Bacterial Biofilms on Electrochemically Active Cathodic Titanium Surfaces

Jin Guo

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Abstract

Bacterial infection is a major concern in orthopedic implants that may lead to implant failure and revision. The resistance of bacterial biofilms to antibiotics increases the difficulties of fighting against infections. In this work, the effects of electrochemical reduction reactions on bacterial biofilm cultured on electrochemically active metal surfaces were investigated to better understand the mechanism of bacterial response to reduction electrochemistry. The influence of voltage and electrolyte were studied on the cellular behavior of *E. coli* HM22 cultured on commercially pure titanium (cpTi) surfaces held at cathodic voltages. Relatively weak potentials at -1 V (vs. Ag/AgCl) could significantly reduce the cell viability in saline solution after 24 hours compared to controls at open circuit potential (OCP, in the range of -0.2 V to -0.38 V vs. Ag/AgCl) (p < 0.05). However, bacterial biofilms cultured on cpTi surfaces in LB media require more negative voltage (below -1.2 V) to induce significant killing efficacy.

On the other hand, the cellular response was correlated with the electrochemical properties of titanium-oxide-solution interface through methods like electrochemical impedance spectroscopy (EIS) and current density monitoring. The electrochemical impedance of the oxide-bacteria-solution interface was dependent on the presence of applied voltage. Sustained voltage treatment at -1 V decreased the impedance of titanium-oxide-bacteria interface in both LB media and NaCl solution at 0.1 Hz than those at OCP (p<0.05). In LB media, the presence of bacterial biofilm significantly reduced the average current density experienced by cpTi surfaces at -1 V compared to controls without cells at -1 V in 24 hours (p < 0.05).
Significant morphological changes were found after voltage treatment in NaCl solution and LB media. In general, ruptured cells after voltage treatment at -1 V in NaCl solution ended in less length, width and height than control cells at OCP (p<0.05). The applied potential at -1 V decreased the length and height of all the cells in LB media after time-lapse photography compared to those of untreated controls (p<0.05).

Finally, time-lapse photography, which could assess cellular movement of bacteria under voltage treatment in real time, was utilized and proved to be an effective method of cellular investigation besides LIVE/DEAD assay, scanning electron microscope (SEM) and atomic force microscopy (AFM). Average bacterial cell velocity significantly increased once -1 V voltage treatment started and then dropped in two hours in NaCl solution (p<0.05), while no such difference was seen during the test in LB media.
BACTERIAL BIOFILMS ON ELECTROCHEMICALLY ACTIVE CATHODIC TITANIUM SURFACES

by

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1. Overview

1.1. Objectives

The focus of this thesis is to characterize how applied electrochemical potentials affect bacterial biofilm growth within a simulated biological environment. The voltage was at -1 V and -1.2 V compared to control groups at OCP vs. Ag/AgCl. The impact of reduction electrochemistry on bacterial biofilm growth is assessed through culturing bacterial biofilm directly on the electrically polarized cpTi samples to determine cell morphology and viability.

On the other hand, this study will also focus on the influences of bacterial biofilm, voltage and electrolyte solutions on the electrochemical properties of titanium-oxide-solution interfaces. A systematic study of the reduction electrochemistry influence on bacterial biofilm behavior would provide solutions for treatments of bacterial infection.

1.2. Hypothesis

1) The viability of *E. coli* HM22 cells cultured on titanium is voltage and medium dependent by way of electrochemical currents and impedance properties at the titanium surface.

2) The electrochemical properties of the oxide-bacteria-solution interface are dependent on electrolyte (LB media or saline solution), the presence of bacterial biofilm and voltage.

1.3. Specific Aims

The specific aims of these research efforts are:
1) Characterize the effects of voltage and medium on the behaviors of *E. coli* HM22 biofilm. Quantify the cell viability and assess the cell morphology of *E. coli* cultured on cpTi in saline solution at the voltage of -1000 mV and in LB medium at -1000 mV and -1200mV vs. Ag/AgCl for 24 hours. Control comparisons are at OCP vs. Ag/AgCl.

2) Quantify the electrochemical changes in titanium-bacteria-media interfaces after 24 hours of voltage treatment at -1000 mV compared to OCP vs. Ag/AgCl in LB media or saline solution.

3) Capture time-lapse photography of *E. coli* HM22 biofilm cultured on cpTi in saline solution or LB media for two hours at the voltage of -1000 mV vs. Ag/AgCl. Quantify the cell viability and morphology changes of bacterial cells cultured on cpTi after electrochemical treatment in 24 hours. Control comparisons are at OCP vs. Ag/AgCl.

1.4 Bacterial Biofilm

1.4.1 Development of Bacterial Biofilm

Studies of bacterial biofilm have been increased in recent years. Approximately 60% of human bacterial infections may involve biofilms. Bacterial cells growing in biofilms are associated with a wide range of human infections. Bacterial biofilm is recognized by its high tolerance to innate host defenses and antimicrobial agents and may cause severe medical problems. While planktonic bacterial cells are susceptible to antibiotics, adherent cells could form bacterial biofilms that are resistant to antibiotics, phagocytes and antibodies. Treatments with regular
concentrations of antimicrobial agents fail to eliminate the bacterial biofilm. Bacterial biofilm requires antibiotics and biocides at levels 500 to 5000 times higher than planktonic cells do\textsuperscript{2-6}.

Bacterial biofilm develops commonly on biomaterial medical devices like joint replacement, contact lenses, artificial heart, vascular prostheses and dental implants\textsuperscript{7}. Common biofilm bacterial species include a wide range of both gram-positive bacteria species like \textit{Staphylococcus epidermidis} and gram-negative bacteria like \textit{Pseudomonas aeruginosa} and \textit{E. coli} that could cause many chronic infections in hosts\textsuperscript{8}.

During the initial attachment, bacterial cells initiate synthesis of an extracellular matrix where extra cells embed. Given their various atomic structures, surfaces tend to present available binding sites for planktonic cell interactions\textsuperscript{7}. For titanium alloy, a thin oxide layer acts as the interface for initial bacterial attachment. Bacterial cell surfaces hold negative charges as most substratum surfaces do. However, the electrochemical environment at the interface of metal-liquid tends to vary because of changes like tissue damage, inflammation and corrosion in the surroundings\textsuperscript{9, 10}. While cells and surfaces naturally repel each other, van der Waals and attractive hydrophobic interactions tend to position bacterial cells near or at the surfaces\textsuperscript{11-13}. At even shorter ranges of distance, chemical interactions (ionic, hydrogen, and covalent bonding) occur within microcolonies. Once microcolonies adhere to the interface, they start to differentiate into extracellular polysaccharide matrix. Finally, adherent microcolonies mature and well-developed biofilms settle on the surface.
When the bacterial community colonizes and settles, fragments of biofilm can break off and disperse to new areas. Planktonic cell could be released from the biofilm into the surroundings. Pieces of a microcolony could simply detach from the biofilm and disperse into the flow until it settles down and initiate a new sessile community.  

1.4.2 Infections of Orthopedics Implants  

Our question focuses on bacterial infection at the surface of orthopedic implants. Hip or knee replacements have recently become commonly used to restore the function of affected joints. Bacterial infection is a major concern in orthopedic implants and may lead to implant failure and revision. In the US alone, total hip and knee arthroplasties account for half a million interventions each year. The rate of infected knee arthroplasties was 0.92%, and that of infected hip arthroplasties was 0.88%.  

Infections centered on biomaterials are difficult to eliminate. As bacterial biofilm protects the infecting bacteria against the host immune system and antibiotics, it usually requires removal of the colonized foreign body to eradicate such infections. An estimate average cost of surgical and medical treatment could reach $30,000 per patient. Consequently, development of alternatives to conventional antibacterial therapy is extremely urgent and desirable.  

1.4.3 Antibiotic Resistance of Bacterial Biofilm
The resistance of adherent bacterial biofilm is not due to conventional antibiotic mechanisms like genetic exchange and expression. However, it is demonstrated to be a peculiar programmed growth\textsuperscript{19}.

*Mechanism of antibiotic resistance*\textsuperscript{1, 8, 20}

![Figure 1.1. Mechanism of antibiotic resistance\textsuperscript{20} (Reproduced with permission of WICHTIG EDITORE S.R.L.)](image)

1) Antibacterial agents may fail to penetrate the full depth of biofilm layers. Antibiotics may be deactivated in the outer layer of the biofilm instead of diffusing into the inside.
2) Certain enzymes in the extracellular matrix may trap and damage the antimicrobial agents.
3) Some of the cells in the biofilm may undergo a slow-growing or non-growing status, like persister cells that are not susceptible to biocides.
4) A distinct biofilm phenotype is programmed where certain resistant genes are initiated and expressed.
1.5 Bioelectric Effect on Bacterial Biofilm

1.5.1 Bioelectric Effect in vitro Studies

A certain number of preclinical physical strategies have been adopted to enhance the antimicrobial agents in their activities against bacterial biofilms, including the applications of electrical fields and current densities\textsuperscript{21}, radiofrequency electrical current\textsuperscript{22} and ultrasound\textsuperscript{23}.

Many in vitro studies have shown that electric fields and current density could enhance the efficacy of biocides and antibacterial agents against bacterial biofilms, defined as the bioelectric effect\textsuperscript{9,19,21,24-28}. Some studies have used relatively high intensity voltage and current density, while others have focused on the efficacy of relatively weak current density on bacterial biofilms\textsuperscript{29}.

\textit{In vitro} studies have shown that direct electric current densities and electric fields could enhance the activities of certain biocides and antimicrobial agents (i.e., aminoglycosides, enthromycin, tobramycin, daptomycin, mosifloxacin, rifampin or linezolid) against certain bacterial biofilms like \textit{Staphylococcus aureus}, \textit{E. coli}, \textit{Staphylococcus epidermidis}, \textit{Pseudomonas aeruginaso} and \textit{Streptococcus gordonii}\textsuperscript{30}.

J. Costerton et al.\textsuperscript{19} reported in 1994 that low-intensity electric fields could significantly reduce the concentration of the antibacterial agent (tobramycin) needed to kill \textit{P. aeruginosa} biofilm. In their study, biofilm bacteria were killed on all the surfaces of conductive coupons that were not
specific to electrodes within electric fields. They indicated that the bioelectric effect did not fully depend on the generations of damaging electrochemical ions and molecules. They suggested that electrically assisted electrophoresis that might induce perturbations of membrane potential and structural changes was the preferred explanation of bioelectric effect.

Jass et al.\textsuperscript{21}, in 1995, grew \textit{P. aeruginosa} biofilms on a dialysis membrane instead of electrodes within the path of electrical current in order to avoid damages from local mechanical disturbance and generations of electrochemical ions and molecules. They discovered that the combination of an antibacterial agent (tobramycin) and electric current could result in a significant reduction of biofilm bacteria population. The increased dissolved oxygen concentration in media resulting from gas production at the electrode surface may increase the metabolic rate of biofilm bacteria and account for the bioelectric effect. This study suggested that changes in metabolic activity and bacterial growth rate besides electrophoresis and iontophoresis could contribute to the bioelectric effect. Later, in 1996, Jass and Lappin-Scott were the first to report the enhancement of electric current on antibiotics instead of aminoglycosides\textsuperscript{31}. They investigated the enhancement of electric current on three different antibiotics and suggested that electric current could enhance antibiotics that were effective against bacteria. The mechanisms of this bioelectric effect were referred to changes of bacterial membrane potential, biofilm structure and exopolysaccharide that might influence bacterial metabolic activities and antibiotic diffusion rate.

Stewart et al.\textsuperscript{32} investigated in 1999 the role of electrolysis products like oxygen, PH, hydrogen, reactive oxygen intermediates and heat in contribution to the electric enhancement of antibacterial efficiency. An increase in dissolved oxygen concentration may reach toxic level that
would weaken bacteria activity and make them susceptible to antibiotic. On the other hand, bacterial growth enhanced from increased oxygen concentration would overcome the reduced susceptibility during a slow growing status within the biofilm. Furthermore, they added sodium thiosulfate that could immediately neutralize reactive oxygen intermediates and showed bioelectric effect remained to be active. Stewart’s recent study in 2013 have shown the killing effect of direct electrical current in *Staphylococcus epidermidis* biofilms in combination with ciprofloxacin at physiologic saline conditions in order to mimic infected artificial joints\textsuperscript{28,33}. They indicated that electrolysis generation of hypochlorous acid from chloride might contribute to the mechanism of bioelectric effect. Thus, a physiologically relevant NaCl solution was crucial in considering environmental conditions.

Patel et al.\textsuperscript{20,30,34}, in 2008, have designed a specific flow chamber with current generation and tested the antibacterial activities of direct electric current with a wide variety of antibiotic agents. They indicated that the enhancement of the activities of antibacterial agents by electric current was not a generalizable phenomenon among different antibacterial agents and biofilm-associated bacteria.

Khoury et al.\textsuperscript{28} have demonstrated that electric field could enhance the *in vitro* killing of *P. aeruginosa*, *E. coli* and *S. epidermidis* biofilms by tobramycin. Although electric field (1.5 V/cm and 15 $\mu$A/cm$^2$) themselves had no damaging effect on adherent biofilm populations, the combination of tobramycin and electric current killed all the bacterial biofilm within 12 hours.
Caubet et al. applied a direct electric current (200 mA) through a standard constant current generator to *E. coli* biofilm with gentamicin or oxytetracycline and showed that the combination resulted in a much great reduction rate of biofilm bacteria.

*Electric current alone has been shown to have a killing effect on bacterial biofilms.*

Some argue that electric current alone does not have a significant detrimental effect on bacterial biofilm growth, however, others indicate that direct electric current results in a discernible effect.

Limited *in vitro* studies have focused on using electric current alone to kill bacterial biofilm. Patel et al. have shown both the killing effect of a prolonged exposure to low intensity electrical direct current (DC) and the enhancement of DC plus antibacterial agents against *Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Staphylococcus aureus* biofilms.

Davis et al. have successfully eliminated both gram-positive and gram-negative planktonic bacterial cells *E. coli, P. aeruginosa* and *Proteus mirabilis* by electric fields and electric current with a variety of electrodes like gold, carbon and platinum in synthetic urine. They suggested this bacterial killing effect in the absence of antibiotics was due to iontophoresis, in which metal ions accreted within bacterial cells or the generation of short-lived chloride compounds.

Patel et al. have also shown the reduction effect of prolonged (up to 7 days) weak electric current (20, 200, 200 µA) on *Staphylococcus* and *Pseudomonas* biofilms in the absence of
antibiotics, a phenomenon they labeled the “electricidal effect.” They demonstrated that higher current density and longer exposure time correlated with greater reduction of bacterial biofilms with either stainless steel or graphite electrodes.

Van der Borden et al.\textsuperscript{40} applied a weak direct electric current (25-125 µA) to initially adherent Staphylococcus biofilms on the surface of surgical stainless steel in a flow chamber and showed that electric current could stimulate the detachment of growing biofilm bacteria from original surfaces and prevent them from colonizing on the surfaces again. They also found that bacteria left on the surfaces after electric treatment were less viable than they were prior to the current application. Van de Borden and his colleagues investigated whether weak direct current could prevent clinical infections around percutaneous pin in goats and found the pin subjected to current was much less infected than the control pin\textsuperscript{41}.

Poortinga et al.\textsuperscript{42} have demonstrated that electric current could stimulate the detachment of bacterial cells from conducting indium tin oxide on glass surfaces within a flow chamber. These results enabled biofilm design or could be used reversibly to prevent biofilm formation in biomedical applications.

1.5.2 Mechanism of Bioelectric Effect

The mechanism of electric enhancement of antibacterial agents and biocides or the killing effect of electric current alone, remains unclear but is still of significant importance. Investigation of this phenomenon would promote the medical applications of bioelectric theory and would also
help us better understand the mechanism of bacterial antibiotic resistance. Some of the hypothetical mechanisms that have been postulated include membrane permeabilization\textsuperscript{28}, reduced capacity of biofilm to antimicrobial agents\textsuperscript{27}, electrophoretic augmentation of antimicrobial transport\textsuperscript{28}, electrochemical generation of potentiating oxidants\textsuperscript{43, 44}, increased delivery of oxygen to biofilm due to electrolysis generation resulting in enhanced bacterial activities and susceptibility to antibiotics\textsuperscript{21, 32}, contraction and expansion of biofilms caused by electric static interactions in electric field and increased effectiveness of antibiotic due to PH shifts and increased transport convective mixing\textsuperscript{45}.

![Proposed mechanism of bioelectric effect](image)

Figure 1.2. Proposed mechanism of bioelectric effect\textsuperscript{20} (Reproduced with permission of WICHTIG EDITORE S.R.L.)

\textbf{a) Membrane structural changes and electrophoretic augmentation of antimicrobial transport}

Membrane potential\textsuperscript{19, 46} was one of the major factors that maintain the basic metabolic activities, and, once disturbed by electric field, cellular electrical equilibria were likely to be unbalanced and orientation and electrophoretic motilities of membrane proteins were altered. Electrophoretic forces could enhance the transport of antibiotic agents through bacterial cell\textsuperscript{47} membranes.
Electrical current could alter not only the individual bacterial cell morphology but also the entire biofilm structure by introducing cations into the exopolysaccharide and cell membrane\textsuperscript{31, 48, 49}. Exopolysaccharide might provide a barrier for molecule diffusions\textsuperscript{50} and current flow could enhance antibiotics and ions diffusion into biofilms and cell membranes. In particular, Berrier et al. investigated whether electric fields could induce changes on membrane potential and outer membrane porin proteins of \textit{E. coli}.\textsuperscript{51} Their results suggested that a specific channel within the inner membrane of \textit{E. coli} stayed open at negative or zero membrane potentials, and increasing voltage would change the native orientation of inner membrane and lead to channel closure.

b) Electrolytic generation of oxygen\textsuperscript{21, 52}

Electrolysis resulting from bioelectric effect and application of electric current alone could increase dissolved oxygen in media, enhance the delivery of oxygen into bacterial biofilm, and finally increase the metabolic rate of bacterial cells and antibiotic susceptibility of biofilms. On the other hand, Stewart indicated in 1998 that increased oxygen concentration might reach toxic levels that would weaken bacterial cells and render higher susceptibility to antibacterial agents. Besides, the physical interruption of bubbles generated at the surface would also contribute to the detachment of bacteria cells from electrode surfaces.

c) Electrochemical generation of potential oxidants

Gilbert and Mali pointed out in their book chapter that reduction reactions happening on titanium surfaces could generate intermediates that were potentially detrimental to microorganisms.\textsuperscript{53}
Davis et al. attributed the killing effect of electric current on planktonic cells to iontophoresis, where the generation of metal ions at the electrode surfaces could be responsible for the antibacterial effect. Besides, the presence of chloride species could also contribute to the results. Costerton et al.\textsuperscript{19} (1994) applied a relatively weak current in a flow experiment and found local generations of electrochemical antimicrobial ions and molecules could partially explain the killing efficacy of bioelectric current. Stewart et al.,\textsuperscript{28,33} in 2013, investigated the killing effect of direct current on P. aeruginosa biofilms in the presence of NaCl and suggested that electrolysis generation of hypochlorous acid from chloride would be a major explanation of the efficacy of direct current.

d) Reduced banding activities of biofilm to antibiotic agents.

The exopolysaccharide matrix of the bacterial biofilms could act as a barrier against antibacterial agents and biocides, bind them and prevent them from reaching their target cells. Some authors suggested that it was possible for electric current to disrupt the electrical charges of the matrix, interrupt the binding sites, and then enhance the permeability of antibacterial agents through the biofilm membrane\textsuperscript{27}.

Other potential mechanisms of the bioelectric effect have been referred to the oxidation of enzymes, membrane damage leading to the leaking of bacterial cells, increase in convective transport because of biofilm contraction and expansion and increased susceptibility of bacterial cells due to temperature increase within biofilms.\textsuperscript{34}

1.5.3 Potential Applications of Bioelectric Effect
Based on the introduction above, applications of direct electric current could be used either alone or combined with various antibacterial agents. Considering the limited knowledge gained from studies so far, investigations of both *in vitro* and *in vivo* studies are necessary before experiment models could be applied to clinical therapies.

Van de Borden has achieved successful treatments of *S. epidemidis* biofilms with weak direct current in a goat model, prevented clinical infections around the percutaneous pin and found that the pin subjected to current was much less infected than the control pin in the absence of electric current\(^1\). Del Pozo et al. have shown that electrical current alone in rabbits significantly reduced *S. epidermidis* biofilms on stainless steel implants over a 21-day exposure\(^3\).\(^9\) Besides, direct electric current has been previously used to introduce antibiotic compounds into the inner ears and other tissues\(^5\). Clinical application of weak direct electrical current could be associated with orthopedic implantations. Furthermore, device-related implants could be modified to apply direct current or electric fields to enhance the delivery of antibiotics and therefore prevent implantation infections.

1.6 Basic Electrochemistry of Metallic Biomaterials with Focus on Cathodic Voltage

While almost all the studies that focused on bioelectric effect were performed though a fixed electric current or electric field, to the best of our knowledge, this study was the first to apply a static electrical potential to bacterial biofilm growth. Rather than fixing the current density which is an indicator of the magnitude of ongoing electrochemical reactions on sample surfaces, we
applied static potential that actually predict the reactions occurring through the metal-biofilm-liquid interface.

Gilbert et al. 55 showed reduction reactions happening on titanium surfaces could deplete the concentration of dissolved oxygen at the interface of metal-liquid interface and thus affect osteoblast cell behavior on implant surfaces. Oxygen concentrations at titanium surfaces decreased significantly within 500 um probe-sample distances in the presence of applied potential. Kalbacova et al. 56 have found that cathodic polarization of Ti-6Al-4V could induce higher generation of intracellular reactive oxygen species and alter the metabolic rate of osteoblast cells. Ehrensberger and Gilbert’s recent work 57 has indicated that static applied negative potential could lead to oxide dissolution and reduced resistance of titanium oxide film which may cause increasing reaction rates on metal surfaces.

1.6.1 Titanium Oxide

Metallic biomaterials have been used in almost all areas of human body implantation like orthopedics, dental, artificial heart valves, spinal and other applications 58, 59. Orthopedic infection is a major concern in clinical therapy that may lead to implant failure and complete replacement. The resistance of bacterial biofilm to antibiotics increases the difficulties of fighting against bacterial infections. It has been shown that the electrical current successfully prevents infections of certain bacterial species. A systematic study of the reduction electrochemistry effects on bacterial biofilm behavior would provide a clear view to the therapeutic treatment of infection. Titanium has been extensively utilized in orthopedic and dental applications because of its
excellent mechanical properties and biocompatibility\textsuperscript{60}. The standard reduction potential of Ti is -1.6 V. When exposed to water or air, bare titanium spontaneously form a thin oxide layer at the surface.

\[ \text{Ti} + \text{O}_2 \rightarrow \text{TiO}_2 \quad \Delta G = -203.8 \text{ kcal/mol} \]

\[ \text{Ti} + 2\text{H}_2\text{O} \rightarrow \text{TiO}_2 + 4\text{H}^+ + 4\text{e}^- \quad \Delta G = -82.92 \text{ kcal/mol} \]

Titanium has several types of alloys utilized in clinical devices, including commercially pure Titanium (cpTi), Ti-6Al-4V, TMZF (Ti-Mo-Zr-Fe) and others. Each alloy shares similarities in chemical and structural features while each has its own disadvantages. Most titanium alloys are resistant to corrosion due to the presence of the spontaneously formed passive oxide film TiO\textsubscript{2-x} (an anion-deficit, n-type semiconducting oxide). Titanium oxide is about 2-10 nm and mostly considered to be nanocrystalline or amorphous\textsuperscript{61}. However, it has been shown that the oxide structure, electrochemical impedance and chemistry change over time with immersion in bioelectrolyte solutions, electrical polarization, and interactions like bacterial cells and proteins. Previous studies have shown that mechanical abrasion of the titanium alloy surface produces cathodic shifts in the open circuit potential. This shift of surface potential can significantly change the oxide properties and in turn change the electrochemical processes ongoing at the interface\textsuperscript{62, 63}.

Two basic types of electrochemical reaction can happen at the surface of electrodes: oxidation and reduction. Each reaction is a half of the entire corrosion process and is considered as a half-cell reaction. Oxidation reactions raise the valence state of chemical species by releasing electrons while reduction reactions reduce the valence state of other species by taking electrons.
An anodic (positive, oxidation) current occurs when metal atoms at the metal surface release electrons and turn into metal ions in the solution and positive charge moves from electrode to electrode. In contrast, a cathodic (negative or reduction) current happens when positive charges move from solution to electrode. The corrosion of the metallic surface is governed by the rule of Mixed-Potential Theory, as the total of currents generated by reduction reactions must balance with the sum of currents generated by oxidation reactions without an external source of applied potentials on a metal surface.  

There are two types of oxidation reactions shown in Eqn 1.1 as follows.

\[
M \rightarrow M^+ + n e^- \text{ (ionic dissolution)}
\]

\[
nM + mH_2O \rightarrow M_nO_m + 2mH^+ + ne^- \text{ (oxide formation)} \quad 1.1
\]

The reduction reactions shown below are just a limited list of possible reduction reactions at the metal surfaces. Reduction reactions involve a variety chemical species across the metal surface including oxygen, water and reactive oxidant compounds like hydroxide radicals and hydrogen peroxide. Furthermore, intermediate species like HO\(_2^\cdot\) may arise from such reduction processes and induce oxidative stress in microorganisms.

\[
O_2 + 2H_2O + 4e^- \rightarrow 4OH^- 
\]

\[
O_2 + 2H_2O + 2e^- \rightarrow 2OH^- + H_2O_2
\]

\[
H_2O + e^- \rightarrow OH^- + \frac{1}{2}H_2
\]

\[
O_2 + H_2O + e^- \rightarrow HO_2^\cdot + OH^-
\]

\[
HO_2^\cdot + H_2O \rightarrow H_2O_2 + OH^-
\]

\[
H_2O_2 + 2e^- \rightarrow 2OH^- \quad 1.2
\]
Figure 1.3. A schematic graph of anodic half-cell (blue) and cathodic half-cell (red) reactions that indicate the current measured (dotted black) in a polarization test\(^\text{38}\). The standard reduction potential of the reduction half-cell is +0.8 V at pH 7. So reduction reactions are favored at lower potentials. The standard reduction potential at the oxidation half-cell is –1.6 V, above which oxidation reactions are favored.

When metal is in contact with electrolytes (body fluids, solution), oxidation and reduction half-cell reactions are present, at which point the metal atoms are oxidized and the water and/or oxygen are reduced. The thermodynamics of these reactions are described by the Nernst equation, which shows that there is a voltage due to the charge separation across the interface, while the kinetics of the half-cell reactions is characterized by exchange current densities. The Nernst potential at which the oxidation and reduction reactions rates are equal is known as open circuit potential (OCP). At OCP, the exchange current densities of oxidation and reduction reactions are equal and opposite. The rate of oxidation reactions will be larger if any voltage greater than OCP is applied to the metal, and vice versa. In the body fluid or simulated body fluid, both metal
oxide formation and metal ion release happen, while reduction of oxygen, water, and proteins also occur\textsuperscript{61}. Based on Fig 1.3, anodic voltage increases from OCP with decreased reduction, but oxidation is not increased while voltage below OCP. Reduction increases and oxidation stays the same until potential below $E_{pp}$.

The interactions between titanium metal and the biological system are largely influenced by the oxide thin films. Titanium is a group 4, period 4 metal in the periodic table. Although metallic titanium is very active with an equilibrium voltage of $-1.6$ V (vs. NHE), a passive oxide film is immediately formed once bare titanium metal is exposed to the air\textsuperscript{61}. The oxide films tightly cover the metal surface with a compact structure, by which the rate of corrosion is reduced. Actually, these oxide films are dynamic and responsive to their environment. The thickness, chemical properties and resistivity of the oxide films are affected by the chemical and electrochemical environment\textsuperscript{61, 66}.

Based on Cabrera and Mott’s theory, strong electric fields are developed across the titanium oxide film and drive its growth in the liquid solution\textsuperscript{67}. With a low electronegativity, titanium atoms tend to lose electrons and form titanium cations at the metal-oxide interface. Meanwhile, oxygen molecules attract the electrons tunneled from the metal and form anions at the oxide–liquid interface. The accumulation of positive charges at the metal-oxide interface and negative charges at the oxide-liquid interface lead to high electric fields across the oxide films (considering the very low thickness of oxide films). The metal cation motion (or cation vacancy motion) and oxygen anion motion (or anion vacancy motion) driven by these electric fields are known as electromigration and can be modeled by the Nernst – Plank equation\textsuperscript{61}. The growth of
oxide films thus happens when the cations and anions are transported and combined. With the charge accumulation at the interfaces and ions’ motion through the oxide films, the oxide films can be modeled as leaking capacitors.

When the thickness of the oxide film is stable, some different theories might explain the electron transportation phenomena in the absence of film growth processes. The band theory can be used to describe the available energy states in the solid. An important concept in the band theory is Fermi level. The Fermi level of metal is the highest occupied energy level and the energy difference between vacuum and it is known as work function. And in the oxides (titanium oxide films), the Fermi level locates between the conduction band and the valence band.

The situation is more complicated in the solution. The concept used here is the equilibrium potential for the redox reaction. It is known that the normal hydrogen electrode potential is around \(-4.5\) eV lower than the vacuum energy. Thus, the redox potential in the solution can be related to the electron energies in the solid.

When the metal phase, oxide phase and solution phase are in contact, the Fermi levels at the interfaces become equal, and the band bending occurs. Due to the difference between the Fermi level of metal and solution, a gradient of electron energy is developed through the oxide film, which drives the electron transfer that provides electrons for redox reactions.

Essentially, two interfaces are formed when putting metallic titanium in the biological system: the interface between bulk metal and surface oxide, and the interface between oxide and body fluids. Additional interfaces should be taken into consideration if coatings or cells are
present on the surface of metal implants. The charge distribution in the solution adjacent to the oxide – solution interface is affected by the net-charge distribution in the oxide films, by which the electrical double layer is established in the liquid phase. So there are three different capacitances in series in the metal-oxide film-solution system. Since the capacitances sum as the reciprocal, the smaller the individual capacitance is, the larger the effect it will have on the overall capacitance. The oxide film capacitance is the smallest of the three in most cases, while the double layer capacitance becomes the smallest at low negative voltages\textsuperscript{69}.

### 1.6.2 Electrochemical Impedance Spectroscopy (EIS)

As discussed above, the net-charge distribution and charge transportation are present at the metal – oxide film – solution interfaces. Thus, each interface can be modeled as a combination of resistive and capacitive elements. Depending on the electrochemistry of the electrode interface, several electrical analog models are available to describe it. Constant phase element (CPE) Randle’s circuit is most commonly used to describe the impedance behavior for the passive oxide film covered systems in the frequency domain and is applied in this study.

In the later 19th century, Heaviside first defined the operational impedance (transfer function, as shown below)\textsuperscript{70}. And with Warburg’s remarkable work\textsuperscript{71}, the mathematical foundation of the EIS was built. The modern EIS technique was made possible with electronics inventions in the 20th century. Due to its linear nature and capability to obtain information over a wide range of frequencies, EIS became a powerful tool in studying the corrosion and electrochemical processes\textsuperscript{70}. Meanwhile, new concepts and techniques were introduced to EIS, and
improvements were made as well. Several groups have developed and applied the Fourier transform EIS, which uses a much shorter scan time to obtain a full spectrum\textsuperscript{72,73}. The impedance imaging technique was developed to measure the local impedance\textsuperscript{74,75}. A large number of works have emerged to address the application of EIS in the cell analysis and biosensors studies\textsuperscript{74-79}.

By characterizing the impedance behavior of an electrochemical interface, the electrochemical impedance spectroscopy (EIS) can be used to characterize the interactions between metal implant surfaces and the biological system. Impedance analysis can be performed in either frequency-dependent mode or time-dependent mode. In this work, we utilized frequency-dependent EIS. This technique involves applying a small sinusoidal voltage overtop of a static voltage and measuring the current response accordingly. A range of frequencies is determined for the sinusoidal voltage, while current amplitude and phase are used to measure the electrochemical impedance. The transfer function is defined as follows to characterize the impedance.

\[
Z' = Z' + iZ'' = \frac{L(V(t))}{L(I(t))}
\]

\[
\tan \delta = \frac{Z''}{Z'}
\]

The electrochemical response measured by EIS can be modeled with an electrically equivalent circuit. In most cases, a certain number of circuit models are able to fit the experimental data, among which the simplest one is chosen for the fitting\textsuperscript{69}. As discussed above, the net-charge distribution and charge transport are present at the metal – oxide film – solution interfaces. So each interface can represent a combination of resistive and capacitive elements. Constant phase element (CPE) Randle’s circuit is chosen as the simplest fitting in this work, in which a constant
phase element is used to illustrate the non-charge transfer processes and a polarization resistor is used to represent the charge transfer processes respectively.

The electrochemical impedance of the titanium implant interface is affected by the components of body fluids. A calcium phosphate apatite layer tends to form on the titanium surface in the body fluid with the adsorption of calcium and phosphate ions. It is shown in the study conducted in the simulated body fluids that the concentration of calcium and phosphate ions could alter the electrochemical impedance of the interfaces. It has also been reported that serum proteins in the body fluids could influence the electrochemical properties of titanium surfaces. It was proposed that the protein adsorption on the titanium surface could impede the transportation of oxygen and lead to cathodic shifts at OCP as observed. Besides, the inorganic ions and adsorbed protein could interact with each other and play a role in the charge transfer processes.
2. Methods and Materials

2.1. Effects of Cathodic Voltage on the Cellular Behavior of *E. coli* HM22 on Cpti Surfaces in Simulated Biological Conditions

2.1.1. Sample Preparation

Disks of grade 4 commercially pure titanium (ASTM-F67) were cut from stock rods to fit the electrochemical chamber with an exposed area of 3.7135 cm$^2$. Surfaces were wet polished with 240, 320, 400 and 600 grit. Samples were then rinsed with deionized water, sonicated in 70% ethyl alcohol for 30 minutes and UV sterilized for 10 minutes.

A custom-made electrochemical chamber previously described was used for cell culture experiments$^{69}$. All components of the electrochemical chamber were rinsed with 70% ethyl alcohol, autoclaved and UV sterilized for 10 minutes.

The discs were placed into a custom-made electrochemical cell culture chamber with electrical contacts to a cpTi disk as a working electrode, a graphite counter and a chlorided silver (Ag/AgCl) wire reference electrode. A rubber stopper with holes for counter and reference electrodes was used to seal the glass chamber. A sterilized O-ring was mounted between the glass chamber and metal sample to prevent cell culture and media from leaking. Air could exchange through the hole for reference wire between the glass chamber and the incubator.

2.1.2. Bacterial Culture and Application of Voltage
E. coli HM22 was first cultured overnight in 25 ml Lysogeny Broth (LB) supplemented with 25 µl DPA at 37 °C with shaking at 200 rpm. LB media consists of 10 g tryptone, 5 g yeast extract and 10 g NaCl per 1L. One ml of overnight cell culture was plated on cpTi surface and kept at open circuit potential (OCP) at 37 °C for 30 minutes. After 30 minutes, the surfaces were gently rinsed with saline solution (0.9 % w/v NaCl) three times. Fresh medium (LB media or saline solution) were then added to immerse the counter and reference electrodes, which were connected to a potentiostat. The cpTi sample was potentiostatically held at -1000 mV or -1200 mV for 24 hours at 37 °C in solution, while the control samples were held at OCP (around -0.20 V in NaCl solution or ranging from -0.30 V to -0.39 V in LB media). Current density of each test was measured in 28-second intervals for 24 hours using an NI9004 A/D board (National Instruments) and Labview software. Average current densities were determined over 24 hours.

2.1.3. Electrochemical Impedance Spectroscopy

Once finished, samples were tested by electrochemical impedance spectroscopy (EIS). CpTi surfaces in the presence or absence of cells were characterized using a potentiostat (Solartron 1280C, UK) with a frequency analyzer. Impedance measurements were performed by introducing 10 mV voltage at -1000 mV after 24-hour voltage treatment. Impedance results were acquired and analyzed through Zplot 2.0 and Zview 2.0 software (Scribner Associates).

2.1.4. Cell Viability Assay
To assess cell viability, a LIVE/DEAD® BacLight™ bacterial viability kit was utilized based on manufacturer protocols. Bacterial cells on Ti surfaces were first rinsed with saline solution three times and then transferred to a six-well plate. The plate was then covered with aluminum foil to prevent photobleaching and kept in an incubator at 37 °C for 15 min. Samples were then taken out, gently inverted and mounted on a petri dish for imaging. Note that there was enough space between the metal surface and petri dish to avoid damaging cells.

Cells were imaged with an inverted microscope (Zeiss Axiovert 40 CFL, Zeiss, Denmark) connected to a CCD mono 12-bit camera (Q-imaging, Canada). Live cells were imaged through FITC filter set and a Texas red dye filter set was used to image dead cells. Cells were picked randomly from at least 5 spots on the sample surfaces to determine the areas of live and dead cells, respectively, using ImageJ software (NIH). Cell viability was calculated from the ratio of live cell area over the total area of both live and dead cells.

2.1.5. Morphological Assessment

The sample surfaces were gently rinsed in phosphate buffered saline (PBS) after voltage treatment and fixed with 4% formaldehyde solution. The samples were then dehydrated in gradients of ethanol (50, 70, 90% and 100% in DI water) and sputtered with gold. Biofilm structure and cell morphology were then assessed using a scanning electron microscope (SEM) (JEOL 5600, Japan).

2.1.6. Statistical Analysis
Data are presented as means and standard deviation (SD). T-test was performed to compare average current density experienced by control samples without bacteria and voltage treated groups with bacteria at -1 V or -1.2 V in LB media or NaCl solution with a p-value of 0.05 to indicate difference.

T-test was also performed to compare cell viability of the controls at OCP and voltage-treated groups at -1 V or -1.2 V in LB media or NaCl solution with a p-value of 0.05 indicating difference.

Two-way ANOVA was performed to indicate the influence of the presence of voltage treatment and NaCl solution on cell viability compared to controls at OCP or in LB media at -1 V at a significance level of P < 0.01 (n=3 for all groups, 5 spots randomly selected from each sample).

2.2. Cellular Dynamics Study on CpTi surfaces Using Time-Lapse Photography

2.2.1. Sample Preparation

Disks of grade 4 commercially pure titanium (ASTM-F67) were cut from stock rod with a diameter of 13.1 mm and thickness of 4 mm. A small cylindrical hole of 1 mm in diameter and 1.5 mm in depth was drilled from the center of one side of the disk. The other side of the disk surface was wet polished through 240, 320, 400 and 600 grit. 0.1 um alumina powder and silica solution were used to yield a mirror-like surface. Samples were rinsed with deionized water (DI), sonicated in 70% ethyl alcohol for 30 minutes and UV sterilized for 10 minutes.
2.2.2. Biofilm growth

_E. coli_ HM22 was first cultured overnight in 25 ml LB media supplemented with 25 µl DPA at 37 °C with shaking at 200 rpm. CpTi sample was placed into a petri dish with 1 ml overnight cell culture on the metal surface and 10 ml fresh LB media to cover the disk completely. Samples were incubated at 37 °C for 24 hours to allow for biofilm growth. After incubation, samples were gently rinsed with 1 ml saline solution and mounted into the electrochemical chamber for voltage treatment and imaging afterwards. All components of the electrochemical chamber were rinsed with 70% ethyl alcohol and UV sterilized for 15 minutes.

2.2.3. Electrochemical Treatment

A custom-made electrochemical chamber previously described\(^{69}\) was used to apply cathodic voltages to bacterial biofilms and to allow bacteria imaging in real time (Fig. 2.1). The chamber consists of the cpTi sample as working electrode, carbon wire as counter electrode and a glass bottom Petri dish with holes on the lid to allow for metallic peg and chloride silver wire electrode to be connected to the potentiostat. The carbon wire was placed into the glass bottom dish and 2 ml media was added into the chamber to cover the entire bottom surface and the carbon wire. The cpTi disk with biofilm on it was removed from the incubator, inverted and connected to the metal peg. The lid was gently placed on the glass-bottomed petri dish. The distance between the metal surface and the glass bottom was about 2 mm to avoid damages to biofilms and to allow for electrochemical reactions happening in the media. Considering the
A metal peg was made from CoCrMo alloy instead of cpTi, electrolyte was carefully handled to avoid touching the peg, which may cause disturbance to the electrochemical behavior of cpTi samples during the test.

![Diagram of electrochemical chamber](Image)

**Figure 2.1.** Custom-made electrochemical chamber for time-lapse photography. A three-electrode system was adopted with working (cpTi samples), counter (carbon wire) and reference (Ag/AgCl wire) electrodes. CpTi samples were connected to the potentiostat with a metal peg through the chamber lid. Air exchange was allowed through the hole of the metal peg.

### 2.2.4. Time-lapse Photography

The cpTi sample was potentiostatically held at -1000 mV in LB media or saline solution for up to three hours at room temperature (18 ± 1 °C), while controls were held at OCP. Images were acquired at 10-second intervals using an inverted microscope and a camera (Leica DMI 6000B, Andor Luca-R). Image acquisition and exposure were controlled by Micro-Manager 1.3 imaging software (NIH). Current density of each test was measured in 28-second intervals for up to three
hours using an NI9004 A/D board (National Instruments) and Labview software. Average current densities were determined afterwards.

2.2.5. Cellular Dynamics Analysis

ImageJ software was used to analyze the movement of bacterial cells at each time point during the voltage treatment in LB media or saline solution. Cell movement was determined by the average speed at which cell centroid moved between 10 consecutive frames.

\[
v = \frac{1}{Nt} \times \sum_{n=0}^{N} \sqrt{(x_n - x_{n+1})^2 + (y_n - y_{n+1})^2}
\]

\(x_n, y_n\): positions of centroids at \(t\)

\(x_{n+1}, y_{n+1}\): positions of centroids at \(t + \Delta t\)

\(N\)= number of cells, \(t= \Delta t\)

2.2.6. Cell Viability Assay and Morphology Analysis

Bacterial cells were stained to determine cell viability according to previously described processes with the LIVE/DEAD® BacLight™ bacterial viability kit after electrochemical test. Sample surfaces were gently rinsed in phosphate buffered saline (PBS) after voltage treatment and fixed with 4% formaldehyde solution. Disks were then dehydrated in gradients of ethanol (50, 70, 90% and 100% in DI water) and sputtered with gold. Biofilm structure and cell morphology were then assessed through contact mode atomic force microscope (AFM) with a Nanoscope scanning probe microscopy controller (Veeco Instruments, Inc.) and SEM.
height, width and length were determined respectively using the Veeco Instruments AFM software.

2.2.7. Statistical Analysis

Results are presented as means ± standard deviation (SD). T-test was performed to compare cell velocities in NaCl solution and LB media during the period when cpTi surfaces were held at OCP before voltage treatment started, -1 V voltage treatment just started and voltage treatment was continued in two hours with a p value of 0.05 to indicate difference. Cell length, width and height were compared respectively by t-test between control cells before the application of potential and voltage-treated cells after two-hour test with p < 0.05.
3. Results

3.1. Effects of Cathodic Voltage on the Cellular Behavior of *E. coli* HM22 on Cpti Surfaces in Simulated Biological Conditions

3.1.1. Electrochemical Testing

Rather than holding current density at a specific value, we applied a static voltage on metallic surfaces. This was done because the type of reaction ongoing depends on the voltage while the current density reflects the rate of reaction. Electrochemical current density, an indicator of the magnitude of ongoing electrochemical reactions on cpTi surfaces, were measured at -1.2 V or -1 V with bacterial biofilms cultured on sample surfaces or not. Note, at -1 V both oxygen and water reduction could occur, however, at -1.2 V the fraction of water reduction increases dramatically. By applying the voltage to cells during the initial attachment and adhesion of biofilm formation, bacterial cells were more sensitive and vulnerable than those in matured biofilm. This was aimed to mimic the clinical procedure of biofilm inhibition and infection prevention before the expansion and maturation of the bacterial biofilm.
Figure 3.1. Current densities experienced by cpTi samples held at -1 V over 24 hours in LB media or NaCl solution, in the presence and absence of bacteria cells. Note: all voltages were versus Ag/AgCl.

Figure 3.2. Average current densities (means ± SD) when cpTi samples were held at -1 V in NaCl solution and -1 V or -1.2 V in LB media, in the presence and absence of bacteria cells over 24 hours. Note: all voltages were versus Ag/AgCl.
Current densities experienced by cpTi surfaces with bacterial cells in both LB media and NaCl solution started at high levels (around 40 µA/cm²) at the beginning of -1 V voltage treatment and gradually decreased as the experiment continued. In NaCl solution with bacterial cells, the current density at -1 V decreased from 32.6 µA/cm² to 15.1 µA/cm² in the beginning 1.5 hours and settled around 10.8 µA/cm² for the rest of the tests. Furthermore, in LB media with bacterial cells, current density of the cpTi surface experienced an approximate 100-fold drop from 35 µA/cm² to 0.48 µA/cm² in 1.5 hours and stayed around 0.54 µA/cm² for over 24 hours. As initial cell attachment and adhesion to cpTi surfaces took up to three hours in biofilm formation, it was interesting to see such a rapid drop of current density during the first stage of biofilm formation in LB media. Bacterial cells were able to protect themselves at the very beginning of electric treatments and hold the current density at low levels for the rest of the experiment.

In LB media, the average current density (0.9 µA/cm²) experienced by metallic samples with bacterial biofilm at -1 V was lower than the current density (10.1 µA/cm²) of controls without cells at -1 V in 24 hours (p < 0.05) (Fig. 3.2). The presence of bacterial cells inhibited the electrochemical current flow and reduced the current density to around 0.5 µA/cm² in the first 5000 seconds (Fig. 3.1).

However, there was no significant difference between current densities in NaCl solution experienced by cpTi samples between with and without bacterial cells at -1 V. The presence of bacteria in NaCl solution resulted in an average current density of 12.2 µA/cm² compared to 12 µA/cm² in the absence of cells at -1 V in NaCl solution. In other words, the overall electrical
current going through the titanium-bacteria-media interface was as high as that through the titanium-media interface without bacteria.

With an increase in voltage level from -1 V to -1.2 V in LB media, the average current density experienced by cpTi samples raised up from 0.9 \( \mu \text{A/cm}^2 \) at -1 V to 3.53 \( \mu \text{A/cm}^2 \) at -1.2 V in the presence of bacterial cells over 24 hours. As the experiment continued, the current density of the cpTi surface at -1.2 V decreased from 56.6 \( \mu \text{A/cm}^2 \) to 8.86 \( \mu \text{A/cm}^2 \) with bacterial cells in the first 5000 seconds and settled around 2.69 \( \mu \text{A/cm}^2 \) for the rest of the tests (data not shown). Besides, the control group at -1.2 V, in the absence of cells, held the highest average current density (15.2 \( \mu \text{A/cm}^2 \)) in LB media.
Figure 3.3. a) Impedance, and b) phase angle results of titanium surface after impedance tests. CpTi samples, 1) with cells at OCP in NaCl solution, 2) with cells at OCP in LB media, 3) without cells at -1 V in NaCl solution, 4) with cells at -1 V in LB media, 5) with cells at -1 V in NaCl solution, 6) without cells at -1 V in LB media at 24 hours. All voltages were versus Ag/AgCl.
In this work, changes of interfacial properties of a cpTi alloy in LB media or NaCl solution were measured at -1 V or OCP with or without bacterial biofilms cultured on metals. The impact of applied static voltage on bacterial biofilms cultured on cpTi partially resulted from changes in the metallic surface oxide layer. Accordingly, it was essential to correspond the biological responses and current densities with the impedance properties of the surface oxide interface by EIS.

Figure 3.3 shows the impedance spectra of cpTi at OCP or -1 V (vs. Ag/AgCl) in the presence or absence of bacterial biofilm in LB media or NaCl solution after 24 hours of electric treatment. In Figure 3.3, voltage treatment at -1 V decreased the impedance and the phase angle of the cpTi-liquid interface in the presence of bacterial cells in both LB media and NaCl solution compared to controls at OCP (around -0.2 V in NaCl solution or -0.35 V in LB media). At OCP, cpTi samples in NaCl solution obtained higher impedance and lower phase angle than those in LB media in the presence of bacterial cells. Considering the higher concentration of NaCl in NaCl solution than that in LB media, more efficient ionic transportation by Na\(^+\) and Cl\(^-\) would lead to higher current flow and lower resistance in the solution.

The existence of bacterial biofilm in LB media on cpTi surfaces held at –1 V increased the impedance compared to controls without bacteria, which verified the results in Figure 3.2 where current density of voltage group with bacteria biofilm in LB media was significantly lower than of controls without cells at -1 V. The presence of bacterial biofilm might contribute to the increased impedance of metal oxide interface. Additionally, in LB media at -1 V in the absence
of bacterial, cpTi surface shows a drop in the impedance and a lower phase angle at frequencies less than 10 Hz indicating a less capacitive behavior after 24 hours.

Furthermore, the impedance of cpTi interface with biofilm in LB at -1 V kept increasing as frequency decreased while impedance in LB media without cells at -1 V almost hit a plateau. Accordingly, Fig. 3.3b shows a higher phase angle of cpTi surfaces in LB at -1 V with bacteria at frequencies less than 10 Hz indicating a higher capacitive behavior after the voltage treatment. This might explain why the interface in LB with bacteria at -1 V did not hold the highest value at lowest frequency of impedance test (0.0032 Hz) although its current density was the smallest after the voltage treatment.

**Figure 3.4. Impedance of cpTi surfaces at the frequency of 0.1 Hz, in the presence or absence of cells, at OCP or -1 V, in NaCl solution or LB media during the EIS test. All voltages were versus Ag/AgCl.**
In Figure 3.4, voltage treatment at -1 V significantly decreased the impedance of the cpTi-liquid interface at 0.1 Hz in the presence of bacterial cells in both LB media and NaCl solution compared to controls at OCP (p<0.05).

3.1.2. Cell Viability and Morphology

![Cell Viability Fraction](image)

**Figure 3.5.** Cell viability fraction of bacterial biofilm measured from LIVE/DEAD assay after -1 V or –1.2 V voltage treatments in LB media or NaCl solution compared to controls at OCP. Note: the large loss of cell viability when cpTi surface was held at -1V in saline solution while high viability resulted in LB medium at -1 V as controls at OCP. All voltages were versus Ag/AgCl.
Figure 3.5 shows the cell viability fraction of bacterial biofilms under different conditions in 24 hours. The cell viability was determined by the area fraction ratio of viable cells over the total of live and dead cells. When immersed in NaCl solution, the voltage treatment of -1 V adversely affected *E. coli* cell viability compared to control groups held at OCP (P < 0.05). The effect of voltage treatment in LB medium was not significant on cell viability at -1 V compared to controls at OCP. One-way ANOVA test shows that cell viability at -1.2 V was significantly lower than that at OCP or -1 V in LB media (p<0.05). The presence of voltage treatment and limited nutrients in saline solution adversely impacted the cell viability on cpTi samples, compared to controls at OCP or in LB media at -1 V (two-way ANOVA, P < 0.01).

Figure 3.6. Fluorescent images of *E. coli* HM22 cells after live/dead stain. a) Cells at OCP in NaCl solution, b) cells at -1 V in NaCl solution, c) cells at OCP in LB media, d) cells at -1 V in LB media, e) cells at -1.2 V in LB media at 24 hours. All images were at 63× magnification. Note: almost all the cells were dead after 24 h voltage treatment at -1 V in NaCl solution,
whereas, cells stayed alive in LB media on cpTi surfaces held at -1 V and were partially dead at -1.2 V. Scale bar were 300 µm on all images. All voltages were versus Ag/AgCl.

Figure 3.7. SEM images of *E. coli* HM22 cells after electrochemical treatment. a) Cells at OCP in NaCl solution, b) cells at -1 V in NaCl solution, c) cells at OCP in LB media, d) cells at -1 V in LB media e) cells at -1.2 V in LB media at 24 hours. All images were at 3000 times magnification. Note: most cells were severely distorted and obviously cavitated after 24 h voltage treatment at -1 V in NaCl solution, whereas, cells on sample surfaces at -1 V in LB media appeared to be spherical and complete as controls at OCP. When samples were held at -1.2 V in LB media, some cavitated cells and cells fragments could be found on cpTi surfaces. Scale bar were 5 µm on all images. All voltages were versus Ag/AgCl.
We examined the *E. coli* HM22 biofilms on cpTi electrodes by live/dead stain and SEM after electric treatments to apply killing efficacy on bacterial biofilms. After 30 min colonization of biofilm growth on cpTi samples, the entire surface of metal electrodes was covered with initially growing biofilm before the application of any experimental protocols.

After 24-hour voltage treatment at -1 V, all the bacterial cells were virtually killed on cpTi surfaces in NaCl solution (Fig. 3.6b), and cells remaining on metals were severely distorted and obviously cavitated (Fig. 3.7b, Fig. 3.8c). Large amounts of cell fragments and broken membranes could be seen on metal surfaces as well. In comparison, bacterial cells untreated by applied potential remained viable (Fig. 3.6a) and intact (Fig. 3.7a) on cpTi surfaces held at OCP in NaCl solution.
Figure 3.8. SEM images of *E. coli* HM22 cells after electrochemical treatment. a) Cells at OCP in NaCl solution, b) cells at OCP in LB media, c) cells at -1 V in NaCl solution, d) cells at -1.2 V in LB media. All images were at 5000 times magnification. Note: most cells were severely distorted and obviously cavitated after 24 h voltage treatment at -1 V in NaCl solution, whereas, some cavitated cells and cells fragments could be found on cpTi surfaces held at -1.2 V in LB media. Scale bar were 5 µm on all images. All voltages were versus Ag/AgCl.

After 24-hour incubation in LB media at OCP, bacterial biofilms on cpTi surfaces, which contained a large number of viable bacteria (Fig. 3.6c), was structurally intact (Fig. 3.7c).
Bacterial colonies formed on sample surfaces after 24-hour incubation in LB media at OCP, which was different than those randomly distributed cells on samples in NaCl solution at OCP. Biofilms treated with -1 V voltage in LB media involved plenty of viable cells in Figure 3.6d, which was similar to untreated biofilm seen in Figure 3.6c. At -1 V in LB media, cells tended to connect to each other although large microcolonies was not found on samples compared to the biofilm populations at OCP in LB media.

An increase of voltage partially killed bacterial cells on cpTi surfaces at -1.2 V in LB media (Fig. 3.6e). Examination of electrode surfaces showed that residual bacteria and a few distorted and cavitated cells could be seen on metal surfaces (Fig. 3.8d). Bacterial cells were dispersed on sample surface while only a few were in contact to the others, which again proved that the application of static potential prevented the formation of bacterial colonies.

We have noted that biofilm bacteria were killed by -1 V voltage treatment in NaCl but remained alive at -1 V in LB media, and that -1.2 V static potential in LB were able to kill some of the bacterial cells.

3.2. Study of Cellular Dynamics on Cpti Surface Using Time Lapse Photography

It has been showed previously that cathodic voltage treatment at -1 V could negatively affect bacterial cell viability in NaCl solution in 24 hours. However, bacterial biofilm grown in LB media required higher voltage (more negative than -1.2 V) to eliminate it from cpTi surfaces. In
this part, we investigated the cellular movements and morphology changes of bacterial biofilm on cpTi surfaces at cathodic voltage (-1 V) compared to control biofilms at OCP (vs. Ag/AgCl).

3.2.1. Analysis of cellular dynamics

![Average Velocity Graph]

Figure 3.9. Average velocities of *E. coli* cells in NaCl solution or LB media, at -1 V or OCP during time-lapse photography. Note: the significant great increase in cell velocity once voltage test began in NaCl solution compared to controls at OCP and large drop in cell velocity as samples undergoing potentiostatic test in two hours (p<0.05). However, no such observation was made on cpTi surfaces at -1 V in LB media.

The changes of cellular movement at -1 V in NaCl solution and LB media provided information for bacterial motility on electrochemically active cpTi surfaces. Cells on sample surfaces at OCP showed an average velocity of about 0.1 µm/s before voltage treatment started in NaCl solution. Once the potentiostatic test started, the average bacterial velocity in NaCl solution suddenly
increased to 0.9 μm/s and gradually dropped to 0.01 μm/s in two hours. No significant changes in cell size were observed virtually through optical microscope during the voltage treatment. In comparison, the application of static potential did not significantly impact the movement of bacterial cells in LB media. Bacterial cells stayed at the velocity of 0.09 μm/s before the application of voltage and slightly increased their velocity to 0.12 μm/s. In two hours, the average cell velocity decreased to 0.07 μm/s.

Although there was no significant difference in average cell velocity before and after voltage treatment in LB media, the average current density measured on cpTi samples at -1 V with bacterial cells was 91.2 μA/cm² in 2 ml LB media during the time-lapse photography, which was much higher than the average (3.73 μA/cm²) on cpTi samples at -1 V with cells in 15 ml LB media in the electrochemical test described before. The higher generation and accumulation of reactive oxidant intermediates raised from increased current density might explain why cells were dead in less volume media but stayed alive in large one at the same potential level of -1 V.

3.2.2. Changes in Cell Morphology

AFM was adopted to visualize the morphology and surfaces of biofilm bacteria and their interactions with the substratum. AFM images in this work show that voltage treatment at -1 V in both LB media and NaCl solution resulted in a certain amount of ruptured bacterial cells on cpTi surfaces, while a few cells remained intact as control cells at OCP after 24 h incubation before the application of potential.
Figure 3.10. AFM images of *E. coli* HM22 after 24 h incubation at OCP before voltage treatment. Note: intact cells with smooth membranes and spherical shape remained on sample surfaces at OCP. Scale bars were 5 µm on all images. All voltages were versus Ag/AgCl.

Fig. 3.11 shows the lysis of bacterial cells under different stages after the voltage treatment at -1 V in NaCl solution. Some cells in Fig. 3.11b revealed a partial disassembly of the bacterial cell wall with a rough membrane and the release of the cytoplasmic materials. Changes in the surfaces of these cells tended to form two bulges, usually in the distal parts of the cell, as compared to untreated bacteria in Fig. 3.10.
Figure 3.11. AFM images of \textit{E. coli} HM22 after time-lapse photography at -1 V in NaCl solution. 
a) 5 \textmu m scale bar, b) 30 \textmu m scale bar. Note: cell lysis and membrane destruction occurred at -1 V in NaCl solution. All voltages were versus Ag/AgCl.
Figure 3.12. AFM images of *E. coli* HM22 after time-lapse photography at -1 V in LB media. a) 5 µm scale bar, b) 30 µm scale bar. Note: cell lysis and membrane destruction occurred at -1 V in LB media. All voltages were versus Ag/AgCl.

A certain amount of completely lysed cells were left on sample surfaces after -1 V voltage treatment in NaCl solution, with collapsed membranes and huge amount of cytoplasmic release (Fig. 3.11). The collapse of bacteria was confirmed by the measurements of cell length, width and height as follows. Lysed bacteria were unable to keep the spherical cell shape as controls and generally obtained a lower cell height.
In Fig. 3.12a, bacteria experiencing different status of cell lysis on titanium surfaces after two-hour voltage treatment in LB media were shown in AFM images. Changes in the top left cell (Fig. 3.12a) led to the appearance of two bulges in the distal parts of the cell, while the top right cell revealed a fully disassembly of the cell wall and a complete cell lysis with the huge amount of cytoplasmic release (Fig. 3.12a).

Unlike voltage-treated cells in NaCl solution, most bacteria undergoing electrochemical treatment in LB media were able to maintain their cell shape with virtually less cytoplasmic release and higher cell height on cpTi surfaces in Fig. 3.12b.

Cells on metal surfaces after -1 V treatment in NaCl solution or LB media were divided into two groups: ruptured and intact cells. Ruptured cells tended to be flattened with a rough and broken membrane inside which organelles distributed unevenly. These cells showed an obviously lower height than untreated control cells. In comparison, intact bacteria after the voltage treatment maintained their cell shape with a higher cell height and a smooth membrane just as control cells (Fig. 3.10).
Figure 3.13. Cell length and width of bacteria on cpTi samples after two-hour voltage treatment in NaCl solution or LB media compared to controls at OCP after 24-hour incubation before voltage treatment. Note: cells were divided into two groups: intact and ruptured cells in NaCl solution and LB media. All voltages were versus Ag/AgCl.

Voltage treatment in NaCl solution at -1 V reduced the length and width of both intact and ruptured cells compared to those of control cells at OCP (p < 0.05). Besides, the height of ruptured cells was significantly lower than that of controls at OCP before the application of potentials (p < 0.05), while the cell height of intact bacteria at -1 V in NaCl solution was not impacted by voltage.
Figure 3.14. Cell height of bacteria on cpTi samples after two-hour voltage treatment in NaCl solution or LB media compared to controls at OCP after 24-hour incubation and before voltage treatment. Note: the cells were divided into two groups: intact and ruptured cells in NaCl solution and LB media. All voltages were versus Ag/AgCl.

In LB media, voltage treatment significantly reduced the length and height of all the cells at -1 V compared to that of the untreated cells at OCP (p < 0.05), whereas, cell width remained to be unaffected by the application of potentials.

In general, ruptured cells after voltage treatment at -1 V in NaCl solution ended in less length, width and height than control cells at OCP (p<0.05). The applied potential at -1 V decreased the length and height of all the cells in LB media after time-lapse photography compared to those of untreated controls (p<0.05).
4. Discussions

In this work, we investigated how bacterial cells cultured on cpTi surfaces respond to changes in surface potential (voltage) and electrolyte (media). By applying static cathodic voltage to the interface of oxide-biofilm-liquid, a series of events occurred and finally resulted in cellular changes of bacteria cultured on metal surfaces.

As the bioelectric effect requires the presence of added antimicrobial agents to inactivate or remove bacterial biofilms, this work remains distinct from the general concept of bioelectric effect since it has demonstrated that applied cathodic potential alone could kill biofilm populations. A few other studies have focused on the killing efficacy of electric field or fixed current alone on bacterial biofilm. As electric field could not promote complete current flow on bacterial biofilm, the role of electrophoretic augmentation of molecule transport could not be well investigated in these studies\textsuperscript{89, 90}. Given the fact that current density could only reflect the rate of ongoing reactions, the application of a static potential could indicate the specific kinds of reduction reactions happening at the interface.

*Generation of reactive oxygen intermediates*

There were several mechanisms related to the killing effect of applied cathodic potential on bacterial biofilm. We reasoned that generation of reactive oxygen intermediates might contribute to the killing efficacy of applied potential on bacterial biofilm growth. This influence may be related to the generation and accumulation process of electrochemically active killing species on the reactive metallic biomaterial surfaces. The amount of generated reactive species was
proportional to current density experienced by cpTi samples held at a static voltage, changes in which could lead to exponential variation of current density.

It was suggested that this killing effect may be due to the toxic substances produced as a result of electrolysis and decreased bacterial metabolic rate. The electrochemical effect on bacterial cells in NaCl solution was obviously higher than that in LB media at -1 V. The presence of bacterial cells in NaCl solution did not impact the average current density compared to that in LB media. There was no significant difference in current densities in NaCl solution between cpTi samples with and without bacterial cells cultured on it at -1 V. Given the limited nutrients in NaCl solution that could just maintain bacterial osmotic pressure, LB media fully promoted the growth of bacterial biofilm with peptides, peptones, vitamins and sodium ions to promote transport and maintain osmotic balance. This impeded killing efficacy of applied potential in LB media may be due to higher metabolic activities and growth rate of bacterial cells in LB media that lead to rapid bacterial propagation, accelerated biofilm formation and strong resistance to electric treatment.

In this study, the effect of applied potentiostatic voltage on bacterial biofilm growth has been demonstrated on cpTi surfaces in simulated biological conditions. Bacterial cells were demonstrated to be sensitive to the electrochemical status of metallic surfaces where cells were cultured. In LB media, the fact that cells were partially killed at -1.2 V but remained alive at -1 V indicated that there was a threshold voltage where bacterial biofilms stopped growing and became vulnerable to electric treatment. Furthermore, with an increase in voltage level from -1 V to -1.2 V in LB media, the average current density experienced by cpTi samples raised up from 3.73 µA/cm² to 12 µA/cm² in the presence of bacteria cells over 24 hours. Additionally, the
amount of water reduction increased in this range as well. Correspondingly, bacterial biofilm population went from an unaffected and viable status to being partially killed by electrochemical treatment. In other words, more negative cathodic potentials resulted in higher current densities and a greater reduction of bacterial biofilm populations. These data imply that the killing efficacy of reduction reactions occurring on sample surfaces was dose dependent. It could be hypothesized that application of voltage more negative than -1.2 V could further reduce bacterial biofilm to even lower levels.

The major reduction reactions on the metallic biomaterial surfaces were hypothesized to be the reduction of oxygen and water as follows.

\[
\frac{1}{2}O_2 + H_2O + 2e^- \rightarrow 2OH^- \quad E^0 = -1.007 \text{ V vs. Ag/AgCl (pH=0)}
\]

\[
H_2O + e^- \rightarrow OH^- + \frac{1}{2}H_2 \quad E^0 = -0.222 \text{ V vs. Ag/AgCl (pH=0)}
\]

It is possible that pH shift due to electrolysis of water could influence bacterial cells and their matrix polymer. When chloride ions are present, generation of hypochlorous acid could contribute to the killing effect of electrolysis. Matsunaga et al.\textsuperscript{91-93} have investigated the electrochemical disinfection of marine bacteria through the generation of free chlorine at appropriate potential.

Intermediate species arising from reduction processes are likely to influence the biological system and induce oxidative stress in cells\textsuperscript{94}. The following reactions were a limited view of reduction half-cell reactions for reactive oxygen species (ROS)\textsuperscript{95}. Previous studies\textsuperscript{96} have
observed the formation of hydrogen peroxide generated from water and oxygen reduction at titanium-oxide surfaces under proper cathodic voltage conditions.

\[
\begin{align*}
O_2 + 2H_2O + 2e^- &\rightarrow 2OH^- + H_2O_2^- \\
O_2 + H_2O + 2e^- &\rightarrow HO_2^- + OH^- \\
HO_2^- + H_2O &\rightarrow H_2O_2 + OH^- \\
H_2O_2 + 2e^- &\rightarrow 2OH^- 
\end{align*}
\]

Furthermore, it has been shown that reduction reactions occurring at the interface of metal-liquid may consume oxygen underneath the osteoblast cell population and deplete dissolved oxygen locally\(^9^7\). Similar reduction of oxygen underneath bacterial cells in biofilm could happen at the metal-biofilm interface as well. Although, decreased oxygen level may result in slower metabolic activities and growth rate of biofilm population and might promote biofilm resistance to antibiotics\(^7\). Davis et al.\(^{25, 26, 36, 37}\) reported that relatively weak electrical currents could kill planktonic cells, and later concluding that this was due to the generation of toxic chloride ions from current\(^26\), which accreted in bacterial cells\(^{25, 36}\). Consterton et al.\(^{21}\) (1994) also demonstrated a decrease in the biofilm population to a less extent. To assess the role of short-lived chloride contents, chloride-containing compounds could be excluded from the electrolyte medium. If the electrical current could still kill the biofilm bacteria, then other causes should be referred in the effect.

Membrane permeability
SEM and AFM images of biofilm populations treated with electrochemical voltage (Chapter 3) demonstrated that cathodic voltage altered the three-dimensional structure of biofilm. Microcolonies formation was only found on cpTi surfaces in the absence of cathodic voltage after 24-hour incubation in LB media. Cell viability was significantly reduced by 24-hour voltage treatment in NaCl solution. Correspondingly, cavitated and distorted cells were distributed randomly on sample surfaces without the formation of biofilm colonies. Although the efficacy of applied potential was not significant at -1 V in LB media, no bacterial colonies were found on samples and only small communities of cells remained on the metallic surfaces. By increasing the voltage to -1.2 V, bacterial cells were further separated to each other and even small communities of cells were less than those at -1 V. This phenomenon indicated that voltage treatment at -1 V in LB media contributed to the prevention of biofilm formation or the destruction of matured biofilms. As it has been shown that biofilm structure is crucial in its resistance to antibiotics and other stimulus, the application of static cathodic voltage would be beneficial to eliminate biofilm in its activities against biofilm formation\textsuperscript{98}. 

In addition, the micrographs also illustrate morphological changes in the bacterial cells in accordance to the effect of voltage treatment in LB media or NaCl solution. The SEM images of bacterial cells experiencing reduction reactions at -1 V in NaCl solution show a certain amount of cavitated and distorted cells on sample surfaces. Lysed cells resulted from voltage treatment exhibited holes in the middle which open them up into two halves. It was possible that after current flow destructed the biofilm structure, reactive oxygen intermediate and other potential toxic molecules were able to reach the outer membrane, bind to outer membrane proteins, and trigger the cell lysis by opening up a hole on it. As a few cavitated cells were shown on voltage-
treated samples at -1.2 V in LB media, more negative voltage was likely to induce greater lysis of more cells as that in NaCl solution at -1 V.

However, there were no obviously cavitated cells on the AFM images of bacteria in NaCl solution or LB media after two-hour voltage treatment. Instead, some cells were completely lysed with a rough flatten membrane while others formed two bulges in its distal parts with a less release of cytoplasmic material. It was suggested that rather than opening up a crack on the cell membrane, voltage treatment may squash and flatten bacterial cells without leaving an obvious cleavage on cell surface. As oxygen reactive substances could react with nucleic acids, lipids, proteins and sugars in or around bacterial cells, the oxidation of lipids, decreased amino acids and sugars could result it the formation of carbonyls and carbonyl adducts. Oxygen species may also react with proteins and cause a serious of events like deamidation and racemization. Such chemical modification could induce disturbance into protein structures and end in protein cleavage, aggregation, degradation and impaired catalytic and structural functions\textsuperscript{99-102}.

It has been demonstrated that electric field could influence the orientation\textsuperscript{46} and electrophoretic motilities\textsuperscript{103} of membrane proteins in eukaryotic cells. Reactive intermediate transport assisted by electrophoresis could disturb membrane potential of individual cells and lead to unbalanced electrical equilibria, altered orientation and electrophoretic motilities of membrane proteins. Furthermore, electrical current could alter the entire biofilm structure by introducing cations into the exopolysaccharide and cell membrane\textsuperscript{31, 48, 49}, and biofilm structural changes could in turn enhance the molecule diffusion into biofilms\textsuperscript{50}. 
J. Costerton$^{2, 3, 8, 16, 21, 48}$ and his colleagues have demonstrated that low-intensity electric fields could significantly reduce the concentration of tobramycin needed to eliminate *P. aeruginosa* biofilm. They indicated that the bioelectric effect did not fully depend on the generations of electrochemical ions and molecules as biofilm bacteria was killed on all the conductive coupons but was not specific to electrodes within electric fields. They suggested the preferred explanation of bioelectric effect was electrically assisted electrophoresis, which might induce perturbations of membrane potential and structural changes. Jass and Lappin-Scott$^{31}$ attributed the enhancement of electric current on antibiotics in killing *P. aeruginosa* biofilms to changes in bacterial membrane potential, biofilm structure and exopolysaccharide that might influence bacterial metabolic activities and antibiotic diffusion rate. Berrier et al. investigated whether electric fields could induce changes on membrane potential and outer membrane porin proteins of *E. coli*.$^{51}$ They suggested that a specific channel within the inner membrane of *E. coli* stayed open at negative or zero membrane potentials and an increase in voltage would change the native orientation of inner membrane and lead to channel closure. Although it was not clear to what extent a relatively weak potential at -1 V or -1.2 V influences these systems, but this was likely to alter the transport and diffusion of reactive oxygen intermediates and other toxic species, which might depend on the outer membrane porin protein for transport into the periplasmic and the cells$^{31}$.

In Chapter 3, Live/Dead images show that certain spots on the metal surfaces in LB media were covered with thick biofilms in the presence of -1 V cathodic voltage, which was different than the appearance of biofilm bacteria in NaCl solution at -1 V. This was possibly due to the influx of charged nutrients in LB media which may induce increasing metabolic rates$^{31}$. It was
hypothesized that the increase in bacterial growth rate may wake up the slow-growing bacteria or persister cells and increase the susceptibility of biofilm populations.

*Bacterial Detachment*

Time-lapse photography was proven to be an effective method in investigating the cellular dynamics under electrochemical stimulus in real time. Bacterial have demonstrated that cell velocity significantly increased once voltage treatment at -1 V started and then dropped after two hours in NaCl solution (p<0.05), while no such difference was seen during the potentiostatic test in LB media. After two-hour voltage treatment, bacterial movement decreased to a level that was even lower than the control cells before the treatment. Given the rate of oxygen and water reduction kept going, the reduction of bacterial motility may be due to the changes in cell activities themselves.

As discussed above, the influx of charged nutrients like sodium ions might promote the bacterial metabolic rate, enhance cell movement once voltage treatment started. Meanwhile, it could wake up the persister cells in the population and increase their susceptibility to electrochemical treatment. Van der Borden et al. 40 have demonstrated that electric currents were beneficial at stimulating the detachment of initially adhering *Staphylococcus* from stainless steel surfaces. Bacteria remaining on metallic surfaces were less viable than those prior to the application of current.

Initially growing biofilm interacted with substratum mainly through Van der Waals, electrostatic and acid base forces. Stimulation from electric current could promote the detachment of initially
growing biofilm from sample surfaces and impair the excretion of exopolysaccharide substances. Once biofilm formation matures, bacteria entrapped in the gel-like exopolysaccharide would restrain the movement of ionic molecules and increase the difficulties of fighting against infections.

As the first stage of bacterial biofilm formation involved bacterial cells attachment and adherent to sample surfaces\(^7\), this work showed that the application of cathodic voltage could stimulate the motility of bacteria, promote detachment of cells from surfaces and thus prevent biofilm growth at the beginning of biofilm formation. At the cathodic electrode surfaces, the evolution of hydrogen gas from reduction reactions could also be responsible for detachment and loss of certain viable cells\(^104\).

This study demonstrates that the cellular behaviors of *E. coli* HM22 cultured on cathodic active cpTi surfaces are affected by voltage treatment and electrolyte solution in accordance to the electrochemical properties of titanium surfaces. Although it provided insights on the mechanism of reduction reactions kill bacterial biofilms, it did not clearly illustrate the way in which applied potential eliminate the biofilm populations. It has been reported that by introducing the lysis gene into bacteria cells, lysis hole as large as cell diameters were generated\(^105\). It was likely that the application of cathodic voltage promoted the expression of such lysis genes, led to the release of cytoplasmic materials and ultimately induced cell death and biofilm destruction. It is also beneficial to investigate the binding sites at which reactive oxygen intermediates interact with exopolysaccharide and individual cell membranes. Studies on the fundamental cellular and electrochemical principles could contribute to the development of practical applications of
electrical effect on bacterial biofilm removal and a better understanding of the fundamental mechanisms by which bacterial biofilm resist antimicrobial agents.
5. Conclusions

This study demonstrates that the behavior of *E. coli* HM22 cells cultured on electrochemically active cpTi surfaces is affected by voltage treatment and electrolyte solution by way of electrochemical currents and impedance properties at the titanium surface.

Relatively weak cathodic potential at -1 V (vs. Ag/AgCl) could significantly reduce the viability of cells cultured on titanium surfaces in NaCl solution in 24 hours (p < 0.05). However, bacterial cells cultured on cpTi surfaces in LB media require more negative voltage (below -1.2 V) to achieve proficient killing efficacy.

Significant changes in cell morphology were found after -1 V voltage treatment in both NaCl solution and LB media. Voltage treatment in NaCl solution at -1 V reduced the length and width of both intact and ruptured cells compared to those of controls at OCP (p < 0.05). Besides, ruptured cells obtained significantly lower height than controls did at OCP before the treatment (p < 0.05). In LB media, voltage treatment significantly reduced the length and height of all the cells at -1 V compared to that of the untreated cells at OCP, whereas, cell width remained to be unaffected by the application of potentials.

Time-lapse photography was proven to be an effective method in investigating the cellular dynamics under electrochemical stimulus in real time. Bacterial cell velocity significantly increased once -1 V voltage treatment started and then dropped after two hours in NaCl solution (p < 0.05), while no such difference was seen during the potentiostatic test in LB media. These
results could be correlated to other cellular performance and electrochemical properties at the metal-bacteria-liquid interface.

On the other hand, changes in cellular responses were corresponded to variation on electrochemical properties of metal surfaces. Current densities were monitored and EIS was used to capture the electrochemical impedance of titanium-oxide-solution interface in the presence and absence of bacteria. The electrochemical impedance of the oxide-bacteria-solution interface is dependent on the presence of voltage. Sustained voltage treatment at -1 V decreased the impedance of titanium-oxide-bacteria interface in both LB media and NaCl solution at 0.1 Hz compared to those at OCP (p<0.05). In LB media, the presence of bacterial biofilm significantly reduced the average current density experienced by cpTi surfaces at -1 V compared to controls without cells at -1 V in 24 hours (p < 0.05). These results could ultimately be used to design new devices with a capability to actively control bacterial infections and inflammation on the implant surface.
6. Future work

This study demonstrates that the cellular behaviors of *E. coli* HM22 cultured on electrochemically active cpTi surfaces are affected by voltage treatment and electrolyte solution and correlated to the electrochemical properties of titanium surfaces. Although it provided insights on how applied potential could influence the electrochemical features of titanium alloy and alter bacterial performance, it gave rise to other questions which need to be addressed in future efforts. Particularly, the mechanism of this killing efficacy of applied potential remains to be established. Investigations on the fundamental cellular and electrochemical principles could contribute to the development of practical applications of electrical effect on bacterial biofilm removal and a better understanding of the mechanisms by which bacterial biofilm resist antimicrobial agents.

When demonstrating the effects of static voltage on the cellular behavior on cpTi in NaCl solution in Chapter 3, only -1 V static voltages were applied to bacterial biofilm cultured on metal surfaces. It might be beneficial to explore the impacts of other voltage levels lower than -1 V to determine the voltage threshold where biofilm populations go from unaffected to dead. Besides, more negative voltage than -1.2 V could be applied on bacterial biofilm in LB media to achieve a significant killing efficacy. Considering the cellular response corresponding to the variation of electrochemical conditions like current densities, there might be a dose-dependence of the cathodic voltage and current density magnitude on bacterial killing and biofilm elimination and a threshold voltage where biofilm population susceptibility greatly increases and bacterial biofilm fully detach from sample surfaces.
Furthermore, application of voltage at intervals shorter than 24 hours might be studied to look at the time dependence of the cathodic voltage on bacterial killing in various biological conditions. Study of cellular response on metal surfaces was not limited to cell viability, SEM and AFM.

There are other biological assays that could be used as a complimentary method in identification of bacterial performance. A DNA microarray could be used to measure the gene expression of bacterial cells after the application of static potential, which might be helpful to explain why the presence of bacterial cells altered the current density experienced by cpTi samples at -1 V in LB media and remained alive afterwards. The application of protein microarray might be helpful to track the interactions and activities of proteins expressed by biofilm bacteria and determine the membrane structure and exopolysaccharide structural response to electrical stimulus. Colony-forming unit (CFU) could be used to estimate the quantity of viable bacterial on a sample surface after voltage treatment to prove the cell viability results from the Live/Dead assay.

In addition, it is worthwhile to consider using other cell lines in the electrical treatment as *Pseudomonas aeruginosa* has been recognized as an emerging pathogen of clinical procedures. Voltage treatment could kill and eliminate other pathogen species as well in order to be applied in practical therapy.
7. Appendices

7.1. Cell Culture Materials

70% ethanol (v/v)

Vials of frozen cells from refrigerator (-80 °C)

Lysogeny Broth (LB) media (10 g tryptone, 5 g yeast extract, 10 g NaCl per 1L) supplemented with 25 µl DPA at 37 °C with shaking at 200 rpm.

7.2. LIVE/DEAD BacLight Bacterial Viability Assay

LIVE/DEAD BacLight Bacterial viability assay was used to monitor bacterial viability based on the membrane integrity of bacterial cells. Dead or dying cells with a compromised membrane will stain red, while live cells with an intact membrane will stain green.

Kit contents:

SYTO 9 dye, 1.67 mM / Propidium iodide, 1.67 mM (Component A), in DMSO

SYTO 9 dye, 1.67 mM / Propidium iodide, 18.3 mM (Component B), in DMSO

Fluorescence microscopy protocols:

a) Combine 997 µL PBS, 1.5 µL component A and 1.5 µL component B separately in a tube, mix thoroughly.

b) Add 150 µL of the dye mixture on bacterial cells cultured on samples.

c) Incubate bacterial cells at 37 °C in the dark for 15 minutes.
d) Mount inverted metal samples on a petri dish with two square coverslip in between to avoid damage to cells.

e) Observe in a fluorescence microscope with filter sets.
8. **Bibliography**


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• Study effects of reduction electrochemistry at electrochemically metallic surfaces on behaviors of bacterial biofilms
• Investigate effects of bacterial biofilms on electrochemical properties of oxide-bacteria-liquid interfaces
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