I, Kolade O Ojo, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Chemistry.

It is entitled:
Development of Sensors for Detection of Magnesium Metal Corrosion

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Development of sensors for detection of magnesium metal corrosion

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Abstract

The need to develop electrochemical sensors and analytical methods for studying the corrosion of magnesium and its alloys in vitro, in real-time, as well as detecting magnesium ions during or after the course of corrosion cannot be over emphasized. Thus, this first part of my dissertation focuses on the use of conductivity as an electrochemical sensor for monitoring, in real-time, magnesium corrosion under cell culture conditions, with enhanced (5%) levels of CO₂ in a cell culture incubator. Because magnesium metal, when pure or as an alloy, has the ability to biodegrade in aqueous, high chloride-containing environments, it has been attracting significant attention in biomaterials research for use as biodegradable implants for bone repair and other applications. However, the interactions between magnesium metal and surrounding cells and tissues as the metal corrodes need to be studied to ensure adequate biocompatibility for each intended application, because cell death occurs as a result of high local concentrations of the solution soluble corrosion products when magnesium reacts with water, which include Mg²⁺, alloying products, OH⁻ and H₂. Because some of these products are primarily ionic, we evaluated whether a conductivity sensor could be used to monitor magnesium metal corrosion in three physiologically appropriate solutions by detecting the changes in ionic strength in real-time. We demonstrated that this sensor, in combination with a pH sensor, recorded very different patterns of real-time changes in the three solutions, over 48 h. This work was based on a collaboration with Dr. Sarah Pixley and Tracy Hopkins of the Department of Molecular and Cellular Physiology, University of Cincinnati.
Current research on altering the corrosion rate of magnesium metals has focused on improving the in vitro tests needed to analyze magnesium metal corrosion rates and identifying new methods of preparing or post-processing magnesium to alter corrosion rates. In this work, we used a conductivity sensor to study and compare the relative corrosion rates of two previously untested magnesium single crystal samples that differed in surface modifications that could alter and change corrosion rates, in serum-containing cell culture medium under cell culture conditions. This second part of this dissertation was also based on a collaboration with Dr. Sarah Pixley and Tracy Hopkins (Department of Molecular and Cellular Physiology, University of Cincinnati), and Dr. Vesselin Shanov (School of Energy, Environmental, Biological and Medical Engineering, University of Cincinnati).

The third part of this dissertation focuses on another method that has the potential to be used to monitor magnesium corrosion, particularly if the magnesium could be alloyed with trace amounts of cerium. The method explored was cathodic stripping voltammetry (CSV) for determination of Ce$^{3+}$ using an indium tin oxide (ITO) electrode. Osteryoung square-wave stripping voltammetry (OSWSV) was used for the stripping step. A detection limit of 5.8 nM was found for a 5 min deposition time. Detection of cerium thus has the capacity to be very sensitive, and thus appropriate for analysis of the amounts that might be released from a magnesium alloy that contained a trace amount of cerium.
Dedication

I dedicate this dissertation to my parents: my late father, Group Captain Andrew Ajayi Ojo and my mother Mrs. Modupe Ojo; my brothers: Olusegun Ojo and Olatunbosun Ojo; my sisters: Mrs. Oluwaseyi Ojo Adeyemo, Mrs. Oluwatoyin Ojo Ososanya and Mrs. Ifelayo Ojo Adediran; my aunties: Mrs. Florence Iyanalu Akerele and Mrs. Yetunde Ayinde; and my uncle: the Honorable Shola Ojo.
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Chapter 1: Introduction

1.1 Overview of this dissertation

This chapter begins by providing a brief introduction to the basics and applications of metallic biomaterials. This section is followed by an introduction to magnesium corrosion and magnesium alloys as biodegradable biomaterials. In this section also, the basics of solution conductivity as a simple electrochemical technique are discussed alongside its potential applications, and the basics of osmolality as it relates to biology will be discussed. The research objectives along with the rationale of this work will also be discussed in this section. The chapter concludes with a brief description of the layout of the dissertation.

1.2 Metallic Biomaterials

According to Williams D.F in 1987, "a biomaterial is a nonviable material used in a medical device, intended to interact with biological systems" [1,2]. Biomaterials are typically made from biodegradable polymers, metals, carbons, glasses, ceramics or composites [3]. Thus, biomaterials should be non-toxic, non-inflammatory, non-thrombogenic, and should not interfere with the regeneration of the body. Just 50 years ago, biomaterials existed but were not recognized. However, at the start of the 21st century, biomaterials became popular, and were used throughout medicine, dentistry, and biotechnology. Prior to World War II, the reason why these materials didn't exist as biomaterials, was because there were no device manufacturing companies then, there were no formalized regulatory approval processes, no understanding of biocompatibility, and there were no academic courses on biomaterials. However, some biomaterials
were in use before WWII for example, materials used as dental implants, sutures, artificial hearts and organ perfusions, and contact lenses.

After World War II, metals, ceramics, and polymeric materials became much more available for use in medicine especially as intraocular lenses, hip and knee prostheses because many crude materials that existed before weren’t successful after their intended applications, because of poor to mixed results [4-6]. The commonly used implant metallic biomaterials today for orthopedic applications include stainless steel, titanium and cobalt-chromium [7-9] based alloys. However, these commonly used implant materials are designed to stay in the human body permanently, and a second surgery is required to remove these materials once implanted if complications develop. There are several disadvantages associated with these kinds of materials. A significant disadvantage is that toxic metal ions or toxic materials might be released during corrosion and during the wear and tear processes occurring in the body, leading to allergies, and chronic infections [10-14]. In addition to these deleterious effects, stress shielding also occurs [15,16]. “Stress shielding, which also leads to “osteopenia” refers to the reduction in bone density as a result of removal of normal stress from the bone by an implant material”. Stress shielding occurs as a result of the mismatch in the elastic moduli of an implant material with that of natural bone. As a result of these negative effects associated with the use of permanent implant materials over the years, biodegradable materials have been gaining interest in medicine and biomedical engineering [17,18]. They exhibit unique properties that could make them potentially outperform currently used implant materials such as stainless steel, cobalt-chromium based alloys and titanium. Many research groups have focused on the development of
magnesium and magnesium based alloys as potential biodegradable materials because they are generally non-toxic, depending on the concentration or amount of the alloying elements associated with them, they are light weight and they corrode rapidly in aqueous environments where there are high levels of chloride ions [19,20]. The potential advantages of magnesium over other non-biodegradable biomaterials, especially for orthopedic applications, are obvious. Based on this background, the development within the last couple of years has been fast and furious. From essentially no papers on the topic several years ago, there are presently 10-15 articles published in international journals each week dealing with different aspects of magnesium application in the field of biomedical materials research. As a result of these findings, a major part of this dissertation focuses on magnesium as a potential biodegradable implant material.

1.3 Overview of Magnesium Corrosion

As mentioned before, the potential advantages of magnesium over other non-biodegradable biomaterials, especially for orthopedic applications, are obvious. Magnesium is commonly used for structural applications in automobile and aerospace industry due to its high strength to weight ratio. Magnesium has also been used as an alloying element, and as a sacrificial electrode for ships or pipelines to slow down their corrosion [19]. However, the low corrosion resistance of magnesium limits its use in structural applications. Due to the high chemical reactivity of magnesium, a lot of attention has been drawn towards the development of magnesium and its alloys as biodegradable implant materials because of their unique physical, chemical and mechanical properties. In addition, the corrosion behavior of magnesium depends on a
variety of factors. These factors include composition of the magnesium alloy, surface treatment and the corrosion environments. Also, the presence of cells and proteins, pH of the corrosion solution, concentration of buffering agents present in the corrosion solution, the rate of diffusion of the corrosion reactants and products, and the composition of the corrosion solution are all important factors [21-23].

When magnesium corrodes in aqueous solution, it is oxidized to magnesium ions and water is reduced to hydrogen gas and hydroxyl ions, and then the two types of ions combine to form an insoluble precipitate, according to the following electrochemical equations [24,25]:

\[
\begin{align*}
\text{Anodic Reaction:} & & \text{Mg} & \rightarrow & \text{Mg}^{2+} + 2\text{e}^- \\
\text{Cathodic Reaction:} & & 2\text{H}_2\text{O} + 2\text{e}^- & \rightarrow & 2\text{OH}^- + \text{H}_2 \\
\text{Product Formation:} & & \text{Mg}^{2+} + 2\text{OH}^- & \rightarrow & \text{Mg(OH)}_2
\end{align*}
\]

However, overall, the aqueous magnesium corrosion process can be represented by the equation below:

\[
\text{Overall Reaction:} \quad \text{Mg} + 2\text{H}_2\text{O} \rightarrow \text{Mg(OH)}_2 + \text{H}_2
\]

In physiological environments, where there is a high concentration of chloride ions (ca.104 mM), chloride ions can react with insoluble Mg(OH)$_2$ to form soluble MgCl$_2$, thereby causing the further dissolution of magnesium according to the following equations [26,27]:
The dissolution of the Mg(OH)$_2$ layer, tends to make the surface more active by altering the protective layer, thereby causing more and more dissolution to magnesium ions. According to Song and co-workers [27], aggressive chloride ions, present in most simulated body fluids, are involved in the intermediate step of magnesium ion release, by increasing the rate of the electrochemical reaction that promotes the conversion of magnesium to univalent magnesium ions [28].

Furthermore, Willumeit and co-workers [29] stated the importance of understanding the corrosion processes of metallic implants in the human body, because understanding these processes is critical in studying biomaterials. However, since the in vivo environment is very complicated, in vitro studies require the analysis of metal corrosion in simulated body fluids that are physiologically relevant corrosion media, in order to further understand and evaluate the underlying mechanisms of biological metal corrosion processes and surface modification [29]. According to Witte and co-workers [30], as well as other groups [7], there is always a discrepancy between in vitro and in vivo corrosion rate. The results of their studies, suggest that the current American Society for Testing and Materials (ASTM) procedure for in vitro testing cannot be used to predict in vivo corrosion rates of magnesium materials because many of the in vitro tests being done by many researchers do not take into consideration the dynamic and complex corrosion environment with high levels of inorganic salts, vitamins, proteins.

$$\text{Mg}^{2+} + 2\text{Cl}^{-} \rightarrow \text{MgCl}_2$$  

$$\text{Mg(OH)}_2 + 2\text{Cl}^{-} \rightarrow \text{MgCl}_2 + 2\text{OH}^{-}$$
Aside from the pitting or local pH changes, which might be the leading causes of the opposite trends observed in the corrosion rates obtained in vitro and in vivo, protein binding can also be a significant factor. Even though the process of protein binding is not well known or well-studied, the presence of proteins is still believed to be one of the most influential factors that determine metal corrosion, because proteins have the tendency to adhere to most solid surfaces in vivo [31]. This statement thereby corroborates the findings by many research groups, that when proteins are added to a corrosion medium, their presence tends to slow down corrosion rates, irrespective of the composition of the corrosion medium used. In order to further mimic the physiological conditions of the human body, and to incorporate bicarbonate/CO₂ as part of the buffering system, comparable to what is found in blood, cell culture conditions are being investigated by many research groups, including our research group at the University of Cincinnati. According to Willumeit and co-workers [29], the presence of environmental carbon dioxide promotes the formation of carbonic acid, which is a precursor for magnesium carbonate formation according to the following equations:

\[
\begin{align*}
\text{H}_2\text{O} + \text{CO}_2 & \rightleftharpoons \text{H}_2\text{CO}_3 & \rightleftharpoons \text{HCO}_3^- + \text{H}^+ \\
\text{Mg} + \text{H}_2\text{CO}_3 & \rightleftharpoons \text{MgCO}_3 + \text{H}_2
\end{align*}
\]  

(1.7)  

(1.8)

The above reactions that promote the formation of MgCO₃ do not occur under normal atmospheric conditions, because the carbon dioxide levels are extremely low at approximately 0.034%. Under cell culture conditions in a cell culture incubator, there is a continuous supply of 5% CO₂, so the equilibrium of the carbon dioxide system will be
shifted to the right according to equation 1.7. This leads to the formation of carbonic acid which then reacts with magnesium to form MgCO₃, which, with a solubility of about 220 mg l⁻¹, is more soluble than Mg(OH)₂. This suggests a higher solubility than Mg(OH)₂, which is formed under normal atmospheric conditions. However, for more complex solutions and systems, it becomes even more difficult to predict the reaction probabilities of corrosion reactions solely on the availability of molecules in the corrosion process. Based on these findings, Willumeit and co-workers [29] suggested a model for in vivo metal corrosion in a biological environment in different steps. These steps include, but are not limited to the initial metal corrosion based on contact with water molecules leading to the release of Mg²⁺, which are capable of reacting with the dissociation products of CO₂, which is abundant in human blood. Permeability layers are also formed at the surface of the metal, which allow formation of intermediate steps in the corrosion process, Lastly, protein adsorption of metal surfaces is possible, leading to cell growth on biomaterials, which further complicates corrosion. Figure 1.1 depicts a summary of some initial steps of corrosion under cell culture conditions, where there is a continuous supply of 5% CO₂, and including the possible corrosion products depending on the composition of the cell culture medium used. In addition, it is important to remember that there are several factors that can affect the corrosion mechanism of magnesium and magnesium based alloys. For example, factors that affect corrosion include the surface area of the magnesium sample, the volume of the media used, the presence of inhomogeneity of the magnesium samples, the kinetics of the alkalization, the presence of impurities, and even the corrosion solution [32]
Figure 1.1. Summary of some of the initial steps of magnesium corrosion under cell culture conditions where there is a continuous supply of 5% CO₂. Adapted from reference [29].

1.4 Magnesium and its Alloys as Potential Biodegradable Biomaterials

As mentioned in the previous section, magnesium and magnesium alloys have attracted significant attention as biodegradable bioresorbable materials due to their light weight, good mechanical properties, greater fracture toughness than bone, elastic modulus very close to that of the human bone (Table 1.1), and their ability to degrade/resorb in an aqueous environment where chloride ion concentrations are very high, as in biological fluids [9,33-35]. The primary product released in this process is the magnesium ion, which is the fourth most abundant cation in the human body, is a major
The major drawbacks in the use of magnesium as a biodegradable implant for bone repair are that a too-rapid corrosion or bioresorption rate can produce negative consequences, felt mainly in the vicinity of the implanted material [26,34,35]. These can be due to the production of excessive levels of magnesium ions, which can be toxic to many different cell types. Or, as more recently described with magnesium ions, an overall increase in osmolality [9,36]. For example, recent studies have suggested that the overall number of particles released during magnesium corrosion leads to an
increased osmolality in the vicinity of the implant. However, none of these species is sodium, which is one of the worst offenders in terms of being damaging because it is osmotically very effective (cellular mechanisms keep this ion from crossing the cell membrane and therefore changes in external levels impact cellular tonicity). However, a high number of particles is still a concern around the magnesium implant because no matter what the nature of the particles, at high concentrations anything can become osmotically active and can overwhelm the ability of the cell to maintain its tonicity. Magnesium also reacts with water, resulting in the production of hydrogen gas and hydroxyl ions. These can impact adjacent cells and tissues via the pressure from the gas or the increase in pH.

Because of these drawbacks and potential problems, controlling the in vivo resorption rate of magnesium is critical to its use as a biodegradable biomaterial in all biomedical applications. Researchers in this field have developed several methods for slowing down the resorption/corrosion rates of magnesium metal, including adding alloying elements that are generally non-toxic, modifying the surface using coatings (like polymers, proteins, anodization, or surface functionalization) or modifying the surface by applying post-processing procedures [9,37-44]. Relatively non-toxic alloying elements that have been added to magnesium include zinc, calcium, manganese, yttrium and rare earth elements [45-50]. The advantages of alloying magnesium with other metals include improving its mechanical properties and adjustment of the corrosion rate by development of a surface film that protects the material from further degradation [27]. The advantages of coatings are that they protect the surface of the implant material from corrosion until the coating breaks down. This has the capability of extending the
lifetime of an implant material after implantation, in order to prevent premature failure. The use of post-processing procedures (like heat treatment or applying pressure) alters the metal structure at the surface in ways that also slow corrosion.

Consequently, adjusting the resorption/corrosion rates of biodegradable magnesium and its alloys still remains a challenge. Issues that contribute to making this a challenge include re-creating the complex in vivo resorption process in a testable in vitro system, and developing tools that can monitor the success of methods designed to alter resorption rates. In terms of developing in vitro systems for testing, despite extensive efforts, it has been extremely difficult to re-create an exact in vivo environment in a testable in vitro system. Even in the most optimal in vitro systems available to date, the corrosion rate of Mg in physiological solutions is still in the range of hundreds of magnitudes different than in the in vivo situation (usually faster corrosion in vitro) [51]. The reasons are that the resorption rate in vivo is dependent on multiple complex reciprocal interactions between magnesium metal and the surrounding fluid and tissue conditions and duplicating these conditions has proven very elusive. Standardized regulations and guidelines such as the ISO10993 for testing biomaterials, and the ASTM G102-89 for testing the corrosion of metals thus are not fully applicable to development of biodegradable metallic biomaterials like magnesium and its alloys. In light of these discrepancies, researchers in this field of study are involved in the complicated process of developing novel guidelines and regulations for biodegradable metals, adapting them where necessary. These discrepancies, in turn, have also resulted in multiple, non-uniform, non-standardized protocols for comparing the results of magnesium corrosion in vitro, in the literature.
In terms of monitoring magnesium resorption rates, difficulties have been encountered in developing appropriate methods to monitor resorption, including difficulties in detecting Mg$^{2+}$ [7]. Weight loss determination after corrosion is one of the simplest methods of monitoring corrosion/resorption rates, and it is still considered one of the most accurate and reliable. The corrosion products are removed by treatment with chromic acid, by following standardized guidelines. This assumes that all of the corrosion products are removed completely by this process and that the un-corroded metal is neither attacked nor destroyed. As simple and easy as weight loss measurements for determination of corrosion rates may seem, it only gives information about the overall loss of material after a corrosion test is complete. But, advantageously, it also allows corrosion monitoring by additional powerful techniques [52-54].

The following methods have been used for the analysis of total magnesium concentration released into the corrosion solution: inductively coupled plasma mass spectrometry (ICP-MS) [55,56], inductively coupled plasma atomic emission spectroscopy (ICP-AES) [47,57,58], inductively coupled optical emission spectroscopy (ICP-OES) [59], and atomic absorption spectroscopy (AAS) [60,61]. Even though, these methods are highly reliable, they all require removal of samples for analysis, they are complicated and are often either inaccessible by many research groups or are expensive techniques. Additionally, optical microscopy [52,62,63] and scanning electron microscopy (SEM) [52-55,60,62,64-67] are often used to study the morphology of the implant material after corrosion, but before removing the corrosion products using chromic acid. Fourier transform infrared spectroscopy (FTIR) [29,60,68], X-ray
diffraction (XRD), energy dispersive x-ray spectroscopy (EDX), and X-ray photoelectron spectroscopy (XPS) [23,49,52-55,58,64,68-74] have often been used to identify surface corrosion products and the structure of a film surface. This provides valuable information about magnesium corrosion.

The overall research described in this dissertation was partially funded by and done in collaboration with the Engineering Research Center for Revolutionizing Metallic Biomaterials (ERC-RMB), which is an NSF-funded Center that involves the following national universities: North Carolina Agricultural and Technical State University (NCAT), University of Pittsburgh (UPITT), and the University of Cincinnati. These aforementioned national universities also partner with the following universities; Hannover Medical School (MHH), Indian Institute of Technology Madras (IITM), and California State University (CSULA), in order to sustain the cutting-edge research partnerships and opportunities that are being fostered among all participating institutions. The vision of this center is to [75] “revolutionize metallic biomaterials and underlying sciences and technologies leading to engineered systems that will interface with the human body to prolong and improve the quality of life, coupled with the development of a vibrant, diverse workforce well-prepared for the multidisciplinary and global challenges and opportunities of the new millennium”. In addition to this vision, the major goal of this center is to “revolutionize metallic biomaterials and smart coatings with built-in responsive biosensory capabilities which can adapt to biological changes to create novel bio-functional engineered systems with applications in craniofacial and orthopedics, cardiovascular and thoracic devices, and responsive biosensors and neural applications, and the overall focus will be on magnesium”.

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While the focus of the partner institutions is in the development of biodegradable metals (magnesium and its alloys), biofunctionalization, surface modifications, and device development for eventual human clinical trials approved by the FDA, the research in the Department of Chemistry at the University of Cincinnati is geared towards the development of electrochemical sensors and devising new ways of monitoring and controlling the release of the corrosion products during magnesium corrosion, both in vitro and in vivo. Working together (as an ERC) is making the research move faster.

Electrochemical sensors that can detect changes in pH, magnesium and other alloying metal ions released, as well as amperometric and potentiometric sensors for following the release of hydrogen gas have been developed and deployed to characterize the corrosion behavior of already available magnesium and magnesium based alloys by our group. Recently, our research group explored the use of a combination of sensors as a convenient and effective means of continuously monitoring chemical species dissolved in solution, particularly in real-time. This combination of electrochemical sensors, called a corrosion characterization system (CCS), was recently shown to allow continuous real-time monitoring of magnesium metal corrosion in vitro [61]. The specific sensors were a glass pH electrode, a water hardness ion-selective electrode (ISE), and a potentiometric hydrogen sensor, to monitor the concentration of solution-soluble corrosion products \( \text{OH}^-, \text{Mg}^{2+}, \) and \( \text{H}_2 \), respectively, of magnesium metal reaction with water. In addition, electrochemical impedance spectroscopy (EIS) was used to monitor insoluble corrosion products accumulating on the surface by measuring changes in the solution resistance of magnesium samples.
during corrosion. This corrosion characterization system (CCS) was demonstrated using in vitro, bench top corrosion of magnesium metal in various buffered and unbuffered salt solutions [61]. However, this system was not designed to monitor magnesium corrosion in the type of complex biological solutions that are encountered in vivo, where the naturally high levels of calcium ions interfere with measurements of Mg$^{2+}$, and a CO$_2$-bicarbonate buffering system and sterile conditions should be maintained. Therefore, we sought to expand this work to explore detection of in vitro magnesium metal corrosion under more physiologically appropriate conditions.

To enable detection of magnesium corrosion products in physiologically appropriate solutions in vitro, we explored the use of a conductivity sensor that essentially detects the concentration of ionic components in a solution [76,77]. Conductivity, the ability of an electrolyte solution to pass an electric current, is concentration dependent as it varies with the sum of dissolved ion concentrations in water. Conductivity is easily measured with an electrochemical sensor by applying a high frequency voltage between two electrodes and measuring the resulting current. To date, conductivity measurements have not been used extensively for monitoring corrosion of magnesium or other metals. When magnesium metal corrodes in pure water, the major solutes released are Mg$^{2+}$ and OH$^{-}$ ions, due to the breakdown of water (as described above). If the solution also contains compounds normally found in vivo, like PO$_4^{3-}$ and CO$_3^{2-}$, then Mg$^{2+}$ reacts with these compounds to form a corrosion layer of precipitated magnesium hydroxides, phosphates and carbonates [23,38,61,65,78]. These corrosion products both deposit on the surface of the magnesium disc and dissolve in the corrosion solution [23,38,61,65,78]. So the absolute amount of released
ions in solution and the rate of change of the concentrations of these ions will vary in
different solutions based on the ratio of production of deposits versus soluble ions.
However, even with substantial deposition of precipitates, at least some of the Mg\(^{2+}\) ions
are typically released into solution. Therefore, conductivity should be a good measure of
the formation of soluble ionic products produced as magnesium metal corrodes after
immersion in a solution. These soluble ions are also the most important factors that
could impact cells and tissues in the vicinity of the magnesium implant, especially if
there are no toxic materials present in the magnesium alloy itself. Therefore, a
conductivity sensor provides an important monitor of the soluble ions that are most likely
to impact biocompatibility of a biodegradable biomaterial, such as a magnesium implant
used for medical purposes.

There are several advantages of a conductivity sensor over other sensors for the
purpose of monitoring the concentration of solution soluble corrosion products during
magnesium corrosion. Compared to measurements made by ion-selective electrodes
(ISEs) for Mg\(^{2+}\) (and Ca\(^{2+}\)), conductivity measurements are less prone to signal drifting
and protein fouling during long term continuous measurements. Also current ion-
selective electrodes lack sufficient selectivity for Mg\(^{2+}\) over Ca\(^{2+}\) [79,80] and they require
routine calibration, unlike a conductivity sensor. Even though conductivity lacks
selectivity for a particular ion, in the case of magnesium corrosion the response is just to
Mg\(^{2+}\) and anions that maintain charge neutrality in the solution (OH\(^-\) and buffer anions
like HCO\(_3^-\), HPO\(_4^{2-}\), H\(_2\)PO\(_4^-\) in buffered solutions). Other techniques currently being used
for measuring or estimating magnesium corrosion include electrochemical impedance
spectroscopy coupled (EIS) coupled with Tafel extrapolation. However, this
measurement has been reported to provide inaccurate estimates of corrosion rates [81]. Measurements of hydrogen gas release during magnesium corrosion has also been used, but this also presents challenges, primarily due to practical aspects like capturing all of the hydrogen, or preventing diffusion through materials used to capture it [35,82]. Other methods of monitoring magnesium corrosion include Mg-sensitive dye assays, like the Xylidyl blue assay [83], which allows detection of soluble Mg$^{2+}$ spectrophotometrically in biological fluids, especially in serum-containing fluids. Determination of corrosion rates by measuring the weight of the metal before and after corrosion and measuring solution osmolality with an osmometer have been mentioned earlier [56]. However, none of these can be done in real-time and many require removing and replacing solution samples at different time intervals, which can impact subsequent corrosion rates. Thus, we propose that conductivity measurements can provide useful capabilities that expand our ability to monitor magnesium corrosion to include real-time measurements in solutions with ion concentrations close to those normally encountered in vivo. Real-time monitoring allows detection and study of changes in magnesium corrosion or degradation during initial phases of exposure to complex biological solutions, which could help us understand both the degradation process itself, as well as what might be occurring in vivo. Thus, we hope to move in vitro studies of magnesium corrosion closer to clinical relevancy.

A conductivity sensor essentially detects the ionic component of osmolality (Figure 1.2), which is the number of osmoles of solute particles dissolved in one kilogram of solvent [84]. Therefore, both conductivity and osmolality are theoretically good measures of magnesium metal corrosion in solutions without living cells. To date,
osmolality, which is a measure of osmotic pressure, has been measured during magnesium metal corrosion using an osmometer that measures solution osmolality based on changes in some physical property of a solution such as freezing point depression, boiling point elevation, vapor pressure lowering, or osmotic pressure [85,86]. Solutions containing the same concentration of particles as are found inside cells are iso-osmotic or isotonic, because the solution causes no movement of water molecules into or out of cells. Thus, no damage to cells is observed when they are in contact with isotonic solutions. In addition, solutions with either no ions or a low osmolality with ions that are impermeable to the cells, are considered hypotonic and cells tend to swell, which, at its extreme can cause cellular rupture. Solutions with higher osmolality than what is inside the cell, again when caused by ions that are not permeable to the cell, are considered to be hypertonic. When cells are in contact with such solutions, they shrink (lose water) and can die [85]. Figure 1.2 depicts the dramatic changes occurring when cells are placed in any of these three solutions mentioned. However, a significant disadvantage of using an osmometer approach is that osmolality measurements are always taken offline during in vitro corrosion and bench top immersion tests of magnesium and magnesium based alloys, which requires constantly changing media.
Figure 1.2. The effects of osmotic pressure on biological cells as they are placed in hypertonic, isotonic and hypotonic salt solutions. Green and yellow arrows depict net flow/movement of water molecules in and out of the cells.

1.5 Objective of this Research

The effect of magnesium on cells and surrounding tissues as it resorbs needs to be studied extensively to ensure adequate biocompatibility for each intended application of magnesium and its alloys as metallic biodegradable biomaterials for the future. Magnesium metal corrosion can produce locally high osmotic levels due to high concentrations of Mg$^{2+}$ and other soluble products of magnesium degradation, which includes alloying products. Because this increase in osmolality in the vicinity of the implant material could cause osmotic shock in and potentially death to cells surrounding the implant, a sensor that detects osmolality in real-time, in physiologically appropriate
solutions close to the implant would aid our understanding of both the degradation and biocompatibility of magnesium and magnesium based implants. Therefore, we propose that a conductivity sensor could serve this purpose.

The initial goal of this research was first to demonstrate that conductivity can be used to estimate osmolality for this application by establishing a relationship between conductivity and osmolality (monitored by an osmometer), under very controlled in vitro conditions. We then further sought to determine if conductivity could be used to monitor the corrosion of magnesium in a set of physiologically appropriate solutions, in real-time, over a set time period, with further comparisons to the osmolality measured by an osmometer. However, as a result of research evolution, we decided to include another major goal, in addition to the initial research goals.

Consequently, the major goal of this research was to demonstrate that conductivity is a valid method to monitor the concentrations of solution soluble corrosion products of magnesium metal in a set of physiologically appropriate solutions, in real-time, over a set time period. This included being able to function in solutions containing calcium and under the conditions normally used for growing cultured cells, which are currently the closest possible approximations of conditions in vivo. By establishing that this sensor can work under conditions that more closely duplicate or simulate the in vivo situation, we propose that the conductivity sensor will help us better understand the magnesium degradation or resorption process, which will help researchers develop methods to better control magnesium and magnesium based alloys resorption rates. This will also importantly provide information about the levels of soluble degradation products that cells might be exposed to during in vitro experiments, which will impact
biocompatibility. In future studies, this might then be useful to help develop in vitro
culture conditions where magnesium resorption rates are more representative of in vivo
rates.

1.6 Conclusion

This introductory chapter gives a broad overview of the history and applications
of metallic biomaterials, magnesium corrosion in different environments, potential
applications of magnesium and magnesium based alloys as metallic biodegradable
biomaterials, significance of the partner institutions involved in this research,
electrochemical sensors that have been deployed for magnesium corrosion monitoring,
and the objective of this research. Chapters two and three of this dissertation focus on
the use of conductivity as a simple and fast electrochemical technique for monitoring, in
real-time, the corrosion of pure magnesium. Chapter two specifically analyzes how the
sensor functions when magnesium is allowed to corrode in three different solutions.
Chapter three focuses on using a conductivity sensor to compare the rates of corrosion
of high purity (single crystal) magnesium samples, treated with different surface
treatments to control the rate of magnesium resorption, in serum containing cell culture
medium and under cell culture conditions, in a cell culture incubator. It is interesting to
say, this is the first time this type of sensor has been used to study metal corrosion
under the conditions mentioned to the best of our knowledge.

Chapter four of this dissertation focuses on cathodic stripping voltammetry (CSV)
of Ce$^{3+}$ ions using a bare optically transparent indium tin oxide electrode (ITO) as a
working electrode, without the need to modify with a thin polymer film for
preconcentration. This is the first time such an electrode has been used for the
detection of Ce\(^{3+}\) using square wave stripping voltammetry in an acidic buffer solution. Because it is so difficult to detect magnesium and other corrosion products of magnesium, then one of the ways that has been suggested to follow corrosion is to include an element that is easy to detect in the magnesium alloying materials, making it an integral part, and something that will be released upon corrosion. The best option is to be able to have an element that is easy to detect and improves the qualities of magnesium as a biomedical implant material. One example has been europium and another is cerium. Cerium is a good element to choose because it has biological properties similar to calcium, and it has been used to make alloys for various metals including magnesium. We chose to look at cerium because previous work in the lab on similar elements suggested that we might be able to develop a sensitive sensing method. Towards that goal, methods were developed and refined to detect very low levels of cerium. Our hypothesis was that we could develop a sensor whose sensitivity to cerium was so exquisite that the amount of cerium needed to be alloyed with magnesium would be extremely low and therefore would not interfere with other physical and corrosion properties of the alloy.

The work presented in chapters two and three were done in collaboration with Dr. Sarah Pixley and Tracy Hopkins from the Department of Molecular and Cellular Physiology at the College of Medicine University of Cincinnati; and Dr. Vesselin Shanov from the School of Energy, Environmental, Biological and Medical Engineering at the University of Cincinnati.
Chapter 2: Conductivity Sensor for Real-Time Monitoring of Magnesium Corrosion in Physiologically Appropriate Solutions

2.1 Brief Overview of Conductivity and Some of its Applications

Conductivity is the ability of an electrolyte solution to pass an electric current, and it is concentration dependent as it varies with the sum of total dissolved ion concentrations in water. Conductivity can be measured by applying an alternating current (I) between two electrodes immersed in a solution and then measuring the resulting voltage (V) as shown in Figure 2.1. During this process, the positively charged sodium ions (cations) migrate towards the negative electrode and the negatively charged chloride ions (anions) migrate to the positively charged electrode, and the solution in question (NaCl) acts an electrical conductor or an electrolyte. As mentioned earlier, conductivity depends on ion concentration. Additionally, it also depends on ion mobility, charge, temperature, and anion size and mass [77]. Consequently, conductivity measurements are very simple in the sense that, one solute solution can be used for solubility determination and the determination of degree of dissociation. However, in multiple-solute solutions, conductivity becomes more complex in the sense that, the contribution of a single ionic solute to overall solution conductivity cannot be determined solely by conductance measurements alone. As simple as conductivity measurements are, they lack specificity and selectivity in the sense that they don’t discriminate between ions in an electrolyte solution, and as a result, the development of conductance measurements as a widely used electroanalytical technique was discouraged [87]. However, with the advancement in technology and electronic
development, highly precise conductimetric instruments have been developed and are being used to follow the course of highly specific chemical reactions or used in chromatographic detection for varying solute concentrations [87].

It should be noted that ions in solution are surrounded by a sphere of oppositely charged ions and water, and when high frequency voltage is applied to an electrolyte solution, the migration of the central ion distorts the cosphere of water and ions of opposite charges thereby causing a drag on ion migration and if this persists, polarization occurs, which causes a halt in the transfer of charge in solution, hence a drastic reduction in the electrical current of the solution [87]. In order to eliminate the problems
Figure 2.1. Description of how solution conductivity works, using a simple NaCl solution as a model electrolyte. Na$^+$ and Cl$^-$ are the major charge carries responsible for an electrical current in solution.

with polarization, modern conductance instruments used for measurement of resistivity or conductivity use alternating potentials with frequencies of 1,000 Hz, which is sufficiently high to prevent polarization [76,87]. Conductivity has been widely used in industries for raw water treatment, for monitoring the buildup of dissolved ionic solids in evaporative cooling water systems and boilers, for leak detection in heat exchangers, in pharmaceutical and food beverage industries as a tool for measuring the concentration of a clean in place solution which is typically sodium hydroxide solution, in interface detection of liquids with different conductivity values, and to monitor how well completely dissolved ionic solids are being removed from brackish water, also known as desalination [88]. To date, and to the best of our knowledge, this is the first time conductivity is being used to study the corrosion of magnesium, in real-time, in vitro under normal atmospheric conditions and under cell culture conditions in a cell culture incubator.

As mentioned in chapter one, our group developed and deployed a corrosion characterization system (CCS) which encompasses the use of a combination of
electrochemical sensors for continuous monitoring of magnesium corrosion in vitro. In this light, we decided to modify the corrosion characterization system (CCS) by replacing the multiple electrochemical sensors with a conductance and a pH sensor, which could then be used in complex physiological solution containing calcium. We also sought to establish a sensor that could detect magnesium corrosion under the conditions normally used for growing cultured cells, which included maintaining sterile conditions, body temperature of 37 °C and high (5-10%) CO₂ levels to maintain a bicarbonate-based buffering system that simulates conditions in human blood. We aimed to identify a sensor that would help, further in the future, establish in vitro culture conditions that provide greater similarities between in vitro and in vivo resorption rates of magnesium.

2.2 Experimental

2.2.1 Chemicals and Materials

The following reagents were used without further purification: sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride hexahydrate (MgCl₂·6H₂O), barium nitrate (Ba(NO₃)₂), silver nitrate (AgNO₃), chromium trioxide (CrO₃); Dulbecco’s modified eagle’s medium/nutrient mixture (DMEM) combined with F12 Ham’s liquid media (DMEM/F12, 1:1 by volume); 100x antimycotic antibiotic mixture, phosphate buffered saline (1X PBS, 0.1 M phosphate), pH buffer standard solutions ranging from pH 4 to pH 10 and conductivity calibration standards with conductivity values ranging from 10 µS/cm to 100,000 µS/cm (Oakton brand). A solution of 0.9% sodium chloride was also prepared by dissolving appropriate amounts of sodium chloride in deionized water. Deionized water with a resistivity value of 18 megohm-cm, was used in preparing all stock solutions.
(Barnstead water purification system, Sybron, Boston MA). Solutions of magnesium chloride with varying concentration ranges between 0 mM and 100 mM were prepared by separately dissolving appropriate amounts of magnesium chloride hexahydrate in PBS, DMEM/F12, and 0.9% sodium chloride in separate 100 mL volumetric flasks. All components (throughout) are from Thermo Fisher Sci., Florence KY, unless otherwise indicated.

2.2.2 Instrumentation

Real time conductivity and pH measurements were performed with a MeasureNet multi-functional chemical analysis network (MCAN®) system [61] consisting of two measurement workstations networked together and managed by a single MCAN® controller and PC (the system can support up to 15 workstations). Each workstation had two ± 2.5 V analog input channels and one high speed serial communication channel. The analog inputs were sampled by a high resolution 24-bit Sigma-Delta A/D with built-in signal conditioning and noise reduction. This was ideal for the low, noisy signals often found in potentiometric measurements of high impedance sensors such as the glass pH electrode. Measurements were stored on the system PC and streamed in real time to the cloud for storage, which could be monitored in real time from any internet connected device to follow the progress while running experiments for extended time periods. Preamplifiers and isolation transformers constructed for sensors used in this work have been previously described [61]. An Accumet excel XL50 Dual Channel pH/Ion/Conductivity Meter (Thermo Fisher Scientific Co.) was used to take offline pH and conductivity measurements during sample preparation as needed. A conductivity probe made of graphite with a cell constant of 1.0 cm⁻¹ (MeasureNet Tech., Ltd, Cincinnati, OH)
was used to take real time conductivity measurements. Osmolality measurements were taken on a Wescor® 5500 vapor pressure osmometer (Wescor Inc., Logan, UT). Kinematic viscosity measurements were taken on a Cannon-Fenske routine viscometer (Cannon Instrument Co., State College, PA). All data analysis was carried out using commercial spreadsheets and graphing algorithms.

2.2.3 Corrosion Cell and Solutions

The corrosion cell used for the bench top part of this work, included a 1000 mL water-jacketed glass beaker with a 105 mm inner diameter. A 65 mL water-jacketed cell with conical sides and a flat bottom was also used for taking conductivity measurements at 37 °C in order to obtain conductivity calibration curves in 0.9% NaCl and PBS. Conductivity measurements were taken under cell culture conditions in order to obtain calibration curves in DMEM/F12. A thermostatic recirculating water bath (MWG Lauda, Lauda, Germany) was used to maintain the temperature of the cell solution at 37 °C as this was physiologically relevant for our application. The solution was stirred constantly using a magnetic stir bar and a magnetic stir plate in order to maintain a homogeneous solution during bench top immersion tests. Parafilm was used to completely seal the top of the corrosion cells and all openings created after sensor (graphite conductivity sensor and glass pH electrode) positioning, in order to reduce evaporation of corrosion solutions during immersion tests. 0.9% NaCl, 1X PBS, and DMEM/F12 were used as model electrolytes as they are typical examples of simulant body fluids used for studying corrosion of metals because they contain inorganic ions with concentrations close to that of human blood plasma. Immersion testing in DMEM/F12 was carried out under cell culture conditions (5% CO₂ in humidified air, 37 °C) in a cell culture incubator (Forma
Scientific) under sterile conditions. The sensors and a Mg pellet were inserted into solution in the upper of two 250 mL plastic tripour beakers nested inside each other, with the top beaker having multiple holes punched in it to allow continuous flow of solutions during immersion. Due to the nesting configuration of the two beakers, it was then possible to have a stir bar in the lower beaker without disturbing the Mg pellet. A volume of 250 mL was used for each immersion test. Figure 2.1 shows the experimental set-up we used for acquiring real-time conductivity and pH measurements.

![Figure 2.2 The experimental configuration for real-time conductivity and pH measurements during magnesium metal corrosion (Not drawn to scale).]
2.2.4 Preparation and Treatment of Pure Magnesium Samples

Mg samples were cut from stock material (99.9% pure, 12.7 mm in diameter, as-drawn, typical impurities 70 ppm Al, 20 ppm Cu, 280 ppm Fe, 170 ppm Mn, <10 ppm Ni, 50 ppm Si, <20 ppm Zn; Goodfellow Cambridge Ltd., Cambridge, U.K) with a lathe to form discs with a thickness of 2 mm. The Mg disc samples were wet polished on both planar surfaces using 600, 800, and 1200 fine grits of SiC paper (3 M 401Q Imperial Wetordry Paper A wt.) and rinsed with deionized water then isopropyl alcohol at the end. This polishing protocol was performed on an AUTOMET® 2 power head and an ECOMET® 4 variable speed-grinder polisher (Buehler, Lake Bluff, IL). An initial polishing time of 60 s was used for the 600 and 800 grit SiC paper type while an initial polishing time of 30 s was used with the 1200 grit SiC paper. A force of 2 lbs and a speed of 100 rpm was applied to the instrument during polishing.

2.2.5 Microscopy and Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) and Corrosion Rate Determined After Immersion

After all bench top immersion tests, samples of Mg were removed from the corrosion solution, rinsed with deionized water and air dried. The surface was characterized by scanning electron microscopy (SEM), energy dispersive x-ray spectroscopy (EDX), and ATR-FTIR. Then the corrosion products on the surfaces of the discs after immersion were removed with chromic acid solution, the pellets were weighed and the corrosion rate was calculated using a previously described procedure [61].

SEM images of Mg discs after immersion but before cleaning with chromic acid, were taken using an XL 30 ESEM scanning electron microscope (FEI, Hillsboro, OR) with an accelerating voltage of 20 kV, coupled with an energy dispersive x-ray spectroscopy
detector (EDAX Inc., Mahwah, NJ) for obtaining elemental composition of the corrosion layer on the Mg discs. Functional groups in the corrosion products layers were determined using a Nicolet 6700 FTIR spectrophotometer (Thermo Scientific, Madison, WI) equipped with a smart orbit diamond attenuated total reflectance (ATR) module. The OMNIC software (Thermo Scientific, Madison, WI) was used to obtain spectra from 4000 to 400 cm$^{-1}$ after 32 scans.

2.2.6 Statistics

Statistical analyses were done with SigmaStat (version 13). All data were normally distributed and ANOVAs (regular or repeated measures, one or two way) were followed by all pairwise multiple comparison (post-hoc) tests using the Holm-Sidak method. Error bars indicate one standard deviation and significance was determined at a level of $p<0.05$. For Mg corrosion time course measurements, three Mg pellets were immersed in each medium at separate times. Other measurements were done at least three times.

2.3 Results and Discussion

In this study, a commercially available conductivity sensor with graphite electrodes was used to record and compare real-time conductivity changes during the corrosion or degradation of pure magnesium metal pellets or discs in three physiologically relevant electrolyte solutions (buffered and unbuffered). The model electrolyte solutions were generally composed of inorganic ions at concentrations similar or close to those in human blood plasma and they have been used by multiple groups for initial pilot testing for biomedical implant applications [9]. The 0.9% NaCl solution is isotonic to blood, while PBS uses the physiologically appropriate phosphate buffering system to maintain the pH at levels required for cell viability. DMEM/F12 is a common mammalian cell culture
medium that contains many additives that are necessary for cell viability and growth, including amino acids, vitamins, organic buffers, and glucose. It also maintains an appropriate pH with a sodium bicarbonate buffering system [37].

2.3.1 Conductivity Calibration Measurements

The measured conductivity of a solution depends on the total concentrations of all of the ions, their rates of movement in an electrical field and their charges (mobility), as shown in the following equation

\[ K = k \sum N_i |q_i| u_i \] (1)

where \( K \) is conductivity, \( N \) is the number of ions (or concentration), \( |q_i| \) is the absolute charge on the ion, \( u_i \) is the ion’s mobility, and \( k \) is a cell constant related to the geometry and spacing of the electrodes. For this application, conductivity was used to track the increase in concentration of Mg\(^{2+}\) and associated anions in the electrolyte solution as corrosion or degradation of pure magnesium metal proceeds. The mobility of Mg\(^{2+}\), \( u_{\text{Mg}^{2+}} \), can be altered or affected by changes in temperature or viscosity of the solution and the charge \( q_{\text{Mg}^{2+}} \) can be altered or changed by forming complexes, for example, with a negatively charged organic ligand. Because conductivity can be altered or changed with differences in the anions available to associate or combine with the released Mg\(^{2+}\) and their concentrations, solution conditions were carefully controlled and monitored.

We first calibrated the conductivity sensor by measuring conductivities after addition of MgCl\(_2\) to solutions of 0.9% NaCl, PBS and DMEM/F12 (which has a basal level of 0.81 mM magnesium). The slopes of the calibration curves were compared as a measure of sensitivity of the sensor. The added MgCl\(_2\) concentrations ranged from 0 to
100 mM. This concentration range both supports growth of many types of cells in culture and covers the range found in magnesium metal extracts made according to ISO standards (up to ~70 mM) [89]. Linear plots (Fig. 2.2) were obtained for all three electrolyte solutions, with intercepts corresponding to the conductivities of the solutions before addition of MgCl₂ was added. Excellent precision was obtained as indicated by the relatively small error bars (n=3) in Fig. 2.2

The sensitivity of a conductivity sensor is its ability to detect a change in ion concentrations and increased sensitivity is defined by an increased slope of the calibration curve of conductivity (mS/cm) versus added ion concentration (mM). Calibration curves of conductivity versus added MgCl₂ concentrations, in each electrolyte solution used, were generated using the method of least squares. The plots (Fig. 2.3) were linear from 0 mM to 100 mM, with these curve parameters:

\[ K \text{ (mS/cm)} = (0.126 \pm 0.004) \times ([\text{MgCl}_2]) + (15.9 \pm 0.1), (R^2 = 0.995) \text{ in } 0.9\% \text{ NaCl (p<0.00001)}, \]

\[ K \text{ (mS/cm)} = (0.113 \pm 0.003) \times ([\text{MgCl}_2]) + (15.4 \pm 0.1), (R^2 = 0.997) \text{ in } \text{PBS (p<0.00001)}, \]

and

\[ K \text{ (mS/cm)} = (0.086 \pm 0.002) \times ([\text{MgCl}_2]) + (14.5 \pm 0.1), (R^2 = 0.998) \text{ in } \text{DMEM/F12 (p<0.00001)} \]

Measurements on a single day were taken in triplicate and each data point of conductivity was an average of three trials obtained on different days. Each error bar on a data point represents one standard deviation on either side of the average of the three trials on those different days. For each solution, the \(R^2\) values were all > 0.9, up to a
conductivity value of 28 mS/cm for 0.9% NaCl, 26 mS/cm for PBS, and 23 mS/cm for DMEM/F12, which corresponds to a very high osmolality of between 500 and 540 mOsm/kg in PBS and 0.9% NaCl and 400 mOsm/kg in DMEM/F12. The detection limits for MgCl₂ in 0.9% and PBS were 0.10 and 3.00 mM, respectively, while the detection limit for MgCl₂ in DMEM/F12 was 1.20 mM.

These results indicate good linear and dynamic ranges with this sensor. Statistically, all pairs of slopes were significantly different (one-way ANOVA, \( p<0.001 \), Holm-Sidak post-hoc multiple comparisons test, \( p<0.05 \)). Although all slopes were significantly different, the slopes for NaCl and PBS were close to each other, while the slope for DMEM/F12 was obviously smaller. We attribute this difference to interactions between Mg²⁺ and the diverse ionic and organic ligand components found in DMEM/F12 that can alter both the ionic charge on Mg²⁺ and its mobility. For example, since Mg²⁺ has an intermediate binding strength to organic ligands, especially those that are base/donor ligands found in DMEM/F12 [90], formation of complexes with a different charge would be expected. Binding to larger structures will also affect ionic mobility.

Conductivity can also be affected or altered by the viscosity of the solution, which could be different in the more complex DMEM/F12 [76,77]. However, the viscosities of each solution were measured and there were no significant differences in viscosity between the solutions before adding pure magnesium metal discs (Table 2.1). These experiments showed that the conductivity sensor functions reliably and effectively in all three electrolyte solutions, providing detection of Mg²⁺ with high precision across a wide but acceptable physiologically appropriate concentration range.
Fig 2.3. Calibration curve of conductivity vs concentration of MgCl₂ added to the respective electrolyte solutions.
Table 2.1. Viscosities of Electrolyte Solutions.

Viscosities were measured 9 times per solution (to give averages ± one standard deviation) and were not statistically different across the four solutions (one-way ANOVA, \( n = 4 \) solutions, \( p = 0.947 \)).

<table>
<thead>
<tr>
<th>Solution Type</th>
<th>Viscosity (cSt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl</td>
<td>0.92 ± 0.04</td>
</tr>
<tr>
<td>PBS</td>
<td>0.91 ± 0.04</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>0.92 ± 0.01</td>
</tr>
<tr>
<td>DI Water</td>
<td>0.91 ± 0.01</td>
</tr>
</tbody>
</table>

2.3.2 Magnesium Corrosion during Immersion Tests

In order to demonstrate sensor applicability for magnesium corrosion monitoring, we followed real-time changes in conductivity and pH during the corrosion of identically prepared magnesium metal pellets in the form of discs over a 48 h time period after immersion in each of the three test electrolyte solutions (Fig. 2.4). Bench in vitro conditions at 37°C were used for immersion in NaCl and PBS and in vitro mammalian cell culture conditions (37°C, 100% humidity, 5% CO\(_2\), and sterility) were used for DMEM/F12. These real-time changes were then compared to the corrosion rate, determined by standard equations after weighing the pellets (discs) before and after
immersion, and osmolality, measured using an osmometer, in samples of the electrolyte solutions taken before and after corrosion.

The major ionic corrosion products for Mg are Mg$^{2+}$ and OH$^{-}$ ions. Because of the OH$^{-}$, the buffering capacity of the solution can have a significant impact or effect on corrosion, affecting both the deposition of reaction or corrosion products on the sample surface and concentrations of soluble ions dissolving in the corrosion solution. Two of our electrolyte solutions are buffered (PBS and DMEM/F12) whereas NaCl is un-buffered. When pure magnesium metal corrodes in an un-buffered solution such as NaCl, an initial increase in pH is expected due to the rapid release of OH$^{-}$ and Mg$^{2+}$ ions into the corrosion solution. When the pH becomes sufficiently high, indicating alkalinity (> ca. 9) the solubility product constant for Mg(OH)$_2$ will be reached, causing precipitation of Mg(OH)$_2$ [91]. The Mg(OH)$_2$ precipitated will not be detectable via conductivity, both because it is neutral and because it deposits on the surface of the magnesium disc, but this precipitate layer could block subsequent Mg$^{2+}$ ion release and therefore slow down magnesium corrosion. This consistent result was clearly observed previously during the measurements made with the CCS [61], and has been documented by others [91].

2.3.3 Real-Time Changes in pH and Conductivity during Mg Corrosion in NaCl

As we expected, when pH was monitored in real-time during the corrosion of pure magnesium metal in NaCl (Fig. 2.4A and 2.4D), the pH increased rapidly within the first 4 h to a high value (at an average of 10.1 pH units) and then maintained a stable value of around 9.5 until the end of the 48 h immersion studies. The conductivity change (Fig. 2.4A and 2.4E) also showed an initial sharp rise and then, as predicted, a distinct plateau
in the slope denoting a slowing of the conductivity rate change. Statistical analysis of the values at 0, 6, 12, 24 and 48 h indicated that the increases in both pH and conductivity were significantly different from the starting value by 6 h, but that no further values were significantly different from 6 h or each other using a one-way repeated measures ANOVA which was used for all immersion studies, indicating significance at the p<0.001 level. Pairwise comparisons for pH are shown in Table 2.2 and for conductivity in Table 2.3. Thus, overall, for both measurements of real-time conductivity and pH, the final values at 48 h were significantly different from starting values. Thus, the combination of measurements gave a clear picture of the dynamics of corrosion as well as the pattern of changes occurring during the release of soluble ionic components over the 48 h time period, which should reflect changes in the dynamics of corrosion, as well as the rates of corrosion.

2.3.4 Real-Time Changes in pH and Conductivity during Mg Corrosion in PBS

As expected due to the greater buffering capacity compared to NaCl [61,92], magnesium corrosion in our second solution, PBS, resulted in smaller pH changes overall, with an initial rise time that was not as rapid nor as high (Fig. 2.4B and 2.4D) as we saw with NaCl. These changes were significantly different (ANOVA, p <0.001) and all pH values from 6 h onwards were significantly higher than the starting value (including the 48 h value), but were not different from the 6 h value (Table 2.2).

The conductivity change over 48 h in PBS, in comparison to the conductivity change in NaCl but in accordance with the smaller change in pH, did not show a rapid initial change. Instead, there was a gradual rise or increase over the entire time period chosen (Fig. 2.4B and 2.4E), with significant changes in the conductivity over 48 h
(ANOVA, \(p<0.001\)). Specifically, the initial change between 0 and 6 h was significant, but conductivity did not change further between 6 and 24 h and then it increased significantly between 24 and 48 h, with the total conductivity change between 0 and 48 h being significant (Table 2.3). Thus, the patterns and dynamics of real-time changes in conductivity and pH over the 48 h time period were very different from those in NaCl, suggesting different corrosion mechanisms.

Interestingly, in PBS, both pH and conductivity measurements showed a higher variability throughout almost the entire 48 h for the three magnesium pellets (discs) compared to NaCl and, as will also be seen below, to values in DMEM/F12 (Table 2.4 gives coefficients of variation for the total 0-48 h changes, in each solution). The same measures used to control experimental conditions were used in all trials of the experiments so this variability appears to be inherent to magnesium corrosion in PBS.

Summary of statistical analyses of conductivity (Table 2.2) and pH (Table 2.3) for repeated measurements taken in each solution at 0, 6, 12, 24, and 48 h for magnesium metal immersed just after the measurement at 0 h. Each measurement (conductivity or pH) in each of the three electrolyte solutions (NaCl, PBS, DMEM/F12) was analyzed with a one-way repeated measures ANOVA. All resulted in \(p\) values of \(<0.001\). The tables below give the results of all pairwise multiple comparison procedures using the Holm-Sidak method and a significance level of \(p<0.05\) was considered significant for these comparisons.
2.3.5 Real-Time Changes in pH and Conductivity during Mg Corrosion in DMEM/F12

The third electrolyte solution, DMEM/F12, is a more complex solution that includes sodium bicarbonate (NaHCO₃), which is the major buffering system in most cell culture media. This compound combines with H₂CO₃ generated from dissolved CO₂ in the cell culture incubator. The concentration of NaHCO₃ in this particular medium is correctly controlled or buffered at physiological pH by using 5% CO₂ in humidified air. The solution also contained antibiotics and an antimycotic solution used to inhibit the growth of bacteria, fungi and yeast in order to maintain sterility throughout the entire immersion test time.

The changes in pH were very small (Fig. 2.4C and 2.4D) but significant (ANOVA, \( p<0.001 \)) showing a continual rise with the 6, 12, 24 and 48 h time points significantly different from 0 h and all other pairs different except for the 12 h versus 24 h values (See Table 2.2). Also, note the very low variability in the real-time pH values in DMEM/F12.
Fig. 2.4. Mg metal corrosion in 3 solutions. Conductivity and pH were monitored during corrosion of pure Mg discs over 48 h, in NaCl (A), PBS (B), and DMEM/F12 (C). Panels D and E compare changes across the three solutions for conductivity (D) and pH (E).

The conductivity values for magnesium metal corrosion in DMEM/F12, like those in PBS, increased steadily over the course of the entire immersion experiment (Fig. 2.4C and 2.4E). Significant differences were seen (ANOVA, $p<0.001$), and the starting value was different from 6 h also, and from all subsequent time points (See Table 2.3). The 48 h value was different also from 6, 12 and 24 h values (See Table 2.3). The real-time conductivity measurements showed an increase in variability over the entire immersion time period, which may have to do with an increasing complexity in the corrosion products being formed over time in this strongly buffered solution.

Thus, in both PBS and DMEM/F12, a combination of conductivity and pH measurements in real-time allow monitoring significant changes in the release of soluble ionic components that will not only reflect, as in NaCl, changes in the dynamics and rate of corrosion, but also changes in ionic components that can significantly impact the interaction of adjacent cells and tissues with an implanted magnesium biomaterial.

2.3.6 Statistics for Comparisons Across solutions

The measures of magnesium metal corrosion were compared across the three electrolyte solutions. Table 2.4 below summarizes the averages, standard deviations and coefficients of variation (relative standard deviations) for 3 magnesium pellets per measurement of magnesium corrosion during immersion in each solution (see also Fig.
Measurements were A) the change in conductivity between 0 and 48 h, B) the change in pH between 0 and 48 h, C) the corrosion rate, determined from the change in pellet weights between 0 and 48 h, and D) the change in measured osmolality between 0 and 48 h (before and after immersion). Data across the three media were compared using a one-way ANOVA (n = 3 media) and p values are given for each ANOVA. All data were normally distributed. If significant (p<0.05), then all pairwise multiple comparisons were done using the Holm-Sidak method and significantly different pairs of values (at the p <0.05 level) are indicated by similar letters, per medium, in the last column.

Table 2.2. Statistics for Changes in Conductivity during Magnesium Corrosion (Time Point Comparisons).

<table>
<thead>
<tr>
<th>Solution Type</th>
<th>Comparison</th>
<th>P value</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl</td>
<td>48 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>24 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>12 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>48 h vs 12 h</td>
<td>0.137</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>48 h vs 24 h</td>
<td>0.302</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>24 h vs 12 h</td>
<td>0.409</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>6 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>48 h vs 6 h</td>
<td>0.043</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>24 h vs 6 h</td>
<td>0.376</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>12 h vs 6 h</td>
<td>0.622</td>
<td>No</td>
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<td></td>
<td>48 h vs 0 h</td>
<td>24 h vs 0 h</td>
<td>12 h vs 0 h</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>PBS</strong></td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>DMEM/F12</strong></td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 2.3. Statistics for Changes in pH during Magnesium Corrosion (Time Point Comparisons)

<table>
<thead>
<tr>
<th>Solution Type</th>
<th>Comparison</th>
<th>P value</th>
<th>P&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl</td>
<td>6 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>12 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>24 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>48 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>48 h vs 6 h</td>
<td>0.138</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>48 h vs 12 h</td>
<td>0.218</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>48 h vs 24 h</td>
<td>0.704</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>24 h vs 12 h</td>
<td>0.407</td>
<td>No</td>
</tr>
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<td></td>
<td>24 h vs 6 h</td>
<td>0.292</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>12 h vs 6 h</td>
<td>0.676</td>
<td>No</td>
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<tr>
<td>PBS</td>
<td>48 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>24 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>12 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>6 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>48 h vs 6 h</td>
<td>0.934</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>48 h vs 12 h</td>
<td>0.757</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>48 h vs 24 h</td>
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<td>No</td>
</tr>
<tr>
<td></td>
<td>24 h vs 12 h</td>
<td>0.778</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>24 h vs 6 h</td>
<td>0.804</td>
<td>No</td>
</tr>
<tr>
<td></td>
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<td>0.705</td>
<td>No</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>48 h vs 0 h</td>
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<td>Yes</td>
</tr>
<tr>
<td></td>
<td>24 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>12 h vs 0 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>6 h vs 0 h</td>
<td>0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>48 h vs 6 h</td>
<td>&lt;0.001</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>48 h vs 12 h</td>
<td>0.005</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>48 h vs 24 h</td>
<td>0.029</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>24 h vs 12 h</td>
<td>0.109</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>24 h vs 6 h</td>
<td>0.006</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>12 h vs 6 h</td>
<td>0.043</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 2.4. Comparisons across Solutions, per Measure of Corrosion.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Measure</th>
<th>ANOVA p value</th>
<th>Ave</th>
<th>SD</th>
<th>Coeff. of Variation</th>
<th>Significant Pairs (a,b or c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>Corrosion Rate</td>
<td>0.033</td>
<td>9.30</td>
<td>2.03</td>
<td>21.8</td>
<td>a</td>
</tr>
<tr>
<td>PBS</td>
<td>Corrosion Rate</td>
<td>13.44</td>
<td>4.88</td>
<td>36.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Corrosion Rate</td>
<td>20.54</td>
<td>4.25</td>
<td>20.7</td>
<td></td>
<td>a</td>
</tr>
<tr>
<td>NaCl</td>
<td>Conductivity 0-48h</td>
<td>0.285</td>
<td>1.12</td>
<td>0.11</td>
<td>9.7</td>
<td>a</td>
</tr>
<tr>
<td>PBS</td>
<td>Conductivity 0-48h</td>
<td>1.08</td>
<td>0.43</td>
<td>39.4</td>
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<td></td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Conductivity 0-48h</td>
<td>1.56</td>
<td>0.47</td>
<td>30.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>Change in pH 0-48h</td>
<td>&lt;0.001</td>
<td>3.00</td>
<td>0.49</td>
<td>16.2</td>
<td>a,b</td>
</tr>
<tr>
<td>PBS</td>
<td>Change in pH 0-48h</td>
<td>1.28</td>
<td>0.25</td>
<td>19.2</td>
<td></td>
<td>a,c</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Change in pH 0-48h</td>
<td>0.39</td>
<td>0.04</td>
<td>10.9</td>
<td></td>
<td>b,c</td>
</tr>
<tr>
<td>NaCl</td>
<td>MsrdOsm 0-48h</td>
<td>&lt;0.001</td>
<td>8.67</td>
<td>3.06</td>
<td>35.2</td>
<td>a</td>
</tr>
<tr>
<td>PBS</td>
<td>MsrdOsm 0-48h</td>
<td>7.33</td>
<td>4.16</td>
<td>56.8</td>
<td></td>
<td>b</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>MsrdOsm 0-48h</td>
<td>25.33</td>
<td>2.52</td>
<td>9.9</td>
<td></td>
<td>a,b</td>
</tr>
</tbody>
</table>

2.3.7 Characterization of the Corrosion Layers on the Surface of Pellets after Immersion by Scanning Electron Microscopy (SEM), Energy Dispersive X-ray Spectroscopy (EDX) and Fourier Transform Infrared Spectroscopy (FTIR)

To further understand conductivity changes in relationship to magnesium corrosion, the surface morphology of the pure Mg discs after immersion was characterized by SEM, EDX (energy dispersive x-ray spectroscopy), and ATR-FTIR. It has been well established that the formed Mg$^{2+}$ ions either dissolve in the corrosion solution or precipitate as an insoluble corrosion product layer on the surface of the discs.
SEM images of the magnesium discs (Figure 2.5: A, B, and C) depict the surface morphology of the corroded Mg discs after immersion in NaCl, PBS, and DMEM/F12 for 48 h. Pitting corrosion was not observed with samples immersed in any of the corrosion solutions. However, a film with uniform cracks was observed on the two samples immersed in PBS and NaCl (Figure 2.5A and 2.5B). The cracks visible on the surface of these two samples are a result of a thick corrosion layer losing water during the process of air drying of corroded samples and then by placement in a vacuum chamber for SEM and EDX measurements. The sample that corroded in DMEM/F12 is very different, showing extensive corrosion that forms a very rough surface with a few cracked and clumped up layers (Figure 2.5C). These images suggest both differences and similarities in the corrosion layers on the discs.

Fig 2.5. SEM images of the corroded surface of pure Mg discs after immersion in (A) NaCl, (B) PBS and (C) DMEM/F12. Bars equal 50 µm.

The EDX spectra were obtained for the samples immersed in PBS and DMEM/F12 but not NaCl, because they were more physiologically appropriate and relevant for
eventual in vivo application. As shown in Fig. 2.6A and B, strong signals for Mg and O were seen in samples immersed in both PBS and DMEM/F12, suggesting that these surfaces have been covered by oxides (MgO and Mg(OH)\(_2\)). Strong signals for P seen in both samples suggest that the corrosion layer in both also contained phosphates. The EDX spectrum of the sample immersed in DMEM/F12 also included strong signals for Ca. This suggests that calcium ions present in this solution were capable of reacting with both CO\(_3^{2-}\) and PO\(_4^{3-}\) to form calcium containing precipitates such as calcium carbonates, calcium phosphates, and possible calcium hydroxyapatite that precipitate on the sample surface during corrosion. The formation of such compounds on the surface of the Mg would place them in a position to interact with adjacent cells and tissues. This is interesting in terms of bone implantation because these particular compounds are capable of inducing bone formation.

ATR-FTIR was used to further examine the functional groups present in the corrosion layers for the samples immersed in PBS and DMEM/F12 for 48 h. As shown in Fig. 2.6C and D, the broad absorption band from 3700 to 2500 cm\(^{-1}\) indicates the presence of hydrogen bonded H\(_2\)O in both samples. Thus, we can say that the corrosion product layer on the sample immersed in both DMEM/F12 and PBS contains MgO or Mg(OH)\(_2\). For the sample immersed in DMEM/F12, the absorption bands at ~1010, and ~550 cm\(^{-1}\) correspond to the presence of phosphates on the surface. For the sample immersed in PBS, the absorption bands at ~ 996, ~782, and ~579 cm\(^{-1}\) indicate that phosphates are one of the major corrosion products on the magnesium surface. For the sample immersed in DMEM/F12, the band at ~1426 cm\(^{-1}\) corresponds to carbonates present on the surface. Thus, we can say that the corrosion product layer on the sample
immersed in DMEM/F12 contains carbonates, phosphates and MgO/Mg(OH)$_2$, while the sample immersed in PBS contains phosphates and oxides, but does not contain the carbonates, as expected. These results are consistent with the results obtained from EDX.

2.3.8 Changes in Corrosion Determined by Weight Change and Metal Characterization

We had speculated that the total change in real-time conductivity over 48 h would be correlated to the actual corrosion rate if the corrosion products are primarily soluble ions that are detectable by conductivity. To analyze corrosion via standard methods, the Mg pellets were removed after the 48 h immersion, rinsed with distilled water, air dried, imaged using SEM, and characterized using EDX and ATR-FTIR. The SEM images (Fig. 2.5) showed the presence of complex precipitation layers on samples in each solution, and the visual appearance of the layers was different between solutions. No evidence of pitting was seen for any of the solutions. The EDX analysis, done only for samples immersed in the more physiologically appropriate and relevant PBS and DMEM/F12 solutions, showed Mg, O and P in both samples, with an additional Ca signal in DMEM/F12, as shown in Fig. 2.6A and 2.6B). This suggests that the precipitates in both contained oxides (MgO and Mg(OH)$_2$) and phosphates and, in DMEM/F12, precipitates also contained calcium. These results were consistent with results from in vivo corrosion of Mg alloys implanted subcutaneously in mice [69]. The ATR-FTIR analysis of samples immersed in PBS and DMEM/F12 (Fig. 2.6C and 2.6D), indicated the presence in both
solutions of oxides (broad absorption band from 3700 to 2500 cm\(^{-1}\)) and phosphates (bands at \(~1010\) and \(~550\) cm\(^{-1}\) for the DMEM/F12 sample and \(~996\), \(~782\) and \(~579\) cm\(^{-1}\) for the PBS sample). The sample in DMEM/F12 additionally showed a band at \(~1426\) cm\(^{-1}\) that corresponds to carbonates present on the surface. These analyses are consistent with complexes forming between Mg and the major anions in each solution.

After these analyses, the pellets were cleaned with chromic acid and weighed to calculate corrosion rates from standard equations. As shown in Fig. 2.7A and detailed in (Table 2.4) the corrosion rates were, in the order of highest to lowest: DMEM/F12 > PBS > NaCl. There were significant differences across the three solutions (one-way ANOVA, \(p=0.033\)), but only the corrosion rates in DMEM/F12 and NaCl were significantly different from each other, while the corrosion rate in PBS was not different from either DMEM/F12
Fig 2.6. (A and B) EDX spectra (CPS = counts per second) of the corroded surface of pure Mg discs after immersion in (A) PBS and (B) DMEM/F12. (C and D) ATR-FTIR spectra of the corroded surface of pure Mg discs after corrosion in (C) PBS, and (D) DMEM/F12.

or NaCl (Table 2.4). Thus, the corrosion rate was highest in DMEM/F12 and was significantly different only from the rate in NaCl.

Fig. 2.7. Measurements of Mg corrosion compared across solutions: NaCl (N), PBS (P) and DMEM/F12 (D). Corrosion rate is shown in (A) and changes across 48 h are shown...
for conductivity (B), pH (C), and measured osmolality (D). Bars with arrows indicate significant differences between pairs of values.

A higher corrosion rate in DMEM/F12 was expected compared to both NaCl and PBS, at least in part due to the total greater buffering capacity, which leads to greater consumption of the released OH⁻, which then leads to a significantly higher release of Mg²⁺ ions into the corrosion solution. In addition to the phosphates that provide buffering actions in both PBS and DMEM/F12, DMEM/F12 has sodium bicarbonate (NaHCO₃), which is also an important buffer due to formation in solution of the bicarbonate ion (HCO₃⁻). The levels of bicarbonate (hence buffering capacity) are kept constant in the medium by using a continuous supply of 5% CO₂ gas concentration (in the culture incubator) to prevent conversion of bicarbonate to CO₂ gas. The concentration of HCO₃⁻ in DMEM/F12 is higher than the concentration of HPO₄²⁻ in PBS. In fact, considering the concentrations of all buffering agents, the total concentration of buffering agents in DMEM/F12 is 30.24 mM and that of PBS is 11.8 mM (no buffering agents in NaCl). During magnesium corrosion, the presence of bicarbonate ions results in the formation of magnesium carbonates. Some will precipitate, as was seen on the magnesium pellets with ATR-FTIR.

The solubility product constant (Ksp) of MgCO₃ is $6.82 \times 10^{-6}$ while the Ksp for Mg₃(PO₄)₂ is $1.04 \times 10^{-24}$, meaning a higher solubility for MgCO₃, suggesting that the magnesium carbonates produced in DMEM/F12 are more soluble than the magnesium phosphates that are the primary products in PBS. Thus in DMEM/F12, the presence of magnesium carbonates would be expected to result in less precipitation due to higher solubility and thus less of a corrosion inhibiting layer, and greater corrosion rates. Thus, we expected
to see greater differences in the corrosion rates in DMEM/F12 than in both other solutions (NaCl and PBS), but we only saw a significant difference between DMEM/F12 and NaCl. One possible factor in this comparison was that there was again higher variability in the corrosion rates in PBS when compared to the other solutions (see coefficients of variation given in Table 2.4), which significantly influences the statistical analysis. Overall, magnesium corrosion in cell culture media, under cell culture conditions, is a very complex process and very different from that in the other solutions, which were done under normal atmospheric conditions.

2.3.9 Comparisons of Mg Metal Corrosion Measures across Electrolyte Solutions

The total change in conductivity over the 48 h immersion of the magnesium pellets is a measure of the total amount of ions released into solution by magnesium corrosion because the solution was not replenished during this immersion period. Although the corrosion rates differed across solutions, but the total conductivity changes over 48 h were not significantly different when compared across the three solutions (Fig. 2.7B) (ANOVA, $p = 0.285$). There was a trend towards an increased change of conductivity in DMEM/F12, however the larger variability again observed in PBS influences to the lack of statistically significant differences (see Table 2.4). However, overall, this suggests that the total ionic mobilities of the Mg$^{2+}$ and OH$^-$ ions released into solution (or changes in the anions of the buffers due to their reaction with OH$^-$) during corrosion of the magnesium metal pellet or discs were approximately the same in each solution.

The total changes in pH over 48 h Mg corrosion were significantly different when compared across solutions (Fig. 2.7C) (ANOVA, $p<0.001$) and each paired comparison was significantly different (Table 2.4). The relative pH change values were (highest to
lowest) NaCl > PBS > DMEM/F12. These relative changes confirm that DMEM/F12 has the highest buffering capacity, as discussed above. The relative pH changes were roughly inversely related to the changes in corrosion rates across solutions. The sample immersed in DMEM/F12 under cell culture conditions exhibited the smallest and least variable pH changes, as expected due to the highest buffering capacity, so it is presumed that no pH-dependent precipitation products formed in DMEM/F12. However, there was precipitation in DMEM/F12, as evidenced by our studies of the corrosion layer, so obviously this precipitation was not sufficient enough to prevent a continued release of ions (ionic corrosion products), as detected by the increase in conductivity.

2.4 Changes in Osmolality over 48 hours of Corrosion.

Another measure of the release of corrosion products is osmolality, which has been used previously to monitor Mg corrosion using an osmometer [89] that measures solution osmolality based on changes in some physical property of the solution such as freezing point depression, boiling point elevation, vapor pressure lowering, or osmotic pressure [84,86,93]. We measured osmolality with an osmometer in each basal solution (0 h values) and after immersion for 48 h (without solution changes) and the data are shown in Fig. 2.7D. Monitoring osmolality in real time during the corrosion of magnesium was not possible because, to the best of our knowledge, no portable osmolality sensor is available that could be used for this application. If osmolality measurements are taken offline during in vitro testing, it requires removing or changing solutions. If solution exchanges are large enough, they can significantly influence the rate of Mg corrosion. In contrast, a conductivity sensor has the capability to continuously monitor Mg corrosion in real-time due to its small size, portability and technological
advancements in the latest instrumentation. The changes in total measured osmolality changes over 48 h were significantly different when compared across solutions (Fig. 2.7D) (ANOVA, p<0.001). The post hoc analysis showed that the NaCl and PBS changes were equal and both were significantly lower than in DMEM/F12 (Table 2.4). Thus, the measured osmolality changes across solutions showed similarities to the pattern seen with corrosion rates (DMEM/F12 > NaCl).

2.5 Discussion

The power of both pH and conductivity sensors is to provide real-time measurements. We have shown that, within each solution, both sensors provided impressive amounts of data that can be used to study the time course of Mg degradation processes. The importance of monitoring conductivity in real time is clearly illustrated by the results in Fig. 2.4 where a significant difference in the course of corrosion between unbuffered and buffered solutions is revealed. In unbuffered 0.9% NaCl the release of Mg$^{2+}$ into solution occurs primarily in the first 4 hours of the experiment (Fig 2.4A). Hypothetically, an implant material that corrodes in this way would expose adjacent cells to an exceptionally large concentration of Mg$^{2+}$ in a relatively short time. By comparison, the release of Mg$^{2+}$ in the buffered solutions (Figs. 2.4A and 2.4C) occurs gradually over the entire time course of the experiment. This slower time course of corrosion would provide time for the removal of excess Mg$^{2+}$ by a combination of lymph and blood flow, as well as uptake by adjacent cells, and would avoid exposing cells to a potentially lethal “burst” of Mg$^{2+}$. Additionally, the results in Fig. 2.4 suggest that essentially all of the corrosion that releases solution soluble Mg$^{2+}$ in unbuffered medium is finished after about 4 hours, whereas the corrosion in the buffered media continues steadily throughout the
experiment. The difference in these two behaviors has important implications for the time required for complete bioresorption of an Mg implant. This detailed information is not revealed by the conventional weight loss measurements, which only give a total mass of Mg lost over the course of the experiment. It would seem reasonable to screen all potential Mg materials that are being considered for implants to determine the time course of Mg\(^{2+}\) release. It might also be useful to run experiments in an unbuffered solution as we did here to determine the “consequences” of fast initial corrosion that overwhelms the buffer capacity immediately adjacent to the implant so that localized pH exceeds the physiological level of 7.2, which then might cause the mode of corrosion to change further. This type of information is important for gaining a detailed understanding of issues that can affect the biocompatibility of Mg implants.

The conductivity sensor has proven to be an effective, relatively easy to use technique for monitoring the solution soluble corrosion products in real time. Its main advantage compared to the ion-selective electrode that we used in the CCS [61] to monitor Mg\(^{2+}\) is the absence of frequent calibration. Because of signal drifting (an inherent problem with ISEs), the ISE in the CCS required daily calibration using a procedure that required removal of the sensor from the solution and immersion in a calibration solution in a separate cell. Performing this step would be much more difficult in the DMEM/F12 experiments in the incubator, where opening the door disrupts both the temperature and the \(\text{CO}_2\) atmosphere used to maintain the buffer. Also, the ISE relies on a selective membrane to detect Mg\(^{2+}\), which has a finite lifetime. By comparison, the conductivity sensor has two solid electrodes that are shielded, making it much more rugged and long-lasting. We also found that the conductivity sensor did not have problems with signal drift.
due to fouling of the electrodes by material adsorbing on the electrode surfaces. We were anticipating problems with this when we moved to the DMEM/F12 medium because of concerns that one or more of the organic material components might adsorb on the electrodes and change the response. However, we found no problems with this in the three media examined.

The conductivity sensor is very precise and covers a wide range of Mg concentrations, as shown by the plots in Fig. 2.3, where the error bars were so small as to be almost totally obscured by the data point symbols. The sensor’s precision is more than adequate for corrosion studies such as these where the main uncertainty is in the corrosion itself as seen by comparing the error bars in Fig. 2.4 with those in Fig 2.3. Calibration curves obtained on the sensor before and after a corrosion run were essentially identical.

We have demonstrated, in parallel studies (see chapter 3), that relative conductivity changes do correlate with actual Mg corrosion rates when all measurements were made in a one type of cell culture medium (containing serum), under cell culture conditions. Thus, within a single solution, conductivity can be a useful sensor to study relative corrosion rates and thereby support work to alter the corrosion rate of Mg and eventually perfect the metal for use in biomedical implants.

Comparisons between absolute corrosion rates and the three measures of solution soluble materials (pH, conductivity and osmolality) are complicated by the fact that the latter do not take into account the precipitation layer deposited on the metal. Comparisons across solutions are also complicated by the complexities of ionic interactions, especially in the solutions with multiple ionic species (PBS and
DMEM/F12). It is also important to note that the conductivity changes in NaCl leveled off after 48 h, but conductivity in both buffered solutions was still steadily rising. Thus, if immersion times were increased, conductivity changes might show greater similarities to both corrosion and osmolality changes, when comparing across solutions. However, longer experiments would require a change in solutions to mimic in vivo conditions, where blood and lymph flow would prevent the build-up of degradation products that is seen in static cell culture conditions. This would require an interruption in sensor readings. One possible solution might be a flow chamber arrangement where solutions could be changed without interrupting continuous sensor readings. While the solution soluble measures (conductivity and osmolality) may not be useful in comparing across different types of solutions, these soluble ionic components formed by Mg degradation are of the greatest biological importance to cells and tissues in the vicinity of a Mg metal implant, as stated previously. Therefore, the more measures that are available to study these species, the better we can understand and further characterize cellular reactions to Mg implants. Here we report that conductivity can be used for such studies.

As stated above, and as seen by comparing the coefficients of variation (Table 2.4), a relatively higher variability occurred when samples were immersed in PBS, compared to the other solutions. As the same methods and rigor were used in all solutions, we propose that the relatively weaker buffering capacity of PBS contributed to the variability in Mg corrosion, and perhaps also the nature of Mg metal corrosion. Variability has been observed previously when comparing Mg corrosion rates in PBS vs NaCl [94]. A higher Mg corrosion rate in PBS vs NaCl was seen over the first two days, but this changed to a lower rate in PBS vs NaCl over six days in solution [94]. The initial
rapid phase of corrosion was proposed to be due, first, to the lack of a precipitation layer on the Mg sample in the buffered PBS, and was also speculated to be due to pitting phenomena that they observed in PBS but not NaCl at the early time points [94]. Pitting might lead to variability, but we did not observe evidence of pitting in PBS in our SEM images. Based on these observations of higher variability, we suggest that it might be prudent to avoid solutions like PBS that have relatively weak buffering systems. This might also apply to solutions like Hank’s Balance Salt Solution (HBSS), which relies primarily on dilution effects to buffer pH changes. It is important to repeat here that even though the changes in conductivity were not significantly different across solutions, there were significant differences in conductivity over the 48 h immersion time within each type of solution.

2.6 Conclusions

In this study, we demonstrated the validity and usefulness of using measurements of conductivity combined with pH to follow Mg metal corrosion in real-time. The conductivity sensor was sensitive over a range of Mg salt concentrations that is both biologically relevant and expected during Mg corrosion. The commercially available conductivity and pH sensors were able to follow temporal changes in Mg corrosion in real-time under conditions used to culture mammalian cells. Thus, it is now possible to follow the time course of release of the soluble ionic by-products of Mg corrosion, which are components that can have a very significant impact on neighboring cells and tissues when a Mg metal implant is placed in the body.

The success of our real-time measurements suggests that conductivity will also be useful for comparing the relative rates of corrosion of different preparations of Mg
metal, as long as the comparisons are made within a single solution. The complexities of the ionic interactions in the different solutions makes comparisons of Mg corrosion rates difficult across solution types. Included in this complexity were variability in several measurements of Mg corrosion in the weakly buffered PBS solution compared to either an un-buffered solution (NaCl) or a strongly buffered solution (DMEM/F12). This supports the use of more highly buffered solutions, similar to those found in vivo, for testing and perfecting methods to alter and regulate Mg corrosion, either by alloying or post-processing treatments. In summary, we propose that the conductivity sensor is an important addition to the arsenal of sensors that are available for monitoring, and therefore studying, the mechanisms of and control of Mg corrosion, with a future goal of developing successful biomedical implants.

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Chapter 3: Conductivity as a Sensor for Monitoring Relative Magnesium Corrosion Rates in Real-Time, in Serum-Containing Media under Cell Culture Conditions

3.1 Introduction

Many recent studies have shown that magnesium and magnesium based alloys are promising candidates for the development of biodegradable metallic implant materials for orthopedic applications [7,33,38,56,61,95]. Using degradable magnesium materials would provide a distinct advantage over permanent metallic implant materials such as stainless steel, cobalt-chromium alloys, and titanium alloys that are designed to stay permanently in the human body during the process of bone healing, unless a second surgery is performed to remove them. Magnesium has other unique properties in addition to its ability to degrade in aqueous physiological environments that should allow it to outperform currently used implant materials. It is light weight and its density and elastic modulus are closer to that of natural bone than any of the metals currently in use [37,96,97]. However, magnesium, in the currently available forms, has very low corrosion resistance, in that it corrodes rapidly in aqueous solution releasing large volumes of hydrogen gas which can affect wound healing. Because of the importance of controlling the corrosion rates of magnesium, researchers in this field are investigating multiple methods to control corrosion rates. Alloing elements that are generally non-toxic, surface coatings with biocompatible polymers, and surface modifications by anodization and plasma electrolytic oxidation (PEO) are currently being tested by some research groups to slow down corrosion rates [98-102]. Another approach is to take
advantage of the fact that, in general, corrosion of polycrystalline materials tends to be higher at grain boundaries because these regions are known to be regions of high energy in these materials. Thus, in this paper, we used a high purity magnesium, single crystal magnesium, as one promising way to achieve corrosion control [103], because of the lack of grain boundaries [104].

To aid the development of an optimal form of magnesium, several methods are currently being used to monitor the corrosion of magnesium and altered forms of magnesium. The commonly used methods include weight loss measurements, recording of electrochemical impedance spectra and potentiodynamic polarization curves, collection of hydrogen gas evolved, pH measurements, microscopy, spectroscopy and measurements of the amount of magnesium released [51,65,67,81,105-107]. The major focus of our research group is to develop electrochemical sensors for the purpose of studying magnesium corrosion. Because the corrosion byproducts of magnesium metal are predominantly ionic, we decided to explore the use of a commercially available conductivity sensor for real-time monitoring of magnesium corrosion. There are several advantages of a conductivity sensor over other sensors for the purposes of monitoring magnesium corrosion. A conductivity sensor has the capability to continuously monitor magnesium corrosion in real-time due to its small size, portability and technological advancements in the latest instrumentation. Conductivity measurements also have advantages over, and are simpler to use, than measurements made by ion-selective electrodes (ISE) for Mg\textsuperscript{2+}. ISEs are prone to signal drifting and protein fouling during long term continuous measurements, especially in the type of serum-containing solutions that are routinely
used in cell culture to duplicate conditions in the body. ISEs also require routine
calibration, which is not required with a conductivity sensor. A conductivity sensor also
can be simpler, in operational terms, than a hydrogen gas evolution system, which
requires careful capture of all gases released during corrosion. Such a device can be
successfully employed to evaluate the effectiveness of different surface treatments such
as anodic oxidation or chemical etching aiming to provide corrosion protection and
control [108-110]

The objective of this research was to use real-time conductivity measurements to
monitor and compare the corrosion behavior of high purity magnesium single crystals
treated with or without a surface modification designed to slow down corrosion. The
conditions used for corrosion were close to in vivo conditions, because they were those
used for culturing mammalian cells in vitro. We then directly compared the relative
changes in real-time conductivity over the set time period of corrosion with several
other, more standard, measures of corrosion to determine how relative conductivity
changes correlated with these measures.

3.2 Materials and Methods

3.2.1 Reagents

CrO$_3$, Ba(NO$_3$)$_2$, and AgNO$_3$ were ACS grade reagents obtained from Fisher
Scientific. Deionized water with a resistivity value of 18 megohm-cm, which was used in
preparing all solutions, was obtained from a Barnstead water purification system
(Sybron, Boston Massachusetts). Conductivity calibration standards with conductivity
values ranging from 10 µS/cm to 100,000 µS/cm (Fisher Scientific & Oakton) were used
for sensor calibration.
3.2.2 Magnesium Sample Preparation

A crystal grower (CVD Equipment Corporation, Central Islip, NY) was employed in this work. The initial poly-crystalline Mg with purity of 99.95% (Alfa Aesar, Ward Hill, MA) was used as raw material to grow single crystals. A graphite crucible with a tapered shape was used to contain the molten material which was enclosed in an external holder made of a special grade stainless steel. The growth process takes place in a vertical quartz tube under argon flow. The tube was surrounded by a vertical crystal furnace. The furnace had two temperature zones to create and control an appropriate temperature gradient in the crucible. The melt was then soaked for several hours to enable complete homogenization. The single crystal was grown by a Bridgman-Stock Barger approach which involves growing the single crystal from the melt by controlled withdrawal of the furnace chamber under a suitable thermal gradient. A typical Mg single crystal size with length of 45 mm, diameter of 6.5mm and orientation close to (0001), was used in this study. A total of six crystals (also called crystal discs) were used in this study. Three were further modified after production by a chemical etching procedure (detailed methods described in [108]). Both etched and non-etched crystals were prepared for immersion by weighing. Sterilization was done by spraying crystal discs with 70% ethanol solution, and then allowing to sit under UV light for 15 min immediately before the immersion test.

3.2.3 Instrumentation

Real time conductivity measurements were performed with a MeasureNet multi-functional chemical analysis network (MCAN®) system consisting of one measurement workstation managed by a single MCAN® controller and PC (the system can support up
to 15 workstations). The work station had two ± 2.5 V analog input channels and one high speed serial communication channel. The analog inputs were sampled by a high resolution 24-bit Sigma-Delta A/D with built-in signal conditioning and noise reduction. Measurements were stored on the system PC and streamed in real time to the cloud for storage, which could be monitored in real time from any internet connected device to follow the progress while running experiments for extended time periods. Preamplifiers and isolation transformers constructed for sensors used in this work have been previously described [61]. A conductivity probe with graphite electrodes (MeasureNet Ltd) was used to take real time conductivity measurements. Osmolality measurements were taken on a Wescor® 5500 vapor pressure osmometer (Wescor Inc., Logan, Utah).

All data analysis was carried out using commercial spreadsheets and graphing algorithms.

3.2.4 Immersion Test

Prior to the immersion tests, the weight of each magnesium single crystal disc was recorded. Immersion testing was carried out in Dulbecco’s modified Eagle’s medium (DMEM, Thermo-Fisher, St. Louis, MO) supplemented with 10% sterile filtered fetal bovine serum (FBS, Thermo-Fisher Hyclone) under cell culture conditions (5% CO₂ in humidified air, 37 °C) in a cell culture incubator under sterile conditions.

Sensors and Mg single crystal samples were inserted into medium in the upper of two 100 mL plastic tripour beakers nested inside each other, with the top one having multiple holes to allow continuous flow of media during immersion, and a stir bar in the lower compartment (resulting from the nesting configuration). A volume of 50 mL was
used for each immersion test for a period of 45 h. After all immersion tests, samples were removed from the corrosion solution, rinsed with deionized water and the surface was then observed by scanning electron microscopy (SEM), energy dispersive x-ray spectroscopy (EDX), attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) and X-ray induced photoelectron spectroscopy (see next section).

3.2.5 Corroded Surface Characterization

High magnification images of magnesium crystal discs after immersion but before cleaning with chromic acid were taken using an XL 30 ESEM scanning electron microscope (SEM) (Fei Co. Hillsboro, Oregon) with an accelerating voltage of 20-30 kV, coupled with an energy dispersive X-ray spectroscopy detector (EDAX Inc., Mahwah, NJ) for obtaining elemental composition of corroded magnesium discs. X-ray photoelectron spectroscopy (XPS) was performed using a Thermo Scientific K-Alpha XPS instrument. The K-Alpha uses Al-Ka x-rays focused to a spot 400 microns in diameter. Emitted photoelectrons were energy analyzed using a 180° double focusing hemispherical analyzer with a 128-channel detector. Survey data were collected at a pass energy of 200 eV and an energy resolution of 1 eV/step, while core level data were collected at 50 eV pass energy and 0.1 eV/step energy resolution. Sample charging was eliminated by using the K-Alpha’s dual-beam charge compensation source, which uses both low energy Ar-ion and low energy electrons. An Ar-ion gun (operated at 2 kV) was used for in-situ cleaning of the sample surface. Data were collected and analyzed using the Avantage data system (v.4.61, Thermo Fisher Scientific, Waltham, MA, USA).
Functional groups in the corrosion products layers were observed using a Nicolet 6700 FTIR spectrophotometer (Thermo Scientific, Madison, WI) equipped with a smart orbit diamond attenuated total reflectance (ATR) module. The OMNIC software (Thermo Scientific, Madison, WI) was used to obtain spectra from 4000 to 400 cm⁻¹ after 32 scans.

3.2.6 Elemental Analysis of Corrosion Solutions

An Agilent 7500 ICPMS (Agilent Technologies, Santa Clara, CA, USA) with rf generator and octopole collision/reaction cell system (ORS) was used to determine the total Mg concentration in the solutions after the corrosion tests for comparison with the results of the conductivity changes from the conductivity sensor. The samples were prepared as follows: a 50 µL aliquot of Mg sample was diluted 200 times in 2% (v/v) Trace Nitric Acid (Fisher Scientific) to a volume of 10 mL. Prior to dilution, 50 µL of 500 µg/L internal standard (composed of ⁷Li, ⁴⁶Sc, ⁷²Ge, ⁸⁹Y, ¹¹⁵In and ²⁰⁹Bi) was added. Calibration was performed using the Claritas (SPEX Certiiprep) multi-element standard solution (5% nitric acid (v/v)) at element concentration levels: 0; 0.05; 0.1; 0.2; 2.0; and 5.0 mg/L and with addition of the internal standards (2.5 µg/L Li, 2.5 µg/L Sc, 2.5 µg/L Ge, 2.5 µg/L Y, 2.5 µg/L In and 2.5 µg/L Bi). Samples were introduced to the Agilent 7500 ICPMS system in no gas mode. The instrumental operating parameters were as follows: rf power 1550 W, carrier gas flow rate 1.08 L/min, make-up gas flow rate 0.10 L/min, nickel sampling and skimmer cones, and performed in no gas mode. The following elements were analyzed for total element study: ⁶⁶Zn, ²⁴Mg, ¹¹¹Cd.
3.2.7 Corrosion Rates

After the corrosion test and the initial analyses mentioned above, the corrosion products were removed with chromic acid and the difference in weight before the immersion test and after the corrosion products were removed was used to calculate the corrosion rate (CR) according to the following equation:

\[
CR = \frac{87.6 \times \Delta W}{Ap t}
\]  

(1)

The weight loss (\(\Delta W\)) in mg is divided by the exposed surface area (\(A\)) in cm\(^2\), the density (\(\rho\)) in g/cm\(^3\), and the time (\(t\)) of exposure in hours.

3.2.8. Statistics

Three etched and three non-etched single crystals were treated to the same immersion and post-immersion protocols. Statistics were t-tests with p<0.05 considered significant (using a Sigma Stat/Sigma Plot version 13 software). All error bars indicate one standard deviation.

3.3 Results and Discussion

3.3.1 Conductivity, Osmolality, and Total Magnesium

In this study, we have used a commercially available conductivity sensor with graphite electrodes to demonstrate an inexpensive and simple method of following the corrosion of magnesium single crystal samples in cell culture medium under sterile cell culture conditions. This sensor has the capability of monitoring an increase in the total dissolved ion concentration as magnesium corrodes in our chosen model electrolyte.
solution. DMEM supplemented with 10% fetal bovine serum (FBS) buffered in 5% CO$_2$ was used as a model electrolyte solution in this study as this is a solution used routinely in cell culture studies to try to recreate in vivo body conditions. This medium contains many additives required for culturing cells in vitro, including amino acids, vitamins, glucose, phenol red as a visual pH indicator, sodium bicarbonate, and sodium pyruvate, amongst others [111]. During the corrosion of magnesium, the corrosion products both deposit on the surface of the magnesium disc samples and dissolve in the corrosion medium [37,61,96,97,111,112]. But the absolute amount of released ions in solution and rate of change of the concentration of these ions will vary from solution to solution based on the ratio of production of deposits versus soluble ions. Thus, in this work, we used just one corrosion solution to compare sensor responses to corrosion rates obtained from differently processed magnesium single crystal samples. One of our magnesium samples was unpolished (as cut) and the other was polished and then chemically etched. Both polishing and chemical etching have the potential to alter the rate of corrosion [113]. The unpolished sample showed a greater change in real-time conductivity of 1.0 ± 0.2 mS/cm (as shown in Figure 3.1) over the entire immersion period when compared to the polished and chemically etched sample, which showed a much smaller change in real-time conductivity of 0.3 ± 0.1 mS/cm over the entire immersion time period. We attribute the higher real-time conductivity change resulting from the unpolished sample to its extremely rough surface and its lack of a protective covering on its surface. Both qualities would be expected to make it more susceptible to corrosion and hence would result in a higher Mg$^{2+}$ release. As for the polished and chemically etched sample, it is believed that a very dense transparent nanolayer of
oxides such as MgO is being formed on the surface [108,109,113], thereby giving it better corrosion protection, resulting in a slower release of Mg\(^{2+}\) and hence a lower real-time conductivity change.

Osmolality of the solutions before and after the immersion test was measured in order to compare the relative change to that with the conductivity sensor, because previous experiments have shown that osmolality is a good measure of magnesium corrosion. Interestingly, corrosion of the unpolished sample, which resulted in the highest real-time conductivity change, also gave the highest osmolality change and corrosion of the polished and chemically etched sample resulted in the lowest change in osmolality, as with the change in real-time conductivity measurements (Figure 3.2). Our findings confirmed that polishing and etching process provided some corrosion protection that resulted in slowing the release of Mg\(^{2+}\).

Since conductivity will only detect free magnesium ion concentration in a solution, we sought to compare this with the total soluble magnesium ion concentration released over the entire immersion period, using ICPMS. We tested magnesium ion levels in the corrosion solution collected at the end of the immersion test. This gives the total soluble magnesium content released over the entire time period because the medium was not changed over that set time period. It was expected that the sample that resulted in the highest real-time conductivity change would result in the highest total soluble magnesium concentration by ICPMS. The total magnesium concentration for the media for unpolished samples was 241 ± 3 p.p.m, which is higher than that measured in the media from the polished and chemically etched samples (19 ± 1 p.p.m), indicative of slower corrosion rate for this sample. This is consistent with the results from the
conductivity sensor, indicating a higher Mg\textsuperscript{2+} release during corrosion for the unpolished compared to the polished samples (Table 3.1).

Figure 3.1. Conductivity sensor response in DMEM + 10% FBS during corrosion of Mg single crystals with differing surface treatments under cell culture conditions
Figure 3.2. Measured osmolalities before and after immersion of Mg samples in DMEM plus 10% FBS under cell culture conditions.

Table 3.1. Overall Results Summary for Comparisons

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Conductivity Change (mS/cm)</th>
<th>Osmolality Change (mOsm/kg)</th>
<th>Total Magnesium Concentration (p.p.m)</th>
<th>Corrosion Rate (mm/y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unpolished</td>
<td>1.0±0.2</td>
<td>45±3</td>
<td>241±3</td>
<td>50±7</td>
</tr>
<tr>
<td>Polished&amp;Etched</td>
<td>0.3±0.1</td>
<td>11±4</td>
<td>19±1</td>
<td>5±3</td>
</tr>
</tbody>
</table>
3.3.2. Surface characterization

Towards determining how well real-time conductivity changes reflect and can be used to study magnesium metal corrosion, we used several other powerful techniques to characterize the corrosion layers of the magnesium single crystals after their 45 h treatment. First, the surface morphology of the magnesium single crystal after corrosion was characterized by SEM, EDX (energy dispersive x-ray spectroscopy), XPS (X-ray photoelectron spectroscopy), and ATR-FTIR. It is well known that the formed magnesium ions either dissolve in the corrosion solution or form precipitates that make an insoluble corrosion product layer on the surface. SEMs of the magnesium single crystal samples (Figures 3.3A-3.3D) depict their surface morphology before and after corrosion. Both samples studied lacked grain boundaries, as was expected due to their single crystal nature. The unpolished sample before immersion in the corrosion medium had a very rough surface, while the surface of the polished and etched sample before immersion in the same corrosion medium was very smooth. After corrosion, the corrosion layer on the chemically etched sample (Fig. 3.3D) did not reveal features that were consistent with pitting corrosion. Instead, a thin film with uniform cracks was observed on the polished and chemically etched sample, as shown in Figure 3.3D. The cracks visible on the surface of this sample are the result of the corrosion layer losing water during the process of drying. The surface of the unpolished sample, treated to the same conditions, was quite different. It showed more extensive corrosion, as evidenced by a much rougher and more complex surface that appears to be thicker, as seen in Figure 3.3B. These SEM photographs definitely shows differences between the corrosion layers formed on the two discs during immersion.
The EDX spectra, as shown in Figures 3.3E and 3.3F, show that Mg and O were present on the surfaces of both samples after corrosion, suggesting that these surfaces have been covered by MgO and Mg(OH)$_2$. Weak signals for Mg, O, P, C, and Ca in the polished and chemically etched sample suggests that a thin corrosion layer of oxides, phosphates and calcium-containing corrosion products was formed on this sample. The EDX spectra of the unpolished sample immersed in DMEM supplemented with serum included much stronger signals for Ca, Mg, O, C, and P, suggesting that a thicker corrosion layer was formed, composed of the same types of oxides, phosphates, carbonates and calcium-containing corrosion products. The presence of signals for Ca in the EDX spectra obtained for both samples suggests that the calcium ions present in this medium were capable of reacting with both CO$_3^{2-}$ and PO$_4^{3-}$ to form calcium-containing corrosion products such as calcium carbonates, calcium phosphates, and possibly calcium hydroxyapatite. These obviously precipitated on the sample surface and it is interesting that this would be happening in the solution immediately adjacent to the magnesium sample surface.

The chemical composition of the corrosion layers on the single crystals after corrosion (but before chromic acid treatment to remove the corrosion layers) was analyzed by XPS, and the results are shown in Fig 3.4. The following core levels were analyzed besides the survey spectra: Mg 2p, O 1s, C 1s, and P 2p. According to the XPS survey scanning spectra of both materials, their elemental compositions included Mg, O, N, C, Ca and P. The high resolution spectra for the Mg 2p, O 1s, C 1s and P 2p in both materials revealed the presence of Mg$^{2+}$, Mg(OH)$_2$, CO$_3^{2-}$, and PO$_4^{3-}$. The presence of the elements Ca and P in the corrosion layers obviously suggests that they
were new corrosion products deposited on both magnesium single crystal surfaces analyzed after immersion, of which these XPS results were consistent with the results obtained with EDX.

ATR-FTIR was used to examine the functional groups present in the corrosion product layers before chromic acid cleaning. FTIR spectra were obtained for both samples after a 45 h immersion. As displayed in Figure 3.5A and 3.5B, the broad absorption band at 3235 cm\(^{-1}\) indicates the presence of hydrogen bonded H\(_2\)O in both samples. For both samples, the bands at 1418 and 1409 cm\(^{-1}\) correspond to carbonates present on both surfaces, while the absorption bands at 1013, 1011, 862, 560 and 550 cm\(^{-1}\) correspond to the presence of phosphates on both sample surfaces. The very low absorption band not clearly visible for both samples corresponds to the presence of a Mg-O bond, which suggests the presence of MgO or Mg(OH)\(_2\) on the surface of the discs. Based on the absorption bands observed in the ATR-FTIR of these samples, we can categorically say that the corrosion product layers deposited on both samples contain carbonates, phosphates and MgO/Mg(OH)\(_2\). These results are very much consistent with the results obtained from EDX and XPS.

3.3.3. Corrosion Rate Behaviors

To better understand our results, we determined the corrosion rate from weight loss measurements of the discs after removing the corrosion products with chromic acid, as previously described [61]. The two types of single crystal samples used in this study were of high purity, and they lacked grain boundary defects, which, if ordinarily present, would tend to reduce the electrical and thermal conductivity properties of these materials and would in turn favor localized corrosion at some preferred sites in a
crystalline material as these are regions of high energy. The average corrosion rate calculated from weight loss measurements was $5 \pm 3$ mm/y for the polished and chemically etched samples. For the unpolished samples, the average corrosion rate was $50 \pm 7$ mm/y. Thus, the corrosion rate was lowest for the polished and chemically etched sample compared to the unpolished sample (Fig 3.6 and Table 3.1). This result is consistent with the concept that the chemical etching procedure gives some type of corrosion protection due to the formation of a dense nanolayer of oxides and hydroxides, which is also consistent with the results from the conductivity sensor.

3.3.4. Statistical analysis

A two-tailed t-test was used to compare the corrosion rates, the measured osmolalities before and after corrosion, and the total soluble magnesium concentration obtained from ICPMS for the two types of magnesium samples studied. Overall, there were statistical significant differences between all values obtained for the unpolished
and polished and etched samples at a significance level of p<0.005. (Table 3.1)
Figure 3.3. Microscopy before and after immersion. SEM images of Mg samples before and after immersion in DMEM plus 10% FBS under cell culture conditions (A, B) unpolished sample, and (C, D) polished and etched sample. EDX spectra of Mg samples before and after immersion (E, F) unpolished sample, and (G, H) polished and etched sample.
Figure 3.4. XPS survey spectra of Mg samples (Unpolished (A), Polished&Etched (B)).

XPS peak fitting for unpolished Mg sample showing the C1s, Mg2p, P2p, and O1s (panels C-F) and XPS peak fitting for the polished and etched Mg sample showing the C1s, Mg2p, P2p, and O1s (panels G-J).
Figure 3.5. ATR-FTIR spectra of the corroded surface of Mg single crystal discs after corrosion in DMEM supplemented with 10% FBS under cell culture conditions.
Figure 3.6. Corrosion rates calculated from mass loss measurements of Mg single crystal samples immersed in DMEM supplemented with 10% FBS under cell culture.

3.4 Conclusion

A conductivity sensor that allows real-time monitoring of magnesium corrosion under cell culture in a physiologically relevant fluid was successfully developed and demonstrated [114]. For the ease of adoption by researchers in this area of study, we selected a commercially available conductivity sensor with graphite electrodes because it is very easy to use, it doesn’t require routine calibration at different time intervals, it resists biofouling in the presence of proteins and living cells, and it can be used with corrosion solutions that contain high levels of calcium and magnesium. As expected, the
sensor was very stable throughout the entire real-time conductivity measurements exhibiting satisfactory performance for our application.

As a proof of concept, we used this sensor to observe the corrosion behavior of two previously untested magnesium single crystal samples that differed in their surface treatments that were designed to slow down their corrosion rates in physiologically appropriate cell culture fluids under cell culture conditions. The results from this sensor showed that the unpolished magnesium single crystal sample released higher concentrations of ions into the corrosion solution when compared to the polished and chemically etched samples. The validity of the use of the conductivity sensor for comparing corrosion rates in real-time was further confirmed by determining that there were also higher levels of soluble magnesium ions (determined using ICPMS) and a higher osmolality (using an osmometer) in the unpolished samples compared to the polished and etched samples. Standard post-immersion analyses showed significant differences in the deposition of precipitation products that were also consistent with a higher corrosion rate of the unpolished samples. Thus, overall, this sensor was shown to be very useful in comparing the corrosion behavior of magnesium materials with different proven corrosion rates. It also has the capability of performing satisfactorily in serum-containing media, which makes the use of some other types of sensor quite complicated. The conductivity sensor not only reliably compared relative corrosion rates, it also added another capability, the ability to measure corrosion in real-time throughout a set immersion period as well as revealing the dynamics of corrosion for the samples tested. This should provide a new dimension to studies of the corrosion behavior of metals in the field of metallurgy. For example, this could be used to study
alterations in the corrosion rate that might be caused by acute exposure of the magnesium to individual components of biological fluids.

In the future, we plan to continue to use real-time conductivity as a valuable tool for studying methods of altering magnesium metal corrosion, as well as to study the effects of exposing magnesium to diverse biologically relevant compounds, under cell culture conditions that are a good in vitro approximation of physiologically relevant conditions.

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Chapter 4: Cathodic Stripping Voltammetric Determination of Cerium Using Indium Tin Oxide (ITO) as a Working Electrode

4.1 Introduction

Cerium is gaining a lot of attention in the area of metallurgy and materials engineering, because of its diverse applications. Some of its applications include and are not limited to the manufacture of television, glass and ceramics, the core for most carbon electrodes of arc lamps, gas lightning, glass polishing and components of catalytic converter for most vehicles [115,116]. Cerium is also being used as an alloying element for magnesium, aluminum, iron, and stainless steel [117]. Cerium being the most abundant of the rare earth elements, makes up about 0.0046% of the Earth’s crust by weight. Although, there are no known biological properties of cerium, the obvious similarities in the chemistry of cerium and calcium has prompted research into its therapeutic applications. It was documented that antihistamine drugs eventually replaced cerium oxalate for treatment of emesis [118]. It has also been documented that cerium is dangerous in the work environment because damps and gases of cerium can easily be inhaled, which can cause lung embolisms, especially during long term exposure [119,120]. Also, cerium from these gases and damps can accumulate in the body and pose a major threat to the liver. Having properties similar to that of calcium, cerium accumulates in the bones in small amounts [120,121]. Some researchers have also documented that cerium is a cofactor for the methanol dehydrogenase of the methanotrophic bacterium methylacidiphilum fumariolicum and cerium salts are also known to stimulate metabolism [122,123].
Instrumental techniques used for cerium detection include: inductively coupled plasma optical emission spectroscopy (ICP-OES) [124,125], X-ray fluorescence spectroscopy [126], spectrophotometry [127,128], neutron activation analysis (NAA) [129], graphite furnace atomic absorption spectroscopy (GF-AAS) [125], chemiluminescence [130], fluorimetry [131], and ion-selective electrodes [132-135]. Some of these techniques require lengthy sample preparation, are complicated and are often either inaccessible or expensive techniques, and they only give measurements of total concentration with the exception of ion-selective electrodes. Thus, the development of simple inexpensive and direct methods for Ce$^{3+}$ detection is necessary.

Stripping analysis is one of the best known electroanalytical techniques because it incorporates an electrolytic preconcentration step, which leads to extremely low detection limits [87]. The excellent detection limits observed are largely due to incorporation of a deposition or preconcentration step during which the target analyte is accumulated onto the working electrode surface either by electrodeposition at a certain potential or by adsorption. During the stripping step for anodic stripping voltammetry (ASV), the working electrode potential is swept in the positive direction (oxidizing) in order to effectively strip the analyte off of the working electrode surface, while the stripping step for cathodic stripping voltammetry (CSV) involves sweeping the working electrode potential in the negative direction (reducing) in order to effectively strip off the target analyte of the working electrode surface [87,136]. This stripping step results in a measureable faradaic current (current due to the target analyte) which is proportional to the concentration of the analyte in or on the electrode, and thus in the sample solution [87,136]. During the preconcentration step for potentiometric stripping analysis (PSA),
the analyte of interest is electrolytically deposited by reduction onto a mercury electrode, while the stripping step is achieved through chemical oxidation with oxygen or mercuric ions present in solution, while adsorptive stripping voltammetry (AdSV) uses the formation of an appropriate metal chelate, accompanied by its controlled interfacial accumulation onto a working electrode of which the adsorbed metal chelate becomes reduced by applying a negative potential scan [87,136].

Anodic stripping voltammetry (ASV), cathodic stripping voltammetry (CSV), adsorptive stripping voltammetry (AdSV), and potentiometric stripping voltammetry are the commonly used stripping analysis techniques [87,136] with adsorptive and cathodic stripping voltammetry being the most frequently found for cerium detection in literature [137,138]. However, anodic stripping voltammetry (ASV) is the most commonly found stripping technique in literature for the determination of a number of trace metals such as lead, cadmium, zinc, mercury, and copper [87,136,139-143]. For cerium determination, the deposition potential required is too negative for detection by ASV, and this can be a challenge for most electrode materials to reach before hydrogen evolution becomes an interference as the Ce³⁺/Ce⁰ redox couple has a very negative standard reduction potential of -2.48 V. Abbas et al. used a novel potentiometric sensor composed of new nano-composite carbon paste electrode fabricated from (Z)-2-((1H-1,2,4-triazol-3-imin)methyl)phenol, as an ionophore, a multiwalled carbon nanotube (MWCNT), nanosilica, and air and water-stable ionic liquid [BMP]Tf₂N for the quantification of Ce³⁺ and achieved a detection limit of 6.45 nM [144]. While this novel sensor only required a response time of ca. 5 s and gave good selectivity, the requirement of a combination of reagents for modification of a carbon paste electrode
makes it less attractive because of extensive sample preparation and probable toxicity issues with organic compounds. Mehran et al. used adsorptive stripping voltammetry on a carbon paste electrode modified with N’-[(2-hydroxyphenyl)methylidene]-2-furohydrazide (NHMF), which is based on the adsorptive accumulation and oxidation of the accumulated Ce(III) to Ce(IV) ions for the determination of cerium and achieved a detection limit of 0.8 nM [145]. A major disadvantage of AdSV is the need to use an organic ligand or a complexing agent and the use of a mercury electrode, which is a requirement of many adsorptive stripping techniques [146], although some have used modified carbon paste electrodes like the ones already mentioned [145]. CSV for cerium has been reported where accumulation of cerium as insoluble Ce₃(PO₄)₄ was studied on a platinum indicator electrode [147].

Indium tin oxide (ITO) is a traditional working electrode material commonly used for spectroelectrochemical studies because it combines both conductivity and optical transparency [148-153], which makes it useful in the development of a spectroelectrochemical sensor that incorporates different modes of selectivity. Another advantage of ITO as a working electrode is that it has a wide positive potential window capable of measurements beyond +1.5 V in water samples and a very smooth background current, making it a suitable working electrode for CSV [154].

The rationale for exploring the detection of cerium by our research group is tied to the fact that cerium, being a member of the lanthanide group has been used as an alloying element for magnesium. Because it can be difficult to detect magnesium and other corrosion products of magnesium when it degrades in aqueous solution, then one of the ways that has been suggested to follow corrosion is to include an element that is easy
to detect in the magnesium alloying materials, making it an integral part, and something that will be released upon corrosion. Another reason is to determine what happens to Ce when the magnesium corrodes in physiologically appropriate solutions. The best option is to be able to have an element that is easy to detect and improves the qualities of magnesium as a biomedical implant material. One example has been europium (Eu) whose detection was explored by our research group [155]. Detection of trace Eu$^{3+}$ was achieved by modifying a glassy carbon electrode with a Nafion film loaded with a novel catalyst-free multi-walled carbon nanotubes (MWCNTs) achieving a calculated detection limit of 0.37 nM [155].

In this study, we explored the detection of Ce$^{3+}$ by CSV using a bare ITO electrode as a working electrode. A series of potential deposition and deposition time optimizations were done using 400 nM Ce$^{3+}$ in 0.1 M acetate buffer with pH 4.5. To the best of our knowledge, this is the first time an indium tin oxide (ITO) working electrode has been used for CSV of cerium. We also demonstrated the possibility of detecting Ce$^{3+}$ in the presence of typical potentially interfering metal ions capable of affecting its stripping peak.

4.2 Experimental

4.2.1 Chemicals and Materials

Cerium(III) nitrate (99.999% purity) was purchased from Fisher Scientific without further purification and was used to make a 10 mM cerium(III) nitrate stock solution in acetate buffer of pH 4.5. Glacial acetic acid (85%, Pharmco Brookfield, CT) and sodium acetate (Fisher Scientific) were mixed in the right proportion to yield the desired pH of
acetate buffer used for our analyses. Deionized water with a resistivity value of 18 megohm-cm, was used in preparing all stock solutions as needed.

4.2.2 Instrumentation

Electrochemical measurements for CSV were executed in a 20 mL conventional three electrode cell consisting of ITO coated glass slides (Corning 1737F, 11-50 Ω/sq, 135 nm thick film on 1.1 mm glass, Thin Film Devices, Anaheim, CA) with 10 mm X 40 mm dimensions as working electrode, a Ag/AgCl reference electrode (3.0 M KCl solution), and a platinum wire auxiliary electrode. An Accumet excel XL50 Dual channel pH/conductivity meter (Fisher Scientific) was used for taking pH measurements. The potentiostat was a BASi 100B electrochemical analyzer (Epsilon, Bioanalytical Systems).

The basic parameters for Osteryoung square wave voltammetry (OSWV) that was used for the stripping step were square wave amplitude = 25 mV, step potential = 5 mV, and frequency = 25 Hz. The extrapolated baseline current method described by Kissinger and Heineman was used to manually measure peak currents ($i_p$) [87,136].

4.2.3 Statistics

Statistical analyses were done with SigmaStat (version 13). All data were normally distributed and ANOVAs (regular or repeated measures, one or two way) were followed by all pairwise multiple comparison (post-hoc) tests using the Holm-Sidak method. Error bars indicate one standard deviation and significance was set at a level of $p<0.05$. 

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4.3 Results and Discussion

The detection of cerium by differential pulse ASV and AdSV have been previously reported. However, to date, in literature, electrodes that have been used include carbon paste electrodes, glassy carbon electrodes, and platinum electrodes. Deposition potential and deposition time optimization experiments were then done to determine the optimal potential and time that gives rise to the highest peak current.

4.3.1 Cyclic Voltammetry of Ce\(^{3+}\) at Bare ITO Electrode

Cyclic voltammetry (CV) was used to initially investigate the electrochemistry of Ce\(^{3+}\) at a bare ITO electrode, because it has proven to be a powerful tool for fundamental and diagnostic studies that provides qualitative information about electrochemical processes under varying conditions. CV experiments were performed in aqueous Ce(NO\(_3\))\(_3\) solution at bare ITO electrode. Ce(NO\(_3\))\(_3\) concentration was 5 mM in 0.1 M acetate buffer of pH 4.5 supporting electrolyte for these studies. The potential of the bare ITO electrode was scanned positively from -0.4 to 2.0 V and back at scan rates of 5, 10, 15, 20, 25, 30, 45, 50, 75 and 100 mV/s. The forward scan of the cyclic voltammograms showed a broad oxidation region with two waves having no well-defined peaks whereas the reverse scan showed a sharp and well defined cathodic peak current for reduction whose potential was considerably more negative than it would be for a reversible process. The sharpness of the cathodic peak is characteristic of reduction of a surface confined material. The overall electrode process has been characterized as follows:

\[
\text{Ce}^{3+} + 2\text{H}_2\text{O} \rightarrow \text{CeO}_2 + 4\text{H}^+ + e^-
\]
where the electrogenerated Ce$^{4+}$ reacts with water to form insoluble CeO$_2$. As seen in Figure 4.1, the positive electrode potential scan from -0.4 V to 2.0 V was sufficient enough to oxidize Ce$^{3+}$ to Ce$^{4+}$ (CeO$_2$) at circa 0.8 V, which preconcentrates cerium at the electrode surface as insoluble CeO$_2$ that then strips off the electrode when reduced during the negative potential scan. Most importantly for CSV, the reduction occurs as a well-defined, sharp peak in a potential region with smooth background current so it would be easily quantified.

During the reverse scan from 2.0 V to -0.4 V, the reduction peaks back to Ce$^{3+}$ at the varying scan rates were observed circa at 0.3 V. A plot of cathodic and anodic peak currents versus scan rate and square root of scan rate (data not shown) yielded a straight line for both, suggesting the electrode chemical reaction might have been either a diffusion-controlled process for the forward scan and surface-controlled irreversible electrode transfer processes for the reverse scan.
Figure 4.1. (A) Cyclic voltammograms (all scan rates) at bare ITO in 5 mM Ce(NO$_3$)$_3$ in 0.1 M acetate buffer, pH 4.5. Potential range: -0.4 V to +2.0 V. (B) Individual Scan rates plot from the lowest to the highest peak current: 5, 10, 15, 20, 25, 30, 45, 50, 75 and 100 mV/s.
4.3.2 Cathodic Stripping Voltammetry (CSV) Optimizations

We used Osteryoung square wave voltammetry (OSWSV) as the stripping step for CSV due to its ability to minimize non-faradaic currents, thereby achieving low limits of detection. For this study, deposition potential and deposition time parameters were examined. Deposition potential optimization is a critical parameter in stripping voltammetry (ASV or CSV). In CSV, the deposition potential is a potential positive of the standard reduction potential to oxidize ions on the electrode surface. Figure 4.2A shows the deposition optimization at a bare ITO with a Ce$^{3+}$ concentration of 400 nM in 0.1 M acetate buffer solution of pH 4.5; a potential range of +1.35 V to +2.0 V was examined. Peak current response initially increased between +1.35V and +1.45 V. Between +1.45 V and +1.65 V, indicative of more positive potentials, the peak current response increase was very gradual. Beyond +1.65 V, a rapid increase in the peak current was observed with the maximum peak current response observed at +2.0 V. During this deposition optimization in our more positive deposition potentials, we didn’t observe any dramatic decrease in the peak current responses obtained, and this suggests non oxidation of water molecules to oxygen gas that generates bubbles capable of interfering with the deposition of CeO$_2$ at the electrode surface. A potential of +2.0 V was chosen as the optimal deposition potential because it yielded the highest peak current and was used throughout our studies.

Deposition time optimization is also very important during CSV or ASV, because deposition time can significantly affect analyte peak current responses. As deposition time increases, sensitivity increases because it increases the degree of
preconcentration of the analyte of interest at the electrode surface, especially during the stripping step. However, deposition time can be increased to a point where analyte concentration gets depleted from the bulk solution onto the working electrode surface. Figure 4.2B shows the deposition time optimization at a bare ITO working electrode. A range of 1-10 min was investigated. A deposition time of 5 min was then chosen and used throughout our studies.
Figure 4.2. (A) The effect of deposition potential on the stripping current of 400 nM Ce\textsuperscript{3+} in 0.1 M acetate buffer (pH 4.5). Deposition time: 5 min. (B) The effect of deposition time on the stripping current of 400 nM Ce\textsuperscript{3+} in 0.1 M acetate buffer (pH 4.5). Deposition potential: +2.0 V.

4.3.3 Dynamic Range and Detection Limits

In order to evaluate bare ITO as a working electrode to quantitatively determine Ce\textsuperscript{3+} concentration, a calibration curve was constructed under optimized conditions in order to determine the dynamic range and detection limit. Ce\textsuperscript{3+} concentrations ranging
from 100 nM to 5000 nM were studied for the purpose of calibration curve construction. For this calibration curve construction, we used just one deposition time of 5 min.

Under the optimized conditions, the ITO electrode was used for the determination of Ce$^{3+}$. Well-defined stripping peaks were obtained in a potential region with a very smooth background current (Figure 4.3A). Peak current increases linearly with Ce$^{3+}$ concentration in the range of 100-700 nM and then essentially plateaus (Figure 4.3B). A reasonably linear response was obtained for concentrations below about 900 nM (Fig. 4.3C): $I_p (\mu A) = (266.2\pm5.1) \times 10^{-4} [\text{Ce}^{3+}] \text{nM} + (2.8\pm0.3)$, $R^2 = 0.998$. The calculated detection limit based on $3\sigma/m$ ($\sigma$ is the standard deviation of the lowest detectable Ce$^{3+}$ concentration measured at least 10 times) method was 5.8 nM. The ITO electrode yielded a lower detection limit compared to several other methods. For example, the PVC-based 1,3,5-Trithiane ISE gave a detection limit of 30 µM under a similar condition [156]. A selective PVC membrane sensor based on $N$-[(2-hydroxyphenyl)methylidene]-2-furohydrazide (NHMF), [4-(4-nitrobenzyl)-1-phenyl-3,5-pyrazolidinedion)] (NBPP), 2-aminobenzothiazole and oleic acid gave detection limits of 7.6 µM, 1.6 µM, and 1.8 µM, respectively [157-159]. The detection limit was 6.45 nM for a potentiometric sensor fabricated with a Schiff base-carbon nanotube–nanosilica–ionic liquid used as a high performance sensing material [160]. A fluorescence sensor for Ce$^{3+}$ based on quenching of glycine dithiocarbamate (GDTC)-functionalized manganese doped ZnS quantum dots (QDs) with a detection limit of 229 nM in phosphate buffer solution (pH 7.5) has been reported [161]. Rounaghi et al. [162] used an ISE fabricated with 2,9-dihydroxy-1,10-diphenoxy-4,7-dithia decane, a novel synthetic ligand for the detection of Ce$^{3+}$, and obtained a detection limit of 2.1 nM which is comparable to what was
obtained in our work with an ITO electrode. Based on our analyses, an ITO electrode yields a very low detection limit for Ce$^{3+}$ without the need for complicated fabrication processes.
Figure 4.3. (A) & (B) Cathodic stripping voltammograms with OSWV and calibration curves of 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000 and 5000 nM Ce\(^{3+}\) in 0.1 M acetate buffer of pH 4.5. Deposition time: 5 min, deposition potential: +2.0 V. (C) Relationship between peak current and Ce\(^{3+}\) concentration in the range of 100-700 nM.
4.3.4 Interference Study

Using the optimized parameters, the detection of 400 nM Ce$^{3+}$ with a 5 min deposition time was investigated in the presence of selected metal ions with magnesium ion being in excess of all other metal ions (Figure 4.4 and 4.5). However, we do not anticipate any serious interference from the metal ions chosen, because most metals do not form insoluble oxides at higher oxidation states, hence CSV provides selectivity against them. We chose a concentration of 5 ppb for all selected metal ions except Mg$^{2+}$, which was at a 100 ppb, and these concentrations were then converted to nM for easy comparison. No interferences were observed for 24 nM Bi$^{3+}$, 33 nM Eu$^{3+}$, 79 nM Cu$^{2+}$, 76 nM Zn$^{2+}$, 42 nM Sn$^{2+}$, and 4114 nM Mg$^{2+}$. However, the presence of 91 nM Mn$^{2+}$ enhanced the Ce$^{3+}$ peak by about 13% (Figure 4.4), but no additional peaks were observed for all metal ions studied. For all peaks compared statistically using a one-way or two-way ANOVA with the overall significance level set at p<0.05, we found no significant differences between the peak currents of cerium in comparison to the peak currents of all potential metal ion interferences except the peak currents observed for Mn$^{2+}$ (p=0.003), meaning the peak current for Mn$^{2+}$ is significantly different from the peak current observed for cerium. This peak current enhancement by Mn was probably due to its ability to also form an insoluble oxide layer (MnO$_2$) at a bare ITO electrode, just as Ce does. Thus, under optimum conditions chosen for our analyses, Ce and Mn are being stripped off the electrode surface at the same time and at the same cathodic peak potential (See Figure 4.6). Since manganese has been found to cause a number of health complications [163], its concentration as an alloying element for magnesium has been kept at trace levels with the intent of developing Mg-Mn alloys as potential
biodegradable implant materials [164], and its use has been discouraged by some researchers because of toxicity issues. Therefore, we do not anticipate any problems from Mn interference. Figure 4.6 shows the stripping voltammetry of Mn$^{2+}$ alone under similar conditions used for Ce$^{3+}$.

We chose an excess concentration of Mg$^{2+}$ as a potential interfering metal ion because cerium is a potential alloying element for magnesium and, most importantly for this application, no interference was observed even with magnesium at high excess concentration. In future, when screening for potential magnesium biomaterials containing alloying elements like cerium, sensors that would be developed for this application will need to exhibit selectivity for cerium over magnesium where magnesium will be present at much higher concentrations. For future studies, since an enhanced peak current was observed in the presence of Mn$^{2+}$, we propose that if for any reason, Mn as reported by Song et al. [165] and Ce are present as alloying elements, a new calibration curve should be made each time with this sensor with the aim of detecting cerium and ensuring reproducibility.
Figure 4.4. Interference study at bare ITO electrode in 400 nM Ce(III) ion in 0.1 M acetate buffer (pH 4.5) solution in the presence of 24 nM Bi$^{3+}$, 33 nM Eu$^{3+}$, 79 nM Cu$^{2+}$, 76 nM Zn$^{2+}$, 42 nM Sn$^{2+}$, 91 nM Mn$^{2+}$ and 4114 nM Mg$^{2+}$.
Figure 4.5. Cathodic Stripping Voltammograms of Ce$^{3+}$ at bare ITO in pH 4.5 acetate buffer with added metal interferences and excess of Mg$^{2+}$. Deposition time: 5 min.

Deposition potential: +2.0 V. [Ce$^{3+}$] = 400 nM.
Figure 4.6. Cathodic stripping voltammogram of Mn$^{2+}$ alone at bare ITO in pH 4.5 acetate buffer. Deposition time: 5 min. Deposition potential: +2.0 V.

4.4 Conclusions

A bare ITO working electrode was successfully used for the detection of Ce$^{3+}$ by CSV for the first time. This sensor is disposable, sensitive and low cost. A CV study shows the electron transfer process is both diffusion controlled and surface controlled. The
effects of deposition potential and deposition time on this electrode were investigated. This sensor exhibits higher sensitivity, selectivity and lower detection limits compared to the ion selective electrodes (ISEs) and fluorescence sensors that have been reported. An interference study shows the ITO electrode provides good selectivity. The presence of ions like Bi$^{3+}$, Cu$^{2+}$, Zn$^{2+}$, Sn$^{2+}$, Eu$^{3+}$, and Mg$^{2+}$ do not affect the performance of this electrode. However, the presence of Mn$^{2+}$ does affect the performance of the ITO electrode. In the presence of Mn$^{2+}$, a new calibration curve needs to be constructed with the goal of detecting cerium free of manganese interference.

The major point of this work is demonstrating the ability to use a bare ITO electrode for the determination of cerium in an acidic buffer without needing to modify this electrode with a thin polymer film for analyte preconcentration or carrying out multiple and complicated fabrication processes. We believe this will be a significant step forward in the application of this sensor to the determination of cerium levels in magnesium based alloys containing rare earth elements like cerium.

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Chapter 5: Summary, Conclusions and Future Work

This last chapter gives a summary of the entire work done in chapters 2, 3 and 4 and proposals for future research that could be done.

In chapter 2, we showed the importance of monitoring the corrosion of pure magnesium in real-time using a conductivity/pH sensor. Corrosion of pure magnesium was studied in three physiological appropriate solutions, a 0.9% sodium chloride solution (NaCl), phosphate buffered saline (1X PBS, pH=7.4), and Dulbecco’s modified eagle’s medium/nutrient mixture (DMEM/F12, 1:1 by volume), a cell culture medium maintained under the standard conditions used for mammalian cell culture. One of our model electrolytes is unbuffered, while the other two are buffered. In an unbuffered 0.9% sodium chloride solution, the release of Mg$^{2+}$ into this solution occurred and finished within the first 4 hours of immersion suggesting that any potential implant material that corrodes in this fashion would potentially expose the adjacent cells and tissues in the vicinity of the implant to very high concentration of Mg$^{2+}$ in a relatively short period of time. However, when pure Mg corrodes in our buffered electrolyte solutions PBS and DMEM/F12, and by comparison to the unbuffered 0.9% NaCl solution, the release of Mg$^{2+}$ into solution was very gradual, suggesting that any implant material corroding in this fashion would provide ample time for the removal of any excess Mg$^{2+}$ by a combination of lymph and blood flow, as well as uptake by adjacent cells and would avoid exposing them to exceptionally high Mg$^{2+}$ concentration. Thus,
this sensor, in combination of a pH sensor, recorded different patterns of real-time changes in the ionic environment created by magnesium degradation, in our three model electrolyte solutions. Statistical analysis of the real-time values at 0, 6, 12, 24 and 48 h immersion time in both pH and conductivity were significantly different from the starting value by 6 h, but no further values were significantly different from 6 h or each other using either a one way repeated measures analysis of variance (ANOVA).

However, several attempts to use real-time conductivity measures during magnesium degradation as a means of estimating osmolality proved abortive in many ways, mainly because conductivity responds to only charged particles in solutions, while osmolality responds to both charged and neutral species in a given solution. It is also possible that the interactions between ions, which can alter ionic mobility and therefore affect conductivity, contributed to the differences between conductivity and osmolality. The findings from this work also reveal the difficulty associated with the ability to make real-time measurements under cell culture conditions. Future work can look to improve the estimation of osmolality from conductivity by further studying magnesium corrosion in a single medium. Also, a more extensive analysis of the changes in conductivity with added MgCl₂ (and perhaps other Mg salts) at a concentration range between 0 mM and 10 mM might further improve the capability of this sensor to be able to estimate real osmolality. Furthermore, we propose studying the effects of adding Mg²⁺ to mammalian cells with this sensor.

In chapter 3, the conductivity sensor alone was used to follow the real-time course of corrosion of two high purity magnesium single crystals with different surface modifications. The modifications were designed to slow down magnesium
release/degradation. These experiments were conducted in a serum-containing cell culture solution and under cell culture conditions in a cell culture incubator. This sensor was also successful in establishing a correlation between conductivity changes, real osmolality changes, corrosion rates calculated from weight loss measurements, and total magnesium released into solution after immersion for 45 h. This was possible because our comparisons were made between samples immersed in the same solution.

For futures studies, for the sensor to be used either in vitro or in vivo, it would be appropriate to shift from a macro-type conductivity sensor to a portable and miniaturized type that can be used to continuously follow the course of magnesium and magnesium based alloys degradation, in cell culture plates with the smallest volume of corrosion solution as possible. We also propose to make these real-time measurements as routine and easy as possible, for easy adoption by many research labs, by developing a simplified and reliable standard operating procedure. In the immediate future, we can continue to use conductivity measurements to compare the corrosion rates of magnesium alloys developed within the ERC. We will continue to compare conductivity, which allows real-time measurements, to osmolality measurements, because osmolality is easier to measure. Understanding this comparison will help us develop a deeper understanding of the magnesium corrosion process.

Cellular metabolism can significantly alter the ionic composition of the cell culture medium (DMEM), which will alter conductivity, even in the absence of any magnesium, be it metallic or ionic. Thus, we propose, in the future, to use a conductivity sensor to study magnesium degradation in the presence of living cells, and examine how the presence of cells alters conductivity. This has the potential to provide information about
how the presence of cells alter degradation rate or the ability to detect the degradation rate using conductivity.

In chapter 4, cerium, one of the most abundant elements of the lanthanide series which has been widely used in the field of metallurgy, especially because of its current use as an alloying element for magnesium, was detected by cathodic stripping voltammetry (CSV) using indium tin oxide (ITO) as a working electrode in an acidic buffer. $\text{Ce}^{3+}/\text{Ce}^{4+}$ showed irreversible electrochemistry with very broad oxidation peaks and a very sharp reduction peak at a bare ITO electrode. Under optimized conditions, a calibration plot for $\text{Ce}^{3+}$ was linear in the concentration range of 100 nM to 700 nM, and a detection limit of 5.8 nM was found for a 5 min deposition time. ITO showed a good positive potential range with a smooth background current for cerium determination. The interesting part of this study was the fact that this is the first time cerium has been detected by CSV using ITO in an acidic buffer solution without the need for modifying this electrode with a charge selective polymer film or the need for a complex fabrication process. Thus, our results suggest that this sensor, because of its good selectivity and sensitivity, can be further developed for practical applications such as monitoring $\text{Ce}^{3+}$ released into solution during the degradation of magnesium cerium alloys being developed as a biodegradable implant material by the ERC.
References


[89] J. Fischer, D. Proefrock, N. Hort, R. Willumeit, F. Feyerabend, **2011**.


# Glossary: Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAS</td>
<td>Atomic Absorption Spectroscopy</td>
</tr>
<tr>
<td>AdSV</td>
<td>Adsorptive Stripping Voltammetry</td>
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<tr>
<td>ASTM</td>
<td>American Society for Testing and Materials</td>
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<tr>
<td>ASV</td>
<td>Anodic Stripping Voltammetry</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CCS</td>
<td>Corrosion Characterization System</td>
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<tr>
<td>CSV</td>
<td>Cathodic Stripping Voltammetry</td>
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<tr>
<td>CV</td>
<td>Cyclic Voltammetry</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>EDX</td>
<td>Energy Dispersive X-Ray Spectroscopy</td>
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<tr>
<td>ERC</td>
<td>Engineering Research Center</td>
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<tr>
<td>ERC-RMB</td>
<td>ERC for Revolutionizing Metallic Biomaterials</td>
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<tr>
<td>ESEM</td>
<td>Environmental Scanning Electron Microscopy</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>FCS</td>
<td>Fetal Calf Serum</td>
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<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
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<tr>
<td>HBSS</td>
<td>Hank’s Balanced Salt Solution</td>
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<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid</td>
</tr>
<tr>
<td>ICPMS</td>
<td>Inductively Coupled Plasma Mass Spectrometry</td>
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<tr>
<td>ISE</td>
<td>Ion Selective Electrode</td>
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<tr>
<td>ISO</td>
<td>International Standards Organization</td>
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<tr>
<td>ITO</td>
<td>Indium Tin Oxide</td>
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<tr>
<td>MWCNT</td>
<td>Multi-Walled Carbon Nanotube</td>
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<tr>
<td>OSWSV</td>
<td>Osteryoung Square Wave Stripping Voltammetry</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PSV</td>
<td>Potentiometric Stripping Voltammetry</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>SBF</td>
<td>Simulated Body Fluid</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<tr>
<td>XPS</td>
<td>X-Ray Photoelectron Spectroscopy</td>
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