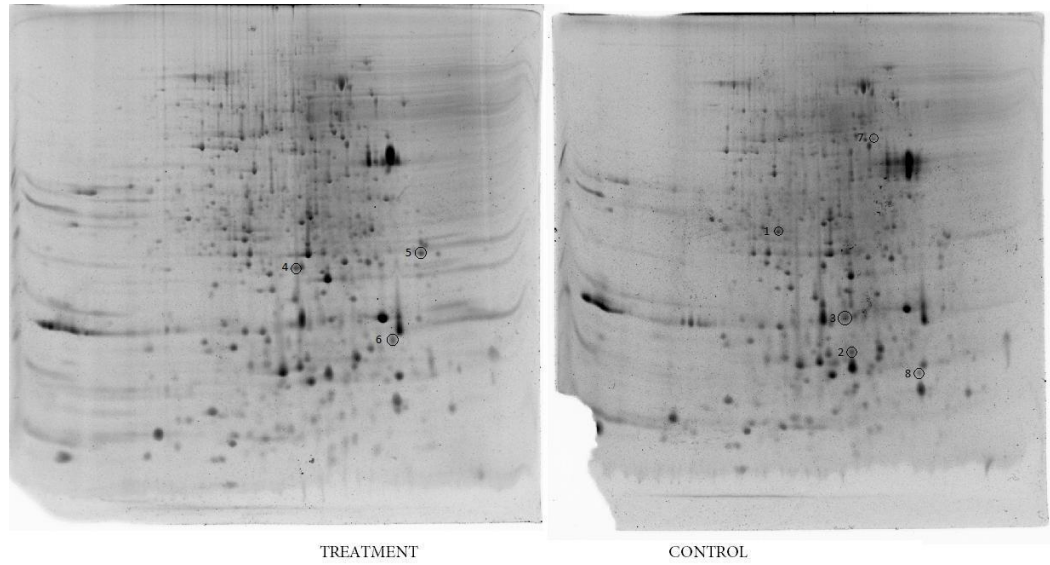


Image 7: The control and treatment gels that show the proteins that were chosen and extracted for protein identification.

#### **4. Discussion**

The application of the bioelectric effect has shown good potential for helping improve the efficacy of antibiotics for the treatment of chronic infections. This is especially important for elderly and young persons with weak or under developed immune systems as well with persons with diseases that decrease the functionality of their immune system such as HIV and AIDS or cystic fibrosis. The advancement in understanding the effects of DC treatment on human cells as well as bacterial cells will help advance this technology against bacteria.

Tests with human cells have shown there to be no major cytotoxic effects from the application of DC current at 500  $\mu\text{A}$  ( $70 \mu\text{A}/\text{cm}^2$ ), but the increase of DC current to 600  $\mu\text{A}$  ( $84 \mu\text{A}/\text{cm}^2$ ) was shown to have noticeable effects on human cells. Thus further experiments with bacteria and tobramycin were centered at 500  $\mu\text{A}$  or  $70 \mu\text{A}/\text{cm}^2$ .

Following the cytotoxicity study, this work progressed to a co-culture model of human cells and bacterial cells treated together with DC and tobramycin. This test was important for understanding the effect of DC treatment and antibiotics on a suspension of PA01 and human cells.

As for the relative cytotoxicity levels of the bioelectric effect, another innovative idea for the treatment of antibiotic resistant bacteria is through the use of nanoparticles. The use of the bioelectric effect has some distinct advantages over nanoparticles. First, the treatment itself does not involve a physical entity that requires degradation by the body. The use of nanoparticles requires there to be research into the biocompatibility and mechanical properties of the material.

There are potential issues with how and where nanoparticles would degrade and what the degraded nanoparticles could cause. One proposed issue is the accumulation of nanoparticles in intracellular areas leading to organelle disruption or gene alterations [12]. A study conducted by Pulskamp et. al. found a 60-80% reduction in cell longevity as a result of the use of carbon nanotubes [13].

The use of the bioelectric effect to control biofilms has been previously studied, but little has ever been researched and understood about the mechanisms of this phenomenon. Previous researchers have found efficacy between the uses of antibiotics with varying electrical currents [17], but the effects on mammalian cells were not tested. A study by Costerton et. al. suggested that killing of bacteria is not reliant the generation of ions or antibacterial particles due to the local electrochemical generation [10]. It has been shown that the stainless steel oxide film helps maintain the electrodes stability. When breakdown of the oxide film occurs, passivation occurs, disrupting the oxide film, and leading to corrosion. This is especially important for applications concerning the bioelectric effect because oxide films are easily disrupted when the electrical current becomes too high or too low and as the pH of the environment decreases. This is not a major issue as the electrodes will only be implanted temporarily, but it is important that any alloys present on the electrodes be safe in the body as a result of corrosion of the electrodes [11]. Furthermore, there is potential that the oxidation and reduction reactions that are occurring could contribute to the efficacy found between antibiotics and DC treatment. To better understand the potential that exists to expand upon this, tests will be run to see how higher

antibiotic concentrations continue to reduce the viability of PA01 and if there is a plateau reached where increased concentrations of the antibiotic have no further effect.

Results from the co-culture study show the drastic increase in microbial killing as the concentration of antibiotics is increased from 1.5  $\mu\text{g/mL}$  to 3.0  $\mu\text{g/mL}$ . This suggests that there may be a threshold concentration of tobramycin for killing PA01.

The proteomic results provide new insight into how the bacteria cells are reacting to DC treatment at the protein level. The 1<sup>st</sup> spot contained was identified to be a 50s ribosomal protein L25 which assists in protein formation, stops peptidyl hydrolysis, and supports peptide bond formation. For spot 3, stress proteins were found. Cells respond to stressful environments by the synthesis of stress proteins such as the one extracted at spot 1. Stress proteins are synthesized to help with the stressful environments, but given that the bacteria would have no genetic information about electrical treatment, the response is probably a standard response not specific to ec treatment.

Protein spots 4 and 5 were found down-regulated and extracted from the control bacteria. Protein spot 4 was found to be outer membrane porin F, which allows for passive diffusion of glucose, nutrients, ions, and water. Protein spot 5 was found to be DsbA, which is a soluble thiol:disulfide oxidoreductase periplasmic protein (responsible for forming disulfide bonds in proteins) and necessary for type III secretion system. Type III secretion system is made of a protein appendage used to detect eukaryotic cells and influence protein expression for

bacterial infection (proteins are directly secreted into bacteria). These spots show that the DC treatment is somehow preventing bacteria from infecting human cells by preventing the formation of the Type III secretion systems. The formation prevention is not from tobramycin as it was not involved in this test. Also it appears that since out membrane porin F is down regulated, the bacterium is working to maintain intracellular homeostasis by preventing the influx of ions, nutrients, water, and glucose.

## **Conclusion**

In this study, we demonstrated that DC treatment at  $70 \mu\text{A}/\text{cm}^2$  is safe to human cells. Furthermore, the co-culture study showed that DC treatment at  $70 \mu\text{A}/\text{cm}^2$  has the ability to selectively kill bacterial cells without causing harm to human cells. The effective concentration of tobramycin for co-treatment with DC was found to be  $3.0 \mu\text{g}/\text{mL}$ . To understand the effects of DC on *P. aeruginosa* at molecular level, the protocol to harvest cellular proteins was optimized to use sonication with lysis buffer. Upon extraction of proteins and comparison between control and DC treatment, ADD NAMES proteins were found to be up- and down-regulated by DC treatment, respectively. Overall, this study provided important understanding to missing information for bacterial control by DC and for developing more effective therapies to control chronic infection.

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## Capstone Summary

The project set up is entitled “Controlling bacterial persister cells with low level electric currents.” The project is designed to understand three different fields related to the study of killing antibiotic resistant bacteria using electrical currents. The three prongs of the study focus on the potential cytotoxicity of electrical currents, viability of human cells treated with an infectious bacteria (*Pseudomonas aeruginosa* strand PA01), and variable protein expression of PA01 due to the prolonged application of electrical currents. So what does each prong really mean?

The first study on the cytotoxicity of electrical currents means the study is designed to measure if using electrical currents on human cells would harm human cells. If something is cytotoxic it is effectively harmful to the cells. It is important to note these human cells come from companies and are professionally and ethically obtained. This is important because if the application of electrical currents, as a way to treat infectious diseases, which are antibiotic resistant, is proven effective, it could eventually lead to use in humans as a last resource fight against bacteria. This study aims to find out whether or not the application of electrical currents is harmful to individual human cells and the overall human body longevity. To put it another way, will the application of electrical currents end up hurting the body more than helping it?

To conduct this portion of the study, different types of cells extracted from the human body are grown in a solution that helps promote cell longevity. Cells are grown for a week in a controlled environment before tests are run. The cells

are grown for a week such that approximately the same amounts of cells are being tested for each testing period. For the tests to be done, cells are put into a cuvette, which is an upright rectangular container that holds the solution that contains the suspended human cells. Electrodes are pieces of electrically conductive material that allow for the flow of electricity from one to another through a nonmetallic material. By placing two electrodes in a cuvette, on opposite ends, it allows for the flow of currents from one electrode to the other through the aqueous medium (human cells suspended in a liquid solution) thus creating an electrical circuit. This experiment effectively puts human cells in the middle of the current flowing between these two electrodes. An Ag/AgCl (silver/silver chloride) electrode is placed between the two electrodes to act as a reference to ensure that the amount of current flowing between the two electrodes is consistent. The reference acts as a feedback mechanism by ensuring that the amount of current flowing from one electrode to the other stays constant. In order to measure whether or not the cells are being harmed, samples are taken from the cuvette before ec treatment starts and then after twenty, forty, and sixty minutes have elapsed. Samples are taken and dyed blue using Trypan blue which serves to dye the cell membrane blue such that cells are easy to count. To count cells a microscope is used with a specialized slide that has a grid network on it. The grid network is set up such that there are four sections of the grid that have a 4x4 grid of squares. By counting the amount of cells in each of the four sections, an average can be taken and the amount of cells in the cuvette can be inferred. The reason there are four sections is to ensure that a good and accurate representation is found. By taking the amount of cells

over the four time intervals (time = 0,20,40,60 minutes), the potential for cytotoxicity from ec treatment is determined. Results have shown that currents less than 600  $\mu$ A have minimal to no cytotoxic effects on the longevity of cells.

The next study is set up to understand how cytotoxicity might be altered due to the infection and presence of bacteria, *Pseudomonas aeruginosa*. This study also aims to see how *Pseudomonas aeruginosa* react to the ec treatment in the presence of human cells and with the presence of an antibiotic, tobramycin. This study is more complicated in that there are more factors involved. The factors being tested include: will the longevity of human cells be affected with the presence of bacteria, ec treatment, and antibiotics? Will killing of bacteria occur with the presence of human cells, ec treatment, and antibiotics? Will killing be more effective because of variable concentrations of antibiotics?

To answer these questions, a similar technique is used as for the first study except a specified amount of bacteria and antibiotic is added. The process of culturing bacteria is done the night before the experiment due to the incredible rate of bacterial reproduction. Bacteria are grown in a liquids solution or medium called lysogeny broth (LB), which is high in nutrients. To prepare bacteria for testing, a set amount of sample is washed with a solution known as a buffer that rids the LB medium. This is done so that bacteria do not rapidly grow during testing. To determine how much bacteria there is in the solution a measurement known as optical density is taken. Optical density is a measure of how much light is able to pass through a sample at a set wavelength (600nm is used). The amount of light that is absorbed by the sample correlates to the population of bacteria.

For example, if the goal OD is 0.6 but an OD of 0.9 is measured, you would add 2/3mL sample and 1/3mL buffer instead of 1mL of sample to make your sample the desired OD. The addition of tobramycin is done at concentrations relative to studies that have shown an effective range for the antibiotic.

Once the experiment is setup, ec treatment is run. At times of zero, twenty, forty, and sixty minutes, samples of cells and bacteria are taken and quantified. The process of quantifying the bacteria is more difficult as it requires the dilution of the sample to a quantity that can be counted then overnight culturing so bacteria colonies can grow and be visible for counting. This process is called plating and tells how the amount of bacteria changes as time passes for the experiment. With the conclusion of this experiment, information was determined about the most important concentrations of trobramycin to effectively kill bacteria alongside ec treatment. This has led to a lot of research being conducted with my mentor and myself as to why and how ec treatment increases the efficacy of antibiotics.

The last part of the study focused on understanding why bacteria are dying due to ec treatment by profiling protein expression. In order to do this, a technique called 2D- SDS Page was used because it creates a two-dimensional map of the membrane proteins. The proteins are shown on a gel material and are separated by the proteins' relative charges by pH and molecular weight. The proteins are displayed as black dots of varying size and darkness and can be extracted and sequenced to determine what specific proteins are. The process to create the protein maps takes six days and starts by creating an overnight culture

and running ec treatment. After ec treatment the cells are broken apart by a lysis buffer then DNA, RNA, and cell membrane fragments are broken up by a machine called a sonicator. The sonicator works by applying sound energy to samples disrupting genetic material so it does not remain in the mixture and alter results. After this, the proteins are put onto a one dimension gel strip and separated by pH gradient using a 24hr period of applied voltage. Protein interactions are disrupted so that proteins do not attach to each other preventing clear results. The last step involves the creation of gels then putting the one dimension gel strip on top and allowing it to run to create a two dimensional map of the process which shows the expression of proteins. For this study, an experimental (ec treated bacteria) and a control test (not ec treated bacteria) showed how the protein expression of the bacteria changed. By extracting proteins that changed in concentrations between the two tests, information about how different membrane proteins are in higher and lower concentrations will provide insight into how the bacteria are reacting to ec treatment.

The importance of this study is to examine new and innovative ways to kill antibiotic resistance bacteria that are becoming increasingly prominent. This problem has seen rise in many hospital settings where there is a constant need to eradicate the presence of any potentially harmful bacteria. The specific strand of bacteria tested, *Pseudomonas aeruginosa*, has been found to be resistant to multiple strands of antibiotics already. *P. aeruginosa* is an opportunistic bacterium that leads to skin infections, issues with cystic fibrosis, and elderly patients with weakened immune systems. *P. aeruginosa* can also lead to the

formation of biofilms in nature and in hospital settings on implants. Biofilms are communities of bacteria that attach to a surface and grow within an extracellular polymeric matrix (EPS) that protects the bacteria from antibiotics and harmful environments until the biofilm bursts and releases the bacteria. Biofilms are especially a problem as they allow for bacteria to trade genes that allow for widespread resistance to antibiotics. Another potential source of antibiotic resistant bacteria is persister cells. Persister cells are dormant cells that are practically metabolically inactive when a patient is taking their antibiotics, but become active again after the antibiotic regime is over and are able to repopulate and cause the symptoms to arise again (this is why it is very important to take antibiotics to completion!). When bacteria are able to repopulate and cause an infection to come back there is a better chance for the bacteria to learn antibiotic resistance and cause potentially life threatening issues to the patients.

Many approaches are being examined to find ways to overcome issues involved with antibiotic resistance, but the focus of this study is to learn more about the use of the bioelectric effect. The bioelectric effect aims to treat bacteria with antibiotics and electrical currents in order to increase the efficiency of the antibiotics. The project has helped prove that no harmful effects have been shown by using the bioelectric effect on varying strands of human cells. In addition, the study has delved into the level of electrical current applied and the amount of antibiotic used to maximize killing of bacteria while ensuring the longevity of human cells. The future of the bioelectric effect is looking into how the use of electrical currents helps improve efficacy of antibiotics by examining how the

antibiotics are transported into the cell. In addition, a sinus infection will be induced on an animal model to test how an actual animal reacts to DC treatment.