2013

Hemocompatibility Of Magnesium Alloys

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Hemocompatibility of Magnesium Alloys

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department: Chemical, Biological Engineering and Bioengineering

Major: Bioengineering

Major Professor: Dr. Donghui Zhu

Greensboro, North Carolina

2013

School of Graduate Studies
North Carolina Agricultural and Technical State University
This is to certify that the Master's Thesis of

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has met the thesis requirements of
North Carolina Agricultural and Technical State University

Greensboro, North Carolina
2013

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Biographical Sketch

Nevija A. Watson was born on July 15th in Santa Monica, California. She received her Bachelor of Science in Chemical Engineering from North Carolina A&T State University in 2010. She has continued her education and is a candidate for the Master of Science degree in Bioengineering at North Carolina A&T State University.

Dedication

I would like to dedicate this work to my mother, Jane Adams, my brother, Evijan Watson and my fiancé, Dalewyn Spinks. They have all been instrumental in my success in all of my endeavors thus far.

Acknowledgements

I would like to acknowledge my advisor Dr. Donghui Zhu. He believed in me enough to select me to be a part of his research team. I also would like to thank Dr. Jenora Waterman, thank you for letting me work in you lab, I learned so much from you. You are a role model for women like me, and I am grateful you took your time to work with me. Dr. Zhigang Xu for providing the novel materials used for experiments and training and use of his lab equipment.

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Abstract

There are over 15 million people living in the United States with cardiovascular disease (CVD). The standard treatment for CVD is stent angioplasty, traditionally bare metal stents have been used for this procedure however they are permanent and can lead to in-stent restenosis. Biodegradable bare metal stents are a suggested solution to this problem because they will degrade over time. Magnesium alloys are the most clinically advanced materials for biodegradable bare metal stents. They have been tested in various clinical trials with promising biocompatibility results; however materials degrade before full healing has occurred. The alloying of magnesium with elements such as calcium and zinc should improve corrosion rate of materials and demonstrate good biocompatibility. This research compares the biocompatibility of four different ternary magnesium zinc calcium alloys and two magnesium-based rare earth alloys to high purity magnesium for cardiovascular applications. Biocompatibility was investigated through *in vitro* testing evaluating the hemocompatibility and cytocompatibility of materials. Tests for hemocompatibility included hemolysis and platelet adhesion and morphology characterization. Cytocompatibility tests were indirect *in vitro* examinations of toxicity measuring human aortic endothelial cell viability and characterizing cell morphology. Tests for hemocompatibility indicated a nonhemolytic response to material and mild platelet activation and aggregation. Cytocompatibility tests concluded that viability was concentration dependent for all materials; however, there was no difference between material types or exposure time. These results concluded that MgZnCa alloys and Mg-based rare earth alloys are promising candidates for cardiovascular stent applications.

CHAPTER 1

Introduction

1.1 Cardiovascular Disease

Cardiovascular disease or CVD refers to any condition that involves narrowed or blocked blood vessels that can lead to a heart attack, chest pain or stroke. The CDC reports that more than 15,800,000 Americans have known coronary artery disease and heart disease is still the leading cause of death in the United States. Roger et al. (2012) reported that more than 40% of the US population is projected to have some form of CVD by 2030, with costs exceeding $1 trillion annually by the year 2030.Cardiovascular disease can result in serious and even fatal problems in the rest of the body as well because reduced blood flow in the heart can result in reduced blood flow throughout the cardiovascular system. This means that some areas will not have enough blood delivering oxygen and nutrients and collecting waste products. Two causes of cardiovascular disease are coronary artery disease (CAD) and congenital heart disease. Congenital heart disease is a condition that is present from birth that results in narrowed or blocked blood vessels. Coronary artery disease is caused by plaque buildup in the arteries that supply blood to the heart. This build up causes stenosis or narrowing of the artery which results in reduced blood flow. This can lead to angina, chest pain and even a heart attack. The surgical treatment for cardiovascular disease is percutaneous coronary intervention (PCI) or angioplasty. This procedure uses a balloon catheter inflated at the blockage site to compress buildup and restore blood flow. A stent is also expanded using the catheter to provide support and prevent restenosis of the blood vessel; healing time takes from 6-12 months. Current stents have a 10% chance of in stent restenosis, and biodegradable magnesium based stents that have conducted

clinical trials have shown beneficial biocompatibility however degradation rate of stents needs to be improved.

1.2 Magnesium Biomaterials

Biomaterials are materials that are used in contact with biological systems. The important characteristics of biomaterials have shifted over time from "passive" biomaterials that highlighted long-term integrity and non-toxic interactions, to materials that actively interact with their biological environment. The first biomaterials were only intended for prostheses and medical device applications. Anderson (2006) reported that the recent emphasis on biomaterials is associated with how the biological interactions with biomaterials. As a change in important characteristics changed so did the selection in biomaterials. Biomaterials have been used since the late 18th century as wires and pins to fix bone fractures. Metals, ceramics polymers and composites have been explored for use as implant biomaterials. Metals are ideal candidates for biomaterials because of their high impact strength, wear resistance and ductility and their toughness compared to other materials.

Some applications for metal biomaterials identified by Moravej and Mantovani (2011) include bone plates, screws, dental implants, suture wires and stents. Traditional metal biomaterials such as titanium, 316 L stainless steel, nitnol and cobalt-chromium alloy, were chosen mainly for their mechanical characteristics. Research (Hench & Polak, 2002; Navarro, Michiardi, Castano, & Planell, 2008) has found that trends in biomaterials research have evolved through three clearly marked generations: the first generation-bio inert materials, the second generation-bioactive and biodegradable materials and the third generation-materials designed to stimulate specific cellular responses at the molecular level. Biodegradable metals are a viable option for biomaterials for surgical procedures that only require the use of a device for a select

period of time or that currently require a second surgery to remove the device. In recent years there has been an increase in research of magnesium and magnesium-based alloys as candidates for biodegradable materials. Some of the most practical applications of biodegradable metals include screws and stents. Research (Witte, 2010; Witte et al., 2005) has suggested biodegradable screws and pins for orthopedic applications because current devices require a second surgery and do not promote bone healing. A study by Waksman (2006) highlighted the benefits of biodegradable stents for cardiovascular applications.

1.3 Thesis Outline

Chapter 2 will be a literature review of pertinent information needed to understand magnesium and magnesium based biomaterials, possible applications for tested materials and how they are tested for biocompatibility. Chapter 3 will discuss methods and materials used to create and evaluate results obtained from this study. Chapter 4 will discuss all results observed in the study regarding the biocompatibility of the materials tested. Lastly, chapter 5 presents a summary of the results discovered and suggested future studies.

CHAPTER 2

Literature Review

Currently the best method to treat cardiovascular disease (CVD) is stent angioplasty. This is an invasive surgical procedure necessary to provide a long term solution for CVD. Once a stent is deployed in the blood vessel it will come in contact with the innermost layer of the vessel, endothelial cells, and with blood flowing throughout the vessel. The interaction between these components of the human body and stent material will be present throughout the time the stent is present in the vessel. Poor interactions with endothelial cells and whole blood can lead to in-stent restenosis, blood clot and emboli formation, kidney failure and heart attacks.

2.1 Human Aortic Endothelial Cells

Endothelial cells are an important link in material biocompatibility, their main function is to synthesize and secrete activators and mediators associated with hemostasis. This role is emphasized in the 2005 study by Bernadini et al., which confirmed that the endothelium represents various groups of cells located in blood vessels of different tissues, and these cells are involved in the roles of antherogenisis, thrombosis and angiogenesis. Some factors that differentiate types of endothelial cells are 1) morphological and functional differences, 2) different response to growth factors, 3) organ specificity and 4) pathological conditions. Aortic endothelial cells are a special type of endothelial cell that form the inner layer of blood vessels as shown in Figure 1. Endothelial cells constitute the natural interface between blood and underlying tissue. A study by Jones and Grainger (2009) states that endothelial cells also represent the barrier used for the transport of particles and other species from the blood and surrounding tissues including the blood brain-barrier. Endothelial cells play a key role in the pathogenesis of atherosclerosis and synthesize and secrete activators and inhibitors of the

coagulation system and influence adhesion and aggregation of platelets. Several researchers (Mani, Feldman, Patel, & Agrawal, 2007; Yoshimoto & Yoder, 2009) found that if endothelial cells react with a biomaterial and successfully proliferate and differentiate, they would release growth factors that can inhibit thrombosis and other adverse effects. Because of the important role of these cells past studies by Jones and Grainger (2009) have used primary human aortic endothelial cells as models of inflammatory response. The reaction of human aortic endothelial cells is linked to other physiological reactions to a biomaterial in the body specifically in the cardiovascular system.

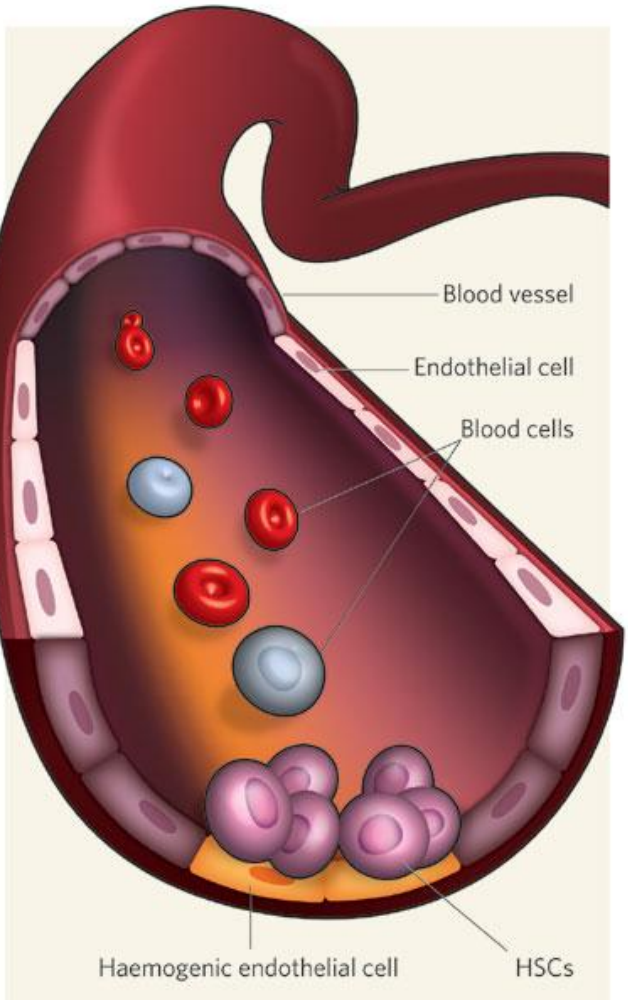

Figure 1. Human aortic endothelial cells (Yoshimoto, 2009).

2.2 Human Whole Blood

Blood is vital to the functioning of the human body. Blood is also constantly interacting with cardiovascular stent materials. The main function of blood is to transport oxygen from the lungs to body tissue and carbon dioxide from body tissue to the lungs. It also transports nourishment from digestion, hormones from glands, and waste to the kidneys. Applegate et al. (2009) reported that the average adult has approximately five liters of blood in their body. Blood is also considered to be alive because it contains living cells. Blood is made up of two main components: plasma and the formed elements. The formed elements of human whole blood are red blood cells (erythrocytes), white blood cells (leukocytes) and platelets. Human whole blood is about 55% plasma and 45% formed elements illustrated in the figure below.

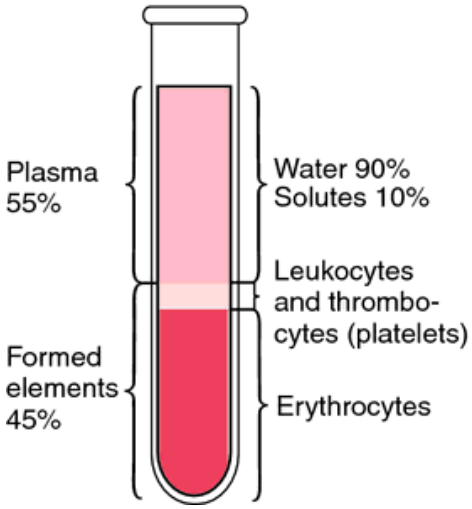

Figure 2. Components of human whole blood (Applegate, 2009).

When the human body is functioning normally whole blood maintains a pH of approximately 7.4 and flows easily throughout the body. However when there is a disruption to blood vessels or surrounding tissues the conditions of blood change. Blood will enter into hemostasis, which is the process of arresting blood loss from the human body. Hemostasis works to stop the flow of blood through vasoconstriction, thrombosis and coagulation.

Vasoconstriction is simply the constriction of blood vessels to slow the flow of blood. Sodium citrate is added to human whole blood after donation from a volunteer to prevent clotting. Sodium citrate is an anticoagulant that stops the process of clotting because citrates chelate calcium so that it cannot participate in the polymerization of fibrin. Understanding the role of human whole blood and hemostasis is vital when analyzing cardiovascular biomaterials.

2.2.1 Platelets. The 2009 study (Varga-Szabo, Braun, & Nieswandt) identified and reviewed the role of platelets in the human body, stating blood platelets are hematopoietic cells produced by bone marrow megakaryocytes. The 1995 study by Blockmans et al. studied the pathology of platelet activation and describes resting platelets as the smallest blood cells, discoid shaped with a diameter of 3 μm and only 1 μm thick. The shape and structure of platelets is affected by several different components including the plasma membrane, cytoskeleton, membrane skeleton, cytoplasm and tubular system. The plasma membrane is composed of a phospholipid bilayer that increases the surface area of the platelet and serves as the site for platelet receptors. The cytoskeleton and membrane skeleton are composed of actin filaments, which play an important role in shape regulation of the platelet plasma membrane. Blockmans et al. (1995) stated that upon activation, microtubular bundles and actin filaments depolymerize and polymerize resulting in shape change and the formation of pseudopods.

Platelet activation occurs when the blood is triggered to begin the process of hemostasis. Upon activation platelets undergo a series of morphological and physiological responses. The study by Varga-Szabo et al. (2009) reported platelets possess various adhesion receptors and sophisticated regulatory machinery in order to adhere in response to a well-defined set of stimuli, such as sub endothelial collagens and ADP released from activated platelets and thrombin generated by the coagulation cascade. Blockmans et al. (1995) reported the two main

categories of mechanisms responsible for platelet activation are physiological and pharmacological. Physiological activators include thrombin, collagen, ADP, and serotonin. Some pharmacological agents are calcium ionophores and cyclin endoperoxide analogues. Both mechanisms are believed to interact with specific receptors on the plasma membrane. Calcium is involved in several different aspects of platelet activation. Researchers (Blockmans et al., 1995; Varga-Szabo et al., 2009) studied the link between calcium and platelet activation. Calcium channels in the platelet membrane are regulated by receptors to promote the influx of stored calcium. Calcium mobilization leads to activation of phospholipases and an increase in intracellular ph. Blockmans et al. (1995) stated that all known platelet agonists increase the intraplatelet calcium concentration. The 2009 study by Varga-Szabo found elevated levels of Ca^{2+} contributes to various steps of cellular activation and can come from the release of compartmentalized Ca^{2+} and the entry of extracellular Ca^{2+} through the plasma membrane.

2.3 Cardiovascular Stents

One of the most advanced clinical applications of Magnesium and magnesium based alloys are as stent biomaterials. Several studies (Heublein, 2003; Mani et al., 2007; Zartner, Buettner, Singer, & Sigler, 2007) reported magnesium stents have been successfully tested in animals and the first clinical human trials have been conducted. Cardiovascular stents are used to treat heart disease combined procedures such as coronary angioplasty, a procedure done to treat patients with clogged arteries. A coronary stent is used for about 60 percent of angioplasty procedures today. During the procedure a small mesh-like cylindrical scaffold is inserted and expanded in the coronary artery to keep the artery open without stenting, restenosis, the re-narrowing of a blood vessel. According to Moravej and Mantovani (2011) restenosis occurs within about 6 months of 30-40% coronary lesions without stents, this number is significantly

reduced with stenting however in-stent restenosis (ISR) can still occur and on average, currently available stents cause in-stent restenosis about 10% of the time.

2.3.1 Characteristics of ideal stents. Several studies (Mani et al., 2007; Moravej & Mantovani, 2011) identify the most common bare metal stent materials as 316 L stainless steel (316L SS), Nitinol (Ni-Ti), titanium (Ti) and cobalt-chromium (Co-Cr) alloy. These materials are corrosion resistant and permanent, which can lead to long-term drawbacks. The main purpose of stenting is scaffolding and remodeling of the artery, which is achieved in about 6 to 12 months. According to Mani et al. (2007) the characteristics of an ideal stent are similar to characteristics of most biomaterials, they should be (1) low profile; (2) have good expandability; (3) sufficient radial hoop and negligible recoil; (4) sufficient flexibility; (5) adequate radiocapacity/ magnetic resonance imaging (MRI) compatibility; (6) thromboresistivity; and (7) drug delivery capacity." Magnesium has been identified as a material that can meet most of the above characteristics; however the thromboresistivity and components of biocompatibility of magnesium and its alloys have yet to be explored. The biocompatibility factor is very important for these biomaterials, when used as a stent they are in direct contact with the endothelial surface and blood. If there is a poor biocompatible reaction with stenting materials restenosis and inflammation will occur, near the implant location causing serious problems in the human body.

2.3.2 Clinical studies of magnesium stents. Several studies have been conducted on magnesium alloys for cardiovascular stent applications. Some materials have entered the phase of animal testing however; some studies have completed large clinical trials for their materials. Heublein (2003) tested AE21 magnesium based alloys implanted in the coronary arteries of 11 domestic pigs. The follow up procedure was performed at 10, 35 and 56 days after implantation and found there was positive remodeling and there was no platelet deposition or thrombus at

endothelial sites however, the stent degraded too fast. A clinical study by Di Mario (2004) of the Lekton Magic coronary stent, shown in Figure 3, was also tested on pigs. This study implanted stents made of WE43 magnesium alloy into the coronary artery of 33 mini-pigs.

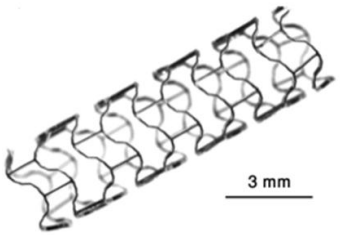

Figure 3. Leckton magic coronary stent (Mani et al., 2007).

The first human clinical study of the Leckton Magic stent (WE 43 Alloy) was reported by Peeters, Bosiers, Verbist, Deloose, and Heublein (2005). Stents were used to treat 20 patients with lower limb ischemia. Preliminary reports showed no adverse reactions to stent materials. Zartner et al. (2007) reported the first successful implantation of a biodegradable human stent in a human. A 3mm length and 10 mm diameter stent by Biotronik Company was implanted in the left pulmonary artery of a preterm baby with congenital heart disease. Follow up procedure during 5 months displayed no adverse reaction and complete degradation of the stent. Results of this study were poisitve, there was no adverse reaction to stent material however the baby did pass away. The PROGRESS-AMS trial of the magnesium stent reported by Waksman (2006) is the only bioabsorbable metal stent investigated clinically as a coronary stent. The PROGRESS study was a clinical trial using 63 patients across 7 European centers to demonstrate safe stent application and absorption follow-ups were done after 4 months. These follow ups displayed no adverse effects biocompatibility, however a majority of stent material had already degraded at the 4 month mark. Although current magnesium-based stents are in clinical stages, new magnesium-based alloys could provide mechanical and biocompatibility improvements

compared to these current materials. Corrosion rate of current biodegradable magnesium stent materials is too fast, and complete degradation occurs before healing is complete.

2.4 Magnesium

The history of magnesium (Mg) as a biomaterial has been covered by several studies (Anderson, 2006; Moravej & Mantovani, 2011; Witte, 2010). Biomaterial applications of magnesium date back to the 18th century, when Edward C. Huse used magnesium wires as ligatures to stop bleeding vessels of three human patients. Later, another surgeon Erwin Payr tested pure magnesium in animals, however, the corrosion rate could not be controlled and therefore magnesium was no longer studied as intensively. Several other surgeons also studied magnesium as a possible biomaterial candidate for cardiovascular applications with promising results. Research by Bernardini, Nasulewic, Mazur, and Maier (2005) highlighted the importance of interactions between magnesium and the human body. They reported that decreased levels of magnesium have been linked to inflammation and promotion of dysfunctions in human umbilical vein endothelial cells, and high levels of magnesium resulted in proliferation, migration and inducing angiogenesis. Even though Mg and its alloys have been investigated for centuries it is still not available as a common commercial biomaterial option.

Moravej and Mantovani (2011) identified the traditional metallic biomaterials as stainless steel, Cobalt (Co)-based alloys and titanium (Ti). Each material has its own advantages, however numerous studies have concluded that with long-term use these metals can lead to significantly elevated metal concentrations in body fluids. Okazaki and Gotoh (2005) found that metals are not normally present in the body can lead to toxicity and other negative side effects. Although these metals are still used for implant purposes researchers are making efforts to find ways to improve on these materials to avoid negative effects and even look into different types of

biomaterials for the same applications. Recently, biodegradable biomaterials have risen to the forefront of materials research topics. Due to it's corrosive properties, several researchers (Staiger, Pietak, Huadmai, & Dias, 2006; Witte, 2010; Yun & Halsall, 2009) have identified magnesium as an ideal candidate. Compared to currently approved and traditional metallic biomaterials magnesium possesses some unique mechanical characteristics and biological advantages.

Not only does magnesium possess desirable physical and mechanical characteristics but also it is naturally present in the human body. Witte et al. (2006) reported that magnesium is the 4th most abundant cation in the body and is essential for good health; it is needed for over 300 biochemical reactions, and stabilizes the structures of DNA and RNA. Magnesium is essential to metabolism, keeps bones strong, supports a healthy immune system, and assists in maintaining normal muscle and nerve function. Magnesium is also present in the food and water we ingest on a daily basis. S. Zhang et al. (2009) found the corrosion of Mg is high in solutions due to its standard electrode potential of -2.37 V; therefore, it is a good candidate for a biodegradable metal. When magnesium biomaterials corrode in the body it is safe because Mg^{2+} ions are normally present and the body naturally excretes excess magnesium though the kidneys. The biological presence of magnesium in the human body indicates that in small levels magnesium is not toxic which makes it an ideal biodegradable material for implants and devices. The recommended dietary allowance set by the Institute of Medicine of the National Academy of Sciences is 310 mg for women and 400mg for males per day.

Huan, Leeflang, Zhou, Fratila-Apachitei, and Duszczyk (2010) outlined the standards for good implantable biomaterials and state that they should possess three main characteristics: acceptable mechanical strength, desirable degradation rate similar to healing rate of surrounding

tissues, and good biocompatibility. The mechanical characteristics of magnesium compared to some traditional biomaterials have been documented by several studies (Mani et al., 2007; Staiger et al., 2006; Witte, 2010) and are illustrated in Table 1 below. Some researchers (Staiger et al., 2006; H. Wang & Shi, 2011; Witte, 2010) have also identified magnesium and magnesium alloys as potential candidates for biomaterials because they are lightweight metals with beneficial mechanical characteristics such as low density, high strength and stiffness. Several studies have researched orthopedic applications of magnesium-based biomaterials because of the similarity in mechanical characteristics to human bones, illustrated below.

Table 1

Mechanical properties of select biomaterials and human bone (Staiger et al., 2006; H. Wang & Shi, 2011; Witte, 2010)

Properties	Human Bone	Magnesium	Co-Cr	316 L Stainless Steel	Titanium Alloy	Nitnol
Density (g/cm^3)	1.8-2.1	1.7-2.0	8.32-9.2	7.9-8.1	4.4-4.5	6.5
Elastic Modulus (Gpa)	3-20	41-45	190-230	189-205	110-117	83
Compressive Yield Strength (Mpa)	130-180	65-100	450-1000	170-310	758-1117	195-690

Magnesium displays some similar mechanical properties to traditional biomaterials however the corrosion rate needs to be improved. Corrosion is the property that enables the use of magnesium for biodegradable biomaterial applications, however if the material degrades too fast it will not sufficiently serve its purpose in the body. One method of improving corrosion rate and possibly other mechanical properties is through alloying. Alloying can change several mechanical properties and improve corrosion rates of magnesium-based biomaterials making these materials viable options for a variety of applications.

2.5 Magnesium Alloys

Past studies (Gu, Zheng, Cheng, Zhong, & Xi, 2009; Kirkland et al., 2010; Witte et al., 2006) have discovered that the addition of various metals such as Aluminum, Silicon, Zinc, and Zirconium have improved the mechanical properties of magnesium-based alloys, making them desirable biomaterials for most orthopedic procedures. Pure magnesium has presented a good biocompatibility with almost no evidence of toxicity; however magnesium-based alloys present the addition of other elements, which can cause changes in biocompatibility. The ideal elements used for alloying would present few potential toxicological problems and produce the desired mechanical improvements from pure magnesium. A variety of elements have been studied for magnesium-based alloy applications. Some elements that have been heavily researched for magnesium biomaterial alloying applications are lithium (Li), zirconium (Zr), aluminum (Al) and calcium (Ca).

Aluminum can effectively improve corrosion resistance of magnesium alloys however it can also lead to harmful effects and is difficult for the body to absorb. Reports vary on the effect of incorporating aluminum into magnesium alloys. The study by El-Rahman (2003) reported that aluminum is a well-known neurotoxicant and its accumulation has been linked to neurological

disorders such as dementia, senile dementia and Alzheimer's disease. However, magnesium alloys containing aluminum have still been investigated for biomaterial applications because of its desirable effect on corrosion resistance. Researchers (Heublein, 2003; Witte et al., 2005) studied the implantation of magnesium-aluminum alloys AZ31, AZ91 andAE21. Both studies reported positive results and identified magnesium-aluminum alloys as possible candidates for biomaterials.

Since aluminum has reported toxicity in the human body, other alloys that improve mechanical properties are also being explored. Feyerabend et al. (2010) study of magnesium alloys reports that zirconium is an interesting candidate for magnesium alloying because it is an effective grain-refining agent in Al-free alloys and contributes to strengthening due to formation of fine-grains by strengthening of grain boundaries. Researchers (Angrisani, 2012; Huan et al., 2010) have incorporated rare earth materials such as zirconium (Zr) into magnesium alloys because it has been shown to increase alloy strength.

Lithium is a heavily studied magnesium alloying material by researchers (Lu, 2010; S. Zhang et al., 2010; Zhou, Zheng, Leeflang, & Zhou, 2013) because it increases ductility, which is ideal for stent applications. However the addition of lithium also compromises other important mechanical characteristics such as strength that cannot be regained. Researchers are now interested in Mg-Li-based materials that are alloyed with other elements. Zhou et al. (2013) researched magnesium based materials alloyed with lithium, aluminum and rare earth elements as candidates for cardiovascular stent applications. They studied the biocompatibility of their magnesium-lithium based alloy and *in vitro* tests concluded there was no significant biological effect from material exposure. Because of the downside of certain alloying elements focus has been brought to alloys containing elements that are virtually harmless such as calcium and zinc.

2.5.1 Magnesium-zinc-calcium alloys. Ternary alloys containing magnesium zinc and calcium have been identified in several studies (Gu et al., 2009; H. Wang & Shi, 2011; Xia, 2012; S. Zhang et al., 2009) as candidates for magnesium alloys. Alloys containing Calcium (Ca), Zinc (Zn), and Manganese (Mn) are identified in the study by (Xia, 2012) as good candidates because they are already present in the body, essential to human health and possess good mechanical properties. Even though they are already present in the body, high levels can cause other toxic effects. Research by (Gu et al., 2009) has suggested that based on corrosion properties of Mg alloys, the concentration of Ca, Zn and Mn should be below 2 wt%, 6 wt% and 1.5 wt% respectively. Based on the general corrosion rate for Mg alloys, 0.2 mg cm^{-2} h^{-1}, the releasing amount of these elements will stay below the daily allowance, depending on the surface area of the device. Research (Xia, 2012) stated that Zinc and Calcium have been shown to successfully improve corrosion resistance and mechanical properties of magnesium; however the biocompatibility has not been extensively studied.

Several studies (Gu et al., 2009; Huan et al., 2010; Xia, 2012) have identified Zinc as a good candidate for an alloying material because it improves the mechanical properties of magnesium. Zinc improves strength and corrosion resistance of magnesium alloys, in levels around 3%. Zinc is also considered a micro-nutrient, and is important for various physiological functions in the human body. The study by S. Zhang et al. (2010) stated that the maximum solubility of zinc in magnesium is 6.2 wt.% at 325 °C. Calcium has been identified by several researchers (Guan et al., 2012; Li, Gu, Lou, & Zheng, 2008; Xia, 2012) as a good candidate as an alloying material for magnesium alloys. Similar to zinc, calcium is an essential element and is also present in the human body. Magnesium-alloys incorporating calcium have been heavily studied for orthopedic applications because the skeleton is mainly composed of calcium

phosphates, has a low density (1.22 g/cm3) similar to human bone and is a major component of chemical signaling with cells. Research (Feyerabend et al., 2010; Guan et al., 2012; Xia, 2012) has found that magnesium based alloys with calcium have improved mechanical characteristics such as a decrease in corrosion rate. Although several studies have tested the biocompatibility of several magnesium-zinc-calcium alloys, every novel alloy composition must be tested.

2.5.2 Magnesium- rare earth alloys. Rare earth metals have been studied by several researchers (Angrisani, 2012; Gu et al., 2009; Huan et al., 2010; Witte et al., 2005; Zhou et al., 2013) as candidates for metallic biomaterial alloys.Feyerabend et al. (2010) identified rare earths as a group of seventeen elements chemically classified in three groups by their ionic radii. The three groups of rare earth elements are as follows: a) light rare earth elements (La-Pr), b) medium rare earth elements (Nd to Gd) and c) heavy rare earth elements (Tb to Lu). Researchers (Angrisani, 2012; Feyerabend et al., 2010) stated that rare earth metals can improve various mechanical properties when alloyed with magnesium they are commonly used for strengthening and to improve corrosion resistance.

Even though rare earth elements can improve mechanical properties researchers Huan et al. (2010) reported, there is controversy concerning the biological effects of these alloys. Researchers (Li et al., 2008) reported, rare earth elements do have a toxic effect on some cells, the study concluded that rats exposed to rare earth elements, yttrium and cerium were diagnosed with severe hepatotoxicity and the study by E. Zhang, Yang, Xu, and Chen (2010) reported excessive yttrium ions have adverse effects on DNA transcription factors in rat genes. Feyerabend et al. (2010) had conflicting results when rare earth elements were tested on various cell lines and primary cells, resulting in cytotoxicity when exposed to low concentrations of light rare earth elements. Toxicity was seen when cell lines were exposed to materials, however no

toxicity was seen in primary cells. However, other studies (Angrisani, 2012; Gu et al., 2009; Zhou et al., 2013) also reported that short term evaluation of rare earth materials, did not have a negative effect on viability of primary cells or cell lines. Li et al. (2008) reported that even the Biotronik Mg absorbable metal stent, which was successful in clinical stages, is composed of 10% rare earth elements. However, this same stent was not well tolerated in other clinical trials. This controversy leads to a deficiency in literature testing magnesium alloys with rare earth materials. Rare earth alloys are attractive materials for stent applications because they possess the ductility necessary for expandable stents. The varying opinions on the biocompatibility of magnesium-based rare earth alloys yields a gap in current research, additional studies are needed to determine the biocompatibility level of new magnesium based rare earth materials.

2.6 Biocompatibility

Biocompatibility is the measurement of the how compatible a material is with a biological system. Biocompatibility testing has been established for current biomaterials; however with addition of alloys and varying concentrations there is still lots of testing to be done on magnesium and Mg based alloys, researchers (Gu et al., 2009; Witte, 2010) stated that there is still a lack of knowledge regarding hemocompatibility, or blood compatibility, of Mg based materials. There are several different tests to measure the biocompatibility of a material that are governed by ISO 10993. The two main categories of biocompatibility testing are material characterization and *in vitro* or *in vivo* biocompatibility. For the purpose of this study biocompatibility was measured by *in vitro* cytotoxicity and hemocompatibility.

2.6.1 Cytotoxicity. The measure of the toxic effect of a material on cells is an important aspect of biocompatibility. *In vitro* cytotoxicity tests are used to evaluate the toxicity of materials with one of the three types of tests: extract test, indirect test or direct test. Jones and Grainger

(2009) outlined the major cell types used for *in vitro* testing as follows: phagocytic, neural, hepatic, epithelial, endothelial, red blood cells and various cancer cell lines. The cell type is chosen based on a typical application for the tested material. Several researchers (Armitage, Homer-Vanniasinkam, & Lindsey, 2004; Gu et al., 2009; Liu et al., 2010; H. Wang & Shi, 2011) used extract tests to measure toxicity of potential metallic biomaterials.Gu et al. (2009) tests various Mg based alloys and reports that there was no toxic effects on fibroblasts (L-929 and N1H3T3), osteoblasts (MC3T3-E1) or blood vessel cells (ECV304 and VSMC). H. Wang and Shi (2011) also stated there was no reduction of cell viability on MC3T3-E1 cells from magnesium alloys compared to pure magnesium. Cytotoxicity of materials is assessed by morphological cell damage, measurements of cell damage, measurement of cell growth, and measurement of cellular metabolism.

Cytotoxicity can be measured in several different forms. Several studies (Huan et al., 2010; H. Wang & Shi, 2011; Xia, 2012) carried out cytotoxicity tests via indirect or extract methods. These same studies also quantify cytotoxicity of cells with the most common method, the MTT assay. However, Fischer (2011) reported that even though these tests are a common assay to use, when testing magnesium materials a false positive or negative can occur with the MTT test. This is because certain levels of Mg 2+ ions also cleave the reagent used to monitor bio reduction in the tested cells. Even though this discrepancy with the MTT test has been reported several researchers still use the MTT assay to determine viability because of the ease of the test method and ability to replicate viability measurements. Data for short exposure times should be considered reliable however, an increase in exposure time leads to the increase in Mg ions that result in false positive results.

2.6.2 Hemocompatibility. Blood compatibility, also governed by ISO 10993, is the aspect of biocompatibility that measures how compatible a material is with blood. *In vitro* methods of hemocompatibility are beneficial because small levels of plasma hemoglobin can be quantified that may not be measureable under *in vivo* conditions. Hemocompatibility is measured with four main categories: platelets, coagulation, thrombosis, hematology, and immunology. This study will be evaluating the effect of the magnesium alloys on hematology and thrombosis. According to ISO 10993-4, some interactions that would be considered undesirable are: activation of platelets, formation of thrombi, injury to circulating cells and injury to cells or tissues. Sheppard, McClung, and Feuerstein (1994) reported that all blood interactions are important because they are a series of events that potentially lead to the formation of a thrombus. Hemolysis testing is the aspect of hemocompatibility that evaluates the damage a material causes to erythrocytes or red blood cells. Guan et al. (2012) reported that hemolysis is the breakage or destruction of red corpuscles, which causes the release of hemoglobin into the surrounding medium. *In vitro* hemolysis of a few magnesium alloys has been researched in several studies (H. Wang & Shi, 2011; E. Zhang et al., 2010; S. Zhang et al., 2010) with varying results this area needs to be studied more if future research especially for biomaterials intended for cardiovascular applications.

2.7 Purpose

The purpose of this thesis is to understand the effects of varying concentrations of alloying materials and rare earth elements on biocompatibility of magnesium alloys. The literature review has identified the progress of current research in addition to the gaps in current knowledge. Several researchers (Gu et al., 2009; Xia, 2012) have studied the effect of various magnesium-based alloys on biocompatibility; however, novel materials of similar compositions

must also be tested since alloy composition will affect biocompatibility. Research (Feyerabend et al., 2010; Witte et al., 2006) of magnesium based biomaterials has focused on the cell compatibility of material, however; endothelial cells are seldom used to measure cytotoxicity of materials. Endothelial cells are important for cardiovascular applications because they will be in direct contact with materials. Very few studies highlight the important aspect of hemocompatibility of magnesium-based materials. Hemocompatibility is an important factor due to the constant interaction with blood for cardiovascular applications. Several different magnesium-based alloys have been identified as good candidates for cardiovascular stent materials; however the corrosion rate of materials is still too fast and needs to be improved.

Dr. Zhigang Xu's research group in the Engineering Research Center at North Carolina A&T State University has developed novel magnesium based biomaterials that can potentially be used for medical implants or devices. These metallic biomaterials are designed to safely degrade in the body at an improved corrosion rate compared to currently available alloys. The hypothesis of this study is that alloying elements that improve the mechanical and corrosion properties of magnesium will not have an adverse effect on material biocompatibility for cardiovascular applications. The objectives are to measure the effect of alloying on cytotoxicity and hemocompatibility of materials. All results from these tests were taken into consideration in order to measure if materials were considered good candidates for cardiovascular stent applications in the human body.

CHAPTER 3

Methodology

Seven different materials were tested in this study: high purity Mg (99.97%), Mg-(1)Zn-(0)Ca, Mg-(2)Zn-(0)Ca, Mg-(3)Zn-(0)Ca, Mg-(4)Zn-(0)Ca, Mg-based RE1 and Mg-based RE-2. These materials were tested for biocompatibility against established guidelines from ISO 10933. Tests included cytotoxicity, platelet adhesion and hemolysis. The cytotoxicity assay measured the toxicity of material exposure to human aortic endothelial cells (HAECs) with an MTT viability assay and characterization of cellular morphologies. Hemocompatibility testing analyzed platelet adhesion and hemolysis using donor human whole blood from a donation center. Platelet adhesion testing measured the number and morphology of adhered platelets from platelet rich plasma, obtained from centrifuged human whole blood. Hemolysis testing measured the effect of materials on the integrity of the red blood cell membrane, using diluted human whole blood

3.1 Sample Preparation

The novel biomaterials provided by Dr. Zhigang Xu's lab are the basis for all experiments performed in this study. Alloys were melted down and as-cast with high purity Mg and commercial pure element granules calcium (Ca) and zinc (Zn) under a mixed gas atmosphere using a mild steel crucible. Materials selected for testing were high purity magnesium (99.97 %), two magnesium based rare earth alloys: (RE 1 and RE 2) and four different compositions of Mg-(x)Zn-(0)Ca alloy (x = 1- 4). The specific concentrations of alloys are not revealed in this study however the general compositions in weight percent are as follows: Mg-(1)Zn-(0)Ca, Mg-(2)Zn-(0)Ca, Mg-(3)Zn-(0)Ca and Mg-(4)Zn-(0)Ca. Each ternary alloy is referred to throughout the literature as labeled in Table 2 below.

Table 2

Material name and respective composition

Material Name	Zn	Ca
MgZnCa 1	1 (wt %)	< 0.5 (wt %)
MgZnCa 2	2 (wt %)	< 0.5 (wt %)
MgZnCa 3	3 (wt %)	< 0.5 (wt %)
MgZnCa 4	4 (wt %)	< 0.5 (wt %)

3.1.1 Cutting sample materials. The Allied TechCut5 Precision sectioning machine, displayed in the figure below, was used to prepare samples for biocompatibility testing. Samples for testing were cut from larger as-cast samples of each material. Final dimensions of each square sample were 10 x 10 x 1 mm^3, as suggested by ISO 10993. Samples were used for cytotoxicity and hemocompatibility tests. A diamond blade was used to cut samples at full rotation and medium force, and various speeds and rates depending on starting sample size. Cutting speeds were approximately 4000 rpm and rate approximately 0.75 inches/minute.

3.1.2 Sample polishing. After cutting samples were mechanically polished, using the Ecomet 3 variable speed grinder-polisher on Silicon Carbon abrasive papers from 400 to 1200 grit in increments of 200. Samples were mounted with wax to polishing stands then loaded into a multi-polisher specimen holder. Polishing was started at a force of 2 pounds for initial contact, and then the force was raised 10-12 pounds for approximately 15 seconds. This procedure was repeated for each silicon carbide grit paper, until the sample surface was void of visible scratches.

3.1.3 Cleaning and sterilization of samples. After cutting and polishing samples were dipped in acetone for degreasing and wax removal, ultrasonically cleaned using the Branson 2510 Ultrasonic Cleaner in the figure below, and then dried in open air. The ultrasonic cleaner uses ultrasonic sound waves, which cause the rapid formation and collapse of ultrasonic bubbles (Branson, 2010). Samples were then stored until used for biocompatibility testing. Immediately prior to all biocompatibility testing samples were sprayed with ethanol and UV sterilized for 30 minutes on each side under the cell culture hood to eliminate contaminants on sample surfaces.

3.2 Cytotoxicity Assay

Cytotoxicity tests are conducted to evaluate the toxic effect of a material on cellular function. *In vitro* tests can be carried out through direct or indirect testing. Tests for cytotoxicity of biomaterials are outlined in ISO 10993-5. Direct tests for cytotoxicity involve the growth of cells in direct contact with the sample material; these tests provide information about how the cells will react when in contact with the material surface. In-direct testing or extract testing uses material extract to test how cells will react when exposed to the material in directly. Extract tests are advantageous because they allow the use of a concentration gradient when testing materials; this allows the researcher to study the effect of different levels of extract exposure.

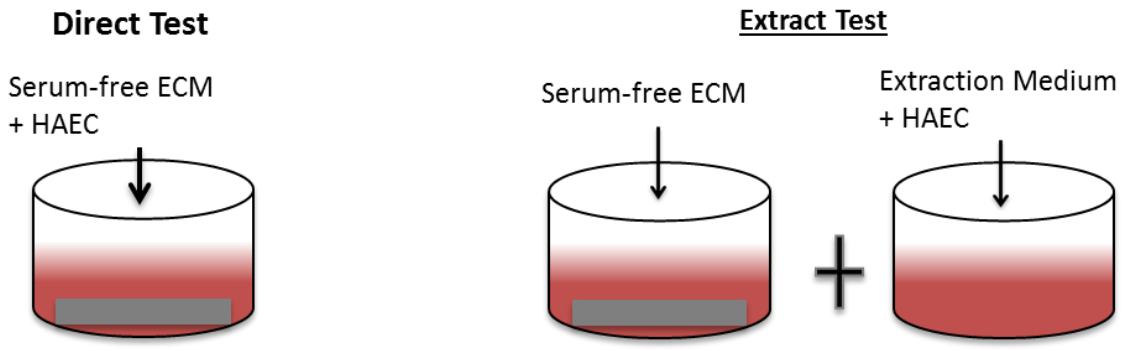

Figure 4. Direct test vs indirect (extraction) test for cytotoxicity.

Human Aortic Endothelial Primary Cells (HAEC) were obtained from ScienCell Research Laboratories (Carlsbad, CA). These cells were cultured at a density of 5 x 10^4 cells per well to evaluate the cytotoxicity of magnesium alloys. HAECs were cultured in a fibronectin coated flask with Endothelial Cell Medium (ECM), 5% fetal bovine serum (FBS), 1% penicillin/ streptomycin solution and 1% Endothelial Growth Supplement (ECGS) at 37°C in a humidified atmosphere of 5% CO_2. Cells were initially seeded from liquid nitrogen storage into fibronectin coated T-75 flasks. After 24 hours fresh ECM media was added to the flask then cells were cultured for a minimum of 72 hours before a media change or subculture. Cells were subcultured by trypsinization when the monolayer reached subconfluence (80%), cells were used for experiments at passage 5.

3.2.1 Extraction media preparation. Cytotoxicity tests were carried out with extraction methods. Extracts were prepared, according to ISO 10993-12, using ECM serum free media as the extraction medium with a ratio of 1.25 ml/cm^2 in a humidified atmosphere with 5% CO_2 at 37°C for 72 hours. The supernatant was removed and refrigerated at 4°C until the cytotoxicity test. Serum free ECM medium was used as a negative control.

3.2.2 Cell morphology characterization. Cell morphology is an important indicator of toxicity in cells. To determine the morphological effect of a solution, cell structure is analyzed and compared under an optical microscope. Morphology is important because once the cellular structure is altered or compromised, cells will begin to deteriorate. Some signs of deterioration for epithelial cells are granular nucleus, cell detachment from surface and cytoplasmic vacuolation. When cells have morphologic signs of deterioration it is an indication of cellular toxicity, contamination or a cell line that is near senesce. ISO 10993 outlines a cytotoxicity scale that is used as a qualitative measurement of cytotoxicity.

Table 3

Scale of morphological indications of toxicity (ISO 10993)

Cytotoxicity Scale	Interpretation
0	Noncytotoxic
1	Mildly Cytotoxic
2	Moderately Cytotoxic
3	Severely Cytotoxic

In preparation for cytotoxicity testing, cells were incubated in fibronectin coated 96-well plates at 5 x 10^4 cells/100 µl and incubated for 72 hours to allow attachment and achieve a high confluence (70%). The medium was then replaced with a concentration gradient of: .1%, 1%, 10%, 25%, 50% or 75% composed of extraction media/serum-free ECM medium. After incubating the cells in a humidified atmosphere with 5% CO2 at 37°C for 2,4, or 7 days the 96-well cell culture plates were observed under an optical microscope shown in Figure 10. Experiments were run in triplicate to ensure repeatability and decrease variation. The optical microscope was used to capture images at different magnifications including 10X, 20X and 40X to observe and compare cell morphologies. Images were captured from each well and compared to the control well. The images were taken in phase contrast, rather than brightfield, in order to better interpret cell morphology.

3.2.2.1 Optical microscope images of cell morphologies. The EVOS optical microscope was used to inspect the morphologies of human aortic endothelial cells after exposure to different concentrations of extraction mediums. The EVOS microscope is a transmitted light, digital inverted microscope. The microscope was used with phase contrast settings to compare the

morphologies of treated and untreated endothelial cells. Images were captured on several different magnifications from 10x to 40x to investigate cell morphologies.

3.2.3 Cell viability assay. Cell viability testing is the section of cytotoxicity testing that measures the effect on cells through viability. Viability is the relative growth rate of treated cells compared to that of cells not treated with the material in question, or the control. Viability is important because it quantitatively measures the effect of a material exposure on cell growth. There are several different tests to quantitatively measure cytotoxicity including, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid), LDH (lactate dehydrogenase) and BrdU (5-bromo-2'-deoxyuridine). Each test focuses on different biomarkers to determine the mechanism of cell death. Some example biomarkers are ATP, LDH and proteases.

In this study an MTT based colorimetric assay was carried out to calculate cell viability in the multiwall plates The MTT assay uses two liquids the MTT labeling reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromid) and solubilization solution composed of 10% sodium dodecyl sulfate (SDS) in 0.01 M HCl. The MTT assay is based on the cleavage of yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells, illustrated in Figure 11. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH(Promega, 2009). An additional row with only MTT and serum free media serves as a black to account for any background noise from the solution when running the assay.

The forzeman crystals formed are solubilized and the resulting colored solution is quantified using a scanning multiwall spectrophotometer as shown in the figure below.
The 96-well plates were kept out of direct light until after crystals were completely solubilized. After 4 hours samples were read with a SpectraMax M5 multi-plate reader at 570 nm and 630 nm, for absorbance measurement. Similar to the spectrophotometer used for hemocompatibility

the multi-plate reader measures the different wavelengths to measure the wavelength of the monochromatic light passing through the liquid in each well.

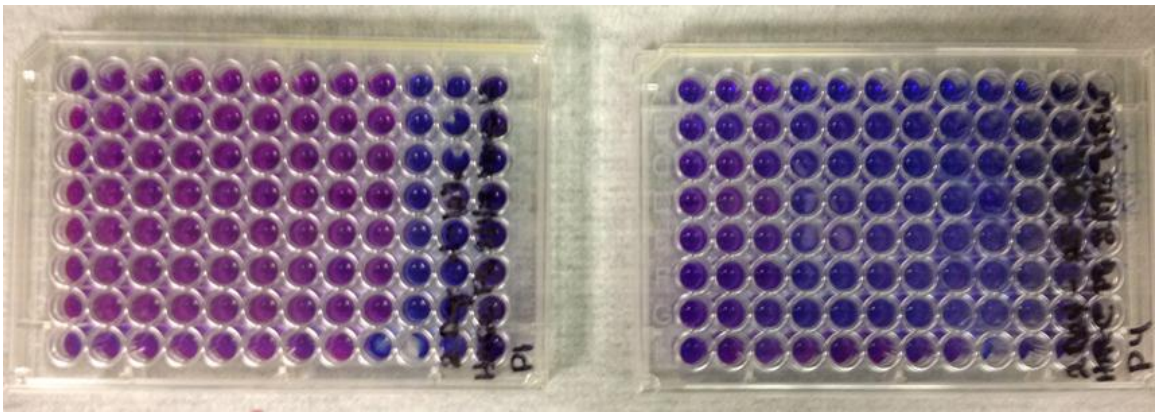

Figure 5. 96-well Plates with solubilized forzeman solution.

A one step cell proliferation kit from Roche (Mannheium, Germany) that combines these solutions was used to carry out these tests. 10 µl MTT were added to each well and were incubated with for 4 hours at 37°C, The absorbance of samples was then measured by microplate reader (SpectraMax M5 microplate reader) at 570nm with a reference wavelength of 630nm. Viability was calculated from the absorbance of each test well and control wells using the equation below. The pH value of each extraction medium was also measured.

$$\%Viability\ (RGR) = \frac{A_s}{A_c} x\ 100$$

Equation 1. Viability compared to control

3.3 Hemocompatibility Assays

Healthy human blood was obtained from Cedar Lane, according to legal protocols, from six different donors and used within the allotted time after the donation. Sodium Citrate was added to the blood as an anticoagulant. The samples used for hemocompatibility studies were the previously identified Mg-(X) Zn-(0) Ca alloys, magnesium based rare earth metals and high

purity magnesium as a control. The number of parallel samples used for statistical count was not less than six.

3.3.1 Static platelet adhesion of magnesium alloys. Platelet adhesion and activation are used to test thromboreisistant properties of materials. Static platelet adhesion tests the number of platelets adhered to a materials surface, high adhesion is an indicator that a material will cause a thrombus to form when exposed to blood. *In vitro* testing for platelet adhesion on a material surface tests the intrinsic pathway of the coagulation cascade. Platelet activation is characterized by the activation stage of platelets that adhere to a material's surface. Platelet activation can be measured by activation of markers from the coagulation cascade as well as through observation of platelet morphologies. Various pieces of equipment where used to carry out these experiments.

Static platelet incubation was performed to analyze the thrombogenicity of samples and to examine the surface interaction between blood and materials *in vitro*. For this test, platelet rich plasma (PRP) was prepared by centrifuging fresh human whole blood, containing 3.2 wt % sodium citrate, at 1000 rpm for 15 minutes. The PRP was overlaid on the top surface of the experimental alloys and incubated at 37°C for 60 minutes as shown in the image below. Samples were then rinsed gently with phosphate buffered saline (PBS) to remove non-adherent platelets. Exposed samples were fixed in 4% paraformaldehyde for 1 hour at room temperature, followed by dehydration in a gradient ethanol/distilled water mixture (50%, 60%, 70%, 80%, 90%, 100%) for 10 minutes each. Lastly samples were dried in hexamethyldislazane (HMDS) solution to ensure all moisture was removed from the sample.

A Hitatchi Scanning Electron Microscope (SEM), was used to observe the surface of platelet-attached samples. SEM was used to capture several images of the sample surface, which

were used to measure levels of platelet adhesion as well as to observe platelet morphology. Prior to placement in the SEM samples were coated with gold using the Polaron E5400 Sputter Coater.

Figure 6. Platelet Rich Plasma overlaid on a 10x10x1 mm^3 piece of material.

Up to four samples were placed in the coating chamber for approximately 90 seconds. The coating time was determined with Equation 2 below.

$$d = KIVt$$

Equation 2. Used to determine thickness of gold coating

Other constant settings inputted into the equation were d- the coating thickness, K-constant based on gold and argon (.17), I- the current (15 mA), V – the volts in kilovolts (1kV) and lastly t-time in seconds. Settings were determined from the equation set forth in the machine manual. Once coated samples were loaded into the vacuum chamber of the SEM and viewed at 10 kV x 5 mA. Different fields were randomly counted and expressed as the number of adhered platelets per mm^2 of surface. Images were also used to express the percentage of platelets in each stage of activation. A minimum of six images was taken per sample. Platelet activation was measured quantitatively using the guidelines established by Goodman (1991) and Sheppard (1994). Activation state was calculated as a percentage to measure the percentage of platelets in each stage. Morphology was measured not only by activation stage of individual platelets but

also by level of aggregation or clumping of groups of platelets on the material surface. The ratings illustrated below in Table 4, were established to identify the level of platelet aggregation on each material.

Table 4

Scale of aggregation measurements for platelet adhesion (ISO 10993)

Aggregation Scale	Interpretation
1	No aggregation
2	Mild Aggregation
3	Moderate Aggregation
4	Severe Aggregation

3.3.1.1 Centrifugation for human whole blood separation. Thermo Scientific Centrifuge, shown in Figure 14, was used to spin and separate human whole blood into platelet rich plasma (PRP) and platelet poor plasma (PPP) and to separate solution components for hemolysis testing. Human whole blood will separate into components based on volume and weight when centrifuged at certain speeds. For hemolysis samples, centrifugation will separate the heavier components of blood such as red blood cells from plasma and other components, in this case released hemoglobin. The supernatant was used for spectrophotometric analysis. Lower centrifuge settings were used to separate whole blood into two components platelet rich and platelet poor plasma. The platelet rich plasma (PRP) was used for static platelet adhesion.

3.3.1.2 Gold sputter coating for SEM sample preparation. The Polaron E54000 Sputter Coater was used to coat the top surface of samples with a thin layer of gold prior to placement in the Scanning Electron microscope. Platelet adhesion samples were covered with a layer of gold

prior to microscopy because even with fixation this is a biological sample. The gold coating makes the sample picture clearer because of the mechanism used for SEM. Rather than a traditional light globe the SEM uses an electron beam that bounces off the sample surface, these bouncing electrons are what make up the image seen by the SEM. Without a thin gold coating, samples will provide a poor signal. A very thin coat is used so that the true sample surface is not compromised. The sputter coater operates using high voltage, a pressurized chamber and argon gas. A DC voltage between 1000-3000 Volts is applied at a reduced pressure. Argon gas ionizes in the chamber producing plasma consisting of positively charged ions and negative charged electrons.

3.3.1.3 Sample surface images with scanning electron microscope. The scanning electron microscope or SEM was used to view the sample surface. The Hitachi SU8000 SEM uses the Semi-in-lens type objective lens and a cold FE-gun to produce ultra-high resolution images. This series of SEM uses beam deceleration to apply a negative voltage to the specimen. This can reduce landing voltage to as low as 100V to view sample characteristics at a low loading voltage. The triple detector SEM system can detect high angle BSE (HA-BSE), surface topography (SE), and low angle BSE (LA-BSE). These three detectors can be mixed to see different surface morphologies. The SE upper detector was used to capture surface information of samples (Hitachi, 2012).

3.3.2 Hemolysis testing of magnesium alloys. Hemolysis is the breakdown or disruption of the integrity of the red blood cell (RBC) membrane. According to ISO 10993 two main factors can cause hemolysis mechanical forces and biochemical factors. Mechanical forces can be pressure or due to flow rates and shear forces from dynamic testing. Biochemical forces include other chemicals, bacterial toxins, pH and metabolic changes. Hemolysis testing is important

because elevated levels of hemoglobin can induce toxic effects that stress the kidneys and other organs and liberate phospholipids that promote thrombosis. Materials can be tested with direct or indirect methods using material extracts. This causes hemoglobin into plasma. Sowemimo-Conor (2002) reports that osmotic changes or changes is pH can cause red blood cells to lysate if the solution because hypotonic or hypertonic. Hypertonic and hypotonic solutions cause cells to lysate because of a difference in concentrations. If the concentration of water outside a cell is greater, water will diffuse into the cell through osmosis. Due to the influx of water the cell will swell and eventually burst. If the concentration of water outside the cell is lower, water will flow out of the cell and it will shrink. In order to remain normal red blood cells must remain in fluids that are isotonic, or at the same osmotic strength, such as sodium chloride. Since osmotic strength is directly related to water concentration it is also associated with the pH of a solution. Changes in pH can result from an increase in ion levels from a degrading material. Hemolysis of RBCs can be visually detected in serum and plasma because of the release of hemoglobin.

Hemolysis testing was carried out according to ISO 10993. Healthy human blood from a volunteer containing sodium citrate (3.2 wt%) at a ratio of 9:1 was diluted with calcium and magnesium free phosphate buffered saline (4:5 ratio by volume). High purity Mg and magnesium alloy samples 10x10x1 mm^3 were dipped in separate standard tubes containing 10ml of phosphate buffered saline that were previously incubated for 30 minutes at 37°C. Then 0.2 ml of diluted blood was added to each standard tube and then incubated at 37°C for 60 minutes. As a negative control 0.2 ml of blood was added to saline, whereas diluted blood was added to distilled water for a positive control. After incubation all tubes were centrifuged at 3000 rpm for 5 minutes. The image below shows samples prior to being run in the centrifuge and after being

centrifuged for 5 minutes. Supernatant from each tube was carefully transferred to a cuvette for spectroscopic analysis at 545nm in the GENYSYS UV-VIS 10 Spectrophotometer.

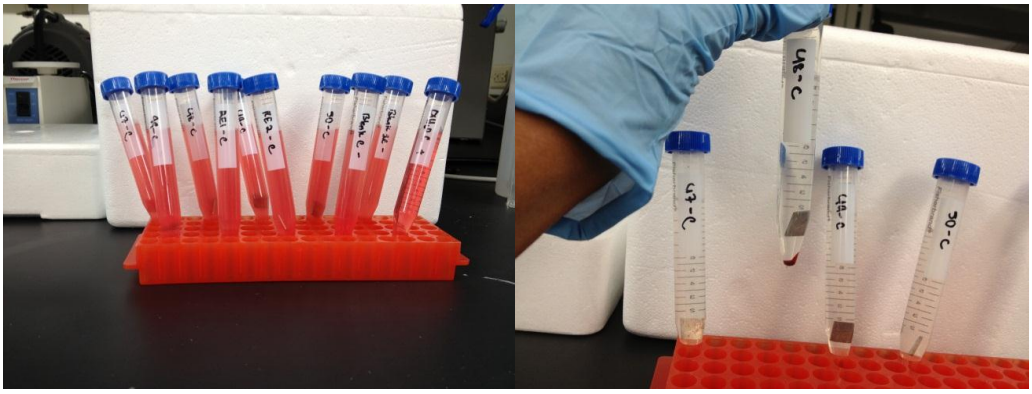

Figure 7. Hemolysis Samples before and after centrifugation.

Supernatant was transferred using a pipette to a polystyrene cuvette. One cuvette containing the negative control, phosphate buffered saline with human blood or the blank was inserted into the blank position. In order to control the level of debris or contaminants in samples aseptic technique was practiced until the last step of transferring supernatant into cuvettes. Hemolysis was based on the average of three replicates and calculated with Equation 3.

$$Hemolysis = \frac{OD_{test} - OD_{negative\ control}}{OD_{positive\ control} - OD_{negative\ control}}\ x\ 100\%$$

Equation 3. Equation for calculation of hemolysis.

Acceptable hemolysis percentage was measured against the grade outlined by ISO 10993. This scale, shown in Table 4, is used to determine if a material is going to causes hemolysis of red blood cells in the body. Materials are classified with three different grades of hemolysis. Materials that fall in the two lowest hemolytic grades, between 0% and 5% are still considered acceptable. This is because these materials will not cause a hemolytic effect that will impact the hemostasis of blood. Materials that are hemolytic are not considered hemocompatible.

Table 5

Measurement scale for hemolytic index (ISO 10993)

Hemolytic Index	Hemolytic Grade
0-2%	Nonhemolytic
2-5%	Slightly Hemolytic
> 5%	Hemolytic

3.3.2.1 Hemolysis absorbance readings. The spectrophotometer is a device used to quantitatively measure the transmittance of visual light in a particular spectrum. The GENESYS 10S uses dual-beam optics and a xenon lamp used to extract and measure a small portion of light (ThermoScientific). Monochromatic light is sent to a material, the solution will then absorb certain amount of light based on the concentration of the solution. Solutions with higher concentrations, usually darker in color to the visible eye, absorb more light than a less concentrated solution. The entire wavelength range of the machine goes from 190-1110 nm. When using Thermo-Scientific GENESYS UV-Vis Spectrophotometer to read absorbance of multiple samples, supernatant of up to 5 samples can be measured at one time.

CHAPTER 4

Results and Discussion

4.1 Cytocompatibility Tests

These tests are indicators of how cells will react when exposed to a material. *In vitro* extract methods were used to test the effect of alloying on the cytotoxicity of magnesium based materials. Cytotoxicity tests measured three main factors and how they were affected: the pH of extraction media, cell viability and cell morphology. The materials tested were MgZnCa 1, MgZnCa 2, MgZnCa 3, MgZnCa 4, RE 1, RE 2 and high purity magnesium as a control. Tests were run in triplicate to ensure repeatability. Statistical analysis was performed by analysis of variance (ANOVA). All pairwise comparisons were performed by the post hoc Tukey test. Significance is defined at three different levels that are indicated by asterisks in relevant figures. Levels of significance and respective symbols are as follows: i) significant $p \leq 0.05$, *; ii) very significant $p \leq 0.01$, ** ; iii) $p \leq 0.001$, ***; and iv) no significance, ns.

4.1.1 Extraction media. Figure 8 illustrates the pH values of extraction medium from magnesium alloys with high purity magnesium pH value as a control. The pH level of extraction medium is important because pH has a large impact on cell growth, the desired pH level would be one as close to normal endothelial cell medium as possible. All extraction mediums had a significantly different pH than normal endothelial cell medium. This is important because normal endothelial cell medium has a similar pH to that in the human body and is ideal for cell growth. High purity magnesium also had a pH significantly different than normal endothelial cell medium even though it is considered a good candidate for biomaterials. The significant increase of pH levels is one of the limitations of static *in vitro* testing. In the body there is a dynamic environment and pH may not be as high.

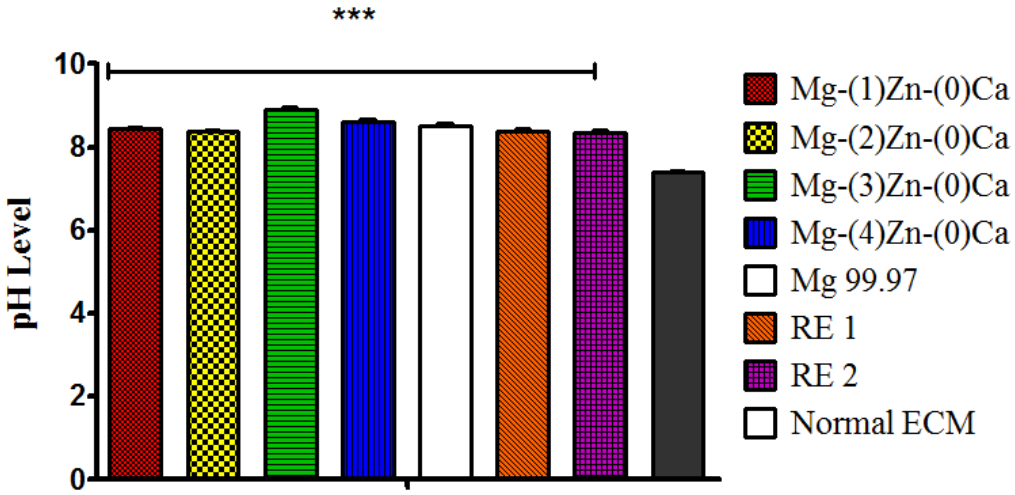

Figure 8. pH levels for extraction mediums compared to Normal ECM.

4.1.2 Cell morphology results. Before carrying out viability testing for cytotoxicity images were captured of each well in each 96 well plate using the EVOS XL optical microscope. Morphology was used to qualitatively measure any changes in human aortic endothelial cell growth. Normal human aortic endothelial cells, displayed in Figure 9 below, are cobblestone or spindle-shaped and have a non-granular cytoplasm.

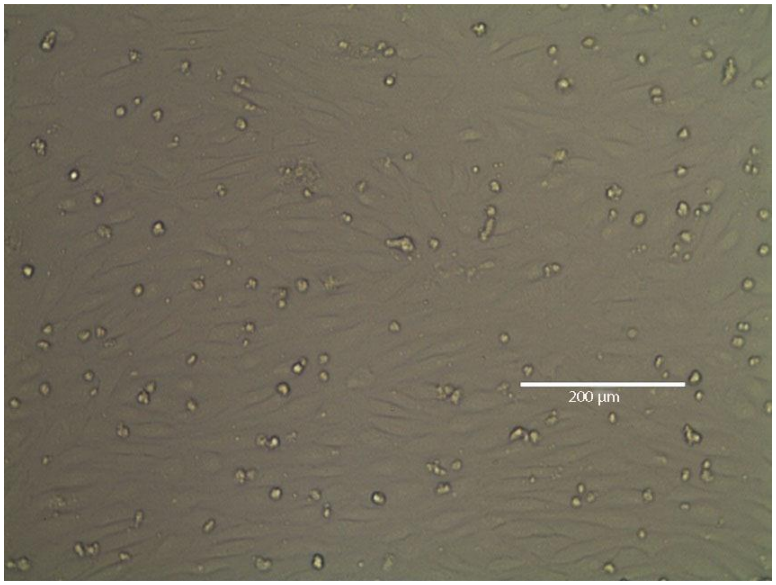

Figure 9. Human aortic endothelial cells without treatment.

Noted morphologic changes were cellular detachment from the surface, membrane integrity, and appearance of cytoplasm and general morphology. Cell morphology was recorded using the cytotoxic scale outlined by ISO 10993. The toxicity rating for cell morphologies is illustrated in Figure 10 below. Morphology was significantly different between extract concentrations. However, there was no significant difference in material or in exposure time.

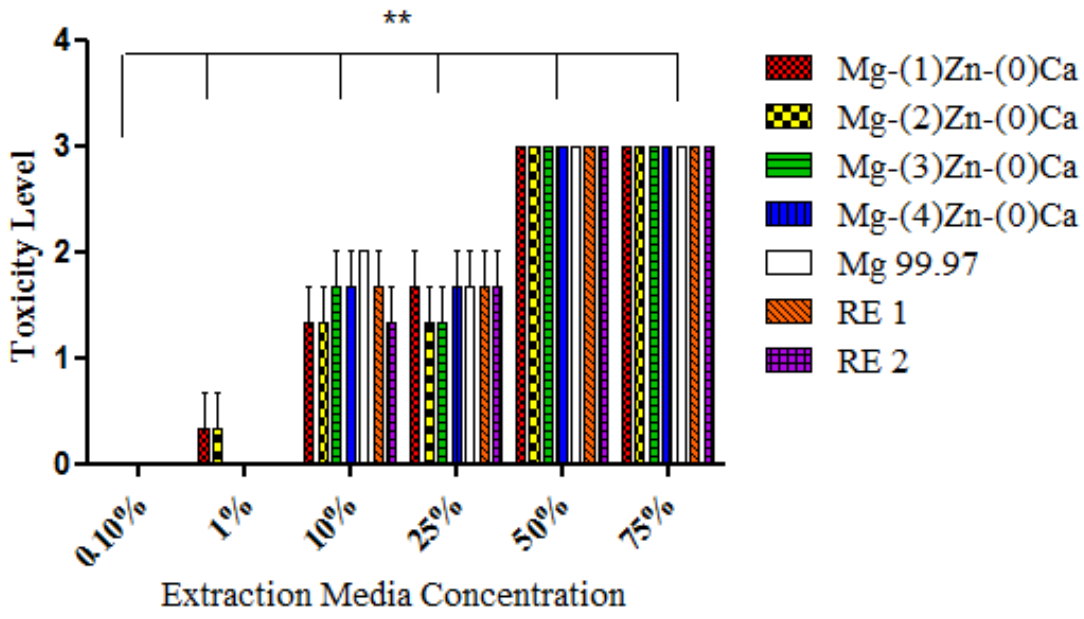

Figure 10. Toxicity ratings of cell morphologies according to ISO 10993.

Since there was no significant difference in material or exposure times, the images used to determine morphological toxicity from only material and exposure time are discussed below. Representative photos of each concentration for MgZnCa 3 are shown below, lower extract concentrations (.1 and 1%), illustrated in Figure 11 below, displayed cytotoxicity scale ratings of 0, meaning the morphology only displayed a mildly toxic effect. Cell morphology was similar to the control well, there were very small levels of detached cells, normal cobblestone shaped general morphology, and similar confluence levels.

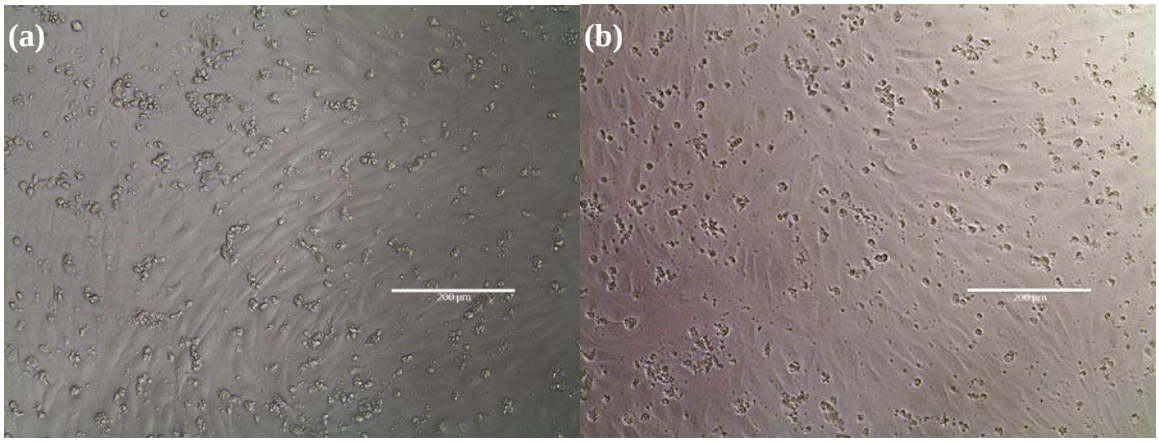

Figure 11. a) MgZnCa 3 .10% extract concentration; b) MgZnCa 3 1% extract concentration.

Middle range extract concentrations (10% and 25%) illustrated in Figure 12 below, displayed a rating of 1 to 2, meaning there was a mild to moderate morphological toxic effect compared to the control. There was a decrease in confluence and an increase in detached and rounded cells floating in the wells.

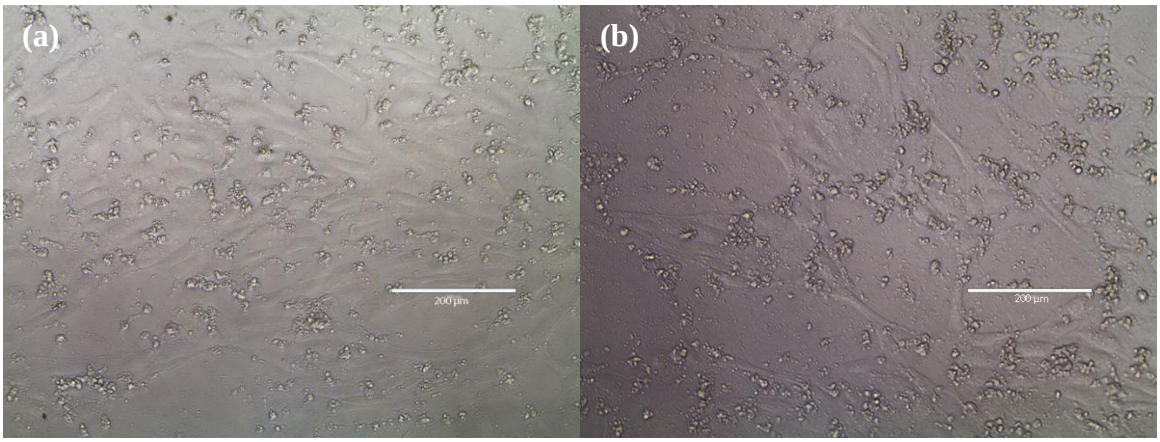

Figure 12. a) MgZnCa 3 10% extract concentration; b) MgZnCa 3 25% extract concentration.

High concentration levels (50% and 75%), illustrated in Figure 13 below, displayed ratings of 3, meaning there was a severe cytotoxic effect compared to the control. The cytotoxic effect resulted in high levels of cell death and detachment from the surface. Cells also had a change in morphology due to the decrease in confluence.

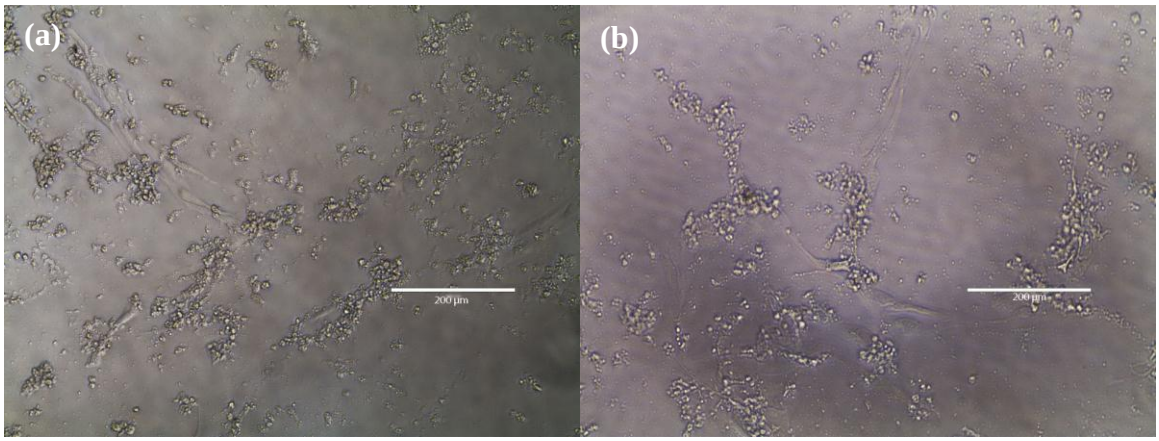

Figure 13. a) MgZnCa 3 50% extract concentration; b) MgZnCa 3 75% extract concentration.

Morphological rating results suggest that alloying resulted in a similar cytotoxic effect compared to high purity magnesium. In low concentrations alloys have no toxic effect on human aortic endothelial cells, however high concentrations are considered severely cytotoxic. There was no significant difference in cytotoxicity between alloys and compared to high purity magnesium. Since alloys produce a similar cytotoxic effect to high purity magnesium they should be considered viable candidates for biomaterials.

Several studies have also analyzed cell morphologies as a measure of cytotoxicity. The study by S. Zhang et al. (2010) concluded tested magnesium zinc alloys and rated morphologies in the 0-1 grade, concluding no toxicity was observed. Li et al. (2008) studied the effect of magnesium calcium alloys on cytotoxicity of L-929 fibroblast cells, observing a healthy morphology even in higher extraction concentrations. Researchers (Guan et al., 2012; Xia, 2012) studied the effect of magnesium zinc calcium alloys on osteoblasts and L929 fibroblasts and observed no change in cell morphology even when exposed to high concentrations of material extracts. These studies concluded there was no cytotoxicity from exposure to material extracts however, pH was not measured or reported to evaluate the increase in media pH due to incubation of materials. The literature did not conclude similar results to this study, however

different cells were used for testing, materials can have a different effect on different cell types. Also cell lines were chosen as opposed to primary cells, which tend to be more robust in comparison to primary cells, which are a more accurate measure of what is going on in the human body.

4.1.3 Cell viability results. Extraction tests were used to quantitatively analyze cell viability *in vitro*. Figures 14-16 illustrate the viability of cells compared to time, material and concentration. Viability results were statistically analyzed using the GraphPad Instant software packages (La Jolla, CA). Results were expressed as the means ± SD. Two-way analysis of variance tests were used to determine interactions followed by the Tukey-Kramer test to determine differences among the groups.

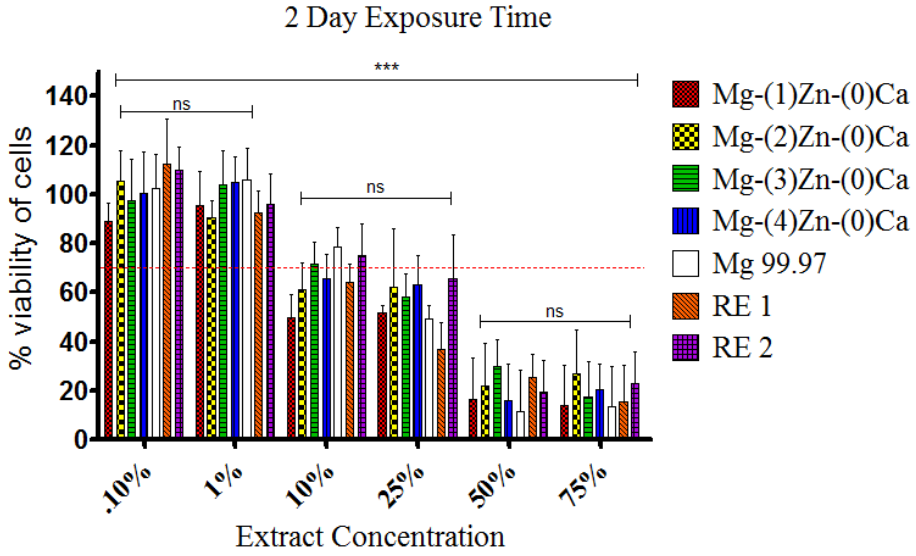

Figure 14. HAEC viability results, 2-day exposure.

Levels of significance and respective symbols are as follows: i) significant $p \leq 0.05$, *; ii) very significant $p \leq 0.01$, ** ; iii) $p \leq 0.001$, ***; and iv) no significance, ns. Exposure that resulted in viability levels lower than 70% are considered toxic to cells. The viability of human aortic endothelial cells was significantly affected by concentration. However viability was not

affected by material type or exposure time. Cell morphologies indicated similar results to the results of the MTT viability tests for 2 day exposure times illustrated in Figure 14.

A previously mentioned limitation of the MTT viability assay for testing magnesium-based materials is that high levels of Mg^{2+} ions can result in false positives for testing. The increase in exposure time of extraction media would lead to an increase in Mg ion presence with the change of media every two days. MTT results, shown in Figure 15 for 4 day exposure times had large amounts of variation because of the range of data due to false positives. By the 7 day exposure time, illustrated in Figure 16, an increase in Mg ions resulted in an incorrect reading of cell viability. Tests indicated that cell growth was increased for some concentrations however morphologies indicated that there was no increase in cell growth for those concentrations or time periods. Based on the experimental results, it can be suggested that in low concentrations magnesium alloys do not result in a cytotoxic effect on human aortic endothelial cells. As reflected from the cell morphology study there was also no significant difference between cytotoxic levels of high purity magnesium extracts and extracts of tested magnesium alloys.

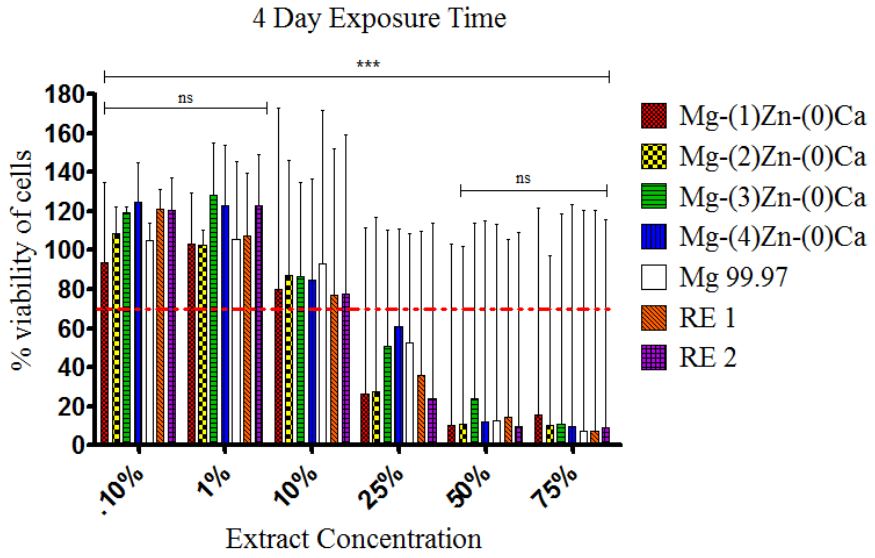

Figure 15. HAEC viability results, 4-day exposure.

The optical images of the morphology study reflected similar results for viability tests until 4 and 7 day exposures. This lack of correlation is due to the limitations of MTT testing for magnesium based alloy samples. This assay suggests that there is no significant cytotoxic effect from magnesium alloys in low concentrations. However, in high concentrations all materials are considered toxic to human aortic endothelial cells.

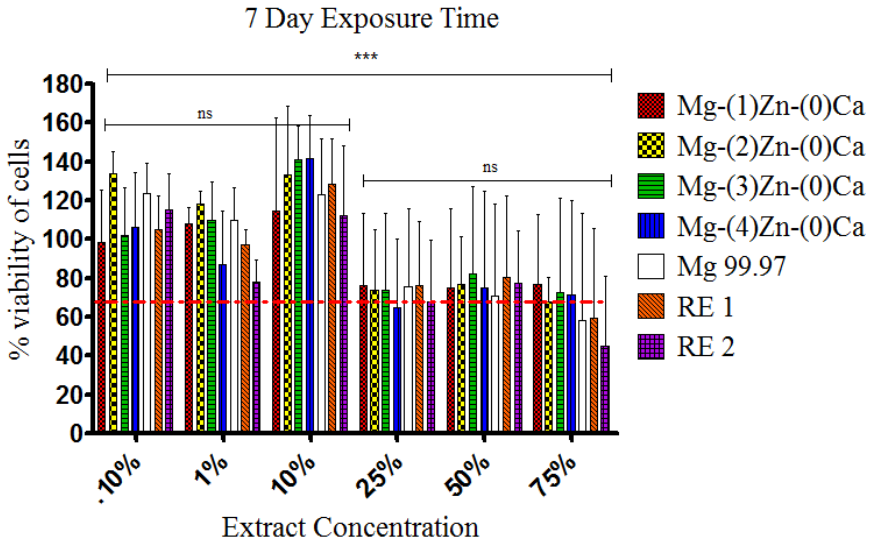

Figure 16. HAEC viability results, 7-day exposure.

As mentioned in earlier chapters, cytocompatibility of various cell types exposed to magnesium alloys have been evaluated. Several studies (Huan, 2010; Wang, 2011; Zhang, 2010) have explored the effect of magnesium, magnesium zinc calcium alloys, and magnesium rare earth alloys on cytotoxicity with varying results, however no studies have studied the effect on human aortic endothelial cells. Gu et al. (2009) tested magnesium zinc alloys and reported that there was no toxic effects on fibroblasts (L-929 and N1H3T3), osteoblasts (MC3T3-E1) or blood vessel cells (ECV304 and VSMC). Researchers H. Wang and Shi (2011) stated there was no reduction of cell viability on MC3T3-E1 cells from magnesium zinc calcium alloys compared to pure magnesium. Researchers Zhou et al. (2013) evaluated the effect of magnesium rare earth

alloys on human umbilical vein cells and vascular smooth muscle cells, results indicated no significant effect on viability. Published literature reflected increased viability of cells and/ or acceptable levels of viability (greater than 70%). However, since both studies used MTT assays, only exposure times of 2 days or less are considered acceptable to compare, increased exposure times also displayed varying results. This variation is most likely due to interactions with MTT tests. Magnesium, magnesium zinc calcium alloys, and magnesium rare earth alloys were tested and cytotoxicity results indicated that viability remained higher than 70%, materials were nontoxic, in short exposure times similar to the results of these studies.

4.2 Hemocompatibility Tests

Hemocompatibility studies were comprised of hemolysis testing, platelet adhesion testing and characterization of platelet morphologies. Six different healthy human volunteers supplied the blood for all hemocompatibility tests. Statistical analysis was performed by analysis of variance (ANOVA). All pairwise comparisons were performed by the post hoc Tukey test. Levels of significance and respective symbols are as follows: i) significant $p \leq 0.05$, *; ii) very significant $p \leq 0.01$, ** ; iii) $p \leq 0.001$, ***; and iv) no significance, ns.

4.3 Mechanisms of Hemocompatibility

The results of hemocompatibility tests are indications of the various actions occurring in the blood. Researchers S. Wang, McDonnell, Sedor, and Toffaletti (2002) studied the link between human blood pH levels and exposures to magnesium and calcium ions. They reported that Mg and Ca could compete for binding sites resulting in an increase in pH and found this change in pH could trigger hemostasis of the blood. Blockmans et al. (1995) reported that platelet activation plays an important role in the first stages of hemostasis. The activation of platelets leads to thrombosis and is subsequently followed by fibrin formation activated by the

coagulation cascade. Although, hemostasis is important to the normal function of the human body, excessive platelet activation is responsible for a variety of arterial blood-clot phenomena.

4.3.1 Platelet adhesion and activation and thrombosis. Platelet adhesion is another method of measuring the hemocompatibility of a material *in vitro*. Platelet adhesion can be measured with two aspects the number of platelets adhered to a materials surface and the morphology of theses platelets. The number of platelets adhered to a materials surface indicates the compatibility of a materials, high levels of platelet adhesion can be considered compatible as long as aggregation and activation do not occur. Sheppard et al. (1994) stated that factors such as material coating, the presence of a platelet activator and time, influence platelet adhesion.

Platelet morphology is indicative of the activation state of adhered platelets. Blockmans et al. (1995) reported that upon activation platelets lose their discoid shape, become spherical in form and extend long, spiky pseudopods and bulky surface protrusions. The main stages of platelet activation are outlined by Goodman (1999) and Sheppard et al. (1994) as "1) Round - Cell is round and raised, absent of pseudopodia; 2) Dendridtic- Visible pseudopodia, average diameter of 2-4 μm; 3) Spread dendritic- platelet body has begun to flatten and increased in diameter, pseudopodia have begun to thicken; 4) Fully spread- platelet is flat and has reached the maximum diameter of 7-10 μm". Platelets that have become activated indicate a risk of a material being thrombogentic. Platelet adhesion and morphology of magnesium and magnesium alloys has been studied by very few researchers (Gu et al., 2009) and there is still a need to evaluate this aspect of several different magnesium alloys. The coagulation cycle plays a key role in platelet activation and thrombus formation.

4.3.1.1 Thrombosis. Thrombosis is the pathologic formation or presence of a thrombus or a blood clot in a blood vessel. Thrombosis is one of the beginning steps in hemostasis. When

there is damage to a blood vessel, blood is exposed to the collagen fibers in the basement membrane of the vessel. Once exposed, platelets will stick to the basement membrane and become activated, these activated platelets release chemicals that cause the aggregation of more platelets to the site. These aggregated platelets will form a platelet clot to stop the loss of blood. Once a thrombus is formed it can cause a blockage at the formation site or it can break lose and become and embolus, and cause blockage in other areas of the body. Thrombosis can inhibit blood flow depending on the size; emboli can cause problems with blood flow in other parts of the body. Some materials can cause thrombosis of platelets on their surface; this can lead to complications of blood flow in the body. Several studies (Blockmans et al., 1995; Brass, 2003) outlined the link between thrombin and platelet activation. Thrombin is a very powerful platelet stimulus, causing shape change, aggregation and secretions from within the platelet. When platelets are exposed to thrombin in vitro it causes them to enter the stages of activation, shown in Figure 17, where the end result is a platelet plug or a thrombus.

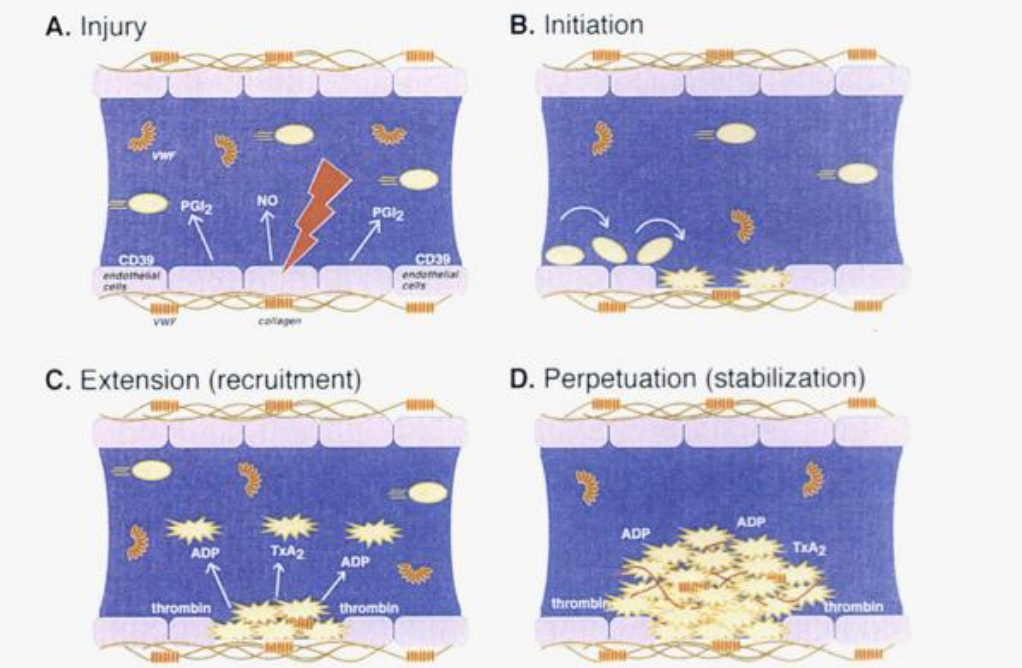

Figure 17. Stages of platelet plug formation (Brass, 2003).

4.4 Platelet Adhesion Results

Figure 18 illustrates the adhesion levels of platelets for tested magnesium based materials with high purity magnesium as a control. High levels of platelet adhesion are considered undesirable for biomaterials because this is an indication of material thrombogeneicity. Platelet adhesion testing was done in triplicate to ensure repeatability. A minimum of six SEM images were taken for each material in different sections of each material, and counted for the number of platelets adhered to the surface. A blind count was done to ensure objective results.

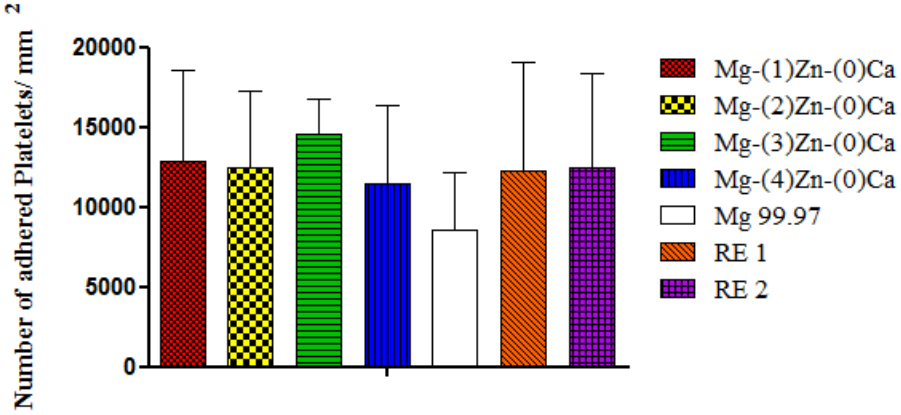

Figure 18. Number of adhered platelets by material.

There was no significant difference between the control high purity magnesium and all six magnesium alloys, or between the magnesium alloys themselves. This indicates that tested magnesium alloys possess good platelet adhesion characteristics and should be considered good biomaterial candidates. Figures 19 a-g below depict SEM images of high purity Mg and all tested magnesium alloys from static platelet adhesion testing. These images show a broad illustration of the different levels of adhesion and aggregation and some different platelet morphologies. To simplify counting of materials, each image was sectioned into four separate sections and the totals of each section were counted for each image. The likely mechanisms behind these results of platelet adhesion and activation are discussed above.

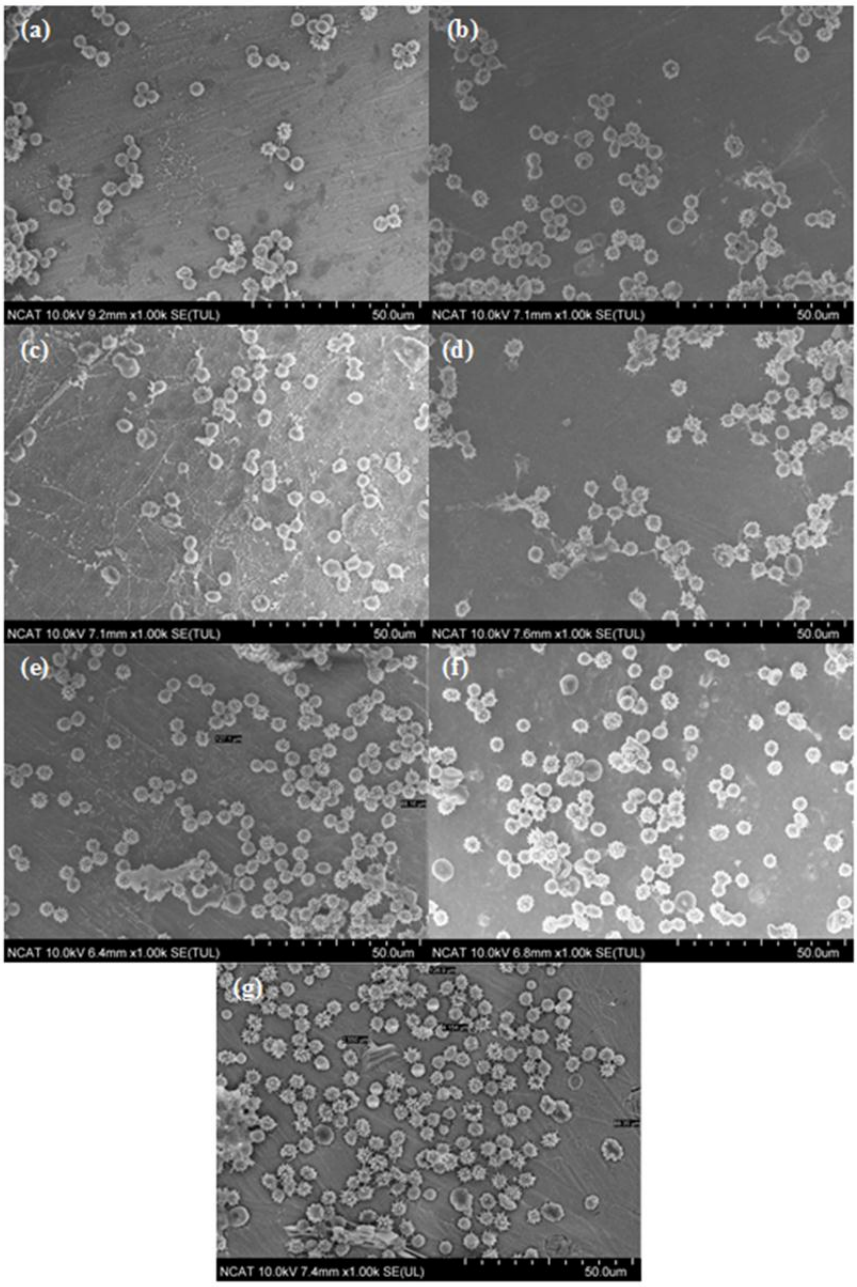

Figure 19. SEM micrographs of a)MgZnCa 1 and b)MgZnCa 2 c) MgZnCa 3 d)MgZnCa 4 e)high purity Mg f)RE 1and g)RE 2 after platelet adhesion assay.

There is no standard level of adhesion considered acceptable for these materials in the ISO Standards, however our findings indicate that there is some adhesion of platelets on the surface of alloys and magnesium indicating the pathway has begun activation. High purity

magnesium had the lowest number of platelets adhered to the surface, even though there was no significant difference. Although insignificant, results do indicate an increase in platelet adhesion with alloying of materials, which suggests alloys are more likely to induce thrombosis. Compared to current literature (Gu, 2009; Wang, 2011), testing of magnesium zinc and magnesium zinc calcium alloys show similar levels of platelet adhesion, with an increase in adhesion with alloying but no significant difference.

4.5 Platelet Morphology Results

Figure 21 illustrates the percentage of platelets in each stage of activation on each sample surface. For platelet morphology measurements, three micrographs were taken of platelets from three different areas of each sample. Platelet morphology testing was done in triplicate to ensure repeatability. Individual platelets were categorized as round, dendritic, spread-dendritic and fully spread according to the criteria determined by Goodman (1991) and Sheppard (1994). Some examples of measure morphologies are illustrated in Figure 20 below.

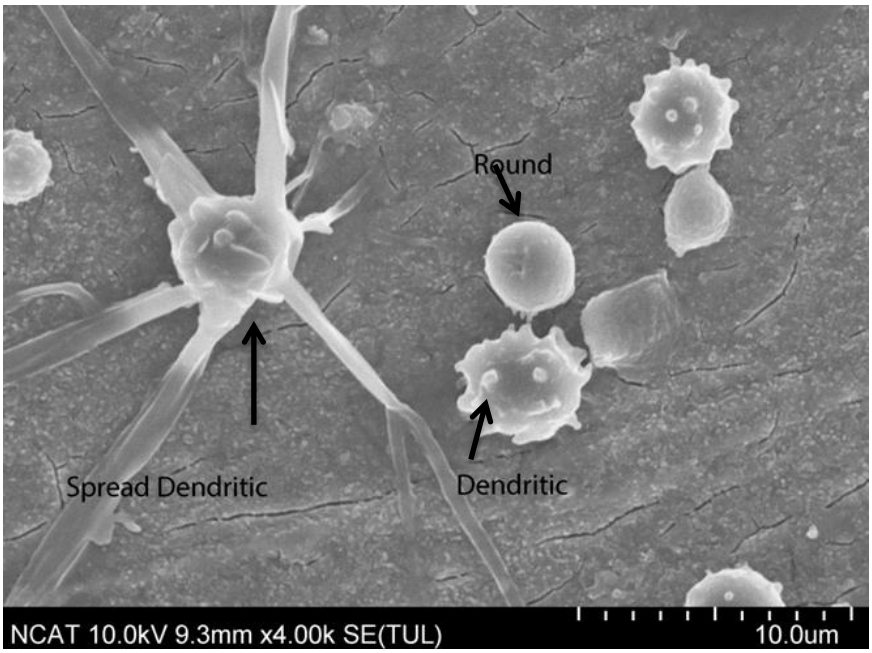

Figure 20. Examples of platelets in various stages of activation.

The percentage of spread platelets was calculated as the number of platelets in its respective category multiplied by 100 then divided by the total number of platelets in each image. There was no significant difference between the activation stages of magnesium alloys compared to the control of high purity magnesium. However there was undoubtedly a significant difference between activation stages, since a majority of materials had the most platelets in the dendritic stage.

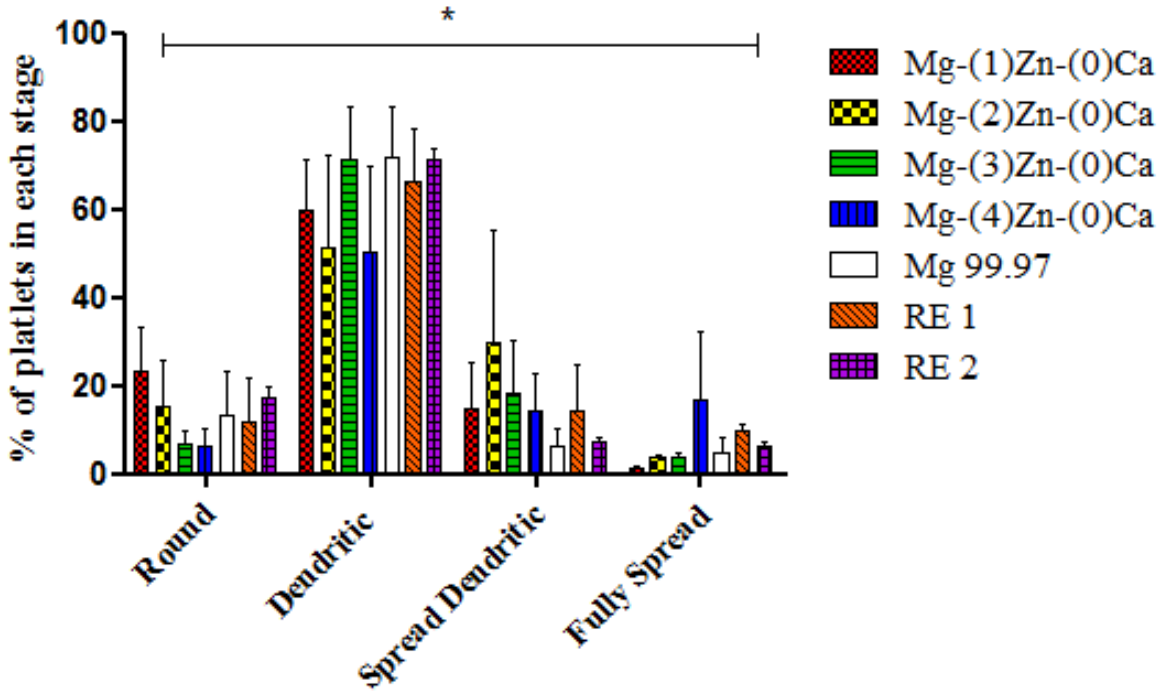

Figure 21. Percentage of platelets in each stage of activation.

Platelet morphologies indicate the stage of activation of adhered platelets. The results suggest that magnesium alloys have a similar activation response compared to the control of high purity magnesium. Platelet morphology or each individual platelet is important to determine activation; however another indication of activation stages is aggregation. Although a majority of platelets were only in the first stages of activation, aggregation and clumping of platelets was observed on material surfaces. Platelet aggregation was measured on a scale of 1-4, based off the

ISO scale outlined in Chapter 3, with 1 being no aggregation and 4 being severe uniform aggregation across the sample surface. Figure 22 illustrates the aggregation level of platelets for each material. There was no significant difference between the material types and aggregation levels remained at 2 and below, indicating that although there was some clumping of platelets there was not a severe response on the material surface.

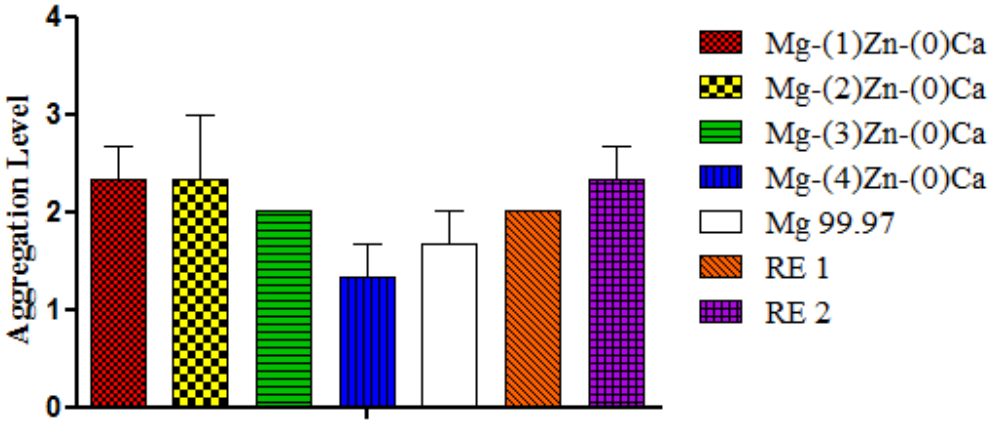

Figure 22. Aggregation level of adhered platelets.

All measurements of platelet morphology indicated that although there was activation of platelets it was only in early stages and aggregation was at a minimum. There was no significant difference in platelet morphology or aggregation between materials or compared to high purity magnesium. These results indicate that tested alloys will not create an adverse platelet response in the body and should be considered good candidates for biomaterials.

4.6 Hemolysis Results

Figure 23 illustrates the hemolysis percentages of magnesium alloys with high purity magnesium as a control. Hemolysis testing was done in triplicate to ensure repeatability. Hemolysis level is an important factor in hemocompatibility tests because an increase of free

hemoglobin can lead to adverse in events in several areas of the body. Statistical analysis was performed by analysis of variance (ANOVA). All pairwise comparisons were performed by the post hoc Tukey test. Levels of significance and respective symbols are as follows: i) significant $p \leq 0.05$, *; ii) very significant $p \leq 0.01$, ** and iii) $p \leq 0.001$, ***. There was no significant difference in hemolysis of magnesium compared to high purity magnesium; there was also no significant difference between hemolysis of magnesium alloys.

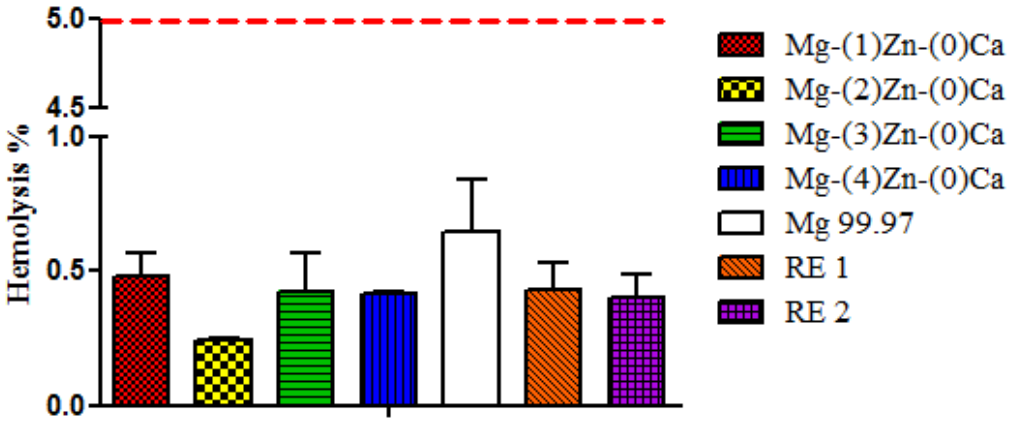

Figure 23. Hemolysis percentages for tested materials.

The similarity between high purity magnesium and tested magnesium alloys suggests that these materials should be considered as good candidates for biomaterials. Also, all tested magnesium alloys were considered nonhemolytic according to the scale set by ISO 10993. Compared to the published studies (Gu, 2009; Zhang, 2010; Guan, 2011) the alloying combinations in our study show decreased levels of hemolysis compared to other magnesium-based alloys.

There are very few publications addressing the hemocompatibility of magnesium alloys. Researchers (Gu et al., 2009; B. P. Zhang, Qiu, Wang, Liu, & Bi, 2011; S. Zhang et al., 2009) studied similar alloys and their blood interactions. Gu et al. (2009) observed a decreased platelet adhesion response and hemolysis percentage of Mg-(1.04)Zn alloys compared to high purity

magnesium. However the magnesium zinc alloy resulted in over 5% hemolysis making materials unsuitable for use. The study by S. Zhang et al. (2009) studied the hemolysis of a magnesium zinc alloy with a higher percentage of Mg, which resulted in acceptable hemolysis of 3.4%. Researchers H. Wang and Shi (2011) investigated the hemolysis of Mg-(1)Zn-(1)Ca alloy and observed extreme hemolysis around 24%. The study of hemocompatibility of magnesium rare earth alloys by Zhou et al. (2013) indicated than 5% hemolysis and non thrombogenicity of materials. Results from this study reflect similar results of some published literature, as samples were considered nonhemolytic and indicated very low levels of thrombogenicity.

CHAPTER 5

Conclusions and Future Research

5.1 Conclusions

The results of this study support the idea that magnesium alloys are good candidates for cardiovascular biomaterials. Specifically, ternary magnesium-zinc-calcium alloys with varying compositions (between 1 wt% and 4 wt%) of zinc and magnesium based rare earth alloys. *In vitro* testing, outlined by ISO 10993, compared the cytocompatibility and hemocompatibility of six different magnesium alloys to a control of high purity magnesium. These *in vitro* tests included cytotoxicity testing, platelet adhesion and activation and hemolysis testing.

Biocompatibility of magnesium alloys was measured by cytotoxicity and hemocompatibility. Cytotoxicity tests measured the morphological effect and viability of HAECs exposed to material extracts. Results from cytotoxicity tests suggested that in there is no significant difference between the toxicity magnesium alloys and high purity magnesium. The cytotoxicity assay indicated that in low concentrations, magnesium alloy extracts showed no significant toxicity to human aortic endothelial cells (HAEC). Cytotoxicity tests reported no significant difference in exposure time however; there was an increase in viability after 7 days. It was concluded that this increase is a reflection of the limitations of MTT tests when analyzing the effect of magnesium alloys because morphological examination of cells after 7 days reflected similar results to cells after 2 day and 4 day exposure.

Hemocompatibility was measured with platelet adhesion and hemolysis testing. Platelet adhesion levels and platelet morphology of magnesium alloys were similar to high purity magnesium. Activation stages and aggregation levels of adhered platelets of magnesium alloys were also similar to high purity magnesium. Platelet morphologies indicated that a majority of

platelets were only in the first two stages of activation. Aggregation levels of adhered platelets were low; no materials indicated high levels of aggregation. Results of adhesion testing need to be further explored since platelet morphology did show some levels of activation and aggregation. Hemolysis levels of magnesium alloys were also similar to high purity magnesium and all materials were considered noncytotoxic. These results suggest that MgZnCa 1, MgZnCa 2, MgZnCa 3, MgZnCa 4, RE1 and RE2 should all be considered as candidates for biodegradable biomaterials.

Compared to current materials in literature, alloys studied in this research have improved corrosion properties due to the inclusion of zinc and calcium or rare earth alloys and good biocompatibility. Literature indicates alloys should improve corrosion rate of materials which will address the need for cardiovascular materials that last longer than 4 months after implantation. Tests indicate these alloys are good biomaterial candidates for stent applications because they exhibited low toxicity to human aortic endothelial cells and good hemocompatibility. These materials should be studied further to analyze mechanisms effecting biocompatibility and should be considered good candidates as biomaterials for cardiovascular applications.

5.2 Future Studies

There are a variety of future studies that should be applied to these same material compositions to further understand the mechanisms behind the results. Also some future studies need to be conducted based on identified limitations. The main limitation of tests is that they are static *in vitro* tests. *In vitro* testing uses a static environment; this has its advantages for testing variations and repeatability, however the human body is dynamic environment. Static testing results in high pH levels for cytotoxicity testing that are not seen in the dynamic environment.

Another limitation surrounds the commonly used MTT viability assay. This is one of the most common assays used to conduct viability tests, however studies have shown (Fisher, 2003) that it can produce incorrect readings when testing magnesium based materials. This was seen in our 4 day and 7 day viability results, which the MTT indicated an increase in viability but morphology did not. In future studies a different assay should be used to measure the effect of cytotoxicity. A BrdU cell proliferation assay that measures the incorporation of BrdU into deoxyribonucleic acid DNA during synthesis may be used to evaluate viability; other Mg-cell interaction studies have used this assay without apparent interference (personal communication with Dr. Jenora Waterman). However, the ideal method of analyzing cytotoxicity of magnesium alloys would be florescence assays (which do not rely upon intracellular enzymes for assay performance) or western blot assay. Western blots will indicate what is going on at a subcellular level and fluorescence assays can indicate viability.

Other future studies are related to tests that are needed to further validate the biocompatibility of the materials tested in this study. Studies associated with hemocompatibility are protein adsorption, clotting time measurement and pathway activation tests. These tests will give further insight to platelet adhesion and activation on materials. Since some level of activation was observed these tests can help to discover the reasons behind the activation. Direct testing with endothelialization of these materials would also be beneficial to further examine the biocompatibility. Direct tests not only measure the cytocompatibility of cells but also the attachment of cells to the material surface. Future studies should also include materials of the same components with varying concentrations in order to further understand the mechanisms behind the compatibilities of these materials.

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