Voltage Effects on Cells Cultured On Metallic Biomedical Implants

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Abstract

Electrochemical voltage shifts in metallic biomedical implants occur *in-vivo* due to a number of processes including mechanically assisted corrosion. Surface potential of biomedical implants and excursions from resting open circuit potential (OCP), which is the voltage they attain while in contact with an electrolyte, can significantly change the interfacial properties of the metallic surfaces and alter the behavior of the surrounding cells, compromising the biocompatibility of metallic implants. Voltages can also be controlled to modulate cell function and fate. To date, the details of the physico-chemical phenomena and the role of different biomaterial parameters involved in the interaction between cells and metallic surfaces under cathodic bias have not been fully elucidated.

In this work, changes in the interfacial properties of a CoCrMo biomedical alloy (ASTM F-1537) in phosphate-buffered saline (PBS) (pH 7.4) at different voltages was studied. Step polarization impedance spectroscopy technique was used to apply 50 mV voltage steps to samples, and the time-based current transients were recorded. A new equation was derived based on capacitive discharge through a Tafel element and generalized to deal with non-ideal impedance behavior. The new function compared to the KWW–Randles function, better matched the time-transient response. The results also showed a voltage dependent oxide resistance and capacitance behavior. Additionally, the *in-vitro* effect of static voltages on the behavior of MC3T3-E1 pre-osteoblasts cultured on CoCrMo alloy (ASTM-1537) was studied to determine the range of cell viability and mode of cell death beyond the viable range. Cell viability and morphology, changes in actin cytoskeleton, adhesion complexes and nucleus, and mode of cell death (necrosis, or intrinsic or extrinsic apoptosis) were characterized at different voltages ranging from -1000 to
+500 mV (Ag/AgCl). Moreover, electrochemical currents and metal ion concentrations at each voltage were measured and related to the observed responses. Results show that cathodic and anodic voltages outside the voltage viability range (-400 < V < +500) lead to primarily intrinsic apoptotic and necrotic cell death, respectively. Cell death is associated with cathodic current densities of 0.1 μAcm\(^{-2}\) and anodic current densities of 10 μAcm\(^{-2}\). Significant increase in metallic ions (Co, Cr, Ni, Mo) was seen at +500 mV, and -1000 mV (Cr only) compared to open circuit potential. The number and total projected area of adhesion complexes was also lower on the polarized alloy (p < 0.05). These results show that reduction reactions on CoCrMo alloys leads to apoptosis of cells on the surface and may be a relevant mode of cell death for metallic implants in-vivo.

On the other hand, we studied how surface oxide thickness of Ti affects its voltage viability range and cellular response and whether anodic oxidation can serve as a means to extend this range. Cellular behavior (cell viability, cytoskeletal organization, and cellular adhesion) on bare and anodized Ti samples, potentiostatically held at voltages at the cathodic edge of the viability range, were assessed. Surfaces were characterized using contact angle (CA) measurement technique and atomic force microscopy (AFM), and the observed cellular response was related to the changes in the electrochemical properties (electrochemical currents, open circuit potential, and impedance spectra) of the samples. Results show that anodization at a low voltage (9 V) in phosphate buffer saline (PBS) generates a compact surface oxide with comparable surface roughness and energy to the starting native oxide on the bare surface. The anodized surface extends the viability range at 24 hours by about a 100 mV in the cathodic region, and preserved the cytoskeletal integrity and cell adhesion. Broadening of the viability range corresponds to an increase in impedance of the anodized surface at -400 mV(Ag/AgCl) and the resulting low
average currents (below 0.1 \( \mu \text{Acm}^{-2} \)) at the interface, which diminish the harmful cathodic reactions.

Finally, cellular dynamics (size, polarity, movement) and temporal changes in the number and total area of focal adhesions in transiently transfected MC3T3-E1 pre-osteoblasts cultured on a CoCrMo alloy polarized at the cathodic and anodic edges of its voltage viability range (-400 and +500 mV(Ag/AgCl) respectively) were studied. Nucleus dynamics (size, circularity, movement) and the release of reactive oxygen species (ROS) was also studied on the polarized metal at -1000, -400, and +500 mV(Ag/AgCl). The results show that at -400 mV(Ag/AgCl) a gradual loss of adhesion occurs over 24 hours while cells shrink in size during this time. At +500 mV, cells become non-viable after 5 hours without showing any significant changes in adhesion behavior right before cell death. Nucleus size of cells at -1000 mV decreased sharply within 15 minutes after electrochemical polarization, which rendered the cells completely non-viable. No significant amount of ROS was released by cells on the polarized CoCrMo at any of these voltages.
Voltage effects on cells cultured on metallic biomedical implants

By

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1. Overview

1.1 Structure of Dissertation

This dissertation is composed of several manuscripts, which either have been or will be submitted for publication in peer-reviewed journals. A general introduction is given at the beginning prior to the presentation of these papers to provide background information, and highlights the significance and objectives of this work for the reader.

The first paper, titled “The voltage-dependent electrochemical impedance spectroscopy of CoCrMo medical alloy using time-domain techniques: Generalized Cauchy-Lorentz, and KWW-Randles functions describing non-ideal interfacial behaviour” was published in Corrosion Science, and is presented as the second chapter of this dissertation. It discusses electrochemical characterization of CoCrMo/phosphate buffer solution (PBS) interface in the time domain and suggests a new time-based equation for the current decay at the interface.

The third chapter consists of the manuscript titled “Electrochemical control of cell death by reduction-induced intrinsic apoptosis and oxidation-induced necrosis on CoCrMo alloy in vitro”, which was published in Biomaterials. In this chapter, the effect of static DC surface potential of CoCrMo at anodic and cathodic voltages on cellular behavior is assessed.

In the fourth chapter “Voltage-controlled cellular behavior on polarized cpTi with varying surface oxide thickness”, the effect of surface voltage on cells cultured on bare and anodized cpTi is studied and correlated to the electrochemical properties of the metal. The focus of the study, however, is mainly on how the thickness of the surface oxide layer affects this interaction at cathodic voltages.
The fifth chapter “Study of cellular dynamics on polarized CoCrMo alloy using time-lapse live-cell imaging” discusses the temporal aspect of the surface-voltage/cell interactions on the surface of CoCrMo alloy via use of transiently-transfected cells.

Each chapter includes the following sections of introduction, materials and methods, results, discussion, conclusion and the references. Some overlap between the content covered in the general introduction chapter and the introduction of each chapters due to the inclusive nature of the manuscripts might exist.

To consolidate the concepts covered in all chapters, synthesis of the results from all chapters are presented in chapter 6, followed by suggestion for future work and an overall conclusions section, containing the conclusions of the entire thesis. Details related to the experimental methods and procedures, software codes, etc will follow this chapter in the appendix section.
1.2 Objectives

The objective of this work is to investigate the effects of surface potential (voltage) on the behavior of osteoblast-like cells cultured on CoCrMo alloy and commercially pure (cp)Ti in simulated body fluids, and correlate the observed cell response with the changes in the electrochemical properties of these alloys.

Alteration of surface voltage of biomedical alloys is known to happen due to oxide disruption events or the presence of oxidants or inflammatory species. This work will help to understand how these voltage changes affect the cellular behavior and correlate with the corresponding changes in electrochemical properties and electrochemical reactions of these biometals and their surfaces. Furthermore, the results could provide insight on designing smart biomaterials, which induce a desired biological effect (e.g., protein expression) through a controlled application of different voltages.

Experimental methods include electrochemical modification and characterization at different voltages using time and frequency based impedance spectroscopy techniques, use of biological assays and molecular biology techniques, fluorescence and electron microscopy imaging.
1.3 Specific Aims and Hypothesis

**Specific aim 1:** To study the effect of cathodic and anodic voltages and their associated electrochemical reactions on the cellular behavior of MC3T3-E1 preosteoblasts cultured on CoCrMo biomedical alloy and to correlate cell behavior with changes in electrochemical properties (polarization resistance, capacitance). Specifically, cellular morphology, viability, organization of cytoskeletal and adhesion molecules (F-actin and vinculin) were correlated with the magnitude of the applied voltages and the corresponding electrochemical currents measured. In cases that voltage-induced cell death occurs, mode of cell death were determined (apoptosis vs. necrosis).

The hypothesis to test under aim 1 are:

- Voltage of the biomedical implants affects the behavior of cells cultured on them via redox reactions occurring at the surface.
- Extreme voltages both in anodic and cathodic directions lead to cell death through different mechanisms (apoptosis and/or necrosis). Accordingly, there exists a voltage viability range for biomedical CoCrMo alloys.

**Specific aim 2:** To study the dynamics of cells cultured on polarized CoCrMo samples using live-cell imaging techniques. Temporal changes to morphology and organization of sub-cellular structures and molecules (Nucleus, vinculin, reactive oxygen species (ROS)) were measured.

The hypothesis to test under aim 2 is:

- The effect of voltage of an implant on the behavior of cells cultured on it is a time-dependent phenomenon, depending on the type of harmful redox reactions present and electrochemical currents measured.
Specific aim 3: To study the effect of varying oxide thicknesses on cellular behavior on polarized cpTi samples at cathodic voltages. Specifically, viability and adhesion behavior of cells at different cathodic voltages were correlated with the impedance spectra of the bare and anodized cpTi.

The hypothesis to test under aim 3 is:

- The thickness of the surface oxide on Ti samples affects the cellular behavior of cells cultured on them, and can broaden its voltage viability range by limiting the level of harmful Faradic currents.
1.4 Background and Significance

1.4.1 Metallic Biomedical Implants

Metallic biomaterials are extensively used in different parts of human body as vascular stents, artificial heart valves, replacement implants in hips, elbows, shoulders, knees, and dental structures [1, 2]. In particular, metallic implants are well suited as orthopedic implants for spinal, hip and knee replacements, where mechanical requirements limit the use of other materials. Despite the frequent use and the growing demand for these implants, issues such as lack of proper implant/tissue integration and implant loosening, infection, formation of pseudotumors, and corrosion still exist and need to be addressed to prevent implant failure in the long term. The biomedical alloys most commonly used as surgical implants include 316L stainless steel (316LSS), cobalt chromium (CoCr) alloys, titanium and its alloys. There are advantages and disadvantages associated with each of these alloys. Titanium, for instance, is well known to be largely inert and provides high biocompatibility, high specific strength, corrosion resistance, low modulus and enhanced osseointegration [3]. However, it tends to undergo severe wear while used as an articulating surface against itself or other metals, and has poor shear strength [4]. Implant loosening and osteolysis due to generation of wear debris in Ti alloys with high friction coefficients have also been reported [5]. On the other hand, CoCr and stainless steel alloys despite having high corrosion and wear resistance, suffer from metal ion (Ni, Co, Cr) release, associated with cytotoxic and carcinogenic effects [6-8]. In addition, due to their high modulus, they are more likely to bring about stress shielding and osteoporosis. The research efforts in the field of metallic biomaterials are therefore orchestrated to provide solutions for the current shortcomings of these alloys by shedding light on the fundamental phenomena governing the
behavior of metallic biomaterials in vivo. A thorough understanding of the surfaces of these implants and the variations present on the surfaces are crucial in achieving this goal.

1.4.2 Conceptual model of polarized metal/semiconductor/electrolyte interface

In order to have a better understanding on how the surfaces of metallic biomaterials interact with their surrounding environment, a conceptual model of such interfaces is presented here. Metallic biomaterials are typically covered with a native surface oxide, which will be the immediate contact point with the biological electrolyte. This thin layer of oxide with semiconducting properties is in contact with the underlying bulk metal on one side and the biological milieu on the other. Since both CoCr alloy and cpTi were used in this work, the nature of bulk and native surface oxides formed on these two alloys are briefly reviewed first. The composition of different grades of cpTi and CoCr alloys, most commonly used for biomedical applications are shown in table 1.1 and 1.2 respectively.

An oxide layer (few nanometers in thickness) is formed on these metals due to the highly active nature of the underlying metal and acts as a barrier towards charge transfer thus limiting corrosion. The cast CoCrMo alloy consists of FCC (γ phase) and HCP (ε phase) phases formed by martensitic transformation from γ to ε during cooling [9,10]. The surface oxide formed on CoCrMo alloy is mainly composed of oxides of Cr and to a lesser extent oxides of Co, and Mo [11-13]. However, it should be mentioned that the composition, ratio of the oxides formed and thickness of the passive film is highly dependent on conditions of formation (e.g., oxidation potential and the medium) [11] (Fig. 1.1). The structural stability of the oxide layer depends on the relative specific volume of metal and the oxide (Pilling-Bedworth ratio). In addition, the surface oxide shows a very dynamic nature
changing constantly over time. The non-stoichiometric nature of the native surface oxides brings about their semiconducting properties. The Mott-Schottky analysis of the passive film on chromium shows a p-type semiconducting character for the oxide [14, 15]. Since the passive film on CoCrMo alloys is primarily composed of chromium, one must expect to observe a p-type semiconducting behavior for it as well. However, depending on the voltage, it may change its semiconducting nature from p-type to n-type behavior [15, 16].

![Graph](image)

**Fig. 1.1:** Profile of changes in the cationic ratio of the passive layer formed on CoCrMo alloy in a simulated physiological solution at different oxidation potentials. The dotted lines show the atomic fraction for Co, Cr and Mo in the bulk alloy [11]. This figure was reproduced from the cited work with permission from the publisher.

The surface oxide on cpTi is primarily made of titanium oxide, but depending on the formation conditions, its stoichiometry and degree of crystallinity varies within a wide range [17].

The passive film on titanium is known to have n-type semiconducting properties [18].
There are two main theories to describe the metal/electrolyte system: the mixed potential theory coming from the corrosion community and the theories in the field of solid-state electrochemistry at semiconductor and oxidized metal electrodes.

For any metal electrode in contact with an electrolyte, a number of electrochemical half-cell reactions are present in balance. The balance requires that the total rate of oxidation and reduction reactions equal each other. The thermodynamics and kinetics of the half-cell reactions are described by the Nernst equation and exchange current densities respectively [19]. Accordingly, there is a Nernst potential associated with any half-cell reaction. According to the mixed potential theory, the potential that is established when a metal electrode is placed in an electrolyte is an intermediate potential that makes the overall rates of anodic and cathodic reactions equal on the surface. This potential is what is known as the open circuit potential (OCP) or $E_{corr}$ of an electrode. The net current at this potential is zero as charges transferred in the anodic and cathodic reactions are equal and in opposite directions. Polarization of a metal or disruption of the surface oxide layer will induce a net current in the anodic or cathodic direction. Examples of the half-cell reactions in metal/electrolyte systems are reduction of oxygen/water [20], proteins [21] at cathodic voltages, and metal oxide growth [22,23] and ion release at anodic voltages.

Table 1.1: Composition of different grades of biomedical cpTi [24].

<table>
<thead>
<tr>
<th>Element</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
</tr>
</thead>
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<td>0.03</td>
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</tr>
<tr>
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<td>H</td>
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Table 1.2: Composition of different types of CoCr alloys used in biomedical implants [24].

<table>
<thead>
<tr>
<th>Element</th>
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<th>Co28Cr6Mo (F1537) Wrought</th>
<th>Co35Ni20Cr10Mo (F562)</th>
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Metallic biomaterials inside the body essentially form two interfaces (Fig. 1.2). The interface between the bulk metallic phase and the native surface oxide with semiconducting properties, and the interface between the surface oxide and liquid phase (body fluids and electrolytes). These interfaces can be modeled as a combination of several double layers, which control the electrical and chemical properties of the surface. A double layer consists of layers of positive charge, layers of negative charge, and regions of high electric fields between or within the charged layers.

1.4.2.1 Double layers at the solid/liquid interface

Three double layers are present at the solid/liquid interface as shown in Fig. 1.2.
These layers consist of a semiconductor space charge double layer, a Helmholtz double layer between the solid and the “outer Helmholtz plane” (ohp) and finally the Gouy-Chapman double layer in the solution next to the solid, in which an excess of ions of one sign exists. There are four regions of excess charge corresponding to these three double layers: the space charge region in the solid, which is in the form of uncompensated impurities or trapped holes/electrons, or mobile holes and electrons near the surface. Second, there is charged planes on the two sides of the Helmholtz region that form the Helmholtz double layer. At the ohp, the charge arises from ions that drift up to the surface, attracted by the excess charge in or on the semiconductor. Finally, there is an extended region of excess space charge in the Gouy-Chapman region of the solution, associated with mobile ions. This charged region together with the countercharge in the Helmholtz region and in the solid, form the Gouy double layer.

Fig. 1.2: The conceptual model of an oxide-covered metal electrode in a solution. The diagram underneath shows the corresponding excess charge density across the interfaces.
In case there are other phases present on the surface such as organic or inorganic coatings including layers of cells, additional double layers must be taken into consideration. Electrical charge can be stored between the oxide and the substrate and in the form of space charge in the oxide, which is related to electrical capacitance. Therefore, each double layer has a capacitance associated with it. Additionally, each double layer especially the Helmholtz and the solid space charge double layers have resistive impedance associated with a dc current flow.

1.4.2.2 Energy band model

Another way to look at the charge density model described above is through energy bands. The band model describes what levels of energy can be occupied by electrons in different solids (conductors, semiconductors and insulators). Accordingly, there are valence and conduction bands separated by a forbidden region of energy called the bandgap (Fig. 1.3). The valence band is normally completely occupied by valence electrons of the solid while the conduction band is normally unoccupied by electrons. In a conductor, the conduction band is partially occupied or overlaps the filled valence band. The distribution of electrons and holes among available energy levels are described by Fermi distribution function. At the Fermi energy level, the probability of occupancy is exactly 50% [22]. Fermi energy can alternatively be described as the electrochemical potential of electrons in an electrode [25]. Transfer of electrons in or out of the semiconductor surface leads to formation of charged regions known as space charge region. The space charge can be in the form of immobile charged impurities or immobile trapped carriers near the surface of the semiconductor or in the form of mobile electrons/holes in the conduction or valence bands.
Description of the energy levels in the solutions due to the polarizing nature of the solvents is more complicated. The energy levels of ions in solutions are described by what is known as fluctuating energy levels (Fig. 1.3). For a simple one-component redox system, the oxidized and reduced forms of the ion have Gaussian distributions of energy levels. The $E_{ox}$ and $E_{red}$ are the most probable energy levels for oxidizing and reducing agents respectively. Thermal fluctuations cause the energy of oxidized and reduced ions drift from the most probable value. These
fluctuations are of importance as the probability of electron transfer between an electrode and an ion in solution depends on the energy level of the ion at the instant of transfer.

### 1.4.2.3 Qualitative description of electrode processes using the band model

We first start by discussing the charge transfer using band diagrams to describe a metal electrode inside a solution with a redox couple (Fig. 1.4) and then proceed to consider the case of an oxide-covered metal electrode with semiconducting properties (Fig. 1.5 and 1.6). At open circuit potential, no current flows through the reference electrode and the electron exchange between the redox couple and the metal will be at equilibrium. Therefore, the Fermi energy levels of metal will be equal to $E_{\text{redox}}$ of the redox couple. Electron transfer from the solution to metal takes place at a rate proportional to the overlap between the density of states of the reducing agents and the density of empty states (above $E_F$) in the metal. Similarly, electrons move to the solution at a rate proportional to the overlap between the density of states of the oxidizing agent and the density of filled states (below $E_F$) in the metal. Helmholtz double layer does not cause any obstruction to current flow as it is so thin (~ 0.3 nm) that electrons can freely tunnel through.

Upon application of a voltage different than $E^0$, an overvoltage $\eta$ (negative in the figure) appears across the Helmholtz double layer. The negative overvoltage leads to a wider overlap between the filled levels in the metal and the levels of the oxidizing agent and the cathodic current from the metal to the solution increases. The decreased overlap between the unoccupied levels in the metal and the levels of the reducing agent lowers the magnitude of the anodic current.

Placement of an oxide-covered metal substrate in an electrolyte leads to establishment of an electric field across the semiconducting oxide, which either raise or lower the band energy in the
space-charge regions. This in itself causes what is known as energy band bending at the semiconductor interface. The band model for a semiconductor differs from that of a metal due to the presence of bandgap and more importantly the space charge double layer (Fig. 1.5). For the sake of simplicity, we only consider the more common case, where the energy $E^0_{\text{redox}}$ is in the bandgap region. Electrons in this case move from the semiconductor conduction band to the overlapping unoccupied oxidizing agent levels. As most of the voltage drop occurs in the space

Fig. 1.4: Schematic diagram combining an energy band model for a metal electrode and its contacting electrolyte with an external electrical circuit. (a) open circuit potential condition (b) an external voltage is applied and current is passed between the sample and a counter electrode. This diagram was reproduced from the cited work [25].
charge region of the oxide, the voltage across the Helmholtz region does not change. Hence, electron injection will always be small and independent of voltage. However, the cathodic current is proportional to the density of levels of the oxidizing agent above the energy $E_{cs}$, and to the density ($n_s$) of electrons at the surface. Therefore, current will depend exponentially on applied voltage since a decrease in $V_s$ is accompanied by an exponential increase in $n_s$. The above model described the current flow across the interface of an n-type semiconductor with a solution. For a p-type semiconductor an analogous model can be applied to the exchange of holes with the valence band [25].

Fig. 1.5: Schematic model for an n-type semiconductor electrode and redox couples similar to figure 1.4. A redox couple with $E^0_{\text{redox}} < E_{cs}$ is shown, with a voltage applied so electrons can be extracted from the semiconductor. Note that $E_{cs} - E_F(\text{ref})$ is constant regardless of $E^0_{\text{redox}}$ and $V_s$. This diagram was reproduced from the cited work [25].
It should be mentioned that in the above descriptions of the metal and semiconductor electrodes only a single half-cell reaction at steady state was considered, where both oxidation and reduction reactions happening in the solution and the electrons flow or are extracted from the metal. In the case of metallic biomedical alloys, there are several factors that significantly add to the complexity of the simplified model of a semiconductor described above. These complications stem from the finite size of the surface oxide, which only spans few nanometers, presence of many surface states and traps in the oxide film, variation of the composition with distance from the metal surface, oxide growth, and presence of several oxidative and reductive half-cell reactions occurring in the solid as well as in the solution. Currently, there is no unifying theory that takes all the above complications into account in order to provide a comprehensive description of the electrochemistry happening at these interfaces [26]. However, the high-field growth model of Mott-Cabrera and the semiconductor electrochemistry theory explain the process of charge transfer in the oxide-covered metals through different perspectives. The Mott-Cabrera theory [27] was originally presented to describe the formation and growth of the surface oxide. According to this theory, electrons tunnel from the metal surface and react with oxygen at the surface to create metal cations, oxygen anions, and a high electric field as a result. The high electric field then causes the motion of the metal cations at the metal/oxide interface and the oxygen anions at the oxide/solution interface and their vacancy counterparts through the oxide. Therefore, the charge transfer in this model occurs due to development of high electric fields initiated by electron tunneling and subsequent electromigration of ionic species. The rate of ion transport depends on the level of the defects and the mobility of the ions in the oxides. As the oxides film grows, the electrical field across the oxide decreases and the transport rates drop.
The semiconductor electrochemistry theory [25], on the other hand, is more focused on the oxide/solution interface and the solution-based redox reactions, and does not discuss any redox processes present in the formation of oxide. A modified format of the previously discussed n-type semiconductor/solution interface (Fig. 1.5) is shown in Fig. 1.6, which could serve as a model for the metallic electrodes covered with a thin–film oxide layer. The principle that drives the magnitude of electron injection or reduction from the electrode is the degree of overlap between the majority carrier band and the energy of reducing or oxidizing agent in the solution as discussed previously. What is mainly different in the case of metal electrodes covered with thin-film oxide layers is a space charge region, which spans the whole oxide thickness.

Fig. 1.6: The band model for an amorphous thin film oxide on a metal. The energy levels A and C result from the higher electron affinity (level A) or a lower ionization potential of some ions with slightly different bonding configuration compared with ions in the conduction and valence bands. The energy level B corresponds to electron traps, while level D indicates an intermediate energy band due to impurities or different oxidation state of the ions. Surface state levels are shown at the interface of metal/oxide and oxide/solution as well (E). This graph was adapted from the cited work [25].
The highly defective amorphous oxide layer has a complex band model caused due to the presence of many sources of energy levels. Main features of the oxide energy band model are the presence of intermediate energy bands associated with impurities or different oxidation states of the ions in the lattice and presence of low-density energy levels next to the conduction ($E_{cd}$) and valence bands ($E_{cv}$) and intermediate bands. Extra energy levels can also be present in the form of surface states and bulk electron traps. Traps block motion of charge carriers across the oxide film. Presence of the surface energy states occurs as a result of ion adsorption, dangling bonds at the interfaces or nonstoichiometry of the oxide in a transition layer between the metal and its oxide. Surface states at oxide/solution interface are usually dominated by adsorbed ions such as H$^+$ and OH$^-$. The density and distribution of the extra energy levels in the oxide affects the probability of the tunneling, which is the dominant electron transfer mechanism in the thin-film oxides.

**1.4.3 Metallic Implants/Cell interactions**

In vivo implantation of medical devices induces a cascade of reactions in the biological micro-environment via interaction of the biomaterial with body fluids, proteins and various types of cells. These interactions, apart from invoking a biological response, may alter the physicochemical properties of the implant as well. Surfaces of biomaterials significantly influence interactions with the body, as the first contact of the body is made with the surface. The parameters that determine the specific interactions taking place at the interface and the long-term integration of the bone-contacting biomaterials with body, include surface chemical composition and topography [28]. They regulate the type and the level of the interactions that occur at the interface such as adsorption of ions and biomolecules (primarily proteins), formation of calcium phosphate layers, and interaction with different cell types (macrophages, bone
marrow cells and osteoblasts). Therefore, the details of the initial interface formed between a biomaterial and the surrounding tissue determines the ultimate success or failure of the materials.

On the other hand, corrosion of metallic implants in vivo and its subsequent effects on the biological behavior and the integrity of the implant itself is one of the most important surface phenomena dictating implant success. All metallic biomaterials are prone to corrosion while in contact with body fluid. Body fluids are considered severely corrosive environments for metallic materials due to the high concentration of chlorine ions, organic electrolytes (amino acids, proteins) and hypoxic conditions. In addition, pH which is normally maintained at the physiological 7.4, can drop to as low as 5.2 right after implantation and only recovers to 7.4 after two weeks [29].

Therefore, biocompatibility of metallic biomaterials may be influenced by the inherent pH and oxygen changes in the microenvironment surrounding the implant [30,31], physical and chemical surface changes [32] (topography, composition, redox states, etc.) and by the release and accumulation of ions in the implant site and/or their distribution throughout body via systemic circulation [33,34]. Currently, the phenomena involved are poorly understood due to the complexity and variability to which they are subjected.

Even in case of in vitro studies of the interactive behavior of cells on the surface of the metallic biomaterials, the metal/semiconductor/electrolyte interface model mentioned earlier will be an oversimplified approximation. The complexity comes from multiple half-cell reactions, ionic transport across the oxide and the presence of several organic and inorganic components in the culture media, extracellular matrix and cell membrane. Considering the significance of surface
interactions in determining the behavior of implants, most of the in vitro studies have been devoted to investigate how simulated body fluids modify the native surface oxide on metallic biomaterials.

1.4.4 Significance of cell culture studies on polarized metals

Although most of the studies devoted to delineate the mechanisms of interaction between metallic implants and cells are focused on the surface chemistry (application of inorganic, organic, or biological coatings) and topography (nano/micro features), there are other phenomena that may influence the overall interaction. Surface voltage shifts is one of the parameters that has received little attention in the context of metallic implants biocompatibility and modulation of cell behavior.

Despite an abundance of research on the effect of metal-based particles and ions released from metallic alloys on cellular behavior [35-40], cell culture studies on the surface of these alloys are not widely performed. Even less studied is how surface voltage shifts and the associated redox currents affect cellular behavior. To date, most of the studies, which have been done on metallic alloys, mainly focused on electrochemical characterization of these alloys in physiological solutions in the absence of cells [41-47]. Few studies reported on cell behavior on polarized biomedical alloys. Gilbert, et al. showed that rat calverial osteoblasts cultured on Ti samples polarized to 0 mV are significantly more spread compared with cells cultured on Ti samples polarized to -1000 mV [20]. Ehrensberger, et al. studied the effect of cathodic and anodic voltages on cells cultured on Ti and found that cathodic voltages more negative than -300 mV (Ag/AgCl) lead to cell death [49].
These results clearly indicate that cell behavior on metallic biomaterials is significantly affected by electrochemical processes (i.e., Faradaic reactions) which can lead to cell death. This is a fundamentally new observation related to metallic biocompatibility and raises fundamental questions about all metallic biomaterial interactions with living systems. Do all metallic surfaces behave similarly in the cathodic potential range? What effects arise in metallic alloys in both anodic and cathodic potential experiments? What is the potential range of cell viability? What are the underlying causes of cell death at high anodic and cathodic voltages? Does the cell death happen by apoptotic or necrotic processes? Answers to these basic questions would be necessary before any potential application can be speculated on use of polarized metallic surfaces. Use of polarized metallic elements with therapeutic effects to treat cancer, alter inflammatory response, improve infection resistance, and prevent neointimal hyperplasia is among such applications.

1.4.5 Effect of polarized electrodes on modulation of cellular behavior and function

The effect of voltage on cells is an interesting area of research, which falls under the general category of electrical stimulation of cells. These studies are mainly focused on either the electrical field effects on cells or the electrochemical processes happening at the cell/electrode interface. The electric effects on cells can be categorized into dielectric, electrophoretic, electrolytic, and cell/electrode interactive effects. Electroporation and electrofusion of cells are well studied examples of the dielectric effect, in which a high voltage pulse of stimulation leads to dielectric breakdown of the membrane and formation of pores or fusion of the cellular membranes. The electrophoretic effect, however, refers to the migration of cells in a solution down a potential gradient between two electrodes. Electrophoresis could be used for both cellular
separation and manipulation. On the other hand, electrochemical effects arise due to redox reactions of electroactive components of the alloys, solution or the cell surface. Irreversible electrochemical reactions of physiologically important substances on the cell membrane or electrolytic formation of active species such as reactive oxygen species (ROS) in the cell culture environment may lead to cell death (electrolytic effect). However, as Aizawa et al. [50] showed, the nature of the cell/electrode interactions is highly dependent on the potential of the electrode and the cellular death is not the only outcome of applying voltage to cells cultured on electrodes. In fact, a proper electrode potential could modulate cellular proliferation, gene expression, protein production, etc (cell/electrode interactive effects).

The study of voltage effects on cells has been done on different electrodes using several cell types in both potentiostatic and galvanostatic modes. Most of the voltage-cell stimulation experiments have been done on transparent indium tin oxide (ITO) electrodes. Yaoita et al. [51] studied the effect of voltages on the viability of Hela cells in culture media for up to 2 hours in a voltage range from +0.5 to +1.5 V vs. Ag/AgCl and found out that high voltages lead to an increase in the number of dead cells. Furthermore, they observed cell swelling at a cathodic voltage of -0.6 V(Ag/AgCl) after half an hour and a change in morphology and actin fiber organization within a voltage range of -0.6 to +0.7 V. More interestingly, they noticed that voltages between +0.5 and +0.7 V have a reversible effect on cells. i.e cells remain alive at these voltages but change into a globular morphology and upon switching the voltage to lower than +0.5 V they spread back to their normal shape. In a study of osteoblast on cpTi samples, Gilbert showed a reduction in cell surface area and rounding up of cells after 2 hours at -1V (SCE) [48]. Zhou et al. [52], however, reported that cathodic voltages are conducive to growth of endothelial cells on ITO electrodes, whereas anodic voltages proved to limit the growth and cause changes in
morphology and actin organization. In another study, Tominaga et al. [53], showed a significant change in morphology and viability of HIV-infected HeLa cells after being exposed to +1.0 V (Ag/AgCl) for an hour.

Gabi et al. [54] studied the viability of myoblasts on ITO microelectrodes in a galvanostatic setup and observed cell death in times as short as 2 minutes when the current density was larger than 57 mAcm⁻². Kalbecova et al. [55], in a galvanostatic study of osteoblasts and monocytes on Ti6Al4V alloy observed shrunken cells and condensed nuclei at high currents, which they attributed to release of reactive oxygen species and a subsequent drop in metabolic activity of cells.

An interesting reversible effect of voltage on the proliferation of human carcinoma cells was observed in the study of MKN45 cells on platinum-sputtered plastic dishes by Kojima et al. [56] They showed that proliferation of cells stops above +0.4 V(Ag/AgCl) but starts again when the voltage is switched back to +0.1 V. The effect of a voltage of +0.74 V(SCE) (applied for 10 minutes) on bacteria cultured on graphite was shown to be loss of respiratory activity and microbial death [57]. Shinohara et al. [58] reported a drastic shift in pH near surface of polypyrrole-coated ITO leading to lysis of erythrocytes at +0.6 V (Ag/AgCl).

In some other studies voltages were applied in the form of sinusoidal or rectangular waves. Koyoma et al. [59] used sinusoidal voltage to induce nerve growth factor production in mouse astroglial cells on ITO electrodes. In a similar study, Kimura et al. [60] used a rectangular wave potential to induce cell differentiation of PC12 cells. Changes to amplitude and frequency of the sinusoidal wave could lead to production of different amounts of LDH, PGI2, TXA2 as reported
by Bouaziz et al. [61] in the study of endothelial cells cultured on a fluoride tin oxide (FTO) electrode.

Some efforts have recently been devoted to stimulation of cultured neurons on planar metallic electrodes [62-65] in order to find out the mechanisms involved in the triggering action potentials and the role of anodic and cathodic potentials on the attached and non-attached parts of the membrane.

1.4.6 Mechanisms of interaction between polarized electrodes and cells

Few studies have investigated the mechanisms of cellular interactions observed on metallic electrodes. The immediate effect of polarization on cells will be alteration of the redox state of electroactive redox couples in cells and consequently the overall redox state of the entire cell. Redox state is important in several cellular processes including differentiation [66], apoptosis [67], gene expression [68,69], and tumorigenesis [70]. The regulation of the redox state is essential in normal functioning of the cells and there are a number of redox couples within the cells to sustain the redox potential at the physiological levels. These molecules include enzymes [71], non-enzymatic antioxidants such as cysteine, GSH, vitamin C and E, and enzymatic antioxidants such as superoxide dismutase (SOD) and catalase (CAT) [72]. Anodic and cathodic potentials may lead to an increase or decrease in the reduced vs. oxidized form of these redox couples hence altering the intracellular redox potential of the system. The ratio of reduced to oxidized glutathione (GSH/GSSG) within cell is often used as a measure of cellular toxicity [73]. Disruption of the normal ratio of GSH/GSSG through polarization of metal electrodes can happen and maybe one of the mechanisms involved in the observed cellular response on polarized metals [74].
At voltages outside the viability range of a metal, cells die either due to electrolysis or an irreversible electrochemical reactions of physiologically important substances in the cellular membranes or electrolytic generation of active species, which are toxic for cells. Kalbecova et al., [55] showed electrical stimulation at cathodic voltages leads to generation of reactive oxygen species (ROS) on the surface of titanium as well as intracellularly. In another study by Aizawa et al. [50], an increase in the expression levels of NGF mRNA after electrical stimulation of astroglial cells, was attributed to activation of protein kinase C (PKC) via mitogen activated protein (MAP) kinase cascade, and AP-1 complex formation. Modulation of ion channels is believed to be another mechanism of voltage-induced effects on cells cultured on metallic electrodes. Through use of calcium channel blockers Aizawa et al. showed that calcium influx via L-type channels plays a role in depolarization induced neurite outgrowth of PC12 cells. Furthermore, cell differentiation could result from application of alternative potential via same L-type channels. Therefore, the exact mechanism of how metal surface potential affects cellular behavior depends on the mode (pulsed, constant) of voltage application as well as its magnitude. While cell shrinkage, decrease in membrane fluidity, and retarded proliferation was associated with constant DC potential, application of a low frequency potential caused activation of PKC, enhanced gene expression and possible activation of SA channel [75].

The effect of varying surface potential of electrodes is not limited to Faradic currents. In a study of individual neurons on an insulated planar electrode using rising ramps as well as falling ramps to provide anodic and cathodic stimulation, Schoen et al. [76] showed that neuronal excitation is elicited under current-clamp. In the current clamp technique, the membrane potential is measured while a current is injected into the cell. The excitation initiates through depolarization of the free membrane, which triggers an action potential (rising voltage ramps case) or via depolarization of
the attached membrane by activation of local ion currents, depolarization of the free membrane and finally triggering an action potential (falling voltage ramps case).

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2. The voltage-dependent electrochemical impedance spectroscopy of CoCrMo medical alloy using time-domain techniques: Generalized Cauchy-Lorentz, and KWW-Randles functions describing non-ideal interfacial behaviour

2.1 Introduction

Electrochemical Impedance Spectroscopy (EIS) is a powerful technique used to study the electrochemical behavior of materials and interfaces. The working principle of EIS is based on perturbing the state of a system by, for example, applying a small oscillating voltage and analyzing the resulting oscillating current response. The electrical stimulus most commonly used in EIS is a sinusoidal voltage applied to the interface at a range of different frequencies, and the sinusoidal current response can be used to find the frequency-domain impedance. However, electrochemical impedance spectroscopy can also take place in the time domain using any arbitrary excitation. Potential and current steps and several noise excitations have been previously used to measure impedance, mainly in AC Polarography [1-3], and have also been applied to corrosion studies [4-6]. In the potential-step current transient method, a small step in potential is superimposed over a fixed voltage condition and the current-transient response is captured with high speed data acquisition methods. This current-transient response can then be converted using Transfer Function Analysis [7-10]. Specifically, numerical integration techniques can be used to obtain the frequency-dependent admittance and impedance of the system [2,6,11,12].

However, the analytical tools available to study time-based electrochemical responses to potential step inputs are somewhat limited particularly when it comes to heterogeneous and dispersed responses of an interface. Simplified electrical analog models like the Randles circuit
or the defected coating surface models which are based on idealized circuit elements are amenable to time-domain analysis. However, difficulties arise when distributed relaxation processes are present that result in stretched decay of the current transient. Two generic types of distributed response can be identified in an electrolytic cell, which requires use of distributed elements in the equivalent circuit employed to fit EIS data. The first type, results from the finite extent of the system. The second type of distributed response, however, is quite different and is caused by the inhomogeneities of the system. To deal with the distributed response of electrochemical systems, equivalent circuits are modified to include Distributed Circuit Elements (DCE) [13] or transmission line models [14,15].

The infinite Warburg impedance was the first distributed element introduced into electrochemistry, which is mathematically analogous to a semiinfinite distributed RC transmission line. Franceschetti and MacDonald published a series of papers on different manifestations of Warburg element under different electrochemical conditions [16,17]. Later on, other distributed circuit elements (DCE) were introduced, which usually have a RC transmission line model analog. To account for the distributed response of porous and rough electrodes, for example, de Levie developed transmission line models for the impedance response [18]. In the frequency domain, the Cole-Cole (or Constant Phase Element (CPE) Randles) model (Fig. 2.1a) is often used to describe the distributed impedance behavior especially in passive-oxide film covered systems. This model, however, has no direct time-domain analog amenable to time-domain analysis.
Moreover, the CPE response \((i\omega t)^{\pm\alpha}\) where \(0 \leq \alpha \leq 1\) is not always constant over all frequencies. Therefore, other DCEs have been introduced to model the behavior over a limited frequency range.

One empirical DCE of the above type is Havriliak-Negami (HN) response [19]:

\[
Z_{HN}(\omega) = \frac{R_{HN}}{[1 + (i\omega \tau)^{\alpha}]^{\beta}}
\]

where \(0 \leq \alpha \leq 1\) and \(0 \leq \beta \leq 1\).

It reduces to Cole-Davidson [20] response when \(\alpha = 1\), and to Cole-Cole response [21] when \(\beta = 1\). The latter shows a symmetric arc in a complex-plane plot and the former an asymmetric arc, which both appear in practice. Another important DCE is that of Kohlrausch-Williams-Watts (KWW) response [22] in the time domain, which yields a stretched exponential proportional to \(\exp\left(\frac{t}{\tau}\right)^n\), with \(0 \leq n \leq 1\).

The KWW stretched exponential have been used in several fields. Description of polymer relaxation behavior is one example, in which the KWW fits the data very well [23,24].

The KWW model was adapted by Gettens and Gilbert to perform current transient-based voltage-dependent impedance analysis of 316L Stainless Steel in fibrinogen containing solution [25]. The time-domain solution to a step in voltage for the Randles circuit (Fig. 2.1a) was modified to include stretched exponentials which provided an ability to deal with the dispersed relaxation processes present.
\[ I(t) = \frac{\Delta V}{R_s} e^{\frac{t}{\tau}} + \frac{\Delta V}{R_s + R_p} \left( 1 - e^{\frac{t}{\tau}} \right) + I_b, \quad \text{where} \quad \tau = \frac{R_s R_p C}{R_s + R_p} \]  

\( I(t) \) is the current transient, \( I_b \) is the baseline current before the step, \( t \) is time, \( \Delta V \) is the voltage step, \( R_s \) solution resistance, \( R_p \) polarization resistance, \( \tau \) the time constant which includes the capacitance of the interface, and \( n \) is an exponent used to represent the dispersion of relaxation times. Birbilis et al. also used a similar modified KWW function in a galvanostatic study of corrosion in concrete-steel systems [26,27].

This approach, while reasonable for electrode systems that are close to Randles-like behavior, has been observed to have difficulty in fitting the observed current-transient decay over the entire time domain for some oxide film-covered alloys. Therefore, alternative time-domain functions need to be considered to better describe and model time-domain transient behavior.

The goals of this chapter are to explore two non-ideal time-transient impedance analysis methods and apply these techniques over a range of voltages (-1000 to +600 mV(Ag/AgCl)) to the behavior of CoCrMo alloy in a phosphate-buffered saline solution that simulates the physiological environment. In particular, a non-ideal time-transient function, the Generalized Cauchy-Lorentz (GCL) function, will be introduced and its dispersion parameter (which is a measure of the non-ideality of the surface) will be compared with the KWW-Randles model published recently [25] over the voltage range along with the impedance parameters.
2.2 Materials and Methods

2.2.1 Sample preparation

CoCrMo (ASTM F-1537) alloy with the composition in wt% listed in Table 2.1 was used to perform the step polarization test. The sample was mechanically mirror polished to 0.05 \( \mu \text{m} \) alumina finish and rinsed with water and ethanol. It was then immersed in Phosphate Buffered Saline (PBS, \( \text{pH}=7.4 \), 0.01 M, Sigma P3813) for 24 hours.

Table 2.1: Composition (wt\%) of low carbon wrought Co28Cr6Mo F-1537 alloy.

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</table>

2.2.2 Electrochemical setup and measurements

The electrochemical setup consisted of a standard three electrode system with an Ag/AgCl reference electrode, and a carbon rod as the counter electrode. All potentials are with reference to Ag/AgCl (4M KCl Orion 900011 Filling Solution, Thermo). A computer program written in LabWindows 2.0 and an A/D board (AT-MIO-16F-5, National Instruments, Inc.) were used to make steps in voltage and record the subsequent current transient response via a model AFRDE4 Bi-potentiostat (Pine Instruments, Inc.) The CoCrMo samples were mounted in a custom EC cell as the working electrode, initially held at -1000 mV(Ag/AgCl) for 10 min, and 50 mV potential steps were applied to the sample every 30 s. Having transformed the time-domain current transients into frequency-domain impedance data [28], the linearity of the system was confirmed by employing Kramers-Kronig transforms [29] and comparison of the response to voltage steps of 10-50 mV in 10 mV increments. The data acquisition started before the voltage step was applied and continued for 30 s after the step (a nominal scan rate of 1.6 mV/s). Current transients
were measured at a sampling rate of 20,000 samples per second, and the process was stopped at +600 mV(Ag/AgCl). The resulting current transient files were then used for fitting of KWW-Randles (Eq. 1) and GCL functions (Eq. 6 see below). Values of $R_s$, $R_p$, and $C$ were determined from the data as a function of voltage and the dispersion coefficient for each model were found through the best fit. The fitting algorithm used in all cases was the non-linear Gauss-Newton algorithm implemented in R statistical language [30]. Two polarization plots were obtained. One by plotting the baseline currents before each step vs. the voltage and the other one from a conventional potentiodynamic test with a scan rate of 1.6 mV/s.

The residual errors of fitting for each function were determined over the voltage range and Student’s t-test was used to determine statistically significant better fits obtained with GCL vs. KWW-Randles. Values of $R_p$, $R_s$, and $C$ related to the oxide were calculated in a similar way described by Gettens et. al [25].

The oxide thickness at each potential was estimated according to [31]:

$$d = \frac{\varepsilon \varepsilon_0}{C_{ox}}$$  \hspace{1cm} (2)

where, $d$ is the thickness of the oxide, $\varepsilon$ the relative permittivity, $\varepsilon_0$ the permittivity of the vacuum (8.854 x $10^{-14}$ F/cm), and $C_{ox}$ the oxide capacitance from Eq. (1). Based on the previously reported values for the oxide film of CoCr alloys, the permittivity was assumed to be 12 [32].

Electrochemical tests of CoCrMo were repeated three times. Best fit time-domain functions were compared to the raw decay data to determine which was best able to fit the decay. Fitting residuals were used to assess the relative best fits and were reported over the voltage range.
2.3 Theory

2.3.1 The Generalized Cauchy-Lorentz Time-Domain Relaxation Function

Presented here is a theoretical development of the generalized Cauchy-Lorentz (GCL) function. The GCL function arises from an analysis of the discharging of a capacitor through what is defined here as a Tafel element (Fig. 2.1b). A Tafel element is defined as an electrical circuit element whose current-voltage characteristic is given by Eq. 3 below

\[ i_t = i_0 e^{-\frac{V_t}{b}} - i_0 \]  

(3)

Here, the Tafel current is defined as the current, above the exchange current, that arises when the Tafel voltage (or overpotential, \( V_t \)) exceeds zero. The second term allows \( i_t \) to be zero when \( V_t \) is zero.

Fig. 2.1: a) Diagram of Randles circuit, where \( R_s \) is the solution resistance, \( R_p \) the polarization resistance and \( C \) the capacitance of the oxide layer or \( Q \), the impedance of CPE. \( C \) is replaced by CPE for non-ideal Randles behavior. b) Tafel-capacitor circuit for finding the GCL function. The Tafel element acts like a non-linear resistor.

The proposed model consists of a capacitor and a Tafel element where the capacitor is fully charged at \( t = 0 \) (Fig. 2.1b). The discharging of the capacitor through the Tafel element obeys Kirchoff’s laws. Noting that the voltages of the capacitor and the Tafel element are equal and opposite yields:
\[ V_T = b \ln \left( \frac{i + i_o}{i_o} \right) \]

\[ V_C = \frac{1}{C} \int_0^t i \, dt + V_{\text{max}} \]

Equating these voltages \((V_T = -V_C)\) and differentiating with respect to time yields

\[ \frac{i}{C} = -b \frac{d}{dt} [\ln i - \ln i_o] \]

\[ = \frac{1}{bC} \frac{d}{dt} [\ln(i)] = \frac{di}{i^2} \]

Integrating both sides, applying the initial condition, defining \(i_{\text{max}} = i_o e^{b \cdot \ln i_{\text{max}}}\), and solving for \(i(t)\) yields:

\[ i = \frac{1}{t} + \frac{1}{bC} e^{b \cdot \ln i_{\text{max}}} \]

which can be rearranged to be in the form:

\[ i(t) = \frac{i_{\text{max}}}{1 + \frac{t}{\tau}} \quad (4) \]

where \(i_{\text{max}}\) is the initial current value and \(\tau\) is the “time constant” i.e. \(\tau = t\) when \(i(t)\) falls to \(\frac{i_{\text{max}}}{2}\)

\[ \tau = \frac{bC}{i_o} e^{b \cdot \ln i_{\text{max}}} \quad (5) \]

Eq. 4 shows a solution for the current transient response which is close to the form of a generalized Cauchy Lorentz function which is:

\[ \varphi(t) = \frac{\varphi_0}{1 + \left( \frac{t}{\tau} \right)^k} \quad (6) \]
where $\varphi_0$ is the transient response at $t = 0$, $\tau$ is the time constant of the decay and $k$ is the parameter which describes the stretched nature of the transient response. The Cauchy-Lorentz distribution is the case where the exponent $k$ is 2, whereas the Tafel-discharging Eq. has $k = 1$. Eq. 6 is an even function about $t = 0$ with a bell-shaped distribution curve often used in spectral fitting algorithms. We have generalized this function to allow for the exponent, $k$, to take on values different than 2 where $k > 0$. When the Eq. 6 is plotted versus the log of time (for $t > 0$), a transient decay function results that is similar in appearance to electrochemical current transient functions that arise from steps in voltage. When the value of $k$ is less than 2, the decay becomes stretched and as $k$ increases above 2 the transient response becomes more step-like. Thus, Eq. 6 is an attractive function for fitting time-transient impedance responses and operates in a very similar way to the KWW stretched exponential function but is, as will be shown, a better fit than the KWW-Randles equation to the raw data.

This is not to say that the capacitive discharge process used to develop this form of the equation is the one ongoing in typical impedance experiments, however, the above analysis does give a rationale and basis for adopting this type of function for electrochemical impedance analyses.

To have the Eq. 6 fit the parameters of a typical electrode interface such that at early time the impedance converges on the solution resistance ($R_s$) and at long times it converges on the sum of the solution and interfacial resistance ($R_s + R_p$) as should be the case for a typical impedance test, one can replace the exponentials in the Randles equation with Eq. 6:
\[ A(t) = \frac{I(t) - I_b}{\Delta V} = \frac{1}{R_s \left[ 1 + \left( \frac{t}{\tau} \right)^{\frac{1}{k}} \right]} + \frac{1}{R_s + R_p \left[ 1 - \frac{1}{1 + \left( \frac{t}{\tau} \right)^{\frac{1}{k}}} \right]} \]  

(7)

When \( R_s \ll R_p \), the above equation can be simplified to give:

\[ A(t) = \frac{1}{R_s \left[ 1 + \left( \frac{t}{\tau} \right)^{\frac{1}{k}} \right]} + \frac{1}{R_p} \]  

(8)

Where \( A(t) \) is the time-based admittance. The rest of the parameters are the same as defined in Eq. 1. The dispersion exponent, however, is \( k \). These functions can be applied to time-transient current data by adjusting \( R_s, R_p, \tau, \) and \( k \) to reach a best fit condition of the experimental data.

Then, the function itself can be numerically transformed to the frequency domain to show the frequency-based impedance behavior of the GCL system.

Two time-based current transient functions (Eq. 1 and Eq. 7) are now available for analysis of the impedance behavior of electrode interfaces. Each equation has a dispersion parameter \( (n, k) \) that reflects the non-ideal and/or heterogeneous nature of the interface. One question that arises relates to the correlation between these parameters. Do these parameters have a relationship between them? In this paper the values of \( n \) and \( k \) can be determined from fitting the current transient responses of CoCrMo held at different voltages where the dispersion behavior varies.

Therefore, the goals of this study are to explore these two different time-based analysis functions of the dispersed current-transient response and apply them to study the voltage-dependent impedance of CoCrMo biomedical alloy in a physiologically representative solution.
2.4 Results

An example of one current-transient response to a potential step is shown in Fig. 2.2a as a log-log plot in the time domain. These data correspond to a step in voltage from -350 to -300 mV(Ag/AgCl). A typical current response shows a peak ($I_0$) and a very fast decay with time. Also shown in Fig. 2.2a are the best fit curves of KWW-Randles (Eq. 1), and GCL (Eq. 7), which gives $n = 0.83$, $k = 1.37$. It is clear based on assessment of residuals of fitting that Eq. 7 does a better job of fitting the raw data than Randles-KWW (Fig. 2.2b). Representative plots of KWW-Randles and GCL functions for similar $R_s$, $R_p$, $\tau$ and varying $n$, $k$ are shown in Fig. 2.2c.

The measured solution resistance ($R_s$) was constant over voltage at about 103 ± 3.6 $\Omega$ cm$^2$.

Polarization resistance of CoCrMo in PBS ($R_p$) versus baseline voltage is shown in Fig. 2.3a. Polarization resistance, which is a measure of the oxide film resistance, shows variation over the voltage range that reflects the changing nature of the oxide film. $R_p$ starts relatively low, 4197 ± 4124 $\Omega$ cm$^2$ at -1000 mV(Ag/AgCl), rises to a plateau of around 9 k$\Omega$ cm$^2$ between -700 mV(Ag/AgCl) and -500 mV(Ag/AgCl), then increases to a peak of 27770 ± 11530 $\Omega$ cm$^2$ around +250 mV(Ag/AgCl). Beyond +250 mV(Ag/AgCl), $R_p$ experiences a sharp fall which is likely due to what we call as the pre-transpassive changes of the surface oxide layer. This term is used to distinguish between the transpassive potential which occurs well above this voltage at around +500 mV(Ag/AgCl).

The interfacial capacitance ($C$) from Eq. 5, which can be rearranged as $C = \frac{i_0}{b} e^{\frac{\nu_{max}}{b}} \tau$, where the constant ($\frac{i_0}{b} e^{\frac{\nu_{max}}{b}}$) in correspondence with KWW-Randles was assumed to be equal to $R_s^{-1}$, was
Fig. 2.2: a) Plots of log of the current density versus log time for the raw data, the best fit KWW-Randles function and the new proposed model (Eq. 7) vs. time on a log-log scale. b) Residuals of fitting across the entire voltage range. c) Schematic plots of KWW-Randles and GCL functions for $R_s = 30 \Omega$ cm$^2$, $R_p = 30$ k$\Omega$ cm$^2$ and $\tau = 0.005$ s. Different exponents for each function are plotted showing the increased dispersion over time.
plotted versus voltage (Fig. 2.3b). The capacitance of the interface has an inverse behavior to $R_p$ and is also due, in part, to the oxide. It shows a complicated behavior with voltage initially being constant from -1000 to -750 mV(Ag/AgCl) at $28 \pm 0.9\ \mu\text{F/cm}^2$. It then falls off steadily as the voltage becomes more positive, decreasing to a minimum at about +250 mV(Ag/AgCl) and then rises rapidly showing a peak at +550 mV(Ag/AgCl) (Fig. 2.3b).

Fig. 2.3: Plots of a) dispersion coefficients ($n$, and $k$) b) polarization resistance c) capacitance d) oxide thickness vs. scanned voltage range.
The dispersion parameters, n and k, over the examined voltage range are shown in Fig. 2.3c. Both dispersion parameters show a relatively constant value over the voltage range of -1000 mV(Ag/AgCl) to +100 mV(Ag/AgCl). Above +100 mV(Ag/AgCl), n and k decrease with increasing voltage showing an increased heterogeneity of the response as pre-transpassive oxide behavior begins.

Fig. 2.3d shows the calculated change of oxide thickness vs. voltage based on capacitance values derived from both Randles-KWW and GCL models using Eq. 2. Both graphs show the same pattern, which consists of three main regions. The oxide has a relatively low constant thickness up to -500 mV(Ag/AgCl) and then it starts to increase in a linear fashion until at around +250 mV(Ag/AgCl) it starts decreasing steadily.

Fig. 2.4: Polarization plots from the step polarization test, where the currents are the baseline currents measured before each voltage step and the conventional potentiodynamic test at 1.6 mV/s.
The polarization plots were obtained by plotting the voltage vs. the baseline current just before
the step. The baseline current plots appear similar to the typical polarization plots obtained for
this alloy in PBS at scan rates on the order of 1.6 mV/s. (Fig. 2.4)

2.5 Discussion

This paper has investigated the use of time-based potential-step current transient methods to
measure the voltage-dependent impedance of CoCrMo in physiological saline solution, PBS.
This alloy’s corrosion behavior is governed by the passive oxide film present on its surface. The
voltage dependant impedance behavior, therefore, is descriptive of this oxide and its structure
and chemistry as the voltage of the interface changes. The results show that there are several
voltage regions where different surface oxide behavior is observed that can roughly be correlated
with the polarization plot (Fig. 2.4) concurrently obtained during testing. In particular, the
variation of $R_p$, $C$ and dispersion coefficients with voltage reflect on the underlying oxide
character.

At voltages more negative than about -700 mV(Ag/AgCl), the surface impedance is low,
capacitance is high and the dispersion is high (low coefficient). This indicates that the oxide, if
present, is thin with low overall resistivity and lower variability. Starting at about -500
mV(Ag/AgCl), the resistance increases and the capacitance decreases indicating the growth of
oxide film. This is consistent with earlier ECAFM studies [33] and electrochemical scratch tests
of CoCrMo alloy [34] which showed a distinct onset of both oxide film domes and
electrochemical response to mechanical abrasion of the surface indicating oxide film onset. The
oxide resistance rises to a peak at about +250 mV(Ag/AgCl) and then falls above this potential and the capacitance follows an inverse trend to $R_p$. Again, these observations are consistent with ECAFM studies which show that the oxide film on CoCrMo undergoes a sudden and distinct change in surface morphology at about +250 mV(Ag/AgCl) with the oxide domes becoming very flat and smooth as the oxide rearranged itself [33]. This transition from platy, dome-like oxide morphology to flat smooth surface morphology was sudden and distinct at this potential. This change of morphology may accompany a change in the chemistry of the oxide layer as well. The surface oxide of CoCrMo alloys is mainly (90 %) composed of Cr$_2$O$_3$ [35-37]. However, chromium oxide is known to be highly non-stoichiometric and it can form intermediate compounds with varying oxidation states at different potentials. Metikos et al., have described the formation of a CrO$_2$ intermediate compound at +200 mV(SCE) on Cr$_2$O$_3$ electrodes [38]. They observed abrupt changes in capacitance and oxide resistance starting at this voltage and correlated them to phase transitions in the film during anodic polarization. The formation of this compound requires oxidation from Cr$^{+3}$ to Cr$^{+4}$ and is consistent with the increase in current and decrease of $R_p$ at this voltage.

This potential is well below the reported transpassive potential for CoCrMo which is between +450 and +550 mV(Ag/AgCl) and indicates changes to the oxide film precede the typically reported transpassive potential by several hundred millivolts. Thus, these results with voltage show clear and distinct voltage regions of oxide behavior and indicate that if, for example, surface mechanical abrasion occurs and the voltage consequently drops, the impedance of this surface will be reduced and will allow greater possibility of charge transport across the interface. It should be noted that the magnitude of $R_p$ found is lower than typically measured with
frequency-based methods. This is due to the relatively high nominal scan rate used (1.6 mV/s).

Reports of severe in-vivo corrosion of CoCrMo including intergranular corrosion and pitting attack as a result of fretting-crevice corrosion show that changes in surface oxide impedance can lead to deleterious processes in-vivo [39-41].

Changes in composition of the oxide layer at different voltages were not directly studied in this work. There are previous studies, however, which investigated the change of the oxide composition in different physiological solutions [36,42] and at different voltages [35,37]. The results from our study correlate well with the changes in chemistry of the oxide that have been mentioned in these studies. The compositional changes occurring at above +250 mV(SCE) are consistent with the increase in current, drop of polarization resistance and dispersion factors that we observed in our study.

It should be pointed out that the voltage-time history of the oxide film surface will influence the structure, chemistry and properties of the oxide film. That is, electrochemical history (the prior voltage-time path) will affect the oxide film character at a particular voltage. In this study, the voltage started at -1000 mV(Ag/AgCl) and was stepped anodically 50 mV every 30 s. The dwell time, the step size and the prior voltages will all have some effect on the detailed behavior. However, there are also clear voltages where transitions occur in behavior that are not as sensitive to prior electrochemical history including the -500 mV(Ag/AgCl) onset of increasing $R_p$ (and decreasing $C$) and the +200 mV(Ag/AgCl) peak $R_p$ voltage. These voltage regions appear to be characteristic of the oxide film that forms. If, however, one were to start at +800 mV(Ag/AgCl) (above the transpassive potential) and step cathodically 50 mV the resultant $R_p$
and $C$ will have different values and the peak voltages will be shifted about 100 to 200 mV cathodic due to the fact that the oxide has been sitting in the transpassive region where the oxide film will have a significantly different chemistry, thickness and electronic properties (Data not shown).

As for the thickness of the oxide layer, an estimate could be made using the values of capacitances obtained at different voltages using Eq. 2. The value of $\varepsilon$ ranges from 9.2 to 13.3 for Cr$_2$O$_3$ and from 12.9 to 13.4 for CoO [43, 44]. The value of $\varepsilon$ depends on the oxide composition, which changes at different voltages. Since the oxide layer is mainly comprised of Cr$_2$O$_3$ over almost the entire voltage range under study [35-37], the average (~12) of the permittivity range for Cr$_2$O$_3$ was chosen to calculate the oxide thickness at different potentials. This value is also close to the permittivity values reported for CoO, so the changes in permittivity associated with entering Co into the oxide layer at voltages above +250 mV(SCE) should not shift this value significantly. B.G. Pound in a similar calculation of oxide thickness of MP35N alloy at different voltages [32] assumed an oxide permittivity of 12.

The derived values of oxide capacitance are in the order of $10^{-5} - 10^{-4}$ F/cm$^2$, which is in the same range of capacitance values reported by Gettens et al. for stainless steel 316 L alloy [25]. This makes sense as the oxide on 316 L SS alloy is also predominantly composed of Cr$_2$O$_3$. The values of oxide thickness derived from Eq. 2 over the voltage range studied in this work show a linear increase in thickness starting at around -500 mV(Ag/AgCl) up to 0 V(Ag/AgCl) (Fig. 2.3d). Oxide thickness then stays constant before it starts to decrease sharply at around +250 mV(Ag/AgCl). The thickness values estimated are close to the values reported for MP35N alloy.
and the thicknesses measured in another work on F-1537 alloy up to +250 mV(Ag/Ag/Cl) [35]. However, our data show a decrease in oxide thickness in the anodic region above +250 mV(Ag/AgCl), whereas other works have reported an ever increasing thickness at these voltages. [32,35,37] This discrepancy could be due to an increase of permittivity, which was assumed to be constant, but indeed is a function of composition and as such will change at different voltages or, as suggested by a similar drop in $R_p$, is due to the early stages of oxide film breakdown starting at +250 mV(Ag/AgCl). The capacitance data of Metikos et al. and Carmezim et al. also show an increase in capacitance values for chromium oxide films in the anodic region above +200 mV(SCE), which corresponds to a thinning of the oxide at these potentials. [38,45]

Finding a simple model which precisely describes the behavior of the current transients proves to be a real challenge. It was previously shown that the solution of Randles circuit only partially matches the current response of the step polarization tests [25]. While fitting the experimental data to the KWW-Randles model provides a better fit, it does not match the data very well at early and late times of the decay (Fig. 2.2a). In particular, towards the end of the current decay in the time range between 0.1 and 0.01 s, the raw data show an obvious departure from the empirical KWW-Randles model. It was observed in most of the cases that the proposed new function (Eq. 7) does a better job in approximating the initial and late-time current transient data. The comparison of these two models using Student’s t-test shows a statistically significant difference ($p$-value < 0.01) between pooled residuals of fittings.

While the capacitive discharge model used to derive the GCL function may not be the mechanism of current transience in these experiments, it does provide a theoretical basis for the
existence of such a function to describe an electrochemical impedance process and provides reasonable justification for its application to these experimental results. The GCL function can be used to find the numerically Laplace-transformed frequency-based impedance of the interface using previously published technique [28].

All impedance spectroscopy methods (even the standard 10 mV sinusoidal input) result in a finite change in the oxide-film on metal surfaces. That is, since oxide films grow and/or shrink, and change chemistry and properties with voltage, any (even small) change in voltage will have a corresponding change in the oxide one is trying to measure. The importance, therefore, in assessing the size of the input stimulus is to assess how much change in oxide behavior (and structure) results from the voltage step in relation to the extant properties (and structure). If the amount that the properties change in the step taken is small compared to the overall measured values, then the size of the step will not have a significant effect on the results and the system will behave linearly [46]. However, if the voltage step results in property changes that are on the order of the property to be measured, then the voltage step, whatever the size, is too large to determine the properties of interest. Thus, the linearity of the system may be affected by the size of the step used in this system.

To determine if the system was linear with the 50 mV step used in these tests, two methods were utilized. First, multiple potential steps were taken from the same starting voltage (-100 mV(Ag/AgCl)) with amplitudes of 10 to 50 mV in 10 mV increments and the response was compared. No differences in $R_s$, $C$ or $R_p$ were observed. Second, the 50 mV step current transients were fit with the GCL equation, numerically transformed to the frequency domain [28].
and subject to a numerical Kramers-Kronig Transformation to transform $Z'$ to $Z''$, and $Z''$ to $Z'$ to determine if one could obtain the real impedance from the imaginary and vice-versa. The resulting transformed data very closely matched the original impedance data when both transformed and starting impedances were compared indicating that the system and function are indeed behaving as a linear system with this voltage step. Finally, it should be noted that Carmezim et al. also utilized 50 mV potential steps in their electrochemical work [45].

2.6 Conclusion

Potential step current transient impedance spectroscopy methods were developed and used to assess the voltage-dependent impedance of CoCrMo surfaces. CoCrMo surface oxides have a voltage-dependent impedance behavior that reflects the properties of the passive oxide film. The oxide film had several voltage ranges where different behavior was observed that may reflect changes in the thickness and chemistry of the oxide.

Several time-based analysis methods were presented including a new “generalized Cauchy-Lorentz” function that provides an ability to better fit current transient data and to calculate impedance values from these data. The GCL function was derived using a Tafel element and capacitive discharge behavior, but is useful as a current transient function for impedance behavior.

Since the degree of non-ideality of the exponential decay process could be indicative of several surface phenomena, the dispersion factors could serve as characterization parameters of the interface.
2.7 References


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3. Electrochemical control of cell death: reduction-induced intrinsic apoptosis and oxidation-induced necrosis on CoCrMo alloy in vitro

3.1 Introduction

The effect of electrochemical processes on cells on or adjacent to metallic surfaces is an important area of research, which falls under the general category of electrical stimulation of cells. These studies are mainly focused on either the electrical field effects on cells or the electrochemical processes happening at the cell/electrode interface. The electric effects on cells can be categorized into dielectric, electrophoretic, electrolytic, and cell/electrode interactive effects [1]. Electroporation and electrofusion of cells are well studied examples of the dielectric effect, in which a high voltage pulse of stimulation leads to dielectric breakdown of the membrane and formation of pores or fusion of the cellular membranes [2,3]. Electrophoretic effect, however, refers to the migration of cells in a solution down a potential gradient between two electrodes. On the other hand, electrochemical effects arise due to redox reactions of electroactive components of the solution at the electrode surface or the constituents of the cell. Irreversible electrochemical reactions of physiologically important substances on the cell membrane or electrochemical formation of active species such as reactive oxygen species (ROS) in the cell culture environment may lead to cell death (electrolytic effect). However, this is not the only outcome of applying voltage to cells cultured on electrodes. In fact, a proper electrode potential could create cell-electrode interactive effects that modulate cell viability [4], cellular proliferation [5-7], gene expression [7], protein production [8-10], morphology [11], and cytoskeletal organization [5,7,12] (cell/electrode interactive effects).
The study of voltage effects on cells has primarily been done on transparent indium tin oxide (ITO) electrodes using different cell types in potentiostatic and galvanostatic modes at anodic and cathodic potentials [4-16].

It is known that metallic implants undergoing mechanically assisted corrosion reactions [17], can experience large shifts in the voltage of the surface in the negative direction. Voltages as negative as -1000 mV have been measured when surfaces of Ti-alloys and CoCrMo alloys have their passive oxide films abraded [18, 19]. Highly oxidizing environments, on the other hand, where hydrogen peroxide is present, as may be the case in highly inflammatory sites in the body, can result in more positive voltages being attained [20]. What role these voltage shifts can have on cell viability have rarely been studied [4, 21, 22]. Additionally, the possibility of using impressed voltages on metallic implant surfaces to modulate and control the biological interactions is an area that has received very little attention. Impressed anodic (positive) voltages typically result in increased oxidation reactions and increased ion release from metallic biomaterials, while impressed cathodic (negative) voltages result in increased reduction reactions. What molecules or species undergo reduction depends on what is present at the implant surface and what voltage ranges are achieved at the surface.

Very little is known about the range of biologically-based electrochemical processes that may be influenced by the proximity of a metallic biomaterial. Cellular processes and mechanisms that may be affected by proximity to a metal surface engaged in redox processes are numerous and central to many cellular activities including adhesion [23], membrane transport [24], actin cytoskeletal stability [25], mitochondrial activity [26], and a host of other cellular redox processes. Additionally, protein structure and stability may be sensitive to redox reactions including reduction of disulfide bonds [27]. Therefore, a better understanding of how surface
voltage of metallic biomedical implants, and the associated electrochemical reactions present, could influence cellular behavior is essential before any attempt is made to design new smart biomedical devices utilizing voltage effects or development of voltage-assisted therapies. In this work, we have studied the effect of impressed cathodic and anodic voltages on the response of cells cultured on the surface of a CoCrMo Alloy. Cellular responses studied include morphology, viability, mode of cell death, and organization of actin cytoskeleton and adhesion complexes. Furthermore, the electrochemical currents and metallic ion levels were measured and related to the observed response. The hypotheses evaluated are that there is a voltage range of viability for CoCrMo alloy, outside of which there are different modes of cell death. In addition, voltage outside of the viable range will affect the adhesion, morphology and cytoskeleton of MC3T3-E1 cells in vitro.

3.2 Materials and Methods

3.2.1 Sample preparation

Disks of high carbon wrought Co28Cr6Mo F-1537 alloy with a diameter of 2 cm were mechanically polished up to 600 grit. The samples were then rinsed with deionised water (DI), cleaned in DI water in an ultrasonic bath for 10 minutes, and autoclaved.

3.2.2 Cell culture and application of voltage

MC3T3-E1 pre-osteoblast cells (ATCC #: CLR-2593) (p6-p25) were cultured in T-75 culture flasks to near confluence inside an incubator (37 °C, 6% CO₂). Cell culture medium was
composed of 90% Alpha Modification of Eagle’s Medium (AMEM, Cellgro) supplemented with 10% (v/v) fetal bovine serum (FBS,Gibco). Metal disks were mounted in custom-made electrochemical chambers [4] and cells were removed from culture flasks for seeding on metal disks using trypsin (Gibco). Culture medium (200 μL) containing 10^4 cells was pipetted on each metal disk and was allowed to settle for 10 min. Additional culture media was added to each chamber to total volume of 20 mL. Chambers were then placed in an incubator (37 °C, 6% CO₂), and metal disks were potentiostatically polarized to -1000, -700, -500, -400, -300, -100, +200, +300, and +500 mV(Ag/AgCl) for 24 h. A standard three-electrode system was used; metal disk was the working electrode, a carbon rod the counter and Ag/AgCl wire the reference electrode. The electrical current of each chamber was measured for 24 hours in 60 s intervals using a NI9004 A/D board (National Instruments) and Labview software and average current densities over 24 hours were calculated. The total amount of electrical charge transferred through the interface was calculated by integrating current density over the duration of voltage application (24h): \( \int_{0}^{24h} I(t)dt \).

Control samples were cell-seeded CoCrMo disks left at OCP (i.e. no voltage was applied) for 24 h. The OCP is time dependant starting from around -400 mV initially and drifts to -200 mV after 24 h. Three independent samples for each voltage were used.

### 3.2.3 Cell viability assay and immunostaining

To assess cell viability on the voltage-treated and control samples, LIVE/DEAD\(^\text{®}\) Viability/Cytotoxicity Kit for mammalian cells (SKU# L-3224, Invitrogen) was used according to the manufacturer’s instructions. Briefly, cells were washed with PBS post treatment and 150
μL of Calcein AM (live probe, 1:5000 conc.) and Ethidium homodimer-1 (dead probe, 1:500 conc.) solution in PBS was pipetted on the samples and incubated for 20 min at room temperature.

To visualize the actin cytoskeleton, adhesion complexes, and nuclei of cells, control and voltage-treated samples at selected cathodic and anodic voltages outside the viability range (-700 and +500 mV) and on the edges of the viability range (-400 and +300 mV) were stained after 24 hours. First, cells were fixed with 4% paraformaldehyde in 4% sucrose containing PBS for 20 min followed by permeabilization with 0.2% Triton X-100 for 5 min, and were blocked with 0.2% gelatin in PBS for 1 h. F-actin and nucleus were detected in fixed and permeabilized cells with 0.5 μM rhodamine/phalloidin (rho/pha) (Sigma-Aldrich, MO) and 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI) respectively.

To visualize adhesion complexes, immunostaining of vinculin was performed with a mouse monoclonal antibody directed against vinculin (clone hVIN-1, Sigma-Aldrich, MO) at room temperature for 1 hr (1:200) followed by incubation with a secondary goat antimouse IgG labeled with Alexa-Fluor 488 antibody (1:300, Molecular Probes) for 45 min. Similarly, in order to determine the mode of cell death (intrinsic or extrinsic apoptosis), control and voltage-treated samples at selected cathodic and anodic voltages of -500 and +500 mV(Ag/AgCl) after 24 hours were immunostained. Rabbit polyclonal antibodies directed against cleaved caspase-3 (Asp175, Cell signaling #9661) and cleaved caspase-9 (Asp353, Cell signaling #9509) were used to visualize the expression levels of caspase proteins.
3.2.4 Imaging and image analysis

Metal disks were mounted on glass slides and analyzed by fluorescence microscopy. For cell viability analysis, cells were imaged with an inverted microscope (Axiovert 40CFL, Zeiss, Denmark) equipped with a CCD mono-12 bit camera (Q-imaging, Canada) and an X-Cite 120 light source (EXFO America, TX). FITC and Texas red dye filter set were used for live and dead cells respectively.

For immunostained samples, images were acquired using a Zeiss Axioplan2 microscope (Carl Zeiss, Inc., NY) equipped with a Hamamatsu Orca II C4742-98 camera, an X-Cite 120 light source, and filter sets for FITC, TRITC and DAPI fluorescence. MetaMorph imaging software (Universal Imaging Corp., PA) was used to control illumination shutters, camera exposure and image acquisition.

The morphology of cells after 24-hour polarization at -1000, -700, -400, -100, +200, +500 mV(Ag/AgCl), and at OCP were investigated using a scanning electron microscope (JEOL 5600). Cells were fixed for 20 min after voltage treatment using 4% paraformaldehyde, dehydrated using alcohol gradients (50, 75, 90 and 100 %) in phosphate buffered saline (PBS, Gibco, pH = 7.4), gold sputtered with a coater and examined in the SEM.

ImageJ software (NIH) was used to quantify morphological characteristics of cells from scanning electron and fluorescence microscopy images. In viability measurements, fluorescent images from samples stained for Calcein AM and EthD-1 were used and a total number of at least 100 cells from 5 different regions of the disks, picked randomly, were counted to determine the percentage of live to dead cells. In analysis of immunostained images, between 20-50 cells were
picked at random and analyzed for nucleus area, total adhesion complex area and count, and caspase expression levels. In order to quantify the adhesion complex size and number, images were converted to 8-bit gray-scale images, inverted, and background subtracted using the sliding paraboloid method. Fast Fourier Transform (FFT) of the images were performed afterwards with subsequent band pass filtering to filter structures smaller than 3 pixels and larger than 10 pixels, and an inverse Fourier transform. Finally, the images were thresholded to highlight the adhesion complexes as particles with a circularity range between 0.0-0.4. All the other parameters were accordingly adjusted to make sure that the adhesion complexes at the periphery of the cells were outlined upon visual inspection. The total area of the outlined adhesion complexes was calculated for single cells and averaged over the total number of cells analyzed. Line scan analysis and fluorescence intensity measurements along one-pixel wide lines across the edges of single cells were performed to measure caspase expression levels as previously outlined [28].

3.2.5 Metal ion concentration analysis

The culture media of voltage-treated samples were collected at the end of each experiment and the level of metallic ions released at different voltages was measured using an inductively coupled plasma mass spectrometer (ICP-MS, Perkin Elmers Elan DRC-e unit). The samples were diluted fivefold with 2% OPTIMA grade nitric acid. Standards from 0.5 to 10 ppb were prepared in the same matrix (culture medium). Results were reported for the following masses: Cr 50, Co 59, Ni 58, Mo 98 (n = 9).
3.2.6 Statistical analysis

Results are presented as means ± standard deviation (SD). One-way ANOVA on ranks (Kruskal-Wallis test) and Dunn’s pair-wise comparison method were performed (SigmaPlot v. 11) to compare the controls and voltage-treated groups with P-value of 0.05 indicating significance.

3.3 Results

3.3.1 Cell morphology and viability

Cathodic and anodic voltages, depending on their magnitude affected cellular morphology. Fig. 3.1 and 3.2 show the SEM images of cells cultured on CoCrMo alloy polarized at different voltages and the corresponding graph of the projected cell areas. The projected cell area for voltages below -400 mV is significantly smaller (p < 0.05) than cells treated at voltages between -100 and +300 mV. Voltages at or below -400 mV cause cells to undergo significant rounding up and reduction in size. In the anodic region, cell sizes do not differ from the cells cultured at OCP up to +300 mV. At +500 mV, however, cells appear smaller and less spread. However, the decrease in cell size at this voltage is not as dramatic as what is observed at cathodic voltages below -400 mV, and cells are still significantly larger than cells on cathodically-treated disks.

The viability voltage range as determined from Live-Dead assay, which is shown in Fig. 3.3, lies between -400 and +500 mV. At -700 mV and below, all cells were rendered non-viable. Anodic voltages at or above +500 mV also had a lethal effect.
Figure 3.1: SEM images of MC3T3 preosteoblasts cultured on CoCrMo alloy polarized to different voltages after 24 hours. The voltage cells were treated at is indicated on the top left of each image. The open circuit potential (OCP) is time dependent starting from around -400 mV and drifts to -200 mV after 24 hours. Note the balled up cells at negative voltages and the flat and well spread nature of viable cells at OCP and +300 mV. Cells at +500 mV despite appearing spread show signs of membrane damage (inset) and are not viable.
3.3.2 Electrical current levels and metal ion concentrations

Fig. 3.4a and 3.4b show average current densities over 24 hours and the average total charge transferred through metal/electrolyte interface at the corresponding voltages respectively. Large currents were measured at -1000 mV (21±3 μAcm$^{-2}$) and +500 mV (11±6 μAcm$^{-2}$). The total amount of electrical charge transfer at these voltages was accordingly high as expected (Fig. 3.4b). Current values between these voltages were as small as ~0.1 μAcm$^{-2}$ at -400 mV and ~3 μAcm$^{-2}$ at -700 mV.

![Graph of average projected cell area as a function of voltage after 24 hours. Cathodic voltages are shown with solid black bars and anodic voltages with gray bars. Note the significantly smaller cell area for voltages below -400 mV compared to other voltages. Cells at +500 mV were also smaller than viable cells, but more spread than cells on cathodic surfaces (*p < 0.05 compared with OCP, n = 100-130). No statistical difference exists between OCP and -100, +200, and +300 mV samples.](image)

Figure 3.2: Graph of average projected cell area as a function of voltage after 24 hours. Cathodic voltages are shown with solid black bars and anodic voltages with gray bars. Note the significantly smaller cell area for voltages below -400 mV compared to other voltages. Cells at +500 mV were also smaller than viable cells, but more spread than cells on cathodic surfaces (*p < 0.05 compared with OCP, n = 100-130). No statistical difference exists between OCP and -100, +200, and +300 mV samples.
The concentration of Cr, Ni, Co, and Mo metal ions is shown in Fig. 3.5. An excessive release of metal ions in the ppm range is observed at +500 mV (Fig. 3.5). At all the other voltages chromium concentration ranges from 18.7±4.3 ppb at OCP to 36±8.3 at-1000 mV. Post-hoc pairwise comparison of different voltage groups reveals that the level of ions released at +500 mV and -1000 mV are different from ion levels at OCP. No significant difference is observed between the level of metallic ions released at OCP and any other voltage within the studied voltage range. At +500 mV the concentration of the four ions measured are statistically higher than the OCP levels. Interestingly, a significant difference (p < 0.05) is also observed between chromium levels at a cathodic voltage of -1000 mV compared with chromium concentration at OCP.

Figure 3.3: The viability graph of MC3T3 preosteoblasts cultured for 24 hours on the surface of CoCrMo disks at different voltages. Cathodic voltages are shown with closed circles and anodic voltages with open circles (*p<0.05 compared with OCP, n= 100-120).
Figure 3.4: Graph of a) average current densities and b) average of total electrical charge transferred through the metal/electrolyte interface at different voltages after 24 hours. Cathodic voltages are shown with closed circles and anodic voltages with open circles. Positive current is from solution to the metal at cathodic voltages and from metal to the solution at anodic voltages.
3.3.3 Effect of voltage on cytoskeletal organization, adhesion complexes, and nucleus size

To investigate the cellular changes in response to electrochemical voltage shifts in more detail, actin and its binding protein vinculin were used as specific marker proteins in combination with fluorescence microscopy. In control samples (OCP), actin filaments were primarily organized in actin stress fibers, and vinculin localized to focal adhesions of actin stress fibers in MC3T3-E1 cells (Fig. 3.6c, OCP). In voltage-treated samples, cells responded by reorganization of the actin cytoskeleton that was accompanied by reduced cell adhesion (Fig. 3.6a-b and 3.6d-e). In
cathodically-treated samples, the cell area had decreased and actin retraction fibers started to accumulate at cellular edges (Fig. 3.6b, –400 mV). Focal adhesion sites were not detectable and vinculin exhibited a diffuse staining pattern within the cell body (Fig. 3.6b). In contrast, cells were rounded up completely and actin retraction fibers and actin filament tangles had formed at the cell periphery at the voltage of –700 mV (Fig. 3.6a, –700 mV). Clusters of vinculin-containing puncta accumulated mainly at the cell periphery, and are likely to present former sites of adhesion (Fig. 3.6a). In anodically-treated samples, the number of actin stress fibers and focal adhesions sites were strongly reduced in MC3T3-E1 cells. Actin stress fibers localized primarily at cellular edges and vinculin was detectable only at residual focal adhesion sites and the perinuclear region (Fig. 3.6d, +300 mV). In the anodic extreme of +500 mV, actin stress fibers had disassembled and immunolocalization of vinculin revealed a diffuse staining pattern throughout the cell body at this voltage (Fig. 3.6e, +500 mV).

Adhesion complexes containing vinculin in cells at different voltages were assessed by measuring their total projected area and number. It can be seen in Fig. 3.7a that the total projected area and number of adhesion complexes on polarized CoCrMo samples are smaller compared to cells cultured at OCP (p < 0.05). Cells treated at +300 mV, despite their high viability, based on the Live-Dead assay results, show low area and number of adhesion complexes compared to OCP. Basically, the further away the polarization voltage is from OCP, the lower is the total area and number of adhesion complexes.
Figure 3.6: Images of MC3T3 preosteoblasts cultured at a) -700, b) -400, c) OCP, d) +300, and e) +500 mV(Ag/AgCl) for 24 hours. Cells were stained to visualize actin cytoskeleton, adhesion complexes, and cell nuclei (red: actin, green: vinculin, blue: nucleus). Note the condensed nuclei, and actin retraction fibers at the cathodic voltages outside the viability range (a and b) and presence of actin filament tangles (b) and a diffuse staining pattern of vinculin (d and e), and irregular nucleus shape (e) at anodic voltages.
Figure 3.7: Graph of a) average of the total area of adhesion complexes per cell, and their average number per cell. Voltages outside the viability range (-700 and +500 mV) and on the edge of it (-400 and +300 mV) were chosen in both cathodic and anodic directions. b) The average nucleus size (projected area) at different voltages after 24 hours. Cathodic voltages are shown with solid black bars and anodic voltages with gray bars. Cathodic voltages below -400 mV have smaller nuclei than cells at OCP and cells at +500 mV (*p<0.05 compared with OCP, n = 23-33).

Similarly, the effect of voltage on the projected area of the cell nuclei is shown in Fig. 3.7b.

Nucleus size is related to cell integrity and function. The average of nuclei projected area of cells cultured at OCP (457±83 μm²) compared to the average cell area (3348±1664 μm²) shows a
karyoplasmic ratio (nucleus size/ cell size) of about 0.13. Except for a voltage of +300 mV, where nucleus size is not significantly different from cells cultured at OCP, cell nuclei at all the other voltages are statistically smaller (p < 0.05, n= 23-33) in size compared to control samples at OCP. Moreover, comparing the nucleus size at the extremes of anodic vs. cathodic voltage range reveals that although cell nuclei are reduced in size at both -1000 and +500 mV, this effect is more pronounced in the cathodic direction.

3.3.4 Mode of cell death (intrinsic or extrinsic apoptosis or necrosis)

Fig. 3.8a and 3.8b show the images of immunostained cells for caspases and the graph of fluorescence intensity of caspase 3 and 9 in the voltage-treated cells. Expression of caspase proteins indicates activation of apoptotic pathways in cells. While expression of caspase 3 ultimately occurs in both intrinsic and extrinsic apoptosis, caspase 9 is solely associated with intrinsic apoptotic pathway. Cells treated at +500 mV show low fluorescence intensities for either caspase 3 or 9, which suggests low expression of caspases at this voltage. Cells treated at -500 mV, however, show high fluorescence intensities, indicating a significant presence of both caspase 3 and 9. No fluorescence signal above the background level was detected from cells cultured at OCP (data not shown).
Figure 3.8: Images of a) caspase 9 (top images) and 3 (bottom images) release in cells cultured on CoCrMo samples and polarized at -500 and +500 mV (Ag/AgCl) for 24 hours (red: actin, green: caspase, blue: nucleus). b) Graph of the corresponding average fluorescence intensities at +500 and -500 mV (Ag/AgCl) (*p<0.001, n = 40-60).
3.4 Discussion

Voltage shifts of metallic biomedical implants could happen due to several factors including fretting corrosion, presence of oxidants or inflammatory cellular response. These voltage shifts could negatively affect cellular behavior and lead to cell death. On the other hand, voltage-induced cell death could be utilized as a potential therapeutic method in fighting cancer or design of smart biomedical devices if different cellular aspects of cell/polarized metal surface interactions become clear [29]. Hence, the effect of surface voltage of CoCrMo biomedical alloy on cellular behavior (cell viability and mode of cell death, morphology, cytoskeletal organization, and cell adhesion) was investigated here.

The viability graph (Fig. 3.3) and caspase protein expression levels (Fig. 3.8) show that extreme voltages both in the anodic and cathodic regions lead to total cell death by apparently different paths, where cathodic voltages appear to induce apoptosis while anodic voltages induce cell necrosis. Therefore, there exists a voltage viability range for CoCrMo outside of which cells cannot remain viable. This viability range for CoCrMo alloy lies between -400 and +500 mV (Ag/AgCl). It should be noted, however, that even at voltages where cells remain apparently viable, the cellular behavior might not be identical to that of cells cultured at OCP, where no voltage was applied to samples. The difference in the number and the projected area of adhesion complexes at OCP and +300 mV, a voltage within the viability range, clearly shows this fact (Fig. 3.7a). Additionally, a range of cell morphology is observed at OCP, which can be related to cell state in the normal cell cycle. On the other hand, other aspects of cellular behavior such as gene expression whose changes were not studied in this work might also show a different profile at voltages that do not compromise cell viability [7-10, 12, 15]. In addition, in this work the
cellular response was only assessed at the 24 hour point and the width of the voltage viability range might become narrower if viability is to be assessed at longer times.

An immediate correlation can be drawn between the current levels and the observed cellular viability throughout the voltage range studied. Cathodic or anodic voltages outside of the viability range lead to cell death, and these voltages correspond to current densities rising to between 0.1 and 10 μAcm⁻². For voltages within the viability range, low currents are present (below 0.1 μAcm⁻²) that do not significantly affect viability. There seems to be a current threshold of around 0.1 μAcm⁻², which drives cells to become non-viable over a 24-hour period. Depending on the initial OCP of the CoCrMo alloy in culture media, the corresponding voltage threshold occurs at -400 mV (Ag/AgCl) or lower. Interestingly, the voltage viability range for CoCrMo and the thin-film ITO electrodes is narrower compared to that of cpTi, where there is no anodic voltage limit to viability. Ehrensberger et al. did not observe any harmful effects on cells cultured on polarized cpTi in the anodic region up to +1000 mV (Ag/AgCl) for 24 h. This is due to the presence of a protective oxide layer on top of the cpTi, which grows indefinitely in the anodic region and impedes current increase and the lack of release of metal ions from Ti at these voltages. In case of the ITO electrodes, there is no such oxide growth process present due to the thin film nature of these electrodes. Therefore, the current steadily increases with an increasing voltage. As for CoCrMo alloy, the surface oxide layer grows in the anodic direction up to +500 mV. Above this voltage, however, a transition to the transpassive state occur, where the oxide becomes unstable. As a result, a high dose of metallic ions is released, and large anodic currents are measured. It is important to note that cell death is primarily caused by irreversible electrochemical reactions of the cellular components and/or generation of active species that are
harmful to cells. While the voltage of a surface determines what kind of electrochemical reactions might be taking place at that voltage, the corresponding current density provides a measure of the magnitude of those electrochemical reactions. The fatal effect of large cathodic voltages and currents are only observed when cells are directly grown on the surface of the electrode. On the contrary, exposing cells to high cathodic currents formed between two electrodes in direct current stimulation experiments results in improved proliferation and cellular orientation along the current direction [15].

Cell death is usually discussed distinguishably as either apoptosis or necrosis. Apoptosis is described as programmed cell death mediated by internal signaling pathways with no or minor inflammation, whereas necrosis occurs due to sudden injury or insult to the cell and leads to plasma membrane disruption, release of intracellular content and ultimately inflammation. Moreover, apoptosis can be initiated through two separate pathways: intrinsic vs. extrinsic. The intrinsic pathway is initiated from within the cell due to DNA damage or some other type of severe cell stress. Extrinsic apoptosis, however, is initiated by attachment of a pro-apoptotic ligand to the death receptor on the cell membrane. What distinguishes intrinsic pathway from the extrinsic pathway in terms of release or activation of proteins is the type of caspase proteins released in each process (Fig. 3.9). Both pathways will ultimately result in the release of effector caspases 3, 6 and 7. However, the initiator caspase proteins, released earlier in the apoptotic cascade are not the same for intrinsic and extrinsic pathways. Caspase 8 is the initiator caspase protein in the extrinsic pathway and caspase 9 is the initiator caspase in the intrinsic pathway. Staining the voltage-treated samples to reveal presence of caspase 3 and 9 proteins suggests different mechanisms for cell death in the cathodic vs. anodic voltage extremes. While the
Figure 3.9: Model of the intrinsic and extrinsic apoptotic cell death. The extrinsic pathway is triggered by attachment of a pro-apoptotic ligand to the death receptor and activation of the initiator caspase 8. In the intrinsic pathway, DNA damage initiates a cascade that leads to release of the initiator caspase 9. Both pathways end with release of effector caspases 3, 6, and 7 (Adapted from Ashkenazi et al. [56]).

caspase 3 and 9 are hardly present in the cells cultured at the extreme of the anodic voltage range, they are observed to be released under cathodic conditions (Fig. 3.8). The minimal
presence of caspases at +500 mV indicates that small populations of cells undergo apoptosis. On the other hand, a closer examination of the cells stained for actin, vinculin and DAPI reveals that cells treated at +500 mV clearly show morphological features of necrosis. These include swollen cell bodies and formation of cytoplasmic blebs (Fig. 3.6e, and Fig. 3.1, +500). Interestingly, these cells look almost as spread as the cells cultured at OCP, where no external voltage was applied to samples. Yet, the actin cytoskeleton looks disrupted and cell nuclei look distorted (Fig. 3.6e). These morphological features make it more reasonable to believe that cells cultured on disks at an anodic voltage of +500 mV after 24 hours have primarily died in a necrotic process. This may be due to the fact that apoptosis and non-apoptotic cell deaths (necrosis) are parts of a set of processes, in which one can dominate the other [30, 31].

In fact, there is a cellular regulatory network, which controls apoptosis, non-apoptotic cell death and survival. Dynamics of this regulatory network determines the cell fate, and pathways leading to these three phenotypes are believed to be highly intertwined. It has been reported that apoptotic and necrotic cell death can concomitantly be activated in ischemic neurons. The relative speed of each process determines which cell death phenotype would be dominant [31]. This could explain why the voltage-treated cells at +500 mV despite showing the morphological features of necrosis stain positive for caspase proteins, which indicates activation of apoptotic pathways.

The activation of both apoptotic and necrotic pathways could be attributed to the release of metallic ions, in particular, Cr(VI) at and above +500 mV. Chromium intake by several different cell types has been well documented. Chromium can exist in different oxidation states, but the most commonly encountered valences are 0, +2, +3 and +6 forms. For CoCrMo biomedical alloys, oxidized chromium is in the +3 valence state at voltages below +500 mV. Above this
voltage, it changes to the +6 form, and in the form of the tetrahedral chromate anions, it can readily be taken up by cells through the general anion transport system [32]. This is in contrast to much less permeable octahedral chromium III, which can mainly cross the membrane through simple diffusion. It is believed that the toxicity of Cr(VI) is due to its intracellular reduction to Cr(III) and generation of unstable Cr(IV) and Cr(V) ions, active oxygen species (hydroxyl, singlet oxygen, superoxide anion), thiyl, and organic radicals [33]. Whether metallic ions induce apoptotic or necrotic cell deaths depends on the dose [34, 35], time [36], cell type [37] and type of metal ion. While low dosage of metal ions leads to apoptotic death, higher doses induce necrosis [34, 38]. Several other groups reported that Cr (VI) leads to apoptosis rather than necrosis [37, 39-43]. In most of these studies, generation of ROS [44] and expression of p53 gene have been mentioned as the mediators of apoptosis induction by Cr (VI). Our results of cells treated at +500 mV for 24 hours show morphological features of necrosis, and presence of minor amounts of caspase-3 and 9, which are indicative of apoptosis. This implies an activation of apoptotic cascades early on when the concentration of chromium is low, and a shift to necrosis later on as the amount of Cr(VI) increases with time. An interesting observation with regard to the release of chromium ion in this work was a higher release of chromium ions at a cathodic voltage of -1000 mV compared with OCP. This observation, which is in contrast with the general notion that ion release is impeded at reducing voltages, can be explained by a drop in impedance of the CoCrMo alloy at cathodic voltages [45]. The impedance of the oxide goes down so much at -1000 mV that even though the voltage driving dissolution decreases, the impedance drop is greater, and the overall effect is an increase in ion release at this voltage. Regarding the metal ion release at +500 mV, it is observed that despite a higher cobalt content of the metal, the amount of measured chromium in the culture media was higher than cobalt. This
could be due to deposition of the cobalt ions on the counter electrode, which depletes the amount of cobalt in the media.

On the other hand, our results show that extreme cathodic voltages (below -400 mV) activate an intrinsic apoptotic cell death process. This was evident from presence of caspase 9 and 3 in the samples treated at -500 mV (Fig. 3.8) and the morphological features specific to apoptosis (shrunken cells, condensed nuclei, and presence of retraction fibers) at cathodic voltages (Fig. 3.6a and 3.6b). What causes the induction of apoptosis in this case is not completely clear. Several factors could play a role in the observed lethal effect of extreme cathodic voltages on cells. Certainly, in terms of electrochemistry, what distinguishes the cathodic extreme of the voltage range studied in this work from the anodic region is the presence of reduction reactions and generation of compounds that could be toxic to cells. Generation of reactive oxygen species (ROS), including super oxide anion (O$_2^-$), hydroxyl (HO'), peroxyl (RO$_2^-$), alkoxy (RO') and hydrogen peroxide is an example of such reactions. ROS molecules could be beneficial or harmful to cells depending on their concentrations [46]. High concentrations of ROS disrupt normal cell function, damage DNA and promote cell death. At low concentrations, however, they take part in intracellular signaling pathways and regulation of cell function. A major source of intracellular ROS generation is the mitochondrion. Moreover, generation of HO' via Fenton and/or Haber–Weiss reactions occurs in the presence of metal ions and causes significant damage on cellular proteins, lipids, and DNA [47]. The effect of ROS on cellular behavior is believed to be highly dose dependant. Within the machinery of a cell, there are enzymes that break down and convert the oxygen radicals to maintain cellular homeostasis. In the presence of excessive amount of ROS, cell death occurs by either apoptosis or necrosis depending on the
severity of the injury to the cell [48, 49]. Kalbacova et al. [22] have reported the electrochemically stimulated ROS generation in osteoblasts cultured on Ti6Al4V samples. Oxidative stress and the generation of H₂O₂ by the cell has been shown to depolymerize actin [25] and alter cellular adhesion [23].

Furthermore, the electro-reduction of proteins present in the culture media and on the cell membrane could very well happen due to the electroactivity of many of these proteins. Stankovich et al. [27, 50] in a series of papers studied the electrochemical behavior of proteins at the surface of electrodes. They showed that at high concentrations of BSA, cathodic polarization leads to breakage of disulfide bonds and formation of an insoluble product on mercury electrode. They also showed formation of a compact film (Hg(RS)₂) at high concentrations of cystine at a polarized mercury electrode. Other studies have focused on the consequences of reduction of disulfide bonds in terms of protein structure and properties, membrane transport, and intracellular behavior (e.g., redox sensitive chaperones, NADH and NADPH behavior, oxidative stress, etc.) Thiol and disulfide redox status also affects the ease of uptake of cell-penetrating peptides (CPP’s) [51] including transmembrane transport of prions, and other short peptides [52]. The redox status of a cell is critical to its oxidative stress state and it seems highly likely that if/when cells are adjacent to a half space (metal surface) that is actively reducing species available at its surface, including disulfide containing molecules, these cells will have their redox balance upset.

Finally, recent studies of metal-on-metal hip prosthesis have shown examples of tissue reactions including osteolysis and pseudo-tumor formation [53-55]. The triboelectrochemical processes present drive the voltage of these devices cathodic and the mechanisms reported here may well be involved in these clinically observed reactions.
3.5 Conclusion

There is a voltage range from -300 mV to +300 mV(Ag/AgCl) where cells remain viable on CoCrMo alloy. For cathodic voltages outside the viability range cells die through an intrinsic apoptotic pathway, based on the release of caspase 3 and 9. For anodic voltages outside the viability range, cell death occurs via necrosis based on morphological hallmarks, including swollen cell body, and membrane damage. The current densities that define the limits of viability are 0.1 μAcm⁻² and 10 μAcm⁻² for cathodic and anodic limits, respectively. Metal ions were released in larger amounts at +500 mV for all ions (Co, Cr, Mo, Ni) and at -1000 mV for Cr ions. These ions likely played a significant role in necrosis, but not in the cathodic range. Adhesions complexes for cells on voltage-controlled surfaces (at all voltages) were fewer (and of less area) than cells on OCP surfaces. Reduced number and size of adhesion complexes at +300 mV indicates altered cell behavior within the viability range of the alloy surface. Nucleus size is a function of voltage with smaller projected areas outside the viability range compared to OCP. Nuclei of cells at the cathodic voltages outside the viability range are smaller than the nuclei of cells treated at +500 mV.
3.6 References


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4. Voltage-controlled cellular behavior on polarized cpTi with varying surface oxide thickness

4.1 Introduction

Titanium and its alloys due to their corrosion resistance and biocompatibility are among the most highly utilized metallic biomedical alloys [1]. The specific surface interactions between these alloys and the body similar to other biomaterials significantly affect their function inside body. In order to elicit a desired response or prevent detrimental interactions, surfaces of implants are often modified using coatings, grafts, or other surface treatment techniques [2-6]. In the case of Ti and its alloys, growing the thickness of the thin native surface oxide layer (2-10 nm) [7] through electrochemical anodization or thermal treatment has been associated with favorable effects on gene expression [8-11], bioactivity [12], cellular migration [13], adhesion [11,14,15], proliferation [16], differentiation [17,18], and osseointegration [19].

On the other hand, the surface voltage of the metallic implants may digress from the open circuit potential (OCP) into more cathodic voltages due to fretting corrosion [20-22], and affect different aspects of the cellular behavior such as cell viability, cytoskeletal organisation, and cell adhesion [23-25]. Negative voltages in the range of -1000 mV(Ag/AgCl) have been reported when the passive oxide films of Ti and CoCrMo alloys have been abraded [21,22]. Likewise, presence of hydrogen peroxide, as in case of inflammation in the body, may lead to a voltage shift in the anodic direction [26]. It is known that there is a voltage viability range outside which, cells cultured on polarized metallic surfaces will be rendered non-viable. The upper and lower limits of the voltage viability range depend on the type of metal and the cell line used among other factors [23, 27-29].
For cpTi, the lower limit is believed to be at -400 mV(Ag/AgCl) and no upper limit has been reported up to 1000 mV(Ag/AgCl) [23]. The surface interactions at play and the parameters that regulate the cellular behavior at these polarized surfaces have not been fully investigated. Cells and the surrounding physiological solutions contain a myriad of active substances that can take part in redox reactions on polarized electrodes. While the voltage determines what type of redox reactions may occur on metallic surfaces, electrochemical currents are a measure of the magnitude of such reactions. The thickness of the native surface oxide on metallic biomedical implants is an important parameter in this regard, as it can enhance or impede charge transfer and subsequently the scale of redox reactions.

To date, there has been no study to investigate how the thickness of the passive oxide influences voltage viability range and cellular behavior on anodized titanium under cathodic polarization. This could be important as it is not clear how the improved cell-biomaterial interactions associated with anodizing titanium and its alloys can be affected by voltage shifts. Furthermore, there could be synergistic effects on directing the cell - metal interactions when cells are cultured on anodized cpTi surfaces at voltages within the viability range.

The objective of this study is to understand the effect of voltages at the cathodic edge of the voltage viability range for cpTi (~ -400 mV(Ag/AgCl)) on cellular morphology, viability, organization of adhesion complexes, and actin cytoskeleton. Furthermore, the surface of the bare and anodized cpTi were characterized at cathodic voltages using electrochemical impedance spectroscopy (EIS), and the impedance parameters were related to the observed biological response. The surfaces of the bare and anodized titanium were additionally characterized using AFM and sessile drop method to measure the roughness and surface contact angle, respectively.
4.2 Materials and Methods

4.2.1 Sample preparation

Disks of grade 4 commercially pure titanium (ASTM-F67) with a diameter of 2 cm were mechanically polished up to 600 grit. After rinsing the samples with deionised water (DI), they were sonicated in DI water for 10 minutes. Bare cpTi samples were mounted in a two-electrode custom-made electrochemical setup and were anodized at a voltage of 9 volts (0.7 ± 0.04 mAcm⁻²) at room temperature in phosphate buffer saline. The anode was the titanium piece and the cathode a carbon rod. Samples were removed from the chambers post-anodization and were rinsed with DI and alcohol. Bare and anodized disks were autoclaved prior to cell culture.

4.2.2 Measurement of surface roughness and contact angle

A Digital Instruments multimode AFM-2 with a Nanoscope IIIa scanning probe microscopy controller (Veeco Instruments, Inc.) was used in contact mode for imaging the bare and anodized disks (N = 3). The standard NP silicon-nitride probes with a thickness of 0.6 μm and a spring constant of 0.06 N/m (Veeco Instruments, Inc.) were used. The roughness of the samples were calculated using the built-in Veeco Instruments AFM software.

To measure the surface contact angles of the bare and anodized samples, a Ramehart 190_F1 goniometer was used. Distilled water droplets (2.0 - 3.0 μl) were suspended from the tip of the syringe while the sample was advanced towards the syringe until the droplets made contact with the disk surface. Images were collected with the camera and the contact angle between the drop
and the substrate was measured from the magnified image. At least three positions were tested at random for each disk, and three independent samples were used for bare and anodized disks. The contact angle readings were recorded 10 times at 0.1 s intervals and were averaged for each droplet.

4.2.3 Cell culture and application of voltage

MC3T3-E1 pre-osteoblasts (ATCC #: CLR-2593) (p6-p25) were cultured on cpTi disks and voltage was applied to the metal disks as previously reported [23]. Briefly, cells cultured (37 °C, 6% CO₂) in T-75 culture flasks were trypsinized and seeded on cpTi disks (10,000 per disks), mounted in custom-made electrochemical chambers. Disks were potentiostatically polarized to -500, and -400 mV(Ag/AgCl) for 24 h using a bipotentiostat inside an incubator. A standard three-electrode system was used. The electrical current of each chamber was measured for 24 hours in 60 s intervals using a NI9004 A/D board (National Instruments) and Labview software, and current densities over 24 hours were measured. Control samples were cell-seeded cpTi disks left at OCP (i.e. no voltage was applied) for 24 h, and OCP values were measured using a NI9004 A/D board (National Instruments) and Labview software. Three independent samples for each voltage and control samples were used.

4.2.4 Cell viability assay and immunostaining

Cell viability and morphology on the voltage-treated and control samples were assessed using LIVE/DEAD® viability/Cytotoxicity Kit for mammalian cells (SKU# L-3224, Invitrogen) as
previously reported [25]. Briefly, cells were washed with PBS post treatment, incubated with a
solution of calcein and Ethidium homodimer-1 in PBS for 20 min, and washed with PBS prior to
imaging.

Visualization of the actin cytoskeleton, adhesion complexes, and nuclei of cells in control and
voltage-treated samples were performed as reported previously [25]. Briefly, cells were first
fixed, permeabilized, and blocked with Triton X-100 and gelatin respectively. F-actin and
nucleus were detected with rhodamine/phalloidin (rho/pha) (Sigma-Aldrich, MO) and 4',6-
diamidino-2-phenylindole (DAPI) respectively. Immunostaining of vinculin was performed with
a mouse monoclonal antibody directed against vinculin (clone hVIN-1, Sigma-Aldrich, MO) and
a secondary goat antimouse IgG labeled with Alexa-Flour 488 antibody.

4.2.5 Imaging and image analysis

Metal disks were mounted on glass slides and analyzed by fluorescence microscopy. For cell
viability analysis, cells were imaged with an inverted microscope (Axiovert 40CFL, Zeiss,
Denmark) equipped with a CCD mono-12 bit camera (Q-imaging, Canada) and an X-Cite 120
light source (EXFO America, TX). FITC and Texas red dye filter set were used for live and dead
cells respectively.

For immunostained samples, images were acquired using a Zeiss Axioplan2 microscope (Carl
Zeiss, Inc., NY) equipped with a Hamamatsu Orca II C4742-98 camera, an X-Cite 120 light
source, and filter sets for FITC, TRITC and DAPI fluorescence. MetaMorph imaging software
(Universal Imaging Corp., PA) was used to control illumination shutters, camera exposure and
image acquisition. ImageJ software (NIH) was used to quantify morphological characteristics of
cells from fluorescence microscopy images. Cell viability, and total adhesion complex area and count were calculated according to the protocol developed previously [25].

4.2.6 Electrochemical impedance spectroscopy

The bare and anodized cpTi surfaces were characterized using a potentiostat (Solartron 1280C, UK) equipped with a frequency analyzer and impedance parameters (impedance, capacitance, phase angle, and dispersion coefficient alpha) were derived assuming a Randle’s circuit for the interface with a CPE component. Measurements were performed by superimposing a 10 mV sinusoidal voltage at -400 mV(Ag/AgCl) at time zero and after 24 h.

Data acquisition/analysis for electrochemical impedance spectroscopy (EIS) was performed using Zplot 2.0/Zview 2.0 software (supplied by Scribner Associates).

4.2.7 Statistical analysis

Results are presented as means ± standard deviation (SD). One-way ANOVA on ranks (Kruskal-Wallis test) and Dunn’s pair-wise comparison method were performed (SigmaPlot v. 11) to compare the controls and voltage-treated groups with P-value of 0.05 indicating significance. Unless stated otherwise, data were collected from at least three independent experiments.
4.3 Results

4.3.1 Anodization and surface characterization

Anodization of cpTi surfaces in PBS at a voltage of 9 V grows a compact non-porous oxide layer whose surface pattern does not differ from the native surface oxide observed in bare samples (Fig. 4.1). The thickness of the formed oxide based on the golden color of the anodized surface and the reported oxide growth rates for cpTi [1] (2 nm/V) is estimated to be about 20 nm. Characterization of the surfaces of bare and anodized cpTi shows that there is no significant difference between these two surfaces in terms of the surface roughness or contact angle (Fig. 4.2). The starting roughness of the bare samples were high (93 ± 13 nm) and the anodized surfaces had a comparable roughness (87 ± 10 nm). Similarly, the wetting behavior of the bare surface with a contact angle of 56° ± 8° was not different from that of the anodized surface with a value of 58° ± 8°.

Fig. 4.1: AFM images of the (left) anodized and (right) bare cpTi. Note that the anodization at low voltage (9 V) did not change the surface topography significantly.
Fig. 4.2: Graph of surface roughness and contact angle of bare and anodized cpTi samples. No statistical difference (p<0.05, n = 9) exists between the contact angle or roughness of the bare and anodized samples.

4.3.2 Cell – material interaction

4.3.2.1 Cell morphology and viability

Staining of cells cultured on bare and anodized cpTi at OCP and under polarized conditions using Live/Dead assay shows high cell viability on both bare and anodized cpTi surfaces at OCP (Fig. 4.3). In terms of morphology, cells cultured on the anodized surface have taken more of an elongated/spindle like morphology, whereas cells on the bare samples seem to have a more spread-out or rhomboid shape (Fig. 4.4). At -400 mV, a significant difference is observed in the viability of cells on the bare vs. anodized samples. While the cells on the bare surface were all dead after the voltage treatment, cells on the anodized surface remained viable (~78%). Nonetheless, they showed signs of distress as evidenced by their rounded and less spread shape than the cells at OCP. Treatment of cells at a voltage of -500 mV, however, rendered cells on both bare and anodized surfaces non-viable. Cells and their nuclei look shrunken and the membrane damage is evident (Fig. 4.4).
Fig. 4.3: Graph of cell viability of MC3T3 pre-osteoblasts at OCP, -400, and -500 mV(Ag/AgCl) after a 24 hour culture period on bare and anodized cpTi samples. Note the sharp drop in viability at -400 mV on the bare sample compared with the anodized cpTi (*p < 0.05, n = 100-130).

4.3.2.2 Effect of voltage on cytoskeletal organization, and adhesion complexes

To investigate how cathodic voltages at the edge of the viability range affect other aspects of cell behavior such as adhesion and cytoskeletal organisation, cells were immunolabeled with focal adhesion-associated protein vinculin and stained to visualize actin stress fibers. Distinct focal adhesions were visible at the periphery of the cells on both bare and anodized surfaces at OCP. Actin filaments were primarily organized in actin stress fibers, and vinculin was localized to focal adhesions of actin stress fibers. No statistical difference in the average size (projected area) of adhesion complexes or their number per cell was found between cells cultured on bare and
Fig. 4.4: Fluorescence microscopy images from the Live/Dead assay of MC3T3 pre-osteoblasts cultured on bare and anodized cpTi at OCP, -400, and -500 mV(Ag/AgCl) for 24 hours. Nuclei of non-viable cells fluoresce red while viable cells only fluoresce green. Note the higher number of non-viable cells on the bare surface at -400 mV compared with the anodized metal.
anodized samples. Cells cultured on bare samples, however, showed a wider size distribution of adhesion complexes compared with the cells on the anodized surface (Fig. 4.5). At -400 mV, cells on bare cpTi samples are reduced in size and have very few distinguishable focal adhesions, and no distinct actin stress fiber is present (Fig. 4.6). On the anodized surfaces at this voltage, however, cells are still well spread with extensive actin stress fibers. Focal adhesions are less frequent and viculin shows a more diffuse pattern compared with the OCP condition. At -500 mV, on both surfaces a disrupted actin cytoskeleton and formation of actin retraction fibers is observed. Moreover, clusters of vinculin-containing puncta are seen at the edges of the cells cultured on bare samples and vinculin is present with a diffuse pattern on the anodized surface.

![Graph of the number and total projected area of adhesion complexes on the bare and anodized cpTi at OCP, -400, and -500 mV after 24 hours. The comparable values of adhesion complex area and number at -400 mV on the bare surface are an artifact related to the algorithm used in outlining the adhesion complexes (*p<0.05 compared with OCP, n = 20-30).]

Fig. 4.5: Graph of the number and total projected area of adhesion complexes on the bare and anodized cpTi at OCP, -400, and -500 mV after 24 hours. The comparable values of adhesion complex area and number at -400 mV on the bare surface are an artifact related to the algorithm used in outlining the adhesion complexes (*p<0.05 compared with OCP, n = 20-30).
Fig. 4.6: Images of MC3T3 preosteoblasts cultured at OCP (top), -400 (middle), and -500 (bottom) mV(Ag/AgCl) for 24 hours on bare (left column) and anodized (right column) cpTi samples. Cells were stained to visualize actin cytoskeleton, adhesion complexes, and cell nuclei (red: actin, green: vinculin, blue: nucleus). Note the spread-out morphology and presence of distinguishable focal adhesions on the bare and anodized samples at OCP. Except for the anodized sample polarized at -400 mV, cells cultured on polarized surfaces show signs of damage.
4.3.3 Electrochemical characterization

4.3.3.1 Impedance spectroscopy

The effect of voltage on cells cultured on polarized cpTi is partly due to the changes that happen to the surface oxide. In order to characterize the changes to the surface oxide layer and relate them to the observed biological response, electrochemical impedance spectroscopy was employed and impedance spectra were recorded for bare and anodized cpTi. Fig. 4.7 shows the impedance spectra of the bare and anodized cpTi at OCP, -400 and -500 mV(Ag/AgCl) at the start of polarization (t=0) and 24 hours after it. For the bare cpTi at OCP, no significant change is observed in the impedance spectra at t = 0 and 24 h. The capacitive/resistive nature of the oxide based on the phase angles measured does not change either. The anodized surface, on the other hand, 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. Comparing the impedance and phase angle spectra of bare vs. anodized samples, shows that anodized cpTi have lower impedance values and lower phase angles at low frequencies (< 10 Hz). This hints at lower dispersion coefficients of the oxide/SBF interface on the anodized surface. At frequencies higher than 10 Hz, anodized samples have higher phase angles, which indicate a lower time constant for the interface. At -400 mV, while a significant drop in impedance is observed for the bare surface, the impedance of the anodized surface rises after 24 hours. Furthermore, the non-ideal nature of the anodized surface is indicated from the phase angle graph. For a completely polarizable electrode showing a purely capacitive character, the phase angle will be 90°. Phase angle values below 90° indicate a non-ideal interface with partial resistive nature. At -500 mV, a drop in impedance values is observed for both bare and anodized samples. The drop is more significant for bare cpTi compared with the anodized surface.
4.3.3.2 Open circuit potential

Fig. 4.7: Electrochemical impedance data for potentiostatically anodized film on cp-Ti formed in 0.1M PBS after different times (0 and 24h) of sample immersion in AMEM+ FBS solution of pH 7.4 at 37 °C in the form of Nyquist plot (left column) and Bode phase plot (right column) at OCP (top), -400 (middle), and -500 (bottom) mV(Ag/AgCl).
4.3.3.2 Open circuit potential

The open circuit potential of bare and anodized cpTi disks were monitored for a period of 24 hours. More positive OCP (-122 ± 6 mV) was measured for the anodized samples compared with the bare surfaces (-434 ± 3 mV) at time zero (Fig. 4.8a). Over time, however, the OCP of both surfaces drift to about -220 mV after 24 hours.

4.3.3.3 Electrochemical currents

Electrochemical current, which is an indicator of the magnitude of the electrochemical reactions occurring at different voltages, was measured on bare and anodized surfaces at -400, and -500 mV. Current levels are initially low (below -0.1 μAcm\(^{-2}\)) for both bare and anodized surfaces at -400 and -500 mV (Fig. 4.8b), and start to gradually rise at longer times. The current increase rate, however, is not the same at different voltages and on anodized vs. bare surfaces. At -400 mV, while current rises from around -0.07 to -1.3 μAcm\(^{-2}\) on the bare surface, it largely stays at around -0.05 μAcm\(^{-2}\) on the anodized surface over 24 hours. At -500 mV, a sharper increase in current is observed on the bare surface compared with the anodized one.

4.4 Discussion

The effect of cathodic voltages on the biocompatibility of metallic biomaterials have recently started to gain importance in the context of corrosion of biomedical implants. The cathodic voltage shifts from the open circuit potential can readily happen due to fretting corrosion, and affect the properties of the surface oxide layer and the implant biocompatibility. That is why a great deal of effort has been exerted into modifying the surface oxide layer of metallic
Fig. 4.8: Graphs of a) variation of the open circuit potential of bare and anodized cpTi in culture media (AMEM+FBS) b) current density as a function of time for bare and anodized cpTi samples polarized at -400, and -500 mV(Ag/AgCl) as a function of time.
biomedical alloys in order to improve their biocompatibility. In case of titanium and its alloys, growing the thickness of the native surface oxide via electrochemical anodization has been shown to be beneficial in improving bone formation and osseointegration. Although the surface and electrochemical properties of the passive films formed on titanium under different conditions are well documented, the changes in the anodized film under cathodic polarization remain unclear. Furthermore, the subsequent response of the cells cultured on anodized titanium surfaces has never been investigated under cathodic polarization.

In this work, we have studied the effect of cathodic voltages at the edge of the viability range to investigate cellular behavior on bare vs. anodized titanium surfaces under cathodic polarization and how it correlates with the electrochemical properties of these surfaces.

Biocompatibility of titanium-based implants is affected by the physico-chemical properties of its passive film such as its thickness, amphoteric nature, semiconducting properties, insolubility, and dielectric constant [30]. Surface treatment methods aim to tailor these properties to elicit an improved physiological response in vivo. Electrochemical anodization is essentially used to increase the surface oxide thickness, but it might also alter surface chemistry, topography, and electronic properties of the passive film in the process. At low voltages, anodization will mainly cause thickening of the surface oxide layer without inducing significant changes in other oxide properties such as the chemistry and structure of the oxide. An increase in the oxide thickness brings about changes in the electronic properties and charge transfer resistance of the passive layer. Fig. 4.2 shows that the bare and anodized cpTi samples used in this work had similar roughness and surface energies. Additionally, it is reported that anodization at low voltages below the dielectric breakdown of TiO₂ results in formation of an oxide layer solely composed of pure TiO₂ [31]. It should be noted that anodization does not always lead to a better corrosion
resistance of cpTi and its alloys. In fact, the polarization resistance of the anodic oxide formed on the surface depends on the electrolyte and anodization potential [32]. Our results also show a lower impedance for the anodized surfaces at OCP, -400, and -500 mV(Ag/AgCl) compared with the bare surface at time zero. This also holds true for measurements of the impedance spectra performed after 24 hours at OCP and -500 mV(Ag/AgCl). At -400 mV, on the contrary, an increase in the impedance values of the anodized surface is observed after 24 hours (Fig. 4.7). This is consistent with the findings of Ehrensberger et al. on changes of polarization resistance \( R_p \) of bare cpTi in culture media at 0 and 24 hours, where \( R_p \) of titanium, having been polarized at different voltages down to -300 mV(Ag/AgCl), increases after 24 hours, but shows a decrease when polarized at more negative voltages [33].

It seems that the important factor influencing cell behavior is the average current densities measured on the polarized surface (Fig. 4.8b). Therefore, rather than the initial starting impedance value, it is how the impedance of the metal surface changes over time that determines the charge transfer through the interface and as a result the magnitude of the cathodic reactions. Impedance of titanium is strongly influenced by the oxidation state of its surface oxide layer, whose stoichiometry depends on the final anodic potential and on the electrolyte used for the oxide growth [34]. Different oxides (TiO, TiO\(_2\), Ti\(_2\)O\(_3\), Ti\(_3\)O\(_5\), TiO\(_3\)) may be present in the oxide film on Ti, with TiO\(_2\) being the most stable [35, 36]. The equilibrium ratio of these oxides changes based on the electrochemical history and the potential of oxide formation [37]. The relative ratio of these non-stoichiometric oxides determines whether the oxide has n-type or p-type semiconducting properties or act as a dielectric. It has been suggested that the outer part of the anodic oxide film converts from n-type semiconductor to a dielectric above 3 V (vs. SCE) and back to an n-type semiconductor below 0.5 V [38]. The mechanisms involved in dictating
the equilibrium electronic state of the surface oxide are not completely understood. However, the
changes in electrochemical currents measured over 24 hours can partly be rationalized based on
the evolution of open circuit potential of the bare and anodized cpTi within this time period.
Monitoring the variation in OCP (Fig. 4.8a) shows that even though the bare surface starts off at
a more negative OCP (~ -440 mV) compared with the anodized surface (~ -120 mV), they
eventually drift towards the same potential of about -220 mV. The larger over-potential that
develops for the bare surface as time progresses explains the rise in current over 24 hours on the
bare surface. On the anodized surface, on the other hand, the over-potential decreases with time
and results in lower currents when the surface is polarized at -400 mV.

The voltage viability range, apart from the magnitude of voltages that set its upper and lower
limits in the cathodic and anodic regions is influenced by other factors such as polarization time.
Our work reveals that the thickness of the surface oxide also shifts the cathodic limit of voltage
viability range for titanium (Fig. 4.3).

Interestingly, despite the significant difference between the cell viability on bare and anodized
surface at -400 mV(Ag/AgCl), the measured adhesion complex number and size (total projected
area) of bare and anodized surface do not differ significantly (Fig. 4.5). This is due to an artifact
related to the algorithm used in outlining the adhesion complexes, where no distinction can be
made between the focal adhesions of viable cells and the clusters of vinculin-containing puncta
at the periphery of the non-viable cells. Therefore, the calculated values for the voltage treated
cells on bare samples at -400 mV(Ag/AgCl) are not a true measure of the number of adhesion
complexes or their size (Fig. 4.5). Furthermore, no difference between the bare and anodized
surfaces were found in terms of the number of adhesion complexes and their size at OCP. This
might seem to be contradictory to what has been reported by others regarding the better adhesion
of cells onto the anodized vs. bare titanium surfaces. However, several physical and chemical properties of the passive oxide such as its roughness, topography, chemistry, and conductivity might be modulated by anodization in general, and it is not clear how much any of these changes contribute individually to the observed cellular response. The anodization parameters in our work were selected in a way to limit the changes to the passive oxide to modulation of its electronic properties as much as possible.

Finally, our results show that anodization of Ti and its alloys as well as inducing the desirable effects on different aspects of cellular response, reported in the literature, could protect cells against cathodic voltage shifts and limits the harmful effect of cathodic redox reaction that might exist in vivo.

4.5. Conclusion

Anodization of cpTi at a voltage of 9 V in PBS shifts the cathodic limit of the voltage viability range into lower voltages. The cathodic limit of the voltage viability range for bare cpTi is at around -400 mV(Ag/AgCl), and anodization shifts it by about 100 mV into more cathodic voltages. Nevertheless, cells show signs of distress on the anodized surface at -400 mV(Ag/AgCl) despite showing high cell viability. Lesser number and smaller size of adhesion complexes on the anodized surface at -400 mV(Ag/AgCl) compared with cells cultured at OCP (p< 0.05) are indicative of this effect. At a higher cathodic voltage of -500 mV(Ag/AgCl), cells were rendered non-viable with a rounded up morphology and numerous actin retraction fibers on both bare and anodized surfaces. The observed cellular response at cathodic voltages correlates well with the electrochemical current levels, where average currents above -0.1 μAcm$^{-2}$ over 24
hours significantly affect cell behavior. Anodized surfaces were less susceptible to the effect of the cathodic voltages due to lower electrochemical current levels and hence limited the magnitude of harmful redox reactions on cells at these voltages. Correspondingly, impedance spectra of the bare and anodized samples showed a significant drop of impedance on bare surfaces at cathodic voltages, whereas the drop in impedance was not as dramatic in anodized samples and even increased when polarized at -400 mV(Ag/AgCl) for 24 hours.

4.6 References


5. Study of cellular dynamics on polarized CoCrMo alloy using time-lapse live-cell imaging

5.1 Introduction

Metallic implants are extensively used to restore or improve function of several body parts and systems [1, 2]. Orthopedic implants, in particular, have structural requirements such as hardness and toughness that can hardly be met by any other class of materials [3]. However, despite being utilized for a few decades, metallic implants still suffer from problems such as lack of proper tissue/implant integration and loosening [4], formation of pseudotumors [5-7], infection [8], and corrosion [9,10] that necessitate a better study of the cellular interactions and electrochemical processes on these surfaces in order to improve their in vivo performance. It was previously shown that the voltage of metallic implants can shift from the resting open circuit potential (OCP) due to micro motions of articulating implant surfaces or inflammatory cellular response [10-13] and significantly affect the cellular behavior and biocompatibility [11,15-17]. Generally, the modified cellular behavior on polarized metallic electrodes includes changes in cell morphology [18], proliferation [19], cytoskeletal organization [20], protein production [21], and gene expression [22]. The details of the polarized metal surface/cell interactions and the mechanisms involved remain largely unresolved to this date. Generation of ROS in osteoblasts is believed to be one of the contributing processes in the observed cell behavior on cathodically polarized Ti6Al4V [15]. Oxidative stress and the generation of H$_2$O$_2$ by the cell has been shown to depolymerize actin [23] and alter cellular adhesion [24]. Adhesion of cells to their substrate plays a significant role in regulating several aspects of cell behavior such as differentiation, growth, migration, proliferation and apoptosis [25-29]. Cell adhesion to any substrate is mediated by the proteins in the extracellular matrix (ECM) and integrins as the major transmembrane ECM receptors. Focal adhesions (FA) are the main subcellular structures that
mediate the regulatory effects of ECM adhesion on cell behavior [30]. Electrochemical polarization of a metal surface alters the biomaterial surface properties [31,32], which can influence protein adsorption and modifies matrix/membrane proteins and cell–ECM interactions in vitro. On the other hand, polarized surfaces may disturb cellular homeostasis, which is regulated by intracellular and intercellular signaling pathways determining the redox state of a cell. Regulated ion movements across the cell membrane is essential for cell survival, and polarized surfaces can disturb the ionic balance and redox state of a cell through membrane voltage-gated channels and other pathways, causing cell death. Previously, we reported the existence of a voltage viability range for CoCrMo biomedical alloy at a fixed time of 24 hours.

In this work, we studied the temporal changes to the adhesion behavior of pre-osteoblasts in response to polarized and non-polarized CoCrMo samples. GFP-tagged vinculin, a cytoskeletal actin-binding protein, was used as a marker for focal adhesions for quantitative analysis of the adhesion sites in the living osteoblast-like cells. Vinculin is one of the most important proteins in focal adhesions, which promotes cell spreading by mechanical coupling of integrins to the cytoskeleton via its actin-binding entity [33]. Moreover, temporal changes in intracellular ROS levels was monitored at different anodic and cathodic voltages. Cell morphology as an indicator of cell state and viability was also assessed. Our results show that loss of adhesion at the voltages outside the viability range of CoCrMo alloy occurs at different time points for cathodic and anodic voltages and the loss of adhesion and viability is not accompanied by generation of ROS molecules.
5.2 Materials and Methods

5.2.1 sample preparation

A bar of high carbon wrought Co28Cr6Mo F-1537 alloy was used to make disks with a diameter of 1 cm and a thickness of 0.2 cm. A small cylindrical hole (0.1 cm in diameter and 0.1 cm deep) was drilled into one side of the disks, and the opposite side was mechanically polished up to 600 grit using sandpaper and 0.1 μm alumina powder afterwards to yield a mirror finish. The samples were then rinsed with deionised water (DI), cleaned in DI water in an ultrasonic bath for 20 minutes, and autoclaved.

5.2.2 Cell culture and transfection of GFP-vinculin

MC3T3-E1 pre-osteoblast cells (ATCC #: CLR-2593) (p6-p25) were cultured in T-75 culture flasks to near confluence inside an incubator (37 ºC, 6% CO2). Alpha Modification of Eagle’s Medium (AMEM, Cellgro) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco) was used as the cell culture medium. Cells were removed from culture flasks for seeding on metal disks using trypsin/EDTA (Gibco). Disks were placed in a 12-well plate culture dish and culture media containing 40,000 cells (~ 100 μl) were pipetted on each disk. Additional culture media (~ 1 ml) was added to each well to cover disks completely, and samples were placed inside the incubator for 24 hours. Cells cultured on disks were transiently transfected with a GFP-vinculin construct (kindly provided by B. Geiger, Department of Molecular and Cell Biology, The Weizmann Institute of Science, Rehovot, Israel) using Optifect™ transfection reagent (Invitrogen). The transfection procedure was followed according to the manufacture’s protocol.
5.2.3 **Reactive Oxygen Species (ROS) detection, nuclear staining, and viability assay**

Image-IT™ LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Molecular Probes®) was used to monitor release of intracellular ROS at different voltages. A stock solution (10 mM) of 5-(and-6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA), a fluorogenic marker for ROS in live cells, was prepared. Cells cultured (100,000 cells per disk) on CoCrMo disks for 24 hours were washed with Hank’s Balanced Salt Solution (HBSS, Gibco) supplemented with Ca and Mg ions once, and a 25 μM solution of carboxy-H₂DCFDA stock was added to each disk and incubated for 30 minutes at 37° C while being protected from light. During the last 5 minutes of incubation, 1 μl/ml of Hoechst in HBSS solution was added to the well containing the sample. The sample was washed three times with HBSS afterwards and was mounted in the electrochemical chamber for subsequent voltage application and imaging. Cells at selected voltages were additionally stained with Calcein AM and Ethidium homodimer-1 (LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells, SKU# L-3224, Invitrogen) according to the previously reported procedure to assess cell viability at the end of polarization test [16].

5.2.4 **Electrochemical setup**

A custom-made electrochemical chamber was used in order to apply anodic and cathodic voltages to CoCrMo disks. A glass bottom Petri dish (50 mm dish, 30 mm glass No. 0 thickness, MatTek corporation) was adapted to construct the electrochemical chamber, where the metal sample served as the working electrode, an Ag/AgCl wire as reference and a Pt wire as the counter (Fig. 5.1). A hole was introduced in the top lid of the glass bottom dish to allow for a connection peg made from the same CoCrMo alloy to go through and fit the hole at the back of
the disk tightly. The CoCrMo disk in the 12-well culture dish was mounted into the electrochemical cell before imaging as follows: First, the Pt wire was placed inside the glass-bottom dish and the dish was filled with 3 ml of culture media. The dish was tilted gently from side to side so that the media covers all the dish surface and the Pt wire. The metal disk, having been kept 48 hours in the incubator post-transfection, was removed from the well, inverted and connected to the peg, and the lid was placed on the glass-bottom dish. The length of the connection peg was slightly shorter (few hundred microns) than the height of the glass-bottom Petri dish leaving space between the surface of the CoCrMo disk and the bottom of the dish in order to prevent any damage to cells and allow for unimpeded ionic and molecular transport.

![Fig. 5.1: Schematic of the custom-made electrochemical cell used for live-cell imaging. A glass bottom dish was adapted to build standard three-electrode system with working (CoCrMo sample), reference (Ag/AgCl wire) and counter (Pt wire) electrodes. A connection peg, going through the lid of the glass bottom dish, was made from the same CoCrMo alloy to fit tightly inside a hole at the back of the sample providing electrical connection.]

5.2.5 Time-lapsed fluorescence microscopy

The glass-bottom dish were then placed inside a stage incubator, where temperature was controlled at 37° C and 5% CO2 air was purged through the chamber via a reservoir filled with
Milipore water to keep the chamber moist and the pH constant. The voltage of the disks were potentiostatically held at -400, and +500 mV(Ag/AgCl) for up to 24 hours or until morphological signs of loss of viability were observed. Cells stained with Hoechst and DCF were additionally polarized to -1000 mV(Ag/AgCl). No external voltage was impressed on the control samples, which were left at the open circuit potential (~ -150 mV(Ag/AgCl)). Images were acquired at 3-minute intervals using a Leica DMI 6000B inverted microscope equipped with an Andor Luca-R camera and a Leica EL6000 external light source with FITC and DAPI filter sets. Micro-Manager 1.4 imaging software (NIH) was used to control illumination shutters, camera exposure and image acquisition.

5.2.6 Analysis of cellular dynamics

Analyses of the cell area, polarity, movement, number of the focal adhesions (FA) and their size (total projected area per cell) of the GFP-vinculin transfected cells at each time point was performed with Imagej software (NIH). Imagej was also used in analyses of size (projected area), circularity, and movement of nucleus, as well as measurement of the levels of ROS-induced fluorescence intensity at different voltages. QuimP11 plugin (T. Bretschneider and R. Tyson) for Imagej was used for semi-automated segmentation and tracking of fluorescent cells and deriving the desired parameters (cell area, polarity, movement). Cell movement at each time point was defined as the speed at which the cell centroid moved between two consecutive frames. Polarity (circularity) was defined by the following equation: \( \frac{4\pi \cdot \text{Area}}{\text{Perimeter}^2} \). A value of 1 reveals the cell’s outline to be perfectly circular. The same plugin was used to quantify the temporal changes in nucleus size, circularity, and movement. Quantification of number and total projected area of focal adhesions were performed via a Fast Fourier Transform (FFT) band pass filtering, and
subsequent thresholding as previously reported [16]. For measurement of fluorescence intensity associated with intracellular ROS generation, Time Series Analyzer v. 2 plugin (J. Balaji) was used. Between 7-14 cells were used to calculate the cellular dynamics and quantify the number and size of focal adhesions at different surface potentials. As for the nucleus and ROS release analysis, at least 50 cells, picked randomly, were analyzed.

5.2.7 Statistical analysis

Results are presented as means ± standard deviation (SD). One-way ANOVA on ranks (Kruskal-Wallis test) and Dunn’s pair-wise comparison method were performed (SigmaPlot v. 11) to compare different time points of each voltage temporal treated group with P-value of 0.05 indicating significance.

5.3 Results

We previously showed how a range of physiologically relevant voltages (-1000 < V < +500) affect cellular behavior after 24 hours of preosteoblast culture on the surface of a polarized CoCrMo alloy. It was observed that anodic and cathodic voltages outside the voltage viability range of CoCrMo alloy (-400 < V < +500) can lead to necrosis and apoptosis respectively [16]. In this work, we examined the temporal changes and dynamics of surface potential-cell interactions at selected cathodic and anodic voltages (-1000, -400 and +500 mV(Ag/AgCl)) and compared them with the dynamics of cells cultured at OCP.

5.3.1 Temporal changes in adhesion, cell size, polarity, and movement

Representative time-lapse images of MC3T3-E1 cells on CoCrMo surfaces at OCP and polarized at -400 and +500 mV(Ag/AgCl) are shown in Fig. 5.2 - 5.4 respectively. GFP-vinculin is mainly
localized around the perinuclear area and in the focal adhesions on the periphery of the cells. Cells exhibit the typical polarized (asymmetrical) form observed on rigid surfaces.

Electrochemical polarization leads to loss of cell viability, while cells cultured at OCP remain viable within the same period. Moreover, unlike cells on surfaces polarized at +500 mV(Ag/AgCl), which exhibit a sudden death, cell death at -400 mV(Ag/AgCl) is preceded by loss of focal adhesions and cell shrinkage.

Fig. 5.5 show the variation of the projected cell area, its circularity, and movement (displacement speed) at -400, +500, and OCP over time. Projected cell area can indirectly serve as a measure of overall pre-osteoblast viability on a surface [16, 17]. Circularity and speed provide information on morphology/polarity and motility of cells on a surface. Transiently transfected pre-osteoblasts on CoCrMo samples, 48 hours after the transfection, show an average projected area of about 2000 ± 1000 μm² with average polarities of 0.6 ± 0.1 and displacement speeds below 0.3 μm.min⁻¹. Application of a voltage of -400 mV(Ag/AgCl) causes cells to undergo a gradual decrease in size and shrink to below 500 μm² after 22 hours. The cells at this point are believed to be non-viable, and the fastest rate of cell shrinkage occurs at 13th hour. On the other hand, the anodic voltage of +500 mV(Ag/AgCl) led to cell death over a period of 5 hours, during which no significant change in cell size was observed right before the loss of viability. In both these cases, cell death was indicated from a major rounding up at the time of death, and formation of membrane blebs. However, cells treated at +500 mV(Ag/AgCl) showed morphological features associated with necrosis. The initial circularity of cells on samples at -400, +500, and OCP is around 0.6 ± 0.1. Later on, in contrast to the cells at OCP, which maintain the same degree of circularity, circularity of cells on polarized surfaces show a varied time profile. At -400 mV, cells begin to grow increasingly apolar 5 hours after the start of polarization reaching
circularities of about 0.83 after 7 hours and drop to the the initial value of 0.6 again after 10 hours. From this point on, the circularity goes up again and a broader distribution of cell circularity is observed. At +500 mV, cell circularity stays at the starting value of 0.6, but shows a broader distribution after about 3 hours post-polarization. Cell movement on samples polarized to -400 mV (0.15 ± 0.11 μm.min⁻¹), +500 mV (0.17 ± 0.14 μm.min⁻¹), and OCP (0.15 ± 0.1 μm.min⁻¹) show an almost identical constant profile with time. On the other hand, size and number of focal adhesions on the polarized surfaces and the samples left at OCP show significant differences (Fig. 5.6). While the number and total area of focal adhesions remain relatively constant for cells at OCP, cells on the polarized samples start losing their FAs after the start of polarization. The point of decreased adhesion (based on significant drop in the number and total area of FAs) occurs after 12 hours on the sample polarized to -400 mV, whereas for the sample at +500 mV, no significant loss of FAs is observed until about 5 hours after polarization, when a sudden loss of viability is observed. The similar time profile of number and total projected area of focal adhesions indicates that the size of each FA remains constant throughout the observed time.

A comparison of the temporal changes in cell area, number of focal adhesions and their size at -400 mV(Ag/AgCl) and +500 mV(Ag/AgCl) indicate that there are different mechanisms involved in the observed biological response. At +500 mV(Ag/AgCl), cells are rendered non-viable after about 5 hours showing a sudden cell shrinkage and loss of adhesion (Fig. 5.4). Cells cultured at -400 mV(Ag/AgCl), however, show a gradual loss of adhesion, cell shrinkage and ultimately rounding up (Fig. 5.3), which corresponds with the lower current levels measured at -400 mV(Ag/AgCl) (~ 0.4 μAcm⁻²) compared with +500 mV(Ag/AgCl) (~ 4.1 μAcm⁻²).

Similarly, prior to cell death at +500 mV(Ag/AgCl), cell size (projected area) does not change
significantly throughout polarization time, whereas at -400 mV(Ag/AgCl) a gradual decrease in cell size is observed. In both cases, polarization causes a reduction in the number of focal adhesions and nucleus size.
Fig. 5.3: Time-lapse images of MC3T3-E1 pre-osteoblasts transiently transfected with GFP- vinculin on CoCrMo alloy and polarized to -400 mV(Ag/AgCl). Cell gradually loses its focal adhesions and retracts until finally balls up and dies. Note the formation of retraction fiber as the cell balls up.
Fig. 5.4: Time-lapse images of MC3T3-E1 pre-osteoblasts transiently transfected with GFP-vinculin on CoCrMo alloy and polarized to +500 mV(Ag/AgCl). Note the sudden loss of adhesion and formation of blebs after 5 hours post treatment.
Fig. 5.5: Graph of average projected cell area (left column) cell circularity (middle column), and speed (right column) of MC3T3-E1 pre-osteoblasts on CoCrMo disks at OCP (top), and polarized at -400 mV(Ag/AgCl) (middle) and +500 mV(Ag/AgCl) (bottom) vs. time. Linear regression analysis of the data shows a non-zero slope for the area vs. time graph at -400 mV(Ag/AgCl) (p<0.05) indicating a gradual decrease with time. No statistically significant increasing or decreasing trend was obtained for the other parameters vs. time.
Fig. 5.6: Graph of average number and the corresponding total projected area of focal adhesions in transiently transfected MC3T3-E1 pre-osteoblasts on CoCrMo disks polarized at -400, +500 mV(Ag/AgCl) and OCP vs. time. Linear regression analysis of the data shows a non-zero slope for the area vs. time graph at -400 and +500 mV(Ag/AgCl) (p<0.05) indicating a gradual decrease with time. Note a shorter time scale for +500mV(Ag/AgCl) treatment, which due to the cell death after 5 hours.
5.3.2 Temporal changes in nucleus size, circularity, and movement

The nuclei of cells cultured on CoCrMo alloy samples for 48 hours show a projected area of 359 ± 51 μm² (Fig. 5.7). Upon application of voltage, a gradual shrinkage in nucleus size is observed at -1000 and +500 mV(Ag/AgCl). Cells cultured on samples left at OCP or at a voltage of -400 mV(Ag/AgCl) show a slight decrease in nucleus size over a time period of 10 hour (Fig. 5.7). The decrease in nucleus size is most significant at -1000 mV, where nucleus size drops to 20% of its initial size only 15 minutes after application of voltage. In addition, the morphology of nuclei changes from round shape with circularity close to 1 to a more elongated and irregular shape (Fig. 5.7). No morphological changes are observed in the shape of nucleus of cells cultured at OCP, -400, or +500 mV(Ag/AgCl) after 200 minute polarization. Similarly, the speed of nucleus displacements is below 0.3 μm.min⁻¹ and maintains the same level at OCP, -400, and +500 mV(Ag/AgCl). At -1000 mV, however, higher speeds are observed as the nucleus rapidly shrinks in size.

5.3.3 Determination of ROS release and cell viability

Measurement of the fluorescence intensity associated with the generation of intracellular ROS, did not show any change above the background levels at any of the voltages applied (-1000, -400, +500 mV(Ag/AgCl)) or at OCP. Exposure of the cells to the ultraviolet light (λ = 340 nm), however, induced generation of ROS after 8 hours on samples polarized at +500 mV(Ag/AgCl) (Fig. 5.8). A similar effect was observed for cells treated at -400 mV(Ag/AgCl) (not shown). Post staining of the samples using Live/Dead assay showed non-viable cells at -1000 and +500 mV(Ag/AgCl) after half hour and 3 hours (Fig. 5.9a and 5.9c), respectively, whereas cells polarized at -400 mV(Ag/AgCl) for 24 hours remained viable (Fig. 5.9b).
Fig. 5.7: Graph of average nucleus size (projected area) (left column), circularity (middle column), and speed (right column) of MC3T3-E1 pre-osteoblasts on CoCrMo disks polarized at -1000 (top), -400 (middle) and +500 mV(Ag/AgCl) (bottom) vs. time. A gradual decrease in the nucleus size is observed (p<0.05) for all the voltage treated groups. Temporal changes in the circularity and speed of the nucleus is only significant (p<0.05) at -1000 mV(Ag/AgCl).
Fig. 5.8: Image of pre-osteoblasts cultured on CoCrMo polarized at +500 mV(Ag/AgCl) while simultaneously exposed to ultraviolet light (\(\lambda = 340\) nm) for three hours in a single spot (d). Cells were stained to reveal nuclei and generation of intracellular ROS, which is indicated by green fluorescence. Note that while the cells present at the UV-lit area generate ROS, voltage-treatment has not caused significant generation of ROS molecules.
5.4 Discussion

The effect of electrochemical polarization of CoCrMo surfaces on the temporal changes in the number and size of focal adhesions of cells cultured on these surfaces was investigated. In addition, the corresponding changes in cell and nucleus morphology and release of ROS was studied. Our results show that depending on the surface potential of the metal, the loss of adhesion and cell death can occur as quickly as 15 minutes after polarization at -1000 mV.
mV(Ag/AgCl) or hours later at +500, and -400 mV(Ag/AgCl). The observed cellular response strongly correlates with the electrochemical current levels measured at the interface, and is in agreement with our previous findings that there is a current threshold associated with loss of cell viability [16,17]. Larger currents increase the magnitude of the redox and other reactions occurring at the polarized interface and lead to a faster cellular response. In addition, magnitude of the voltages impressed on the metal and whether they are cathodic or anodic determines the type of reactions that can occur on the surface.

On the other hand, we did not observe a significant change in the amount of intracellular ROS molecules generated at cathodic or anodic voltages. This was not exactly expected as reduction of oxygen and generation of hydrogen peroxide should readily happen at -1000 mV(Ag/AgCl) at the metal/electrolyte interface from a thermodynamics point of view [15]. Likewise, at +500 mV, release of Cr^{6+} and its subsequent intracellular reduction could induce DNA damage and cell stress, triggering ROS generation [34]. ROS molecules act as a two-edged sword being lethal to cells at high concentrations while playing regulatory roles at low concentrations. Considering the permeability of cell membrane to hydrogen peroxide, one might expect to observe a rise in the level of intracellular ROS accordingly. Cell stress can also lead to production of intracellular ROS. On the other hand, signs of cell stress including an abrupt drop in nucleus size were evident at -1000 mV within 10 minutes and a subsequent Live/Dead assay showed a completely non-viable state of the cells treated at this voltage post treatment (Fig. 5.9a). Therefore, the absence of elevated levels of intracellular ROS at cathodic and anodic voltages in this work indicates that there must be other processes involved which lead to cell death before intracellular ROS generating mechanisms become activated. In other words, the scale of injury to the cell due to polarizing the sample at this voltage (current densities around 30 μAcm^{-2}) must have been so
pronounced that cells died through a different mechanism. The exact nature of these processes will be the subject of future studies. On the other hand, at -400 mV(Ag/AgCl), average current densities (below 0.4 μAcm⁻²) may not have been large enough to cause a measurable change in the amount of ROS molecules generated. It is possible that disruption of Ca⁺² entry into cells plays a major role in the observed response of cells cultured on polarized metallic surfaces.

Considering the presence of different types of Ca⁺² channels on the cell membrane including the voltage-gated Ca⁺² channels, modulation of these channels as a result of surface polarization seems likely. Changes in the normal influx of Ca⁺² into cells even at non-disruptive levels could have adverse effects including cell proliferation, differentiation and modulation of apoptosis. It is, in fact, believed that Ca⁺² dependent processes are closely intertwined with mainstream apoptosis executioners (caspases) [35]. Similarly, it will be worthwhile to investigate how redox reactions occurring on polarized metallic surfaces may modify different proteins, which have important regulatory function in cell adhesion and survival. Focal adhesion kinase (FAK), an important component of the focal adhesion complex, and the pro-apoptotic death associated protein (DAP) kinase are examples of such proteins.

Finally, it should be mentioned that the cellular response observed in this work cannot solely be attributed to the effect of surface polarization, and there are other factors that may contribute to the overall behavior observed. The GFP-vinculin produced in the cell as a result of transfecting cell with a synthetic DNA, for instance, is not identical to the wild type vinculin, and can modify the cell behavior. In addition, depending on its concentration, GFP-vinculin can have cytotoxic effects if expressed in large quantities. Combined with the phototoxicity effect, which is always present in fluorescence imaging to a greater or lesser extent, the actual time to death at any voltage can be reduced as a result. To what extent phototoxicity contributes to the observed
cellular behavior depends on the type and concentration of the fluorescent probes utilized and imaging parameters such as illumination intensity and the interval of image acquisition. This explains the discrepancy in the time-scale of loss of viability in the transiently-transfected cells and those stained with Hoechst at -400 mV(Ag/AgCl). While all the transfected cells are completely rounded up and non-viable after about 13 hours post-polarization, cells stained with Hoechst remained viable up to 24 hour within the same time at this voltage (Fig. 5.9b). Time-lapse microscopic imaging serves as a powerful tool for investigations into different aspects of cell behavior such as motility, migration, intracellular protein trafficking, and cell signaling, and adhesion among others. It provides invaluable hints for delineating the mechanisms of cell response to different stimuli. It was found in this work that the loss of viability outside the voltages viability range for CoCrMo alloy occurs at different time scales on the anodically vs. cathodically polarized surfaces. Furthermore, right before the major morphological changes associated with the loss of cell viability (loss of adhesion, cell shrinkage), there is no difference between the motility of cells on polarized vs. non-polarized surfaces. The time between the onset of cell death indicated by the sudden rounding up or formation of blebs in the membrane and the complete cellular arrest only takes few minutes. This shows that in order to pinpoint the exact mechanism of interaction between cells and polarized metals, the sequence of intracellular events happening such as release of regulatory and signaling molecules and proteins should be brought into light. This might require using different plasmids, which express the fluorescent protein of interest. Studies into the release of fluorescent Cytochrome c or Caspases, which initiate the process of cell death should be considered in this regard.
5.5 Conclusions

Time-lapse study of transiently transfected MC3T3-E1 pre-osteoblasts with GFP-vinculin on CoCrMo alloy polarized at -400 and +500 mV reveals a reduction in the number and total area of vinculin-rich focal adhesions at -400 mV with time. At the anodic voltage viability edge (+500 mV), there is no significant change in either the number or total projected area of focal adhesions right before cells die at this voltage after 5 hours. Similarly, the projected cell area follow the same trend showing a gradual decrease with time at -400 mV, while maintaining the same size right before cell death at +500 mV. Surface potential influence nucleus size of cells cultured on samples polarized at -1000 mV within 15 minutes post-polarization leading to an abrupt shrinkage (80%) of nucleus and alteration of morphology (circularity). Generation of ROS molecules, which is hypothesized to be partially responsible for the observed cell response on polarized metals, was not observed to be the case at any of the voltages investigated in this work. This shows that there might be other processes involved in the observed loss of adhesion and cell death outside the voltage viability range of CoCrMo.
5.6 References


6. Synthesis

6.1 Overview

The aim of this section of the dissertation is to put the findings of this work into perspective by highlighting the importance of the study of cellular behavior on polarized metallic alloys, and to remark on how this work contributes to bringing different aspects of cell-polarized metal interactions to light.

Metallic implants have been used to repair human body parts and restore their function since ancient times. However, similar to other artificial materials used in the body, they may invoke undesired reactions compromising the biological functions of cells and tissues as well as the integrity of the implant. There are several physicochemical properties (e.g. shape, size, surface chemistry, morphology and porosity) of the metallic biomedical devices that determine the type and magnitude of their interactions in vivo. The effect of surface potential and the subsequent Faradic reactions that ensue on metallic surfaces is an area left largely unexplored. Considering the multitude of the redox reactions occurring in the physiological systems that involve charge transfer, it is worthwhile to study how the Faradic reactions on metallic implant surfaces may influence these reactions and the biological system. The fact that voltage shifts on the surface of metallic implants can happen due to micromotions and/or physiologically relevant phenomena provides further motivation. Moreover, findings of such studies could shed light on mechanisms of failure in metallic biomedical devices and may suggest ways to improve their biocompatibility and function.

6.2 Discussion

In this work, we observed how cells cultured on metallic surfaces (CoCrMo and anodized cpTi) respond to changes in the surface potential (voltage). Upon application of voltage to an oxide-
covered metal, a sequence of events occur which ultimately lead to the observed cellular
response. This work and the similar works performed previously on other biomedical alloys tried
to determine the specific nature of these events and their time scales.

Referring to the conceptual model discussed in chapter 1, polarization modifies the passive oxide
film of the metal, may bring about redox reactions in the solution/cell and trigger a biological
response. This work answered some specific questions regarding the events taking place at the
metal oxide/solution/cell interface when the metal is polarized.

In chapter 2, we examined the changes in the charge transfer resistance (polarization resistance),
capacitance, and the degree of ideality of the oxide layer at different surface potentials (Fig. 2.3).
Three voltage regions were identified within the the studied voltage range from -1 to +0.6
V(Ag/AgCl) range: a region of high polarization resistance (high impedance) in between of two
regions with low polarization resistance. The low polarization voltage range in the cathodic
region is due to the thinning of the passive oxide layer due to the reduction of this layer, whereas
the low polarization voltage range in the anodic region starts at above +0.5 mV(Ag/AgCl) when
the oxide becomes unstable and loses its integrity as it nears the transpassive voltage. The higher
electrochemical currents at these low polarization resistance regions correspond well with the
drop in the viability of cells cultured at these voltages (Fig. 3.3). The drop in the impedance of
the oxide layer at the extremes of the voltage range (-1 and +0.5 V(Ag/AgCl) may also be the
reason for an increased amount of the chromium ions (main constituent of the passive oxide
layer) released from the surface at these voltages compared with OCP (Fig. 3.5). In particular,
the increased chromium ion concentration at -1 V is interesting as due to the highly reductive
nature of this voltage, release of chromium ion is not expected to exceed that of the OCP
condition. As for non-Faradic effects, charging can occur in different regions across the
interface. It is believed that except for extreme cathodic voltages, changes in the capacitance are dominated by the passive oxide layer. Capacitance of oxide may be due to changes in dielectric constant, thickness of the oxide or its roughness. It is not clear, though, that how much each of these factors contribute to the measured capacitance at each voltage. The estimated oxide thickness at different voltages, therefore, might be slightly different from the actual values as we assumed the changes in the capacitance are solely due to the changes in the oxide thickness.

In chapter 3 it was found that there is a voltage viability range above -400 mV(Ag/AgCl) and below +500 mV(Ag/AgCl) for CoCrMo alloy. This shows that cells only remain viable if the potential of the surface of CoCrMo alloy stays within a lower cathodic (-400 mV(Ag/AgCl) and an upper anodic voltage (+500 mV(Ag/AgCl) range in a 24 hour time period. Outside of this voltage range, the reduction and oxidation reactions will induce cell damage. This is the first report of existence of a voltage viability range for cells cultured on CoCrMo biomedical alloy. Aizawa et al. previously reported a voltage viability range for HeLa cells cultured on ITO electrodes, which bear some resemblance to the voltage range obtained in this work. They reported cell swelling and detachment at voltages below -200 mV(Ag/AgCl) and a gradual cell shrinkage and death above +400 mV(Ag/AgCl) leading to complete cell death within an hour at +1200 mV(Ag/AgCl). These findings emphasize the importance of the Faradic reactions in affecting the behavior of cells cultured on polarized metals. The comparable voltage viability range observed hints at the presence of possible common redox reactions of the constituents of the cell membrane or the physiological solution at the edges of the cathodic or anodic regions. The exact nature of these reactions are unknown and should be investigated in future efforts. What is known from this work based on the levels of electrochemical currents at different voltages is that there is a direct correlation between the magnitude of the Faradic reactions and
the degree of cell damage. Higher current levels, associated with higher rates of Faradic reactions cause more damage to cells. More interestingly, at voltages just below the cathodic edge of the viability range the current threshold that was observed to cause loss of viability is well below 1 μAcm$^{-2}$.

The effect of the voltages outside the viability range was further assessed on the mode of cell death and the adhesion behavior and cytoskeletal integrity at these voltages. Interesting differences in the mode of cell death were observed at cathodic vs. anodic voltages (apoptosis or necrosis). Apoptosis is described as programmed cell death mediated by internal signaling pathways with no or minor inflammation, whereas necrosis occurs due to sudden injury or insult to the cell and leads to plasma membrane disruption, release of intracellular content and ultimately inflammation. This finding is important with regard to applications intended for cancer therapy to induce cell death in the tumor site for example. Ideally, any therapy that involves induction of controlled and localized cell death as part of its remedial principle should bring about death via apoptosis rather than necrosis in order to minimize the inflammatory response.

Another interesting aspect of this work is drawing the attention to the possible effects of voltages within the viability voltage range that are not lethal to cells (-400 mV < V < +500 mV). A lower number of focal adhesions on the surface polarized at +300 mV(Ag/AgCl) was an example of voltage effect in reduction of cell adhesion. Other interesting changes could be induced in the cells cultured on metallic biomedical devices through application of controlled voltages. Therefore, the study of voltage effects on the cell behavior within the voltage viability range of CoCrMo or other alloys merits a further investigation. These efforts will constitute a major step in generating knowledge required for the design of smart biomedical devices in the future.
The motivation to study the effect of cathodic voltages on the behavior of cells cultured on anodized cpTi was the effect of surface oxide thickness in limiting the rate of Faradic currents. The study of cell behavior on bare vs. anodized titanium (chapter 4), which has a thickened oxide layer provides a basis to apply a same voltage to two surfaces of oxide-covered cpTi (native oxide and grown oxide) but cause different currents to flow. The hypothesis was that voltage is a key parameter in determining what redox reactions could take place on the surface, whereas the currents are a measure of the rate of those reactions. In fact, the results of this chapter further confirmed the role of Faradic reactions as the dominating factor in the observed cell response. From the findings of this chapter, it is implied that an increased oxide layer only delayed the effect of the voltages harmful to cells. This was due to lower average currents in the 24 hour period of polarization. The average currents above the threshold current of 0.1 μAcm⁻² affected the cells in the same way regardless of the culture on the bare or anodized surface. This suggests that whatever redox reactions occurring at -400 mV on the bare cpTi surface must still be present on the polarized anodized surface but at a lower magnitude. This is indicated from the higher impedance of the anodized surface at -400 mV and the corresponding lower currents.

What is missing from the general picture of interactions between polarized metallic alloys and cells cultured on them up to this point is the time scale of the cell response at different anodic and cathodic voltages. In chapter 5, the time scale of the voltage effects on cells were the focus of attention. Time together with the voltage and current magnitudes constitute the three major interrelated factors of a triangle that inform the cell behavior on polarized metals (Fig. 6.1). A major difference was found in the sequence of events leading to cell death at cathodic and anodic voltages out of the viability range, which further confirms different course of actions caused by reduction vs. oxidation reactions. While at -400 mV(Ag/AgCl) a slow gradual reduction in cell
size and loss of adhesion proceeds cell retraction and rounding up within 24 hours, at $+500$ mV(Ag/AgCl) the onset of death occurs suddenly without a significant change in the cell adhesion prior to cell death.

Fig. 6.1: The interrelated factors influencing the cell response on polarized metals.

Considering these three factors, the voltage viability range of the metallic alloys can be extended or narrowed depending on the the culture time or exposure times, and surface oxide resistance towards charge transfer.

Fig. 6.2: Hypothetical voltage viability graph as a function of time.
It stands to reason that there is a voltage viability range that is independent of time. In other words, a narrower voltage range within the range found here, as the viability range might exist that does not affect cell viability regardless of the culture time (Fig. 6.2). Nonetheless, other aspects of the cellular behavior may still be modulated in a time-dependent manner.

6.3. Suggestions for future work

This work built upon the findings reported previously on how polarized surfaces can affect the electrochemical properties of the metallic implants and the resulting cellular behavior on these surfaces. Although it provided new insights into how surface voltages can affect the electrochemical properties of CoCrMo alloy and influence the cell behavior, it brought about many more questions which need to be answered in future efforts. Particularly, a great deal could be explored regarding the fundamental cellular and molecular principles governing the cell behavior observed in this work.

In the study of voltage effects on the electrochemical properties of the CoCrMo alloy in chapter 2, only static DC voltages were applied in a step-wise manner. Considering the complexity of the surface potential profile that the metallic biomaterials may undergo in vivo, it is beneficial to explore application of other voltage profiles to these surfaces. These profiles should ideally represent the fluctuating nature of the surface potentials that might occur on articulating surfaces as a result of disruption of the surface oxide. In addition, other simulated body fluids apart from PBS, used in this work could be utilized. Another aspect that is missing from this work is an investigation into the changes in the surface chemistry of the CoCrMo alloy as a result of voltage changes. X-ray photoelectron spectroscopy (XPS) is one method that could be utilized in this
regard. Using this method, the oxide chemistry across the thickness of the oxide and presence of electrochemically formed products at the interface can be determined.

The electrochemical properties measured in this work reflect an average response of the surface due to the heterogeneous nature of the oxide. It is suggested to employ micro-impedance techniques to limit the area under investigation to a feature of interest (single grain, grain boundary, etc).

Study of voltage effects on the cellular behavior (chapter 3) is an area with a lot of potential for further investigation. Study of the molecular basis of the cellular response observed on polarized surfaces is a case in point. In this work we confirmed activation of an apoptotic pathway at cathodic voltages below -400 mV(Ag/AgCl) on the surface of CoCrMo alloy. There is an array of other biological assays to identify apoptosis and distinguish it from necrosis that could act as complimentary methods to provide further confirmation and to delineate the specific pathway that is activated. In addition, there are other aspects of cell response such as a modified gene expression profile, changes induced in the proteins present in the membrane or in the cytosol that can be additionally studied. RNA lysis of the voltage-treated cells and use of PCR arrays that can detect changes in the gene expression for multiple genes simultaneously is one of the ways to proceed with this effort.

On the other hand, the focus of this work was on the voltages outside the viability range of the cells at relatively short time (24 hours). A longer cell culture study on the surface of CoCrMo alloy at voltages within the viability range can provide clues on how to modulate the cell behavior in order to induce a desired effect without the loss of viability as have been reported by others in culture studies of several different cell lines on ITO electrodes. The results of such
studies could provide invaluable clues on the design of smart biomedical devices which can purposefully control cell behavior.

Also, it is worthwhile to consider use of other cell lines in the voltage studies as the implants will be exposed to other cell types like macrophages. Voltage could also show an effect on controlling the differentiation path of stem cells, opening a deluge of other possibilities in terms of tissue engineering applications.

The cell culture study on bare and anodized cpTi under cathodic polarization (chapter 4) showed the relative protective effect of the increased oxide layer on the anodized metal, which diminishes the harmful effects of the cathodic reactions on cell viability. It would be very interesting to investigate how cells react to patterned anodized surfaces under cathodic polarization. Our preliminary results did not reveal a significant difference in the behavior of the cells on such patterned surfaces compared with the bare ones within 24 hour post culture and voltage treatment. However, this should be studied in more details as any interaction with a patterned surface may occur at longer culture times or higher cell densities.

In the live-cell studies of this work (chapter 5), a number of other plasmids could be used to study the effects of voltages on other cellular compartments such as mitochondria, endoplasmic reticulum, etc. Furthermore, double-transfection could be tried to study two proteins simultaneously. Examples of plasmids for this purpose would be GFP-vinculin and mcherry actin to study the mechanisms involved in the adhesion behavior and cytoskeletal changes.

Moreover, the release of extracellular and intracellular ROS should be investigated at a wider range of voltages. In this work, only two voltages were studied, and the results showed a
discrepancy with others. A more comprehensive study of ROS release could confirm the results obtained here or bring the shortcomings of this work to light.
7. Conclusions

The overall objective of this work was to expand on the previous efforts carried out investigating the effect of Faradic currents on the behavior of cells cultured on polarized metallic biomedical alloys. Furthermore, the observed cellular response was correlated to changes in electrochemical properties of these surfaces. In order to study the electrochemistry as well as the cell biology taking place at these interfaces new techniques and methods were developed or employed and the following conclusions were drawn:

- Potential step current transient impedance spectroscopy method was used to assess the voltage-dependent impedance of CoCrMo surfaces. CoCrMo surface oxides have a voltage-dependent impedance behavior that reflects the properties of the passive oxide film (polarization resistance, capacitance, and degree of non-ideality). The oxide film had several voltage ranges where different behavior was observed that may reflect changes in the thickness and chemistry of the oxide.

- A new time-domain “generalized Cauchy-Lorentz” function that provides an ability to better fit current transient data and to calculate impedance values from these data was developed. The GCL function was derived using a Tafel element and capacitive discharge behavior, but is useful as a current transient function for impedance behavior.

- There is a voltage range from -300 mV to +300 mV(Ag/AgCl) where cells remain viable on CoCrMo alloy. For cathodic voltages outside the viability range cells die through an intrinsic apoptotic pathway, based on the release of Caspase 3 and 9. For anodic voltages outside the viability range, cell death occurs via necrosis based on morphological
hallmarks, including swollen cell body, and membrane damage. The current densities that define the limits of viability are 0.1 μAcm$^{-2}$ and 10 μAcm$^{-2}$ for cathodic and anodic limits, respectively.

- Metal ions were released in larger amounts at +500 mV for all ions (Co, Cr, Mo, Ni) and at -1000 mV for Cr ions. These ions likely played a significant role in necrosis, but not in the cathodic range.

- Adhesions complexes for cells on voltage-controlled surfaces (at all voltages) were fewer (and of less area) than cells on OCP surfaces. Reduced number and size of adhesion complexes at +300 mV indicates altered cell behavior within the viability range of the alloy surface.

- Nucleus size is a function of voltage with smaller projected areas outside the viability range compared to nucleus size of cells cultured at OCP. Nuclei of cells at the cathodic voltages outside the viability range are smaller than the nuclei of cells treated at +500 mV.

- The cathodic limit of the voltage viability range for bare cpTi is at around -400 mV(Ag/AgCl), and anodization shifts it by about 100 mV into more cathodic voltages. Nevertheless, cells show signs of distress on the anodized surface at -400 mV despite showing high cell viability.

- Lesser numbers and smaller sizes of adhesion complexes on the anodized surfaces at -400 mV compared with cells cultured at OCP (p< 0.05) are indicative of the unfavorable effect of this cathodic voltage on cell adhesion. At a higher cathodic voltage of -500 mV,
cells were rendered non-viable with a rounded up morphology and numerous actin retraction fibers on both bare and anodized surfaces.

- The observed cellular response at cathodic voltages correlates well with the electrochemical current levels, where average currents above -0.1 μAcm\(^{-2}\) over 24 hours significantly affect cell behavior. Anodized surfaces were less susceptible to the effect of the cathodic voltages due to lower electrochemical current levels and hence limited the magnitude of harmful redox reactions on cells at these voltages.

- Impedance spectra of the bare and anodized samples showed a significant drop of impedance on bare surfaces at cathodic voltages, whereas the drop in impedance was not as dramatic in anodized samples and even increased when polarized at -400 mV(Ag/ACl) for 24 hours.

- Time-lapse study of transiently transfected MC3T3-E1 pre-osteoblasts with GFP-vinculin on CoCrMo alloy polarized at -400 and +500 mV reveals a reduction in the number and total area of vinculin-rich focal adhesions at -400 mV at different time scales.

- At the anodic voltage viability edge (+500 mV), there is no significant change in either the number or total projected area of focal adhesions right before cells die at this voltage after 5 hours. Similarly, the projected cell area follow the same trend showing a gradual decrease with time at -400 mV, while maintaining the same size right before cell death at +500 mV.

- Surface potential influences nucleus size of cells cultured on samples polarized at -1000 mV within 15 minutes post-polarization leading to an abrupt shrinkage (80%) of nucleus and alteration of morphology (circularity).
• Generation of ROS molecules, which is hypothesized to be partially responsible for the observed cell response on polarized metals, was not proved to be the case at any of the voltages investigated in this work. This shows that there might be other processes involved in the observed loss of adhesion and cell death outside the voltage viability range of CoCrMo.
8. Appendices

8.1 Generalized Cauchy Lorentz (GCL) Model Development

The equivalent electrical circuit with the Tafel element (Fig. 2.1) was solved to obtain the voltage and current equation as follows:

Voltage equation:

\[ i_t = i_0 e^{v_t} - i_0 \]

\[ C = \frac{q}{v_c} \rightarrow i_c = C \frac{dv}{dt} \]

\[ v_c = v_{\text{max}} \quad @ \ t = 0 \]

\[ i_c + i_t = 0 \rightarrow i_c = -i_t \]

\[ C \frac{dv}{dt} = -i_0 e^{v} + i_0 = -i_0 (e^v - 1) \]

\[ \int \frac{dv}{e^v - 1} = \int -\frac{1}{C} i_0 dt \]

\[ b \left( -\frac{v}{b} + \ln \left( \frac{v}{e^b - 1} \right) \right) = -\frac{i_0 t}{C} + k \]

@ \ t = 0 \quad V = V_{\text{max}} \rightarrow k = -v_{\text{max}} + b \ln \left( \frac{v_{\text{max}}}{e^b - 1} \right)

Rearrangement gives:

\[ -(v - v_{\text{max}}) + b \ln \left[ \frac{v}{e^b - 1} \right] = -\frac{i_0 t}{C} \]

The above expression could be simplified to:
\[-(v - v_{\text{max}}) + b \ln \frac{v}{v_{\text{max}}} = -\frac{i_0 t}{C}\]

By assuming $v << b$ and using $e^x \approx 1 + x$

Current equation:

\[v_c = \frac{1}{C} \int_0^t \text{id}t + v_{\text{max}}\]

\[v_t = b \ln \left(\frac{i}{i_0} + 1\right) = b \ln \left(\frac{i'}{i_0}\right)\]

Where $i' = i + i_0$

\[v_c + v_t = 0 \rightarrow v_c = -v_t\]

\[\frac{1}{C} \int_0^t (i' - i_0)dt + v_{\text{max}} = b \ln \left(\frac{i'}{i_0}\right)\]

Differentiating

\[\frac{1}{C} (i' - i_0) = -b \frac{d}{dt} \left[\ln i' - \ln i_0\right]\]

\[-\frac{1}{bC} dt = \frac{1}{(i' - i_0)} d\left(\ln i'\right)\]

**8.2 Laplace Transform of the Cauchy Lorentz Model**

\[Z(\omega) = \int_0^{+\infty} Z(t)e^{-i\omega t} dt\]

\[v = b' \ln \frac{I}{I_0}\]
\[ Z(t) = \frac{dv}{dl} = \frac{b'}{I} \]

\[ I(t) = \frac{I_{\text{max}}}{1 + (\frac{t}{\tau})^k} \]

\[ Z(t) = b \left( 1 + (\frac{t}{\tau})^k \right) \]

\[ b = b' \cdot I_{\text{max}} \]

\[ Z(\omega) = \frac{b}{i\omega} + \frac{b \Gamma(k + 1)}{\tau^k (i\omega)^{k+1}} \]

\[ = -\frac{bi}{\omega} + \frac{b \Gamma(k + 1)}{\tau^k \omega^{k+1} e^{\frac{\pi}{2}(k+1)i}} \]

\[ = -\frac{bi}{\omega} + \frac{b \Gamma(k + 1)}{\tau^k \omega^{k+1}} \left( \cos \frac{\pi}{2}(k + 1) - i \sin \frac{\pi}{2}(k + 1) \right) \]

\[ = \frac{b \Gamma(k + 1)}{\tau^k \omega^{k+1}} \cos \frac{\pi}{2}(k + 1) - i \left( \frac{b}{\omega} + \frac{b \Gamma(k + 1)}{\tau^k \omega^{k+1}} \sin \frac{\pi}{2}(k + 1) \right) \]

If \( k = 1 \)

\[ Z(\omega) = -\frac{2b}{\tau \omega^2} - i \frac{b}{\omega} \]

8.3 R statistical language code written for analysis of the step polarization data and least square non-linear fitting (Newton method)

The input is the DAT file for each voltage step, which will be read into R and fed into a matrix structure. Different parameters are calculated and the best fit of the experimental current graph to the GCL current decay equation is obtained and saved to file. A “for” loop is used to repeat the steps for all the voltage steps.
for(w in 1:40)
{
  data<-read.table(paste("C:/Users/Morteza/Documents/CoCr step pol data/CC4/CC10",w%%10, 
  w %%10,".dat",sep=""),skip=1)
  area=0.2
  I=0; step.time=0
  dv=0.05
  imax=max(data[,2]) /area*1e-6
  for(i in 1:dim(data)[1])
    if(data[i,2]==max(data[,2])) imax.row=i
  ib<-ave(data[1:(imax.row-20),2])/area*1e-6
  ib<-ib[1]
  iinf=data[ave((dim(data)[1]-50): dim(data)[1]),2] /area*1e-6
  iinf<-iinf[1]
  rs=dv/(imax-ib)
  rs<-rs[1]
  rp=dv/(iinf-ib)
  rp<-rp[1]
  for(j in imax.row:(dim(data)[1]))
    {
      I[j-imax.row+1]<-data[j,2] /area*1e-6
      step.time[j-imax.row+1]<- data[j,1]-data[imax.row,1]
    }
  x<-step.time
  y<I
d <- data.frame(x=step.time, y=1)

model <- nls(y ~ imax*m/(x^k+m),d,start=list(k=1.3,m=0.0001))
model2 <- nls(y ~ dv/rs*exp(-(step.time/t0)^n)+dv/(rs+rp)*(1-exp(-(step.time/t0)^n)),d,start=list(n=0.5,t0=0.001))
model3 <- nls(y ~ dist22(alpha),d,start=list(alpha=0.7))

plot(log(x),fitted.values(model),col=5)
par(new=TRUE)
plot(log(x),fitted.values(model2),col=6)
par(new=TRUE)
plot(log(x),fitted.values(model3),col=7)
par(new=TRUE)
plot(log(x),y)

dd<-data.frame(ib,iinf,rs,rp,coef(model),coef(model2))
write.table(dd,file= "parameters.txt ",sep="   ",append=TRUE)
write(coef(model), file = "data1.txt",ncolumns = 2, append = TRUE, sep = " ")
write(coef(model2), file = "data2.txt",ncolumns = 2, append = TRUE, sep = " ")
write(coef(model3), file = "data(alpha).txt",ncolumns = 1, append = TRUE, sep = " ")
write(sum(resid(model2)^2),file="resid.randle.txt",append=TRUE)
write(sum(resid(model)^2),file="resid.HG.txt",append=TRUE)
write(fitted.values(model),file="fittedvalsHG.txt", sep="   ", append=TRUE)
write(sum(resid(model3)^2),file="resid.cole-cole.txt",append=TRUE)
8.4 Kramers-Kronig Transform Visual Basic Macro code

Kramers-Kronig transform is used to check the linearity of the impedance data obtained from the step polarization method.

Private Sub CommandButton1_Click()
    Dim i As Integer
    Dim w(100) As Variant
    Dim Zp(100) As Variant
    Dim Zpp(100) As Variant
    Dim Zppk As Variant
    Dim Zpk As Variant
    Dim Zpk2 As Variant
    For i = 0 To 93
        w(i) = Cells(9 + i, 2)
        Zp(i) = Cells(9 + i, 5)
        Zpp(i) = Cells(9 + i, 6)
    Next i
    ' take Zp and make Z"'
    For j = 1 To 92
        Zppk = 0#
        Zpk = Zp(0)
        Zpk2 = Zp(92)
For k = 1 To 92
If k = j Then GoTo 9999
Zppk = Zppk - 1 * (2 * w(j) / 3.141592) * (Zp(k) - Zp(j)) / (w(k)^2 - w(j)^2) * (w(k + 1) - w(k))
Zpk = Zpk + (2 * w(j) / 3.141592) * (w(j) / w(k) * Zpp(k) - Zpp(j)) / (w(k)^2 - w(j)^2) * (w(k + 1) - w(k - 1)) / 2
Zpk2 = Zpk2 + (2 / 3.141592) * (w(k) * (Zpp(k)) - w(j) * Zpp(j)) / (w(k)^2 - w(j)^2) * (w(k + 1) - w(k - 1)) / 2

9999 Next k

Cells(9 + j, 13) = w(j)
Cells(9 + j, 14) = Zppk
Cells(9 + j, 15) = Zpk
Cells(9 + j, 16) = Zpk2
Next j

End Sub
8.5 Cell culture procedures

All solutions and materials coming into contact with cells must be sterile. Aseptic techniques should be practiced accordingly.

8.5.1 Material

- 70% ethanol (v/v)
- Vials of frozen cells from the nitrogen tank
- Sterile PBS (1X) or HBSS/Ca/Mg, 37° C
- Trypsin/EDTA, 37° C
- Sterile Pasteur pipets
- Water bath, 37° C
- Tissue culture flasks (Petri Dishes)
- Cryovials, and DMSO
- Hemacytometer with cover slip and counter
- Complete medium (AMEM) with serum (10% FBS), 37° C

8.5.2 Starting cell culture from frozen cells

- Remove a 2-ml vial of frozen cells from the nitrogen tank
- Place the vial in the water bath for 1-2 minutes. Wipe the vial with 70% alcohol before moving it under the tissue culture hood.
- Using a 1-ml pipette tip, remove the cell suspension from the vial and transfer it to a 15-ml sterile tube and add 8 ml of complete pre-warmed culture media.
- Centrifuge the cells at 1000 rpm for 5 minutes.
- Remove the supernatant (containing DMSO) and add 10 ml of warm media. Make sure the cell pellet becomes completely suspended in the media.
- Remove the cells from the 15-ml tube and plate them in a culture flask or Petri dish using a Pasteur pipette. Place the culture flask in the incubator (37 °C, 6% CO₂) and change medium every two days until they become confluent.

8.5.3 Cell removal from the flasks

Once the cells become completely confluent cells must be subcultured or passed. For plating the cells on the metallic samples, it is also necessary to detach the cells from the culture flasks.

- View cultures using an inverted microscope to check confluence and confirm absence of bacterial infection.
- Remove the spent medium and wash the cells with PBS in order to remove serum.
- Add trypsin/EDTA (2 ml for T-75 flasks) and tilt the flask to spread trypsin over all the surface and incubate for 5 minutes.
- Cells must be floating at this point and can be removed and transferred to a 15-ml tube.
- Centrifuge for 5 min (1000 rpm)
- Remove the supernatant and add new medium (10 ml).
- Cell suspension can be plated on a metal surface or in a new culture flask for passaging.

8.5.4 Cell counting

In order to determine the number of cells cultured on a substrate, cells need to be counted as the cell density is a factor in affecting the cell response on a substrate.
- Remove 10 μl of cell suspension and pipette it into the edge of hemacytometer counting chamber. The suspension will be drawn under the glass cover slip due to capillary force.

- View the slide under microscope and find the central area of the grid. This area is bordered by a set of three parallel lines.

- Count the cells in each of the four corners of the central grid and use the following formula to count the number of cells.

\[
\text{Cells/ml} = \text{average count per square} \times \text{Dilution factor} \times 10000
\]

8.5.5 Cryopreservation of cells

- Trypsinize cells and centrifuge to remove medium.

- Resuspend cells with freezing medium at a concentration of 2-4 million per ml and add 1 ml of cells into labeled cryovials.

- Fill up the the freezing container, Nalgene® Mr. Frosty with ethanol to the marked level and place cryovials into it and transfer it to the -80° C freezer overnight.

- Transfer the vials to liquid nitrogen for long term storage.

8.5.6 Immunostaining procedure

- Wash cells with PBS and fix using a solution of 4% PFA + 4% sucrose for about 20-30 min.

- Add 50 mM NH4Cl for 10 min.

- Wash with PBS and add 0.2 % Triton X100 in PBS for 5 min.
- Wash with PBS and 0.2 % gelatin twice (5 and 10 min respectively).
- Add 0.5 μM rhodamine phalloidin to each well.
- Incubate coverslip with PBS and 0.2% gelatin for an hour.
- Add first antibody and incubate for 1-2 hours at room temperature.
- Wash with PBS+ 0.2 % gelatin twice (2 and 10 min respectively).
- Add second antibody (FITC or TRITC) and incubate for 30-45 min.
- Wash with PBS+0.2% gelatin twice( 2 and 10 min).
- Wash with PBS twice.

8.5.7 LIVE/DEAD viability assay

The Live/Dead viability assay (SKU# L-3224, Invitrogen) contains a probe for dead cells (Ethidium homodimer-1) and a probe for live cells (Calcein AM).

- Prepare a solution of Ethidium homodimer-1 (1:500) and Calcein AM(1:500) in PBS and mix thoroughly.
- Apply enough of the solution to the samples to cover the surface of the cell culture area.
- Incubate for 20 minutes while protecting samples from the light.
- Wash with PBS twice and image.

Care should be taken at all stages before imaging to protect dye solution from exposure to intense light.
8.5.8 Transfection protocol

- Plate cells on the substrate of interest and incubate over night.

- Dilute the transfection agent (Optifect®, Invitrogen) in serum free media (1:20 v/v) in an 1.5 ml Eppendorf tube.

- In a separate 1.5 ml Eppendorf tube dilute the plasmid (DNA) in serum free media to get to a concentration of 10-30 μg/ml.

- Add the contents of one of the tubes to the other and incubate for 5 minutes at room temperature.

- Add 200 μl of the mix to each well. (Note: this is for a 12-well plate. In the case of larger well sizes the amount of the volume needed should be adjusted accordingly)

- Incubate for 4-6 hours and change medium after that.

- Allow for protein expression to happen for 24-48 hours post transfection.

8.5.9 ROS detection assay

Image-IT™ LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Molecular Probes®) was used to detect intracellular ROS.

- Prepare a stock solution (10 mM) of 5-(and-6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (carboxy-H$_2$DCFDA) by adding 50 μl of DMSO to 275 μg H$_2$DCFDA, prepare aliquots and store in freezer (-20 °C).
- Make the working solution by adding 5.0 μl of the 10 mM stock to 2.0 ml of warm HBSS/Ca/Mg or other buffer.
- Wash cells once.
- Apply the solution to the samples making sure the solution covers cells and incubate for 30 minutes at 37°C while protecting it from light.
- Add 1 μl/ml of Hoechst (optional) to the working solution during the last 5 minutes of incubation.
- Wash cell three times.
- Mount in warm buffer and image.
9. Vita

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Education:

- PhD in Biomedical Eng., Syracuse University (08/07-09/12) (GPA: 4.0)
  Selected courses: Advanced Biomechanics & lab, Surfaces of Biomaterials, Tissue
  Engineering, Mathematical Modeling in Physiology, Cardiovascular Engineering.
- MS in Biomedical Eng. (Biomaterials), AmirKabir University of Tech., Iran (09/04-02/07)
- BSc in Materials Science and Engineering, Iran University of Sci. & Tech. (09/98-10/03)

Research Interests:

- Biomaterial-cell interactions
- Bioelectrochemistry
- Biosensors
- Mathematical modeling in physiology
- Image processing

Research Experience:

Biomaterial/Cell interactions

- Characterized corrosion behavior of biomedical alloys (CoCrMo and cpTi) in simulated body fluids
  (SBF) using time and frequency-based impedance spectroscopy and developed the equivalent
  electrical circuits (EEC) of the interface.
- Developed a new model to describe the transient current response at metal/electrolyte interface.
- Implemented a micro-impedance measurement technique to study local corrosion behavior of
  implants.
- Modified an electromechanical system to setup a micro-anodization unit for generation of
  micropatterns of oxides on metal samples.
- Performed cell culture and applied biochemical assays such as viability assay, immunostaining,
  TUNEL, ROS detection to assess response of cells cultured on polarized biomedical alloys.
- Studied cellular dynamics on CoCrMo alloy using live-cell time-lapse fluorescent microscopy.
- Proficient is using scanning electron microscopy and energy dispersive spectroscopy (EDS) in study
  of morphology, microstructure and chemical composition of metals and cells.
- Experienced in using AFM in different modes (contact, tapping, fluid mode, electrochemical) to
  image the surface of metallic implants, fixed and live cells under electrochemical control.

Biosensor design

- Constructed a disposable biamperometric capillary-filled biosensing device by forming thin-film
  electrodes on a PET film using physical vapor deposition and photolithography techniques.
• Formulated and immobilized a mediator-based detection reagent on the electrodes and studied the efficiency of the mediator using cyclic voltammetry.
• Characterized sensor linear range, response time, reproducibility, and stability using chronoamperometry.
• Proposed a new design for an electrochemical glucose test strip to lower production cost by eliminating the costly photolithography step in forming the electrodes.

Mathematical modeling and computer simulation

• Utilized Matlab and C++ to model the diffusion profile of a substance released from an interface.
• Developed a VB code to model the molecular dynamics and elasticity of solids.
• Utilized R statistical language to analyze data from DNA microarray chips and derived the list of differentially expressed genes through analysis of variance (ANOVA).
• Developed codes in XPP and AUTO to simulate and numerically solve equations of dynamical systems.
• Automated data analysis of large data sets by writing VB macros in Microsoft excel.
• Modified a C++ program to simulate electrical wave propagation and pattern formation in cardiomyocytes.
• Simulated fluid flow in steel ladles using Fluent and Gambit package to optimize the working parameters.
• Developed Labview programs to read, monitor, filter and record the voltage output of potentiostats and atomic absorption spectroscopy (AAS) devices.

Research Training:

• GEM4 Summer School on Mechanics in Developmental Biology, Massachusetts Institute of Technology (MIT), Cambridge, July 9-20, 2012.
• National Biomedical Computation Resource (NBCR) Summer Institute, University of California, San Diego, August 1-5, 2011
• Nanoscale Science of Biological Interfaces summer school, University of California, Santa Barbara, June 19 - July 1, 2010
• Microarray Data Analysis, Mathematical Bioscience Institute (MBI), Ohio State University, 7–25 July, 2008
• Fabrication of an electrochemical glucose test strip, Materials and Energy Research Center (MERC), Iran, 2006-2007
• Application of Nanotechnology in Drug Delivery, joint workshop held by Universite de Geneve and Iran Polymer & Petrochemical Research Center in Tehran 11-12 September

Technical Skills:

• Electrochemistry
  Impedance spectroscopy, chronoamperometry, potentiogalvano static tests, and cyclic voltammetry
• Cell biology
Cell culture and biochemical assays, immunostaining, transfection, live-cell imaging

- **Microscopy and image analysis**
  Atomic Force Microscopy, Scanning Electron Microscopy (AFM, SEM), Fluorescence microscopy, ImageJ

- **Material Characterization**
  Energy Dispersive Spectroscopy (EDS), Atomic Absorption Spectroscopy (AAS), Mechanical Testing Systems (MTS), UV-Vis spectroscopy

- **Mathematical modeling and simulation**
  C++, R statistical language, Matlab, Visual Basic, Labview, XPP

**Publications:**


**Presentations:**

- 9th World Biomaterials Congress, June 1-5, 2012, Chengdu, China
- Annual meetings of Society for Biomaterials (2009-2012)
- National Biomedical Computation Resource summer institute (University of California at San Diego, 2011)
- Nanoscale Science of Biological Interfaces summer school (University of California at Santa Barbara, 2010)
- Materials and Processes for Medical Devices (MPMD) Conference (Minneapolis, 2009)
- Boulder School for Condensed Matter and Materials Physics (University of Colorado at Boulder, 2009)
- Ohio State University summer program in mathematical modeling in Biomedical Engineering (2008)
- Annual Nunan Lecture & Research Day (Syracuse University, 2009-2011)
- Alliance for Graduate Education and the Professoriate (AGEP) Symposium (Syracuse University, 2008)

**Awards:**

- Winner of Nunan poster presentation award (2011), and best oral presentation award (AGEP 2008)
Recipient of Syracuse Biomaterials Institute (SBI) fellowship (2009)
Outstanding Teaching Assistant award (2009)
2nd place, Panasci business plan competition, Whitman school of management, Syracuse University (2008)
Distinguished student (ranked first among four thousand participants in Iran’s nation-wide graduate school entry exam, 2004)

Teaching Experience:

- Teaching assistant (ECS 326, Engineering materials, design and applications)
  Fall 2007 and spring 2009, Instructor: Prof. Jeremy Gilbert
- Teaching assistant (ECS 326, Engineering materials, design and applications)
  Fall and spring 2009, Instructor: Prof. Yoshiki Oshida

Language Fluency:

- Fluent in Farsi and English, good in Arabic and French.

Personal & Hobbies:

- Painting, skiing, tennis, surfing, music