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ESCHERICHIA COLI HM22 BIOFILM VIABILITY WHEN IN CONTACT WITH IMPLANT-GRADE, TITANIUM-6ALUMINUM-4-VANADIUM ALLOY UNDER VARIED ELECTROCHEMICAL CONDITIONS

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ABSTRACT

This study was undertaken to investigate whether inducing electrical currents in Ti6Al4V alloy implants in organic systems holds promise as a means to combat the growth of biofilm infections. Prior research has shown the electrochemical reactions occurring on the surface of titanium alloys allow implants placed in contact with ionic solutions found in the body to negatively affect the viability of MC3T3 cells. If this holds true for bacteria, then the study of biofilms and their electrochemical interaction with titanium alloy implants may therefore lead to novel approaches to combating multidrug tolerant infections in implant patients.

Imaging analysis was conducted utilizing a Scanning Electron Microscope (SEM). Live/Dead cytotoxicity staining assay and fluorescent microscopy were also performed. *E. coli* HM22 was cultured and plated on Ti6Al4V discs, then inserted into custom-made electrochemical cell culture chambers under fresh LB media, and further incubated. Electrical contacts were attached to the Ti6Al4V disc and used to expose the sample to a voltage potential. Samples were tested at static potentials between -800 mV and +800 mV for a period of 24 hours versus Ag/AgCl. Samples were either fixed and dehydrated with formaldehyde and ethanol for SEM imaging or stained for live/dead imaging.

The results of this study indicate that cathodic polarization below -400 mV dramatically reduces the viability of *E. coli* biofilm cultured directly upon Ti6Al4V within 24 hours. Anodic polarization above -400 mV did not display statistical differences in viability as compared to OCP conditions after 24 hours. SEM imaging found no statistical difference in surface coverage between OCP and samples treated with anodic potentials below -200 mV. Cathodic potentials above OCP did, however, display increased surface coverage as compared to samples at OCP.

These outcomes may have clinical significance for titanium modular orthopedic implants. Via corrosion, the potential of such implants can shift down into cathodic ranges that exhibit poor biofilm viability and performance. These outcomes indicate that it might be possible to design improved metal alloy implants that naturally generate or through added active electronics generate specific cathodic potentials that might in the future be used to reduce or eliminate bacterial infections in implant patients.

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INTRODUCTION

The majority of surgery-related infections in developed countries are caused by bacterial colonies called biofilms. Biofilms are composed of bacterial cells that attach to a surface and release a sugar matrix that surrounds the colony with a protective coating helping to make them resistant to common antibiotics[1]. This antibiotic resistance, often referred to as multidrug tolerance, makes bacterial biofilm infections extremely difficult to treat.

Bacterial infections occur in approximately 1,000,000 medical device implant surgeries a year in the United States[2,3]. This represents approximately 5% of all implant surgeries in the United States[4]. Implant-centered infection rates have not significantly declined over the past decades, making this one of the most significant challenges in medical devices today. There are numerous reasons for device-related infections, such as non-compliance with hygiene protocols by hospital personnel and bacterial colonization from a patient's own skin. The medical community has much to learn about avoiding and controlling infectious growths in patients who receive surgical implants.

Implants are said to be composed of biomaterials, which are defined as materials used in the construction of devices that interact with biological systems. Metal alloys are the most commonly used biomaterials in the construction of dental and orthopedic devices including implants. Titanium is often a primary component of these alloys. Titanium is utilized because of its high biocompatibility with bone tissues. This biocompatibility is partly attributed to the relative corrosion resistance of titanium. When titanium is exposed to air or

an oxygenated solution like those found in the human body, the metal surface will spontaneously form a titanium oxide layer. This oxide layer acts as a barrier between the titanium implant and the human body, limiting corrosion kinetics.

The study of bacterial colonies and their interaction with titanium alloy biomaterials is essential to developing safer medical devices and implants. To combat or eliminate multidrug tolerant infectious biofilms and improve the lives of implant patients, researchers must develop novel approaches to destroying these drug resistant bacterial colonies that do not rely on ineffective antibiotics. The research described herein was undertaken to explore one such potential approach to reducing bacterial infections in implant recipients.

Prior research has shown that electrochemistry – the electrochemical (oxidation and reduction) reactions that occur on the surface of metallic biomaterials placed in contact with simulated biological fluids (SBF) similar to those found in the human body – can negatively affect the health and viability of MC3T3 preosteoblast cell populations[5,6]. Positive (anodic) and negative (cathodic) voltages that arise from these electrochemical reactions can result in either increased oxidation reactions that break down oxide layers that form on "biocompatible" alloys allowing metal ions to enter the biological system and potentially inhibit biofilm formation under the right conditions, or increased reduction reactions resulting in solution-based changes near the implant surface. These voltages can be induced artificially or they can occur naturally due to abrasion of titanium surfaces against bone, fretting at modular implant connections, or in-vivo biochemistry. It is possible that these same

electrochemical effects may be capable of negatively affecting bacterial populations. One approach might include embedding electronic components into implants, or galvanically coupling to an actively corroding metal that can proactively generate potentials to adversely affect bacterial growth. These approaches may prove to be methods to eliminate biofilm-based infections in implant recipients.

This study was undertaken to investigate and understand how electrochemical changes in and around titanium alloy implants in biological systems affect the growth and viability of bacterial biofilm colonies cultured on their surfaces. *E. coli* (HM22) bacteria were cultured in an ionic solution on Ti6Al4V metal alloy samples. The samples were then electrically polarized to a range of static potentials between -800 mV and +800 mV for a period of twenty-four hours. Control samples were allowed to freely establish their resting open circuit potential (OCP). If certain voltages were shown to be effective in killing bacterial colonies, this would be a positive indication that inducing specific electrochemical currents around metallic implants might prove effective within humans. Such knowledge could lead to improved implants or implantation techniques to reduce the occurrence of acute infections in patients recovering from implant and other orthopedic surgeries.

METHODS AND MATERIALS

Sample and Chamber Preparation

Discs of grade 5, titanium-6-aluminum-4-vanadium (Ti6Al4V) were prepared for use through sequential wet sanding to a final finish of 600 grit. Disc samples were then rinsed with ethanol and ultrasonically cleaned in 95% ethanol for a period of 30 minutes. These samples were then placed into fresh ethanol for sterilization. The samples were mounted in the bottom of custom-made glass electrochemical cell culture chambers (Figure 1). A threaded metal rod was connected to the unpolished underside of the Ti6Al4V sample. This rod passed through a similarly threaded nylon bushing to provide an external electrical contact to the Ti6Al4V sample. When the glass chamber was lowered and threaded onto the nylon bushing, it compressed a rubber o-ring which provided a water-tight seal around the surface of the Ti6Al4V sample. After compression of the o-ring, this set-up exposed a 3.8 cm^2 area of the Ti6Al4V sample to the interior of the electrochemical chamber. This chamber could be sealed at the top of the glass by way of a rubber stopper. The rubber stopper had access holes for the insertion of a graphite rod counter electrode, the insertion of a chloride silver wire (Ag/AgCl) reference electrode, and for gas exchange. Thus, the Ti6Al4V sample disc could be used as a working electrode.

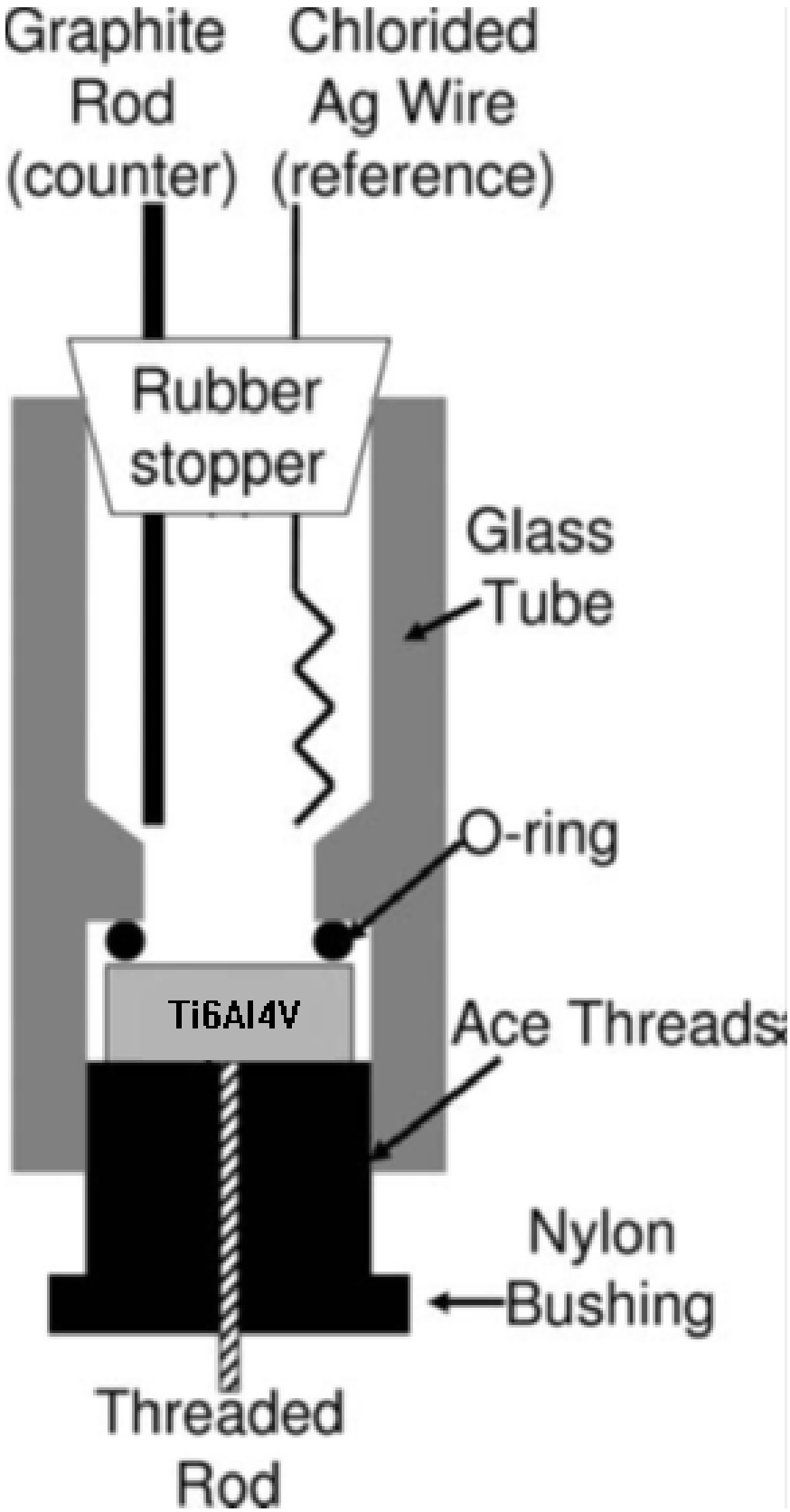

Figure 1 - Schematic of the electrochemical testing chamber. (Taken from Ehrensberger and Gilbert, 2009)

Cell Culture

In all performed experiments, a sterile loop was used to withdraw the HM22 *Escherichia coli* cell line from a glacial stock to inoculate a 25 mL lysogeny broth media composed of 5 g tryptone, 5 g NaCl and 2.5 g yeast extract (all components per 500mL millipore water) supplemented with 25 μL diphenyl phosphorazidate (DPA) in T-75 culture flasks. The inoculated cells were cultured for a period of 18 hours at 37°C with shaking at 200 rpm. Next, the electrochemical chambers were seeded with the *E. coli* cells. 20 μL cell suspensions were then dispensed onto the Ti6Al4V sample surfaces. An additional 20 mL of fresh lysogeny broth was then added to the chamber to immerse the chloride silver reference and carbon counter electrodes. These chambers were placed into an incubator at 37°C. The samples remained in the incubators for 20 hours under static conditions to allow for the growth of biofilm on the Ti6Al4V sample surfaces.

Electrochemical Testing

Electrode connections were made from the electrochemical chambers to a potentiostat (EG&G 263, Princeton Applied Research). The Ti6Al4V samples were potentiostatically held at -800 mV, -400 mV, -200 mV, +200 mV, +500 mV, +700 mV, or allowed to sit at OCP (-100mV). All voltages used in this study were with respect to a chlorided silver wire reference electrode. Current was observed before and after testing.

Cell Morphology and Surface Coverage

After 24 hours in culture, the potentiostat was disconnected from the electrodes and the Ti6Al4V samples were rinsed with phosphate buffered saline to remove the lysogeny broth media and any non-adherent cells. Cells remaining on the Ti6Al4V surface were then fixed using 4% formaldehyde and 96% PBS solution and dehydrated using graded ethanol mixtures of 50%, 75%, 90%, 100% ethanol:PBS. The formaldehyde solution and ethanol solutions each were allowed to rest on the samples for a period of 15 minutes, then removed. After fixation and dehydration, the samples were allowed to dry in an incubator at 37°C for a period of twenty hours. To prevent charge build-up on the sample surfaces during imaging, the Ti6Al4V disc samples were freeze-dried and gold sputter-coated for a period of thirty seconds. These samples were imaged on a Joel 5600 scanning electron microscope (SEM). Images of the Ti6Al4V surface were captured at fixed magnifications of 500X, 1000X, 2500X and 5000X in the secondary electron mode. Secondary electron mode images captured topographical features of the sample surfaces. Images were assessed both quantitatively and qualitatively. Quantitative assessment consisted of exporting SEM images for surface coverage calculations using ImageJ image analysis software. Qualitative analysis consisted of general characterization of *E. coli* bacteria morphology. A statistical analysis to compare each test group with the control group was performed using a T-Test, with a P-Value less than 0.05 against the control of open circuit potential considered to be significant.

Cell Viability

Cell viability was determined using live-dead staining at selected potentials (OCP, -800, -400, -200, +800) after twenty-four hours to assess viability with fluorescent imaging. An Invitrogen L-7007 Live/Dead Bacterial Viability Kit was used to assess live/dead viability/cytotoxicity. Bacteria were imaged using an inverted microscope (Axiovert 40CFL, Zeiss, Denmark) that was fitted with a CCD mono- 12 bit camera (Q-imaging, Canada). This microscope was further fitted with Image-Pro 3D suite version 5.1 (Media Cybernetics, MD), which was used to acquire and then overlay green (live) and red (dead) images. Voltage potential was removed from the samples. The voltage-treated lysogeny broth was removed, the electrochemical testing chambers were disassembled, and the Ti6Al4V samples were transferred into six well plates. The samples were washed with 5mL of PBS, after which the PBS was immediately removed. Dye was prepared using a mixture of 997 μL PBS, 1.5 μL live-staining dye and 1.5 μL dead-staining dye. 150 mL of dye mixture was pipette directly onto each Ti6Al4V sample's surface. The samples were held at room temperature and kept from light for a period of fifteen minutes to dry. Excess solution was blotted off of sample surfaces using a kimwipe. Samples were then placed inverted onto a petri dish utilizing glass coverslip spacers to protect the cells on the surface for imaging. Data was generated to directly show the viability or cytotoxicity of *E. coli* bacteria at various potentials.

Images were analyzed using ImageJ (NIH), an image processing program. Composite images of sample surfaces were submitted to a threshold to display

live cells (green) of hue 50 – 255, and brightness 42 – 255. Dead cell (red) images were thresholded to a hue of 0 – 40 and a brightness of 42 – 255. These images were converted to binary and analyzed by measuring area fraction of the total image. This was done to determine the ratio of dead cells to live cells. To calculate the total percent live of the population of cells in an image, the following equation was utilized:

$$\% \text{ Live} = (G / (G + R)) * 100$$

G represents the surface coverage of the total image by only live (green) cells. R represents the surface coverage of the total image by only dead (red) cells.

Statistical Analysis

Three Ti6Al4V samples were tested at each voltage for each cell culture measurable outcome. A single control Ti6Al4V sample at OCP was run alongside each voltage level. The surface coverage and cell viability outcomes at twenty-four hours were compared across voltages through one way analysis of variance (ANOVA) techniques, and a statistical difference was considered to be significant at $P < 0.05$. ANOVA was followed by Bonferroni-Holm ad-hoc analysis. All statistical calculations were performed using XL Toolbox (2012 Daniel Kross).

RESULTS

Morphology

Displayed in Figure 2(a-g) are low magnification (500x) secondary electron micrographs of *Escherichia coli* HM22 cells cultured on electrically polarized Ti6Al4V samples for a period of twenty-four hours at different voltages. The morphology of the cultured cell samples polarized at -800 mV and -400 mV are noticeably different from the cells cultured at all the other potentials. The

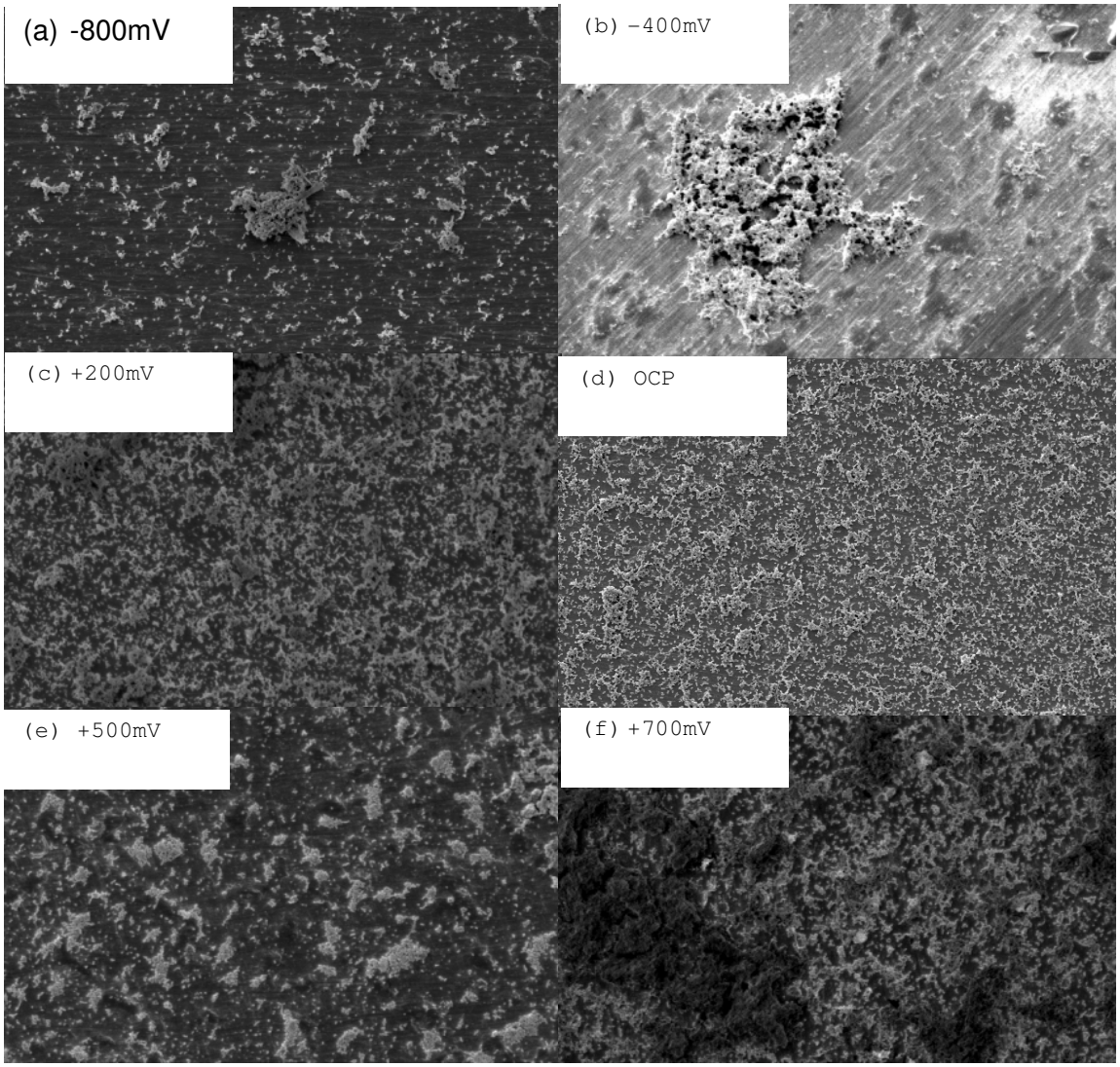

Figure 2. A panel of secondary electron micrographs of *E. coli* HM22 cells on electrically polarized Ti6Al4V samples after 24 hours (500x magnification). Voltages are labeled in each image.

cells at -800 mV and -400 mV [Fig. 2(a-b)] appeared smaller and irregular in shape and more scattered in appearance, as compared to the larger, more elongated cells at the other potentials [Fig. 2(c-f)]. The large globular biofilm forms in the -800 mV and -400 mV images look almost charred.

Figure 3(a-c) displays *E. coli* HM22 cells cultured on electrically polarized Ti6Al4V samples at -800 mV, -400 mV and OCP for twenty-four hours at 1000x

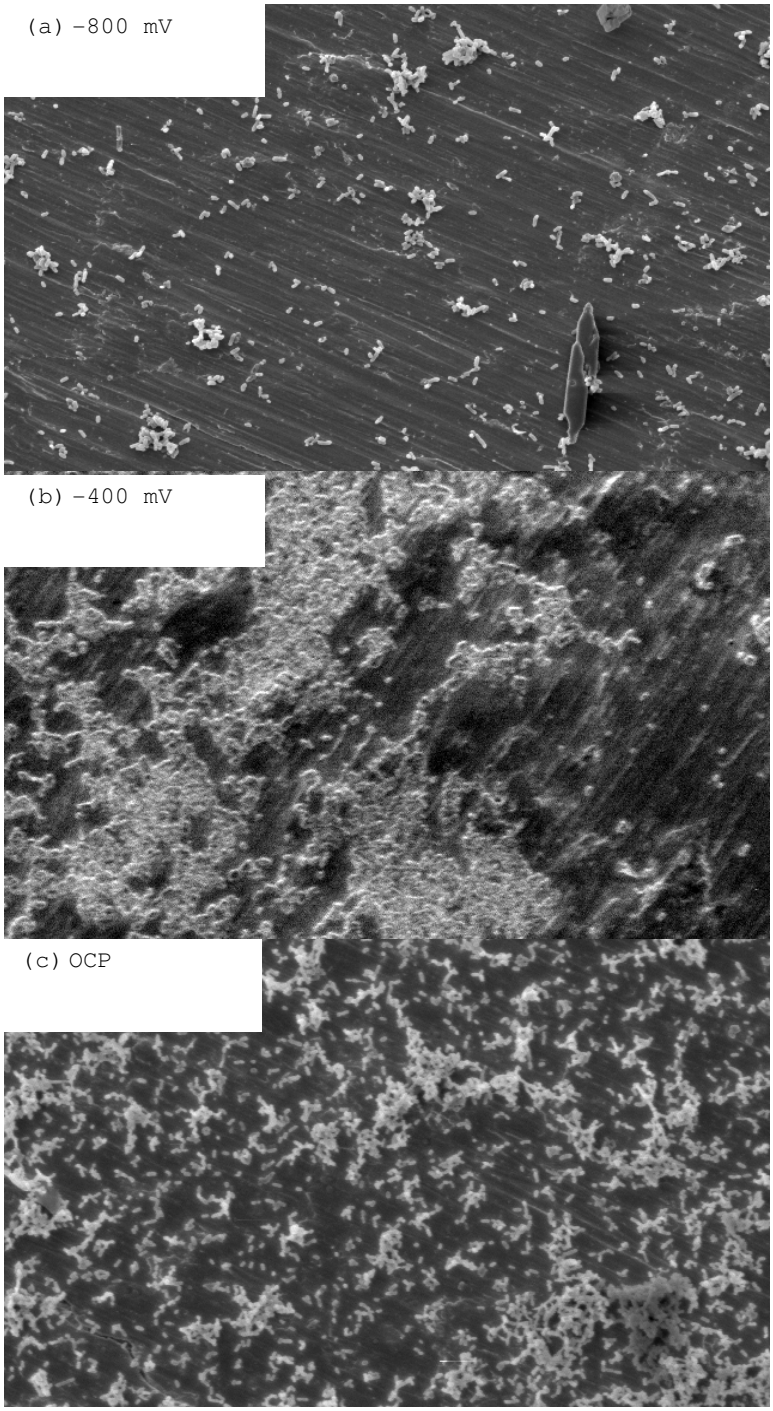

Figure 3. A panel of secondary electron micrographs of *E. coli* HM22 cells on electrically polarized Ti6Al4V samples for 24 hours (1000x magnification). Voltages are labeled in each image.

magnification. These images show more clearly the contrast in cell morphology and coverage. Cells cultured at -800 mV and -400 mV [Fig3(a-b)] are more round, with less cells possessing the familiar *E. coli* aspect ratio of 2:1 seen at OCP [Fig3(c)], or voltages above OCP (-100 mV). Also, there was less coverage of bacteria at -800 mV compared to OCP.

Surface Coverage

The quantitative analysis of cell surface coverage is displayed in Figure 4 as a plot of average *E. coli* HM22 percent coverage of the Ti6Al4V samples for each voltage condition for images at 500x magnification. These values include surface coverage attributable to *E.* coli biofilm. The surface coverage of cells cultured on electrically polarized Ti6Al4V at voltages of -800 mV, -400 mV, and +200 mV for twenty-four hours had no significant variation ($P > 0.05$) compared to cells cultured at OCP. *E. coli* HM22 bacterial cells cultured above +200 mV displayed a statistically significant ($P < 0.05$) difference in Ti6Al4V surface

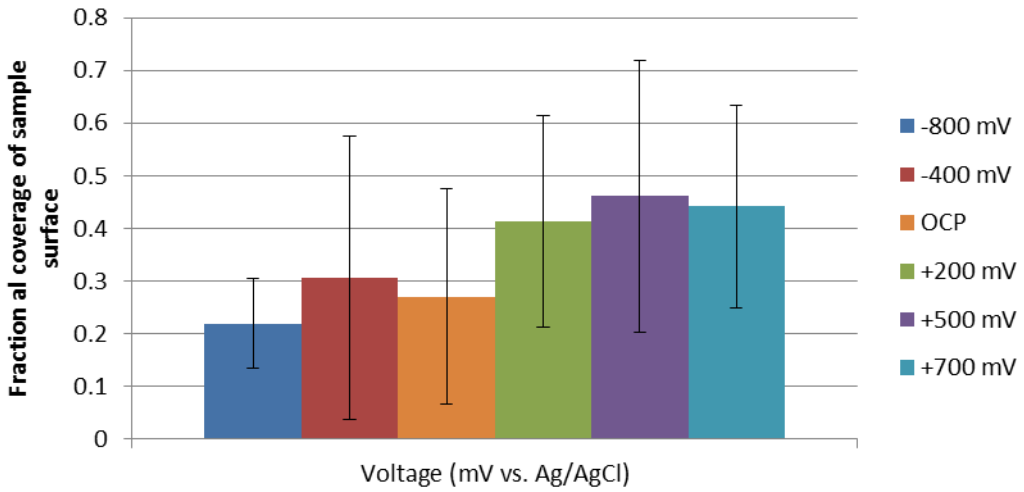

Figure 4. A plot of average Ti6Al4V surface coverage (error bar = ±1 SD) versus voltage at 24 hours. Note the increase in surface coverage above OCP.

coverage as compared to those cultured on OCP.

Cell Viability

Figure 5(a-f) shows the live-dead viability/cytotoxicity results for *E. coli* HM22 cells cultured on -800 mV, -400 mV, -200 mV, OCP, and +800 mV at twenty-four hours. Note that the cells at the most cathodic voltage (-800 mV) [Fig5(a)] are largely dead (red cell bodies) at this potential. Cells at voltages more anodic than -800 mV [Fig5(b-f)] were still viable (green cell bodies).

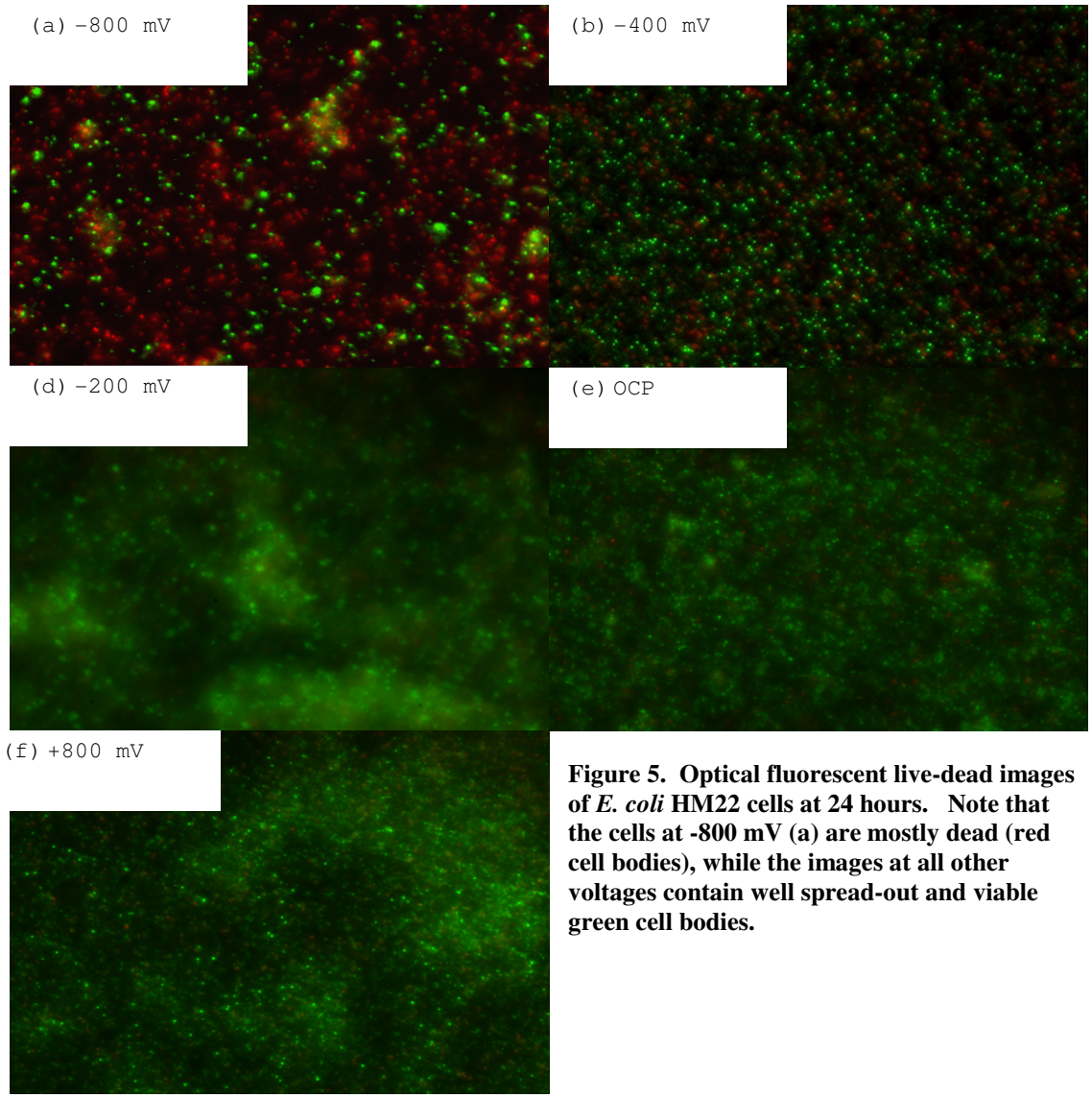

Figure 5. Optical fluorescent live-dead images of *E. coli* HM22 cells at 24 hours. Note that the cells at -800 mV (a) are mostly dead (red cell bodies), while the images at all other voltages contain well spread-out and viable green cell bodies.

Figure 6 shows the twenty-four hour live/dead viability/cytotoxicity assay outcomes at the various chosen electrically polarized Ti6Al4V conditions. Analysis showed that viability was significantly reduced ($P < 0.05$) at -800 mV when compared to OCP and all the other potentials. -200 mV was also shown to have nearly 100% viability ($P < 0.05$) as compared to OCP. There were no significant differences in cell viability or toxicity for *E. coli* HM22 at -400 mV, OCP, or +800 mV. Taken together, the data appears to show that *E. coli* HM22 undergoes significant cell death at -800 mV by twenty-four hours.

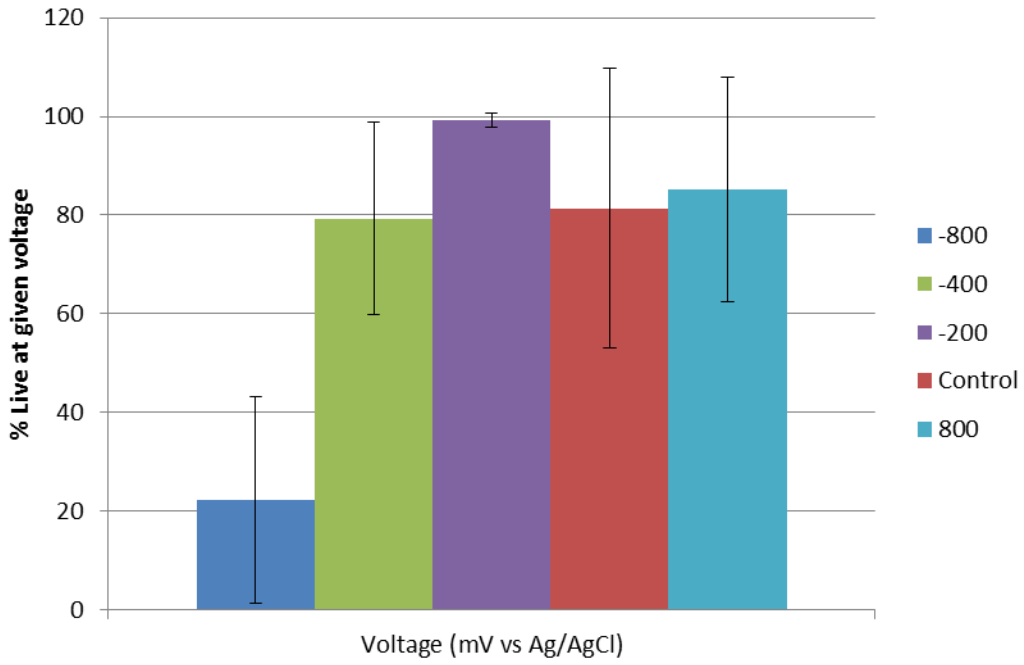

Figure 6. Plot of the average 24 hour live/dead viability/cytotoxicity assay for cells cultured at each potential on Ti6Al4V (error bar = +/-1 SD). Take note of the steep drop in viability at --800 mV relative to cells at the other potentials.

DISCUSSION

This study demonstrated that cathodic electrochemical voltages have a significant effect on bacterial viability for HM22 *E. coli*. However, there was no real effect on biofilm surface area coverage of Ti6Al4V by cathodic voltages below +200mV, in relation to OCP.

The results of this study have shown that cathodic polarization below -400 mV drastically reduces the viability of *Escherichia coli* HM22 bacterial cells when cultured directly upon Ti6Al4V surfaces within twenty-four hours. Anodic polarization (-400 mV to + 800 mV) appears to have no significant difference in viability after twenty-four hours. The mechanism of the antibacterial activity of electrochemical stimulation has been suggested by del Pozo et al.[8] to be the result of membrane damage leading to the leakage of essential cytoplasmic constituents, the formation of electrolysis products (such as chlorine molecules and H_2O_2), decreased bacterial respiratory rate, induced drag of hydrated ions along with an electrical field which places an extra force stimulate the detachment of bacteria, along with other mechanisms. Another possibility is that cathodic voltages result in a flux of electrons through the surface directly into the biofilm and cells, altering biochemical pathways within the cell or membrane voltage dependent structures[6]. Proteins in the solution may be irreversibly altered by reduction reactions on sample surfaces that might alter bacterial behavior[6].

It is important to point out the distinct plunge in viability that occurs at some voltage between -400 mV and -800 mV, as observed in this study. This small window of voltage produces an almost 60% drop in viability. It is very

likely that this transition is much narrower than -400 mV. Further work focusing on this window to pinpoint a transition point would deliver valuable information regarding electrochemical thresholds in relation to biofilm growth. This result is similar to that seen for mammalian MC3T3 cells cultured directly on commercially pure titanium (cpTi) implant surfaces[6].

This study suggested that bacteria cultured at -200 mV experienced a 99.27% viability rate, significantly higher than that of cells cultured at OCP, about -100 mV ($P < 0.05$). It is possible this may be an error, due perhaps to either an error in red dye application for samples at -200 mV, or an issue involving the low brightness levels of the images taken. Both of these issues could cause the methodology utilized by this study to inaccurately detect the hues representing dead cells, causing an error in calculation. Further research may be necessary to validate these results.

It is likely that the negative impacts of cathodic effects on biofilm viability are not limited to bacteria in the vicinity of Ti6Al4V alloy. It is possible that cathodic voltages will have similar effects on bacterial growth in proximity to other metallic biomaterials. . Future work to determine the effects of electrochemistry on the surfaces of other common metallic alloys (e.g. CoCr) would provide insight into the potential of cathodic voltages to retard bacterial growth on a variety of conductive biomaterials.

The findings of this study regarding surface coverage agree with research stating that applied voltages have little influence on the biofouling of surfaces by biofilm[7]. The current study did, however, find statistically significant evidence

suggesting that anodic potentials above +200 mV cause an increase in surface coverage of approximately 20% as compared to *E. coli* cells cultured at OCP on Ti6Al4V. Further testing is warranted to validate this finding, as it may have some influence on biofilm growth around Ti6Al4V implant sites experiencing anodic OCP shifts via corrosion.

There are several concerns that should be addressed associated with the present study. While the intended study sought to discover the effects of electrochemistry on *E. coli* biofilm growth and viability cultured directly on the surface of metallic implants, it did not attempt to evaluate the impacts to planktonic bacteria that were suspended in the media. The impact of electrochemistry on bacteria not in immediate proximity to implants may require further study. As an *in-vitro* test, only cells attached to the sample are under the observed influence of the adjacent electrified material. Only a small proportion of a given bacterial population is in a biofilm phase at a given time. If attempts are made to determine the full efficacy of Ti6Al4V-based electrochemistry in reducing infectious viability, especially in *in-vivo* studies, they will need to take planktonic and otherwise remote biofilm growth into consideration.

There may be some potential error in results due to the design of the electrochemical testing chambers. It is possible that the presence of the two electrodes may have influenced results. The reference electrode (the Ag/AgCl wire) was unlikely to have affected the results given its standardization and presence amongst all testing setups. The Ag/AgCl wire would have also been unaffected by the applied voltages as it is isolated by the electronics of the

potentiostat. The carbon counter was a more likely candidate for producing error. This electrode was required to complete the given electric circuit. Its participation in the circuit may have forced its surface to undergo electrochemical reactions separate from those occurring on the Ti6Al4V sample surface. While this effect would have been lessened due to its distance from the sample surface, it is unclear whether this would have had a marked effect on cell viability. While possible, it is unlikely that slight variations in the exposed surface area of the carbon counters would have had a significant enough effect upon potential charge density to effect viability. This phenomenon could have produced variation amongst different electrochemical testing chambers. It is also possible, but unlikely, that carbon counter debris (having detached from the counter) made its way to the sample surface and had any appreciable effect on surface coverage data. It is unlikely that carbon residue present during live/dead assay testing would have any effect on viability data.

CONCLUSION

This study was undertaken to investigate whether inducing electrical currents in Ti6Al4V alloy implants in organic systems holds promise as a means to combat the growth of biofilm infections. An electrochemical cell culture chamber was used to promote cell growth on electrically polarized Ti6Al4V alloy surfaces. This system was employed to assess the interactions between Ti6Al4V surfaces and *Escherichia coli* HM22 cells. Results indicated that anodic polarization above +200 mV had a positive effect upon the spreading of biofilms cultured directly on Ti6Al4V at twenty-four hours. Cathodic polarization below -400 mV was shown to dramatically reduce the viability of cells compared to the OCP condition. These outcomes may have clinical significance for titanium modular orthopedic implants. Via corrosion, the potential of such implants can naturally shift down into cathodic ranges that exhibit poor biofilm viability and performance. Improvements in the composition of implant alloys may be used to promote this natural generation of potentials that discourage biofilm growth. These outcomes also indicate that adding active electronics to metals may allow implants to generate specific cathodic potentials that could act to reduce or eliminate bacterial infections in implant patients. Utilizing the positive influence of electrical stimulation coupled with Ti6Al4V could very well be a promising method to combat orthopedic device-related infections.

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SUMMARY OF CAPSTONE PROJECT

The majority of surgery-related infections in developed countries are caused by bacterial colonies called biofilms. Biofilms are composed of bacterial cells that attach to a surface and release a sugar matrix that surrounds the colony with a protective coating helping to make them resistant to common antibiotics. This antibiotic resistance, often referred to as multidrug tolerance, makes bacterial biofilm infections extremely difficult to treat.

Bacterial infections occur in approximately 1,000,000 medical device implant surgeries a year in the United States[1,2]. This represents approximately 5% of all implant surgeries in the United States[3]. Implant-centered infection rates have not significantly declined over the past decades and it is one of the most significant challenges in medical devices today.

The medical community has much to learn about avoiding and controlling infectious growths in patients who receive surgical implants. These implants are said to be composed of biomaterials, which are defined as materials used in the construction of devices that interact with biological systems. Biomaterials are used in a wide variety of medical procedures in addition to metal alloy implants. The study of bacterial colonies and their interaction with metal alloy biomaterials is therefore essential to developing safer medical devices and implants. To combat or eliminate multidrug tolerant infectious biofilms and improve the lives of implant patients, researchers must develop novel approaches to destroying these

drug resistant bacterial colonies that do not rely on ineffective antibiotics. The research described herein was undertaken to explore one such potential approach to reducing bacterial infections in implant recipients.

Prior research has shown that electrochemistry – the electrochemical (oxidation and reduction) reactions that occur on the surface of metallic biomaterials placed in contact with the types of ionic solutions found in the human body – can negatively affect the health and viability of bacterial cell populations including biofilms[4,5]. Positive (anodic) and negative (cathodic) voltages that arise from these electrochemical reactions can result in either increased oxidation reactions that break down oxide layers that form on "biocompatible" alloys, allowing metal ions to enter the biological system and potentially inhibit biofilm formation under the right conditions, or increased reduction reactions resulting in solution-based changes near the implant surface. These voltages can be induced artificially or they can result naturally at implant sites when alloyed implant components rub against each other. The magnitude of the naturally occurring voltages is usually not sufficient to create adverse bacterial cell growth conditions. Perhaps, however, embedding electronic components into implants that proactively generate potentials able to adversely affect bacterial growth may prove to be a method to eliminate biofilm-based infections in implant recipients. This study was undertaken to investigate and understand how chemical and electrical changes in and around titanium alloy implants in organic systems affect the growth and viability of bacterial biofilm colonies. *E. coli* (HM22) bacteria were grown in the lab under artificial conditions ("in vitro") and

placed in contact with a metal alloy implant of the type used in hip replacements immersed in a solution designed to mimic human body chemistry ("an ionic solution"). The construct was then subjected to electrochemical currents at varied voltages and analyses were performed to determine whether electrochemical conditions could be established that inhibit bacteria growth. If certain voltages were shown to be effective in killing bacterial colonies, this would be a positive indication that inducing specific electrochemical currents around metallic implants might prove effective within humans.

The specific implant alloy used in this study is called Titanium-6-Aluminum-4-Vanadium or Ti6Al4V for short. Ti6Al4V alloy is one of the most commonly used hip replacement implant materials. The first stage of this study required the preparation of a usable bacterial population. The strand *Escherichia coli* HM22 (*E. coli* HM22) was chosen for its safe, non-pathological nature and ease of use. *E. coli* HM22 was cultured for 18 hours in a 25mL lysogeny broth (LB) media with 25uL diphenyl phosphorazidate (DPA) at 37°C. Lysogeny is a nutrient rich solution commonly used for growing bacteria in the lab. The solution was mechanically shaken at 200 rotations per minute throughout its incubation. The bacteria were then plated on sterile, polished Ti6Al4V discs and inserted into custom-made electrochemical cell culture chambers under fresh LB media. These electrochemical chambers were further incubated for 20 hours to grow a biofilm layer. Electrical contacts to the Ti6Al4V disc from a potentiostat functioned as a working electrode, along with a graphite counter and chloride silver wire reference electrode inserted into the media. The potentiostat is a device that

controls the voltage of the working electrode. Samples were tested at six different voltages: 700mV, 500mV, 200mV, OCP (-100mV), -400mV and -800mV for a period of 24 hours. Once voltage was cut, samples were either fixed and dehydrated with formaldehyde and ethanol for scanning electron microscope (SEM) imaging or stained for live/dead imaging to determine the fractions of live and dead bacteria present.

In this study, imaging was conducted utilizing a Joel 5600 Scanning Electron Microscope. Live/Dead cytotoxicity staining assay (Invitrogen L-7007) and fluorescent microscopy was conducted using a Zeiss Axiovert 40 CFL Inverted Fluorescence Microscope and Imaging System with Phase Contrast. Image analysis to assess cell surface coverage and viability was conducted utilizing an image analysis software package that allowed one to quantify the fraction of living and dead bacteria in each image (ImageJ).

The results of this study showed that cathodic voltages below +200 mV had no real affect upon *E. coli* HM22 biofilm surface coverage of Ti6Al4V samples. Anodic voltages above +200mV were shown to increase sample surface coverage by biofilm growth. This may warrant further testing, as corrosion near Ti6Al4V implants can produce anodic voltages in this range. This would potentially increase biofilm formation on these surfaces.

Significantly, cathodic potentials below -400 mV were shown to drastically reduce the viability of *E. coli* HM22 biofilms on Ti6Al4V surfaces after twenty-four hours of voltage exposure. No other tested potential had a

significant effect upon biofilm viability. This increase in bacterial killing near -800 mV may be due to a number of different electrochemical effects on cell surfaces. This sharp drop in viability occurs somewhere between -400 mV and -800 mV, and further research could be performed to attempt to pinpoint the voltage at which viability begins to significantly decline. This would return additional valuable information.

These outcomes may indicate that adding active electronics to metals may allow implants to generate specific cathodic potentials that could act to reduce or eliminate bacterial infections in implant patients. Utilizing the positive influence of electrical stimulation coupled with Ti6Al4V could very well be a promising method to combat orthopedic device-related infections.

Summary References:

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