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The Effect Of Magnesium On Angiogenesis Evaluated Utilizing The Chick Chorioallantoic Membrane Assay

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The Effect of Magnesium on Angiogenesis Evaluated Utilizing the Chick Chorioallantoic Membrane Assay

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Biographical Sketch

Lauren Ashlee Douglas-Byrd is the daughter of Ms. Doris Lee Byrd and the granddaughter of the late Mrs. Vestula Lowry Byrd. Born in Greenville, SC, raised in Clover, SC and Gastonia, NC Lauren has sought academic success since pre-school. Graduating number 27 in a class of 237 students from Clover High School, Lauren was enthusiastic about furthering her education on a full academic scholarship at Tuskegee University. As a 2005 cum laude Bachelor of Science, Biology, minor in Bioethics, graduate of the magnificent Tuskegee University, Lauren formed several lifelong bonds and cemented the idea of becoming a lifelong learner. In August 2010, Lauren was introduced to Bioengineering, the National Science Foundation's Engineering Research Center for Revolutionizing Metallic Biomaterials, and has not looked back. Lauren viewed the opportunity to pursue a Master's degree at the World Renowned North Carolina Agricultural and Technical State University College of Engineering as a privilege and honor. There have been highs and lows, bumps and smooth sailing, but ultimately, her experience at North Carolina A&T State University has been extremely rewarding.

Lauren is a member of Alpha Kappa Alpha, Sorority, Incorporated, being inducted in the Gamma Kappa Chapter in Fall 2003. She is a member of Alpha Kappa Mu General Honor Society, Beta Kappa Chi Scientific Honor Society, and the Golden Key National Honor Society.

Dedication

This thesis is dedicated to my late grandmother, Vestula Lowry Byrd. She instilled in me many morals and values that developed me into the woman that I am today. I would also like to dedicate this thesis to Dr. Helen Benford, who was my mother away from home at Tuskegee University who pushed me towards my goal and never let me give up. I dedicate my thesis to all the friends, family members, sorority sisters, and acquaintances that motivated me to push me toward my goal when life handed me a lemon orchard. I would most importantly like to dedicate this thesis to my mother, Ms. Doris Lee Byrd who always loved me, believed in me, encouraged me, and supported me. I am forever indebted to her.

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Nomenclature and Symbols

3D	Three dimensional
ATP	Adenosine triphosphate
Al	Aluminum
Ang	Angiopoietins
AZ31	Aluminum:Zinc 3:1
bFGF	Basic fibroblast growth factor
C	Carbon
Ca	Calcium
CAM	Chorioallantoic membrane
Cl	Chlorine
EDX	Electron dispersive x-ray
Mg	Magnesium
Mg-5Zn-0.3Ca	Magnesium:5% Zinc: 0.3% Calcium
MMP	Matrix metalloproteinases
O	Oxygen
P	Phosphorus
PBS	Phosphate buffer solution
PDMS	Polydimethylsiloxane
PLGA	Polylactic-co-glycolic Acid
PLLA	Poly-l-lactide acide
SBF	Simulated body fluid
SEM	Scanning electron microscope
VEGF	Vascular endothelial growth factor
Zn	Zinc

Abstract

Magnesium and magnesium alloys are emerging as possible novel biodegradable materials for medical implants such as cardiovascular stents. Understanding the angiogenic properties of magnesium would increase the level of knowledge regarding the healing process once the material is implanted. The chick chorioallantoic membrane assay is an effective, inexpensive method of assessing magnesium's angiogenic properties. The assay is more informative than an in vitro model, in terms of magnesium degradation environment, and provides similar results of an in vivo model. This model can be used as a preliminary method of assessing results before investing labor and finances into larger in vivo models. The corrosion results of magnesium alloys in an in vivo environment and in vitro environment yield insight towards the byproducts of the implant, once degradation has occurred. Immersion test results compared to the in vivo chorioallantoic membrane assay results have shown the behavior of two specific magnesium alloys-AZ31 and Mg-5Zn-0.3Ca. Full comprehension of the angiogenic process stimulated by magnesium will assist in the development of magnesium implants that are more effective treatment methods for many medical applications.

CHAPTER 1

Introduction

1.1 Introduction

At the helm of developing implants, biodegradable materials are at the forefront of new science initiatives. Aimed at reducing subsequent surgeries and providing beneficial elements to the body, biodegradable implants comprised of magnesium are the front runners as a new implantable material that is beneficial in the treatment of a various medical conditions, as well as providing an important element to the body. The focus of the research conducted was to evaluate the effects of magnesium, magnesium alloys, and degradation products on angiogenesis, utilizing the chicken chorioallantoic membrane assay. The aim was to show that magnesium, magnesium alloys, and degradation byproducts are capable of upregulating angiogenesis in the tissues that are in direct contact with the research sample.

1.2 Objective

The focus of the research plan proposed by the Engineering Research Center for Revolutionizing Metallic Biomaterials is to fabricate and commercialize magnesium based biodegradable biomaterials that will regenerate biological functions in craniofacial, orthopedic, cardiovascular, and responsive biosensor applications. The objective of this study was to utilize the chick chorioallantoic membrane assay as a viable method to analyze the effects of magnesium and magnesium based alloys on angiogenesis. Post implantation angiogenesis is critical to healing at the site, the research performed in this study will provide ground level knowledge of the interactions that occur between developing vasculature and Engineering Research Center fabricated magnesium based alloys.

CHAPTER 2

Literature Review

2.1 Biodegradable Metals

Biomaterials for implants can be comprised of a variety of materials. Material selection is dependent upon site, use, and duration the implant is required. Biomaterials that have been used to fabricate implants include, but not limited to, ceramics, composites, metals, and polymers. Metals have a high wear resistance, increased strength, increased toughness, and high ductility, in comparison to other biomaterials. Traditionally, scientist and surgeons opted for metals that were corrosion resistant, such as titanium and stainless steel. Interest has increased in the scope of supplying implants that are beneficial to the healing and health of the patient. The combination of health consciousness with modern developments has paved the way for biodegradable implants. Materials that can be fabricated to provide strength and integrity, in addition to beneficial corrosion by products, have shifted the focus of biomaterial development.

The material for biodegradable stents is requested to have at least the following characteristics: it must be biocompatible, degradation products of the material must also be biocompatible, the material must stay in the placed for several months before its complete bioabsorption and the radial force of the resultant stent must be enough for scaffolding effect during the request period (Moravej & Mantovani, 2011). The purpose of biodegradable implants and coatings is to support tissue regeneration and healing in a specific application by material degradation and concurrent implant replacement through the surrounding tissue (Witte et al., 2008). Degradable metallic materials could potentially replace corrosion-resistant metals currently used for stent application as it has been shown that the role of stenting is temporary and limited to a period of 6-12 months after implantation during which arterial remodeling and

healing occur (Mantovani, 2011; Moravej & Mantovani, 2011). The candidate materials for biodegradable stent application should have properties in comparison to stainless steel 316L, which is the current gold standard.

Table 1

Mechanical and physical properties of implant materials in comparison to natural bone

Properties	Natural Bone	Magnesium	Titanium	Stainless Steel
Density ($\frac{g}{cm^3}$)	1.8-2.1	1.74-2.0	4.4-4.5	7.9-8.1
Elastic modulus (GPa)	3-20	41-45	110-117	189-205
Compressive yield strength (MPa)	130-180	65-100	758-1117	170-310
Fracture toughness ($MPam^{1/2}$)	3-6	15-40	55-115	50-200

Development of biodegradable metallic implants has revolutionized the concept of biomaterials from purely mechanical replacement devices towards true biological solutions (Moravej & Mantovani, 2011). Although corrosion is generally considered as a material failure, when the resultant corrosive byproducts are advantageous to the patient, corrosion of the material becomes acceptable (Moravej & Mantovani, 2011). The ability of magnesium to corrode into beneficial by products is advantageous to scientist, surgeons, and most importantly, patients. The purpose of these biodegradable implants is to promote tissue regeneration and optimal healing, facilitated by the degradation by products. It is desirable for these implants have minimal impact on the surround healthy tissue and not elicit a toxic response of the immune system.

2.2 Magnesium

Magnesium has become a candidate for biodegradable stent application based on the fact that it is found in several tissues and biochemical reactions in the body. Magnesium is the second most abundant intracellular cation and fourth most abundant mineral in the body. Fifty percent of the magnesium that is found in the body is located in bone, with the rest being dispersed amongst tissues and cells of the body. One percent of magnesium is found in the blood, yet this is very critical to regulating magnesium levels throughout the body. The essential element is a substantial intercellular cation which is involved in more than 300 biological reactions of the cell(Moravej & Mantovani, 2011). The magnesium intake for a normal adult should be 300-400 milligrams per day, with excess magnesium efficiently being excreted to the body without causing deleterious effects (Song, 2007). Magnesium maintains normal muscle and nerve function, helps to maintain a healthy heart rhythm, aids in maintaining the immune system, and it keeps bones strong. Magnesium is also known to play a role in energy metabolism, or the regulation of ATP and protein synthesis.

In 1808, elemental magnesium was discovered by Sir Humphrey Davy. Dr. Edward C. Huse began using magnesium wires in 1878 to fabricate ligatures for his patients. Based on his observation of the degradation of the wires, dependent upon the size of the wire, the application of metallic biodegradable implants began shortly thereafter(Moravej & Mantovani, 2011). Magnesium is a desirable material for biodegradable metals used for implants due to its high biocompatibility. The mechanical properties of magnesium are similar to the mechanical properties of cortical bone. Maier et al researched the effects of magnesium on endothelial cell migration and proliferation to discover that high levels of magnesium facilitates the reendothelialization of vascular injuries, thus preventing excessive subintimal proliferation of

smooth muscle cells and reducing the risk of thrombosis, when human umbilical vein endothelial cells were exposed to magnesium (Maier, Bernardini, Rayssiguier, & Mazur, 2004). Reendothelialization is a requirement for healing of tissues following vascular injury which may result from surgery or any other impact to the vascular system.

Magnesium is highly corrosive in environments with high chloride content. The degradation of magnesium and magnesium alloys occurs via corrosion. Corrosion is a chemical or electrochemical reaction that occurs between a metal and its environment. Corrosion is triggered by varying temperatures, metal heat treatment during fabrication, metal surface conditions, exposure to radiation, exposure to environmental contaminants, pressure and stress to the metal, and other miscellaneous factors. Corrosion may be uniform, galvanic, crevice, or occur via pitting. The corrosion of a metal may be induced by stress cracking the surface, erosion of the metal, or damage caused by the presence of hydrogen. The surface ions of the metal become oxidized, or they lose electrons, while the exposed agent gains those electrons, thus being reduced.

Galvanic corrosion is the most commonly known form of corrosion and is a result of differing oxidation potentials for metallic ions. The larger this difference, the increased amount of galvanic corrosion that occurs. The degradation of magnesium occurs primarily by galvanic corrosion. The degradation of magnesium alloys always leads to hydrogen evolution and alkalization of the solution (Song, 2007). The pH of a solution must be greater than 11 for magnesium to form a protective layer to halt corrosion. This very basic pH level is not ideally found in normal body conditions. Hastened degradation of magnesium due to the chemical composition of the body has a significant impact on the mechanical properties of magnesium (Mantovani, 2011). In the human body, the evolved hydrogen bubbles from a corroding

magnesium implant can be accumulated in gas pockets next to the implant, which severely impacts healing times of the site. Tissue necrosis can has been shown to increase due to hydrogen gas formation.

Around 1900, Payr already proposed that tissue oxygen and water content, carbon dioxide, the dissolved salts in blood and the chemical processes in cells were mainly responsible for the corrosion of magnesium *in vivo* (Witte, 2010). In an effort to control the rate of degradation of magnesium, the material is can be coated with a biocompatible material. Biodegradable layers of PLGA and PLLA mixed with a drug is a potential coating for magnesium implants. The PLGA and PLLA allow for slow release of the drug, while allowing the magnesium to remain in place for mechanical and structural support. Lu et al. reported the fabrication of biodegradable AZ81 magnesium alloy stent coated with a composite multi-layer film for control of the biodegradation rate and drug release rate of the magnesium alloy (Moravej & Mantovani, 2011). The *in vitro* experimentation concluded that the layer of poly-l-lactic acid composite increased the corrosion resistance and controlled the biodegradation rate of the material. Magnesium can also be alloyed with aluminum, manganese, and rare earth elements to decrease the rate of corrosion for the material (Mantovani, 2011). Due to magnesium's capability to quickly degrade in chlorinated environments, magnesium is often alloyed with zinc, aluminum, and calcium to halt degradation. Magnesium and its alloys are generally known to degrade in aqueous environments via an electrochemical reaction which produces magnesium hydroxide and gas (Witte et al., 2008). Song reported that there are two main groups of magnesium alloys, those containing aluminum and those that do not. The biocompatibility of magnesium alloys that contain aluminum is poor. Calcium is often alloyed with magnesium to improve strength, decrease the corrosion rate, and improve the biocompatibility of the material.

Research has shown that magnesium based biodegradable stents are more biocompatible than current permanent stents, but due to the fact that the corrosion rate of the material has successfully been controlled, the ideal alloy is yet to be developed (Mantovani, 2011). In fact, the objectives targeted in the development of the next generation of biodegradable metallic stents are controllable degradation rate, prolonged mechanical stability and further reduction of impact on developing tissues (Mantovani, 2011).

2.3 Angiogenesis

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing vessels. It is a normal and vital process in growth and development, wound healing, and tissue repair. Angiogenesis is also pathogenic, in that it is responsible for tumor malignancy. The physiological process of angiogenesis is maintained by the balance of growth and inhibitory factors. Abnormal vessel growth is seen in cancer, skin disease, macular degeneration, diabetic ulcers, cardiovascular disease, and stroke.

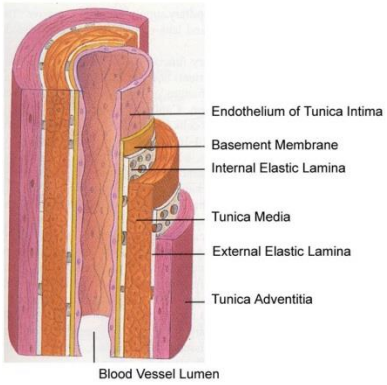


Figure 1. Blood vessel anatomy. From *http://bme.ccny.cuny.edu/faculty/jtarbell/SMC%20images.htm*

Blood vessels are comprised of three layers-tunica intima, tunica media, and tunica adventitia, as outline in Figure 1. The tunica intima is the innermost layer formed by endothelial

cells that are held together by polysaccharides and connective tissues. The tunica media is the vascular smooth muscle layer of the blood vessel that contains large quantities of connective tissue and polysaccharides. The tone of the blood vessel is regulated by the tunica media. The tunica adventitia is the connective tissue layer that is responsible for the nerve supply to the vessel walls. Angiogenesis occurs from the tunica intima layer. The endothelium is the thin layer of cells that line the interior surface, or lumen, of blood vessels and lymphatic vessels, which forms the interface between the circulating blood and lymph. The cells that form the endothelium are endothelial cells and line the entire circulatory system. Endothelial cells are responsible for fluid filtration, blood vessel tone-the resistance to passive stretch during resting state, neutrophil recruitment, and hormone trafficking. Damage to the endothelium by injury, whether deliberate, such as in surgery, or unintentional, requires repair. Healing has to occur after any type of injury or surgery.

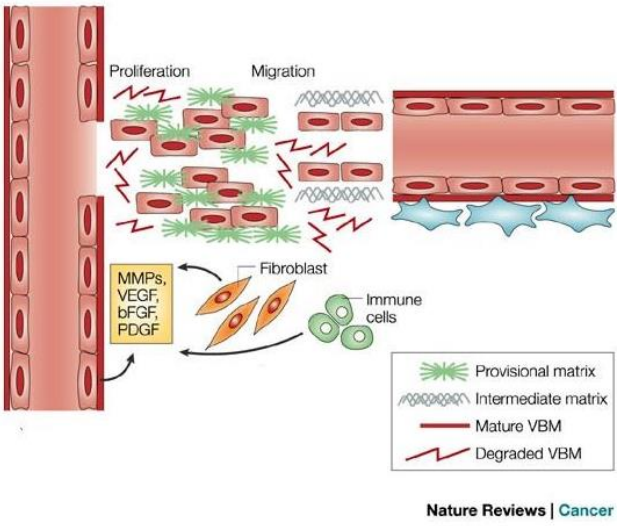


Figure 2. The angiogenic process. (Kalluri, 2003).

The process of cutaneous wound healing is a complex and carefully orchestrated cascade of overlapping events that involves changes in extracellular matrix protein composition, cell migration, and proliferation in response to numerous cytokines, Figure 2 (Grzesiak &

Pierschbacher, 1995). Crucial to the repair process is the migration of inflammatory cell types such as macrophages and neutrophils, epithelial cells, fibroblasts, and endothelial cells into the wound site over the course of the first three days after injury, where they have multiple responsibilities resulting, ultimately, in a healed wound (Grzesiak & Pierschbacher, 1995). This sequential process of restoring form and often function to the injured tissues is vital. The cascade of events begins with hemostasis, followed by inflammation, cell proliferation, and tissue maturation. Hemostasis is the process of stopping blood flow by clot formation. When tissues are exposed to an initial injurious stimulus, a blood clot is formed from circulating fibrin. Circulating chemokines and cytokines are released to recruit cells that phagocytize the damaged tissue.

Following injury, blood flow to the area must be stopped. Hemostasis occurs in three notable phases: vascular phase, platelet phase, and coagulation phase. The vascular phase occurs up to 30 minutes after injury and involves vasoconstriction of the smooth muscle cells of the endothelium to slow blood flow. During the platelet phase, the damaged endothelial cells release the blood clotting factor, von Willebrand's Factor. Von Willebrand's Factor allows the circulating cells to attach to the surface of the endothelial cells, causing aggregation. Aggregation of platelets forms a plug for the blood vessel. Aggregated platelets function to stimulate the release of platelet thromboplastin and thromboxane, which further activates the clotting process. The surfaces of these platelets now serve as a vasoconstrictor. During the coagulation phase of the hemostasis process, fibrinogen is converted into insoluble fibrin which attracts circulating aggregated platelets in addition to red and white blood cells to form a more stable clot. Dependent upon the duration of injurious stimulus, it is determined if the tissues are in a state of chronic or acute inflammation and which cellular components are recruited to the

area. The intrinsic and extrinsic pathways of coagulation allow fibrinogen to be converted to fibrin and coalesce with circulating platelets to form a clot. The extrinsic pathway is activated by extraneous materials to the blood and damaged endothelial wall. Coagulation Factors III-Tissue Thromboplastin activates Factor VII, which is calcium dependent, which proceeds to activate Factor X-Prothrombin Activator. The extrinsic pathway is initiated within 12 to 15 seconds, but due to the rapid duration, the effect is fast acting and not effective in maintaining a stable clot. The intrinsic pathway is stimulated by blood receiving signals from the damaged vessel wall. Clotting Factor XII is activated by the blood cells contacting the damaged endothelium, and in turn activates Factor XI. These two clotting factors activated Factor IX, which proceeds to activated Factor VII. Factor VIII, Factor III, and the required calcium ions activate Factor X-Prothrombin Activator. Despite the fact that both pathways lead to activation of Factor X-Prothrombin Activator, the intrinsic pathway is capable of eliciting a long standing result. The clot functions to maintain tissue structural support until the original form can be restored. The platelets are responsible for releasing cytokines and chemokines that biochemically attract pro-inflammatory factors, such as prostaglandins, thromboxane, and histamine, which cause vasoconstriction or vasodilation of the intact vasculature.

During the inflammatory phase, damaged tissues are removed by immune cells-neutrophils, basophils, eosinophils, and macrophages. Growth factors are also released in the area, to allow for cell proliferation and migration. This permeability of the blood vessels allows the recruitment of the aforementioned immune cells.

The inflammatory phase of wound healing is then followed by proliferation of cellular components to restore the damaged area. The hallmark characteristics of this phase are angiogenesis, collagen deposition, tissue formation, and wound contraction. Angiogenesis is

required to allow the tissues to become oxygenated and continue with growth and development. The neovascularization of the tissue assists in the restoration of not only form, but function to the area. This is not to say that is process is always perfect, because aberrations do occur during maturation and post-translational modification of the tissues.

At the cellular level, angiogenesis is initiated by degradation of the basement membrane of the parent vessel by the responding endothelial cells (Guedez et al., 2003). Endothelial cells then migrate toward an angiogenic stimulus and proliferate (Auerbach, Lewis, Shinners, Kubai, & Akhtar, 2003). Angiogenesis occurs in four steps: stimulation of endothelial cells by angiogenic factors, degradation of the capillary basal lamina by activated endothelial cells via extracellular proteinases, capillary formation and migration of endothelial cells, and new vessel maturation. Angiogenic growth factors activate receptors present on endothelial cells. Angiogenic growth factors stimulate blood vessel growth. Under normal physiological condition, angiogenic growth factors are produced in balance with angiogenesis inhibitors. Physiological imbalance of angiogenic growth factors and inhibitors encourage aberrations.

Table 2

List of growth factors and the function they have on angiogenesis

Growth Factor	**Function**
Fibroblast Growth Factor	Promotes proliferation and differentiation of endothelial cells, smooth muscle cells, and fibroblasts
Vascular Endothelial Growth Factor	Affects permeability
Angiopoietins (Ang1 and Ang2)	Stabilize vessels
Platelet Derived Growth Factor	Recruit smooth muscle
Integrins	Bind matrix macromolecules and proteinases
Basic fibroblast growth Factor	Induces endothelial cell migration, proliferation, and tube formation

Basic fibroblast growth factor (bFGF) is the angiogeneic growth factor that is active during the stimulation of endothelial cells by other angiogenic factors. bFGF is a potent inducer of endothelial cell migration, proliferation, and tube formation. bFGF is a tyrosine kinase receptor. Vascular endothelial growth factor (VEGF) is specific for endothelial cells. VEGF is responsible for inducing extracellular proteinase expression, expression of specific integrins for migration. VEGF is capable of initiating cell proliferation and migration by binding specific endothelial cell receptors-Flt-1 and Flk-1. Flt-1 is a protein kinase receptor responsible for cell proliferation and differentiation. Flk-1 is also a tyrosine kinase receptor that is expressed on endothelial cells, but is responsible for other processes such as neurogenesis and vasculogenesis. The inactivation of p53 and experiencing levels of decreased oxygen inactivate VEGF. Research has shown that using drugs to coat biodegradable stents results in injured arterial walls and severely impacts surrounding tissue, delaying reendothelialization of the area. Vascular endothelial growth factor is a potent endothelium-specific angiogenic factor, which may assist in promoting reendothelialization of the denuded arterial wall (Wang et al., 2010).

The activated endothelial cells release proteases that degrade the basement membrane to allow endothelial cells to escape. These newly freed endothelial cells are then allowed to proliferate, or increase in number, into the surrounding matrix, connecting with neighboring vessels. Degradation of the capillary basal lamina by activated endothelial cells via extracellular proteinases matrix metalloproteinases and urokinase. Matrix metalloproteinases (MMPs) are 22 zinc dependent endopeptidases that degrade extracellular matrix components. The specific metalloproteinases activated during angiogenesis are: MMP1, MMP2, and MT1-MMP. MMP1 is a collagenase that breaks peptide bonds found in collagen. MMP2 is a gelatinase, which is a proteolytic enzyme that hydrolyzes gelatin into polypeptides, peptides, and amino acids. MT1-

MMP is responsible for the actual basal lamina degradation. Urokinase is responsible for converting plasminogen to plasmin. Plasminogen is an 88 kilo Dalton single stranded plasmin precursor synthesized in the liver, stored in eosinophils, and forms complexes with fibrinogen and fibrin. During coagulation, large amounts of plasminogen are integrated in the clot that is formed by fibrin, to prevent premature lysis of the clot. Plasmin is present in the blood and functions to degrade blood plasma proteins, namely fibrin. Plasmin is responsible for activating collagenases. The cleavage of fibrin, fibronectin, thrombospondin, laminin, and von Willebrand factor occurs because of plasmin. The binding of urokinase to the urokinase receptor induces cell proliferation and regulation of cell migration.

Integrins are transmembrane receptors on the surface of many cells that mediate the cellular interactions of the angiogenic cascade. Integrins require magnesium and calcium ions to function (Grzesiak & Pierschbacher, 1995). Extracellular magnesium has been well established as a receptor-mediated chemoattractant for endothelial cells. Neovascularization occurs when the endothelial cells of a microvessel receive an angiogenic stimulus, resulting in the development of sprouts from the parent vessel in the general direction of the signal (Guedez et al., 2003). Integrins are receptors that mediate the attachment between a cell and the tissues that surround it, communicating with the extracellular matrix of the surrounding tissues. The information that is communicated by the integrins to the extracellular matrix allows rapid biochemical responses to the tissue environment. Sprouts, as the newly formed vasculature is termed, grow in length and diameter, forming from the lumen outward. It is the length and diameter of the vessel that differentiate angiogenesis from vasculogenesis. Vasculogenesis is the formation of new blood vessels from stem cells, it mainly occurs during embryogenesis. The location and size of the blood vessel corresponds to the influence of the growth factors, the

specificity of post-translational modification by neighboring organs, and occasionally pathogenic factors, such as tumor formation. Integrin expression present on newly forming vessels allow the migration of endothelium cells into the surrounding matrix. Extracellular proteinases, matrix metalloproteinases and urokinase, facilitate the migration of endothelial cells into the surrounding matrix. In order for the new vessel formed from the sprout to mature, angiopoeitin, a growth factor whose tyrosine kinase receptors mediate cell signaling to induce tyrosine phosphorylation, leads to the binding and activation of other enzymes. Platelet derived growth factor, which is produced by the endothelial cells of the newly formed vessels, are responsible for the recruitment of smooth muscle cells.

Bioengineers have been charged with the task of developing ideal biodegradable magnesium based implants. A promising strategy to increase the success of tissue healing, regeneration, and integration of the developing implant lies in the key process of revascularization which is crucial in improving the successful integration of implants both *in vitro* and *in vivo (*Poh, Shi, Lim, Neoh, & Wang, 2010). Understanding the angiogenic properties of magnesium would increase the level of knowledge regarding the healing process once the material is implanted. It is important to have a vast comprehension of the impact of magnesium on further growth and development of the tissue. Improving the revascularization of the area often requires delivery of angiogenic growth factors to localized tissues to stimulate angiogenesis. Vascular endothelial growth factor is the most potent and widely used key regulator of angiogenesis (Wang et al., 2010). Vascular endothelial growth factor is not only critical to angiogenesis, but is also known to function during bone repair by promoting the cellular functions of osteoblasts-proliferation, migration, and differentiation-and indirectly affecting the function of osteoclasts in the cellular environment (Poh et al., 2010).

Immobilization of angiogenic growth factors onto the surface of metal substrates may be a viable approach in promoting revascularization and enhanced implant integration in a controllable manner (Poh et al., 2010).

A number of in vivo systems have been developed to understand angiogenesis, including the rabbit cornea model, the chick chorioallantoic membrane (CAM) assay, and vitelline membrane (VIM) assays (Scott, 2004). These systems are impractical for the study or screening of large number of samples and are far removed from angiogenesis observed in a human system (Scott, 2004).

2.4 Research Models

2.4.1 In vitro research models. *In vitro* research methods involve simulating a biological environment. *In vitro* research methods serve as a foreshadowing to what is to be expected when an experiment is conducted in a biological environment. Simulating biological conditions prior to conducting *in vivo* experiments provide insight on expected results. The chemical composition of the environment where the implant will be placed is important in pondering the effects of the degradation byproducts. Exploring what those products are in an *in vitro* environment allows better understanding of how the material will perform in an *in vivo* experiment. If the desired results are not garnered, this research methods aids in developing new alloys that have the ideal degradation properties. Immersion test of magnesium alloys AZ31 and Mg-5Zn-0.3Ca were conducted in Hank's simulated body fluid. Hank's simulated body fluid is a highly saline solution that has been used repeatedly to assess a materials ability to bind with bone. When held to physiological temperature of 37°C, this solution causes the formation of hydroxyapatite on the surface of the material. The assessment of the presence and extent of

hydroxyapatite formation on the surface of the metal enables conclusions to be drawn on the effectiveness of the research sample.

2.4.2 In vivo research models. *In vivo* research models encompass a broad variety of specimens. From zebra fish to human subjects, *in vivo* experiments can provide a large amount of information. When financial and available resource limitations impact a desired research plan, smaller animal models are available. Many researchers desire to evaluate their research on smaller, inexpensive methods prior to investing large amounts of time and financial resources into costly large animal or human models. Currently the department of Bioengineering does not have the financial or laboratory resources available to conduct large animal model research. The chicken chorioallantoic membrane assay is an ideal *in vivo* research model to utilize under these conditions. The Institutional Animal Care and Use Committee does not recognize developing embryos as an animal model, since the embryo is not technically an animal at this point. The information provided by smaller animal models often provide enough preliminary data to determine if it is ideal to pursue larger animal models.

Table 3

Comparison of the Chicken Chorioallantoic Membrane Assay to large Mammalian Models

Chick Embryo CAM Model	Mammalian Models
Low cost (eggs, time, labor, etc.)	Moderate to high cost (rats-dogs, per diem, time, labor, etc.)
Complete in vivo environment	Complete in vivo environment
Very simple surgery to place implants	More complex surgery to place implants (i.e., requires anesthesia, operating room)
Allows direct, continuous visualization of implant site through window	Does not allow visualization of implant site
Animals do not need to be restrained	Animals often have to be restrained
Maximum implantation time of about 2 weeks	Implantation time can be several years
Allows evaluation of both acute and chronic inflammation (up to 2 weeks)	Allows evaluation of both acute and chronic inflammation
Allows evaluation of granulation tissue in response to injury, including neovascularization.	Allows evaluation of granulation tissue in response to injury, including neovascularization.
Fibrosis seen in response to tissue injury and implants.	Fibrosis seen in response to tissue injury and implants.
Tissue and blood sampling is easily done (i.e., through window) but amount is limited	Tissue and blood samples are available in larger quantity but require more invasive procedures
Biology/physiology of this model is well known (e.g., been used for toxicity, tumor, angiogenesis studies)	Biology/physiology of this model is well known
Cost-effective model for utilization of expensive and limited reagents (e.g., cytokines, antibodies)	Cost-ineffective model for utilization of expensive and limited reagents (e.g., cytokines, antibodies)

*(*Valdes et al., 2002)

2.5 Chorioallantoic Membrane and the Chick Chorioallantoic Membrane Assay

The original chorioallantoic membrane assay was developed by experimental embryologists more than 50 years ago and has long been a mainstay for the study of embryonic organ development (Auerbach et al., 2003). The underlying principle of the chorioallantoic membrane assay is the measurement of time dependent occurrence of effects on the

chorioallantoic membrane when exposed to a test item (Scheel et al., 2011). Requiring twenty one days to develop from fertilized embryo to chick, makes the chick chorioallantoic membrane assay a quick, inexpensive, efficient research model that provides information similar to small animal research models. In researching ideal models for in vivo studies, it is important that the model mimic what may occur in large animal models and human clinical trials. The use of a developing embryo allows for understanding of the reaction that a biomaterial will have with this environment. The chicken chorioallantoic membrane provides an environment that is vast with proteins, growth factors, and a dynamic environment. The fact that the developing embryo lacks a mature immune system means the tissue responses will be similar to an acute and chronic inflammatory response that would be elicited in mammalian models. Current models that are used for assessing tissue response to biomaterials are expensive, laborious, and may not offer direct visualization of the changes that are being modulated by the presence of the material being tested. The chorioallantoic membrane assay was discovered in the 1970's as an alternative to the controversial Draize's eye assay that was conducted from rabbit specimens. The chorioallantoic membrane assay is an inexpensive method of assessing biological processes such as transport, gas exchange, tumor transplant, toxicity and angiogenesis. The advantages of using a mammalian model are the same with the chorioallantoic membrane assay. The chorioallantoic membrane assay is beneficial in being able to achieving similar results in a shortened time period.

Upon fertilization of the egg by the rooster, the yolk enters the oviduct of the hen. Albumen is added to the yolk and the yolk and albumen are covered by the shell. The egg is released from the oviduct into the external environment. The temperature change from inside the

hen to the external environment, causes the contents of the egg shell to retract away from the shell, and form an air sac, which provides space for the embryo to develop.

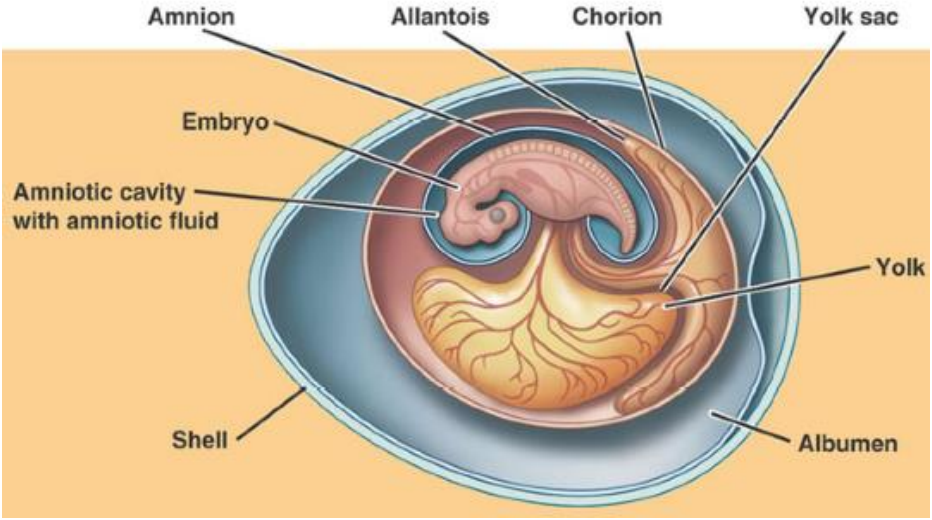


Figure 3. Illustration of the developing chick embryo depicting the representative membranes and egg contents. (Carothers, 2011).

The contents of the fertilized chicken egg are depicted in Figure 3. The chicken embryo is comprised of several embryonic membranes that are vital in the growth and maturation of the embryo into a chicken. The yolk sac is responsible for providing the embryo with nutrients and food, the amnion encloses the embryo and provides protection, the allantois is the respiratory organ for the embryo. The allantoic vessels are responsible for carrying sodium and calcium to the embryo and waste away from the embryo into the allantoic membrane. This membrane is pivotal in the developing embryo's vascularization. From the point of fertilization, the chorioallantoic membrane is forming via vasculogenesis within the embryo and is fully developed during days eight through ten. The embryo becomes fully encased by the chorioallantoic membrane by day twelve. Days four through eight are when most angiogenic activity is occurring within the developing embryo. This is the most ideal time periods to assess the interaction between a biomaterial and angiogenesis.

Angiogenesis is indicative of protein adsorption and its dependence on the chemical structure of the biomaterial. Even though the avian model may elicit a different cell type than a mammalian model, the cells essentially have the same function. The inflammatory process for the avian model is similar to that of the mammalian model. Both models release chemotactic triggers to recruit inflammatory cells to the region. Since this model can be used to measure incorporation of the biomaterial, this model is also useful in assessing surface modifications of the biomaterial that would promote cell adhesion and tissue growth.

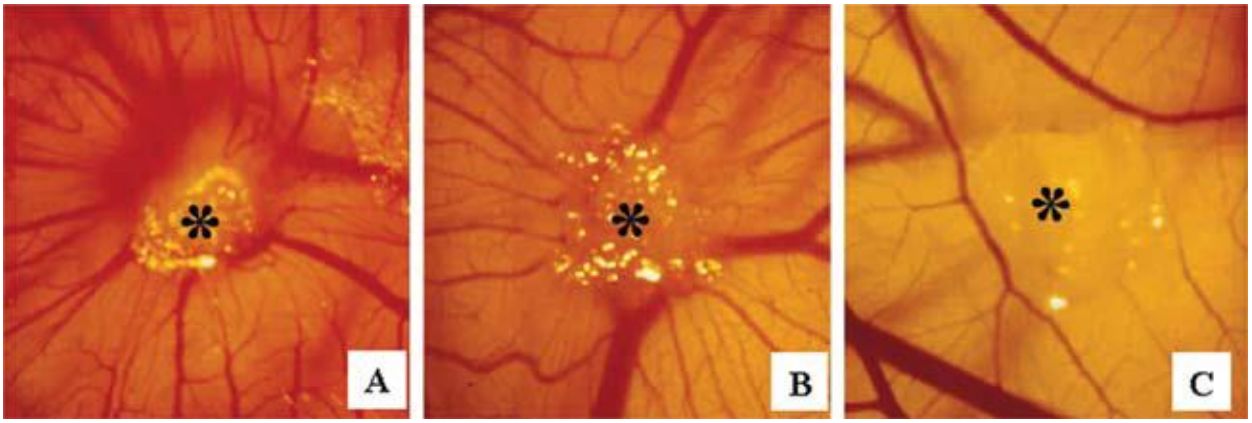


Figure 4. Angiogenic changes of the CAM after exposure to gelatin sponges that are urokinase plasminogen activator-transfected endothelial cells (uPA-R5) seeded (A), parental endothelial cells seeded (B), and the gelatin sponge without cells (C). (Ribatti, 2010).

Figure 4 shows the surface modifications of the chorioallantoic membrane that has been infiltrated with gelatin sponges. The gelatin sponges contain urokinase plasminogen activator-transfected endothelial cells and parental endothelial cells. An unseeded gelatin sponge was used as the control. Angiogenic changes towards the asterisk are displayed in Figure 4A and 4B, in response to the cell seeded gelatin sponge. There were no notable angiogenic changes observed in Figure 4C. Sequential photographic images were analyzed to formulate these conclusions.

The chorioallantoic membrane assay is relatively inexpensive, in that few laboratory supplies are required to prepare the egg for implantation. Fertilized eggs are sterilized and allowed to incubate for three days prior to placing any material on the allantoic membrane. Albumen is extracted from the fertilized egg to increase the air space and provide room for the desired materials to become implanted. Two to three microliters of albumen are extracted via syringe. This opening is then sealed. Research specimens are placed on the surface of the chorioallantoic membrane for investigation.

Previous studies have shown that the chorioallantoic membrane assay is capable of eliciting a mild acute inflammatory response and angiogenic response. These responses are required for increased healing after implantation of a biomaterial into the body. Rough surfaces of the material aid in incorporation into the chorioallantoic membrane. Although polishing makes the surface of the sample relatively smooth, the surface of the oxide layer is often present in most samples used during experimentation.

The limitation of the chorioallantoic membrane assay in understanding the effects of an alloy and its degradation properties on angiogenesis is that development of the membrane and angiogenic activity is highest during days 3 through 12. When a sample requires extensive amounts of time to degrade, those longer than 9 days, assessment does not provide a complete picture of the full extent of the correlation. Materials that are not quickly dissolved may not be ideal for this research model. Distinguishing between angiogenesis that occurs innately from angiogenesis in response to a test material, without the use of histological testing, is also a limitation of the chorioallantoic membrane assay (Ribatti et al., 2001). Table 2 lists a few advantages and disadvantages of the chick chorioallantoic membrane assay.

Table 4

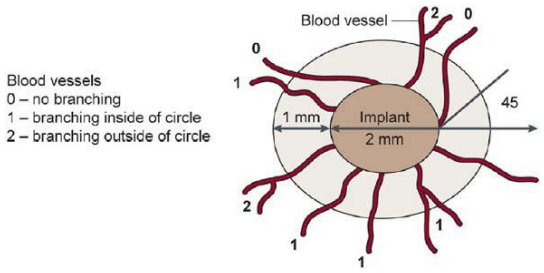
Advantages and disadvantages of the chorioallantoic membrane assay

Advantages	Disadvantages
Easy methodology	Difficult monitoring
Sterility is not required	Non-specific inflammatory reactions
Reproducibility	Preexisting vessels
Reliability	Sterility is not required

*(*Ribatti, 2010).

The methodology of utilizing the chick chorioallantoic membrane assay is relatively easy, with difficulties arising from egg opening methods. The lack of a required sterile environment can be considered as advantageous and disadvantageous. It is an advantage because the eggs can be opened in any location, not requiring the use of specialized laboratory equipment, if it is not available. Lack of sterility can be considered a disadvantage for experimental methods that provided the ideal environment and nutrient source for bacteria and mold, which is provided by the incubator and the developing embryo. Dependent upon the development period at which the implant is placed on the chorioallantoic membrane surface, the presence of preexisting vessels may affect analysis of vasculature that has developed as a result of the test material. It is vital to consistently document angiogenic changes. Consistent methods of quantifying angiogenesis are also a limitation of use of this model.

a



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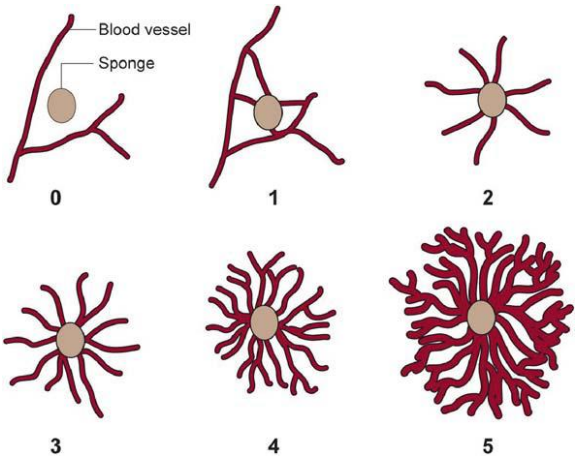

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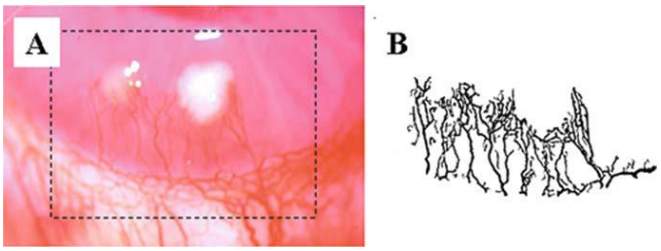


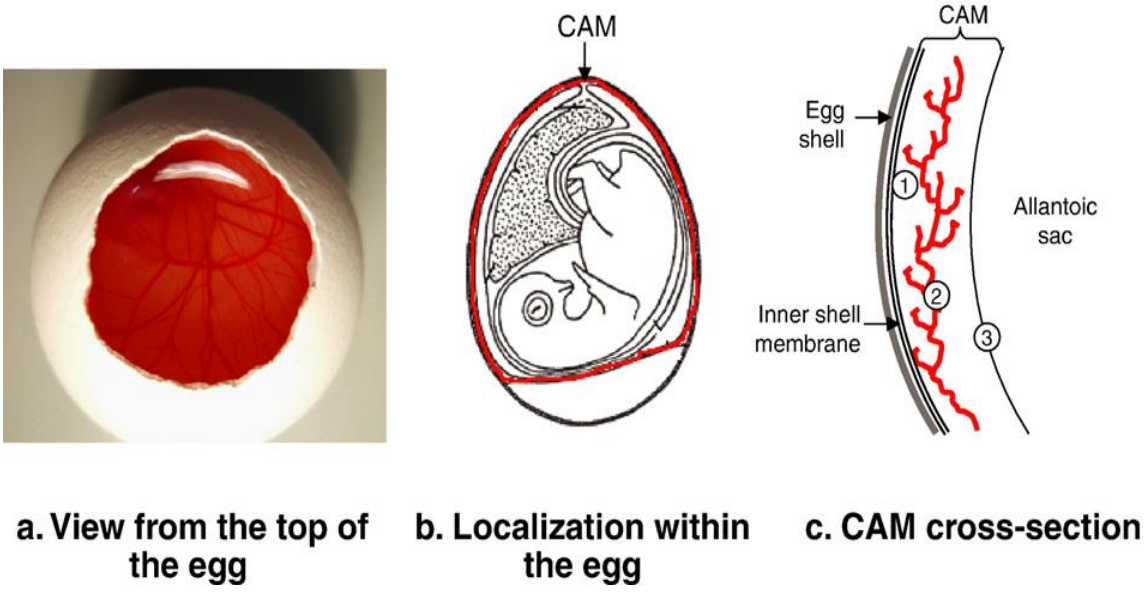
Figure 5. Methods of quantifying angiogenesis based on angiogenesis that occurs within a defined area (a), the intensity of angiogenic changes that occur (b), and skeletonized image reconstruction of the chorioallantoic membrane for software analysis (c). (Ribatti, 2010).

Figure 5 depicts three methods for quantifying angiogenic changes that occur. When angiogenic changes are observed within a defined area, they can be scored based on the branch points that occur within or outside of the defined area. Many researchers have used a scoring system 1-5 that denotes the angiogenic changes that occur, based on the intensity or the number of newly formed blood vessels that are present. The use of specialized computer software to quantify angiogenesis is also an option.

2.5.1 Embryo incubation. Embryos used for this study were received from Mountaire Farms of Siler City, NC, the North Carolina Agricultural and Technical State University poultry farm of Greensboro, NC, and Sunrise Farms of Catskill, NY. Upon receipt of embryos from the vendor, excrement and dirt should be removed from the surface of the egg with a dry towel, by vigorous, but gentle rubbing. Development of the embryo is halted when the egg is placed in an environment under 65°F, therefore transport from the respective farm did not impact embryonic developing. Placing the embryo in an incubator, whether desktop or cabinet, at temperatures above 85°F with more than 75% humidity is the ideal condition for development. The experiments conducted in a Lyon Turn-X incubator and a GQF Digital Sportsman cabinet incubator. Maintenance of ambient temperature and humidity are important factors for conducting the chicken chorioallantoic membrane assay.

2.5.2 In ovo chorioallantoic membrane assay. The in ovo chorioallantoic membrane involves allowing development of the embryo to occur inside the shell.

a



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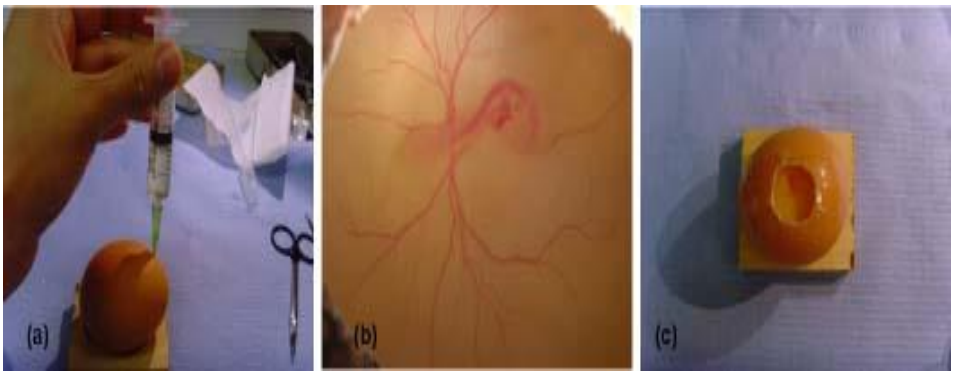


*Figure 6.*Method of performing the in ovo chick chorioallantoic membrane assay. The embryo may be stored vertically (a) or horizontally (b). (Vargas, 2007).

After 3 days of incubation, 3-4 mL of albumen is extracted from the egg to increase the amount of air space, as shown in Figure 6b-(a). Once the membrane has settled, the shell of the embryo is perforated to create a window. This window allows for access to the chorioallantoic membrane and direct visualization of the tissue response to the implanted material. The window can be created on the vertical or horizontal axis of the egg shell, as depicted in Figure 6a and b respectively. Creating a window on the horizontal axis allowed for perforated embryos to be stored in the desktop Lyon incubator without modification to the incubator. Sealing the window is also vital. An improper seal to the eggshell allows for contamination, which is deleterious to

the experiment. The fertilized eggs must be stored in an incubator that allows the eggs to be rotated on a consistent basis. The eggs are rotated every hour to prevent nerve damage to the developing embryo. It is ideal to assess the tissue response 24 hours after implantation and on a consistent schedule for the duration of the experiment. The benefit of allowing the embryo to develop inside the shell is the nutrients that are provided to the developing embryo from the shell. The duration of the experiment conducted was not extended to the point when the embryo required the calcium and minerals from the shell for bone development.

2.5.3 Ex ovo chorioallantoic membrane assay. The ex ovo chorioallantoic membrane assay completely removes the developing embryo from the shell.

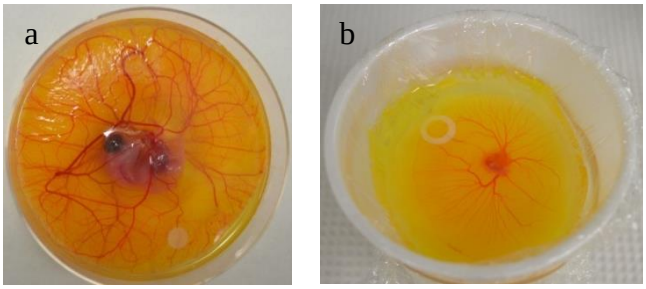


Figure 7. Methods for performing the ex ovo chorioallantoic membrane assay. (a) Petri dish method. (b) Plastic sling method.

Figure 7 shows the different methods of conducting the ex ovo chorioallantoic membrane assay. The contents of the egg can also be placed directly inside a petri dish and allowed to develop, Figure 7a. The embryo is also allowed to develop in a plastic sling that is placed inside a cup of water, which creates a humidor for the embryo, Figure 7b. The sling method is better for experiments that require more than one day. The petri dish is an effective model, but an increased mortality rate was observed with this method, in comparison to the sling method. The water allows the embryo to rotate at will, similar to what would occur in nature. The water also provides a source of humidity, similar to the gas exchange that would occur from the hen sitting

on the eggs. A sterilized petri dish encloses the cup. Without the limitation of the square cut into the shell of the embryo, the ex ovo method allows for complete visualization of the entire embryonic development process. As previously stated, the shell is useful in providing nutrients that are required for bone maturation and development, the duration of this experiment does not warrant the necessity of the shell.

Table 5

Comparison of in ovo and ex ovo chick chorioallantoic membrane assay

	In ovo	Ex Ovo
Advantages	The sources of calcium for building skeletal elements is kept	Large chorioallantoic membrane area available for testing
	Normal development of the embryo	Direct visualization of the entire chorioallantoic membrane
	High embryo survival rate	Evaluation of several samples in one single embryo
	Easy methodology	No eggshell falling on chorioallantoic membrane
	Sterility is not required	Easy access to chorioallantoic membrane vasculature
	Embryos can reach hatching	*In vivo* observation of embryo development
Limitations	Small surface is exposed	Difficult methodology (e.g. transfer the embryo to plastic dish, sterility requirements).
	Difficult monitoring	Low embryo survival rate
	Risk of angiogenesis induced by eggshell pieces	Do not reflect physiological conditions
		Embryos cannot reach hatching

(Vargas, 2007).

When comparing the in ovo and ex ovo methods as demonstrated in Table 5, no advantage or limitation is more prevalent to consider the use of one method over the other. The decision of use of one method should be determined by the length of time the specimens are required for experimentation, the extent to which the researchers would like the embryos to develop, and methods of post experimentation analysis. Based on the research conducted in this study, utilizing both the in ovo and ex ovo methods, the plastic sling method provides better results in

comparison to simply opening the contents of the fertilize egg into a sterilized petri dish when using the ex ovo method; the in ovo method should be used when post experimentation analysis involves reconstruction and impact of the implant on the complete chorioallantoic membrane is to be assessed, versus assessment of a localized region. If embryonic development requires the embryo to develop calcified tissue structures, it is more ideal to allow development to occur in ovo, with the shell in place to provide nutrients.

CHAPTER 3

Materials and Methodology

3.1 Implant Preparation

The effect of magnesium on angiogenesis was evaluated by several research methods. Pure magnesium, magnesium alloys, and salt solutions of expected corrosion products were used.

3.1.1 AZ31 discs and rods. Magnesium AZ31 was purchased from Alfa Aesar. The 1.87 mm diameter wire was cut into 2 mm rods. The rods were polished to a smooth finish and sterilized with 70% ethanol. The rods were further cut into 0.5-1 mm rods and polished to 0.125 micrometers, which was confirmed with digital calipers. The polished discs were sterilized with ethanol prior to being placed on the surface of the chorioallantoic membrane. These samples were also used for the immersion test.

3.1.2 Lead wire. Lead wire was used as a negative control. .125mm diameter lead wire was cut into 2-3 mm length rods, sterilized with 70% ethanol, and directly placed on the surface of the membrane.

3.1.3 Magnesium wire. Pure magnesium wire (0.125mm) was purchased from Goodfellow. Chromium acetate was used to clean the surface of the pure magnesium wire for 3 minutes. The wire was then cut into 2-3 mm samples. The wire was either directly placed on the chorioallantoic membrane or affixed to a polystyrene ring.

3.1.3.1 Polystyrene rings. 101 to 1000 µL Polystyrene pipette tips were cut into rings, polished to a smooth finish, and used to mark the location of implanted materials.

3.1.3.1.1 Polydimethylsiloxane. Polydimethylsiloxane (PDMS) is a silicone based polymer that is inert and biocompatible. Sylgard 184 silicone elastomer base and Sylgard 184

silicone elastomer curing agent was used in a 10:1 ratio, respectively. The materials were properly mixed for 2 minutes. The PDMS is placed in the oven for 2-3 minutes to remove the bubbles from the mixture. The mixture was used to affix the sample to the polystyrene ring. Once the desired specimen was affixed, it was then cured. The PDMS was cure at 60°C for 1 hour.

3.1.3.1.2 Glass cover slips. Five millimeter diameter glass cover slips were affixed to polystyrene rings using polydimethylsiloxane. The glass cover slips were used as markers for implanted materials.

3.1.3.1.3 Filter paper. Whatman filter paper was hole punched to form a ring and serve as a marker for implantation sites for various materials during the course of the experiment.

3.1.3.2 Vascular endothelial growth factor and polylactic-co-glycolic acid. Fifty six milligrams for polylactic-co-Glycolic Acid (PLGA) was dissolved in 1000 microliters of chloroform. The solution was hand swirled until the PLGA was fully dissolved. The wire was then allowed to air dry in the flow hood. Twenty micrograms of vascular endothelial growth factor (VEGF) was purchased from Fisher Scientific and reconstituted in 1X phosphate buffer solution (PBS). Twenty microliters of the VEGF and PBS solution was used to microdip the magnesium wire in an altered polystyrene pipette tip. Each wire was dipped three times in the solution. The VEGF wire was placed in a petri dish and stored at -20°C to prevent protein denaturation. The samples were placed directly on the surface of the chorioallantoic membrane.

3.1.4 Expected corrosion product solutions. Salt solutions were prepared of aluminum chloride, calcium chloride, magnesium chloride, and zinc chloride at 1 millimole, 10 millimole, and 20 millimole concentrations in 1X phosphate buffered solution. Phosphate buffered solution (1X) was used as an experimental control. Twenty microliters of the solution was placed on a

glass cover slip that had a polystyrene ring attached to denote where the salt solution was placed on the glass cover slip. The cover slip was then allowed to air dry, and then the sample was placed on the surface of the membrane. Filter paper was also used as a marker in experimentations in which the 20 microliters of the denoted salt solution was directly placed on the surface of the membrane. These solutions were chosen based on the composition of the magnesium alloys.

3.1.5 Titanium rods and wire. Titanium was used as a positive control. Titanium wire (1.86 mm diameter) was cut into 2-3 mm length rods, polished to a smooth finish, and sterilized with 70% ethanol. The titanium wire was directly placed on the surface of the membrane. 0.125 mm diameter titanium wire was cut into 2-3 mm length rods, sterilized with 70& ethanol, and affixed to polystyrene rings for implantation on the chorioallantoic membrane.

3.1.6 Mg-5Zn-0.3Ca. Two millimeter and five millimeter diameter magnesium alloy composed of magnesium, 0.3% calcium, and 5% zinc was cut into 0.5-1 millimeter rods. The rods were polished to 0.125 micrometers, which was confirmed with digital calipers. The formed discs were sterilized with ethanol prior to being placed on the surface of the chorioallantoic membrane. These samples were also used in the immersion test.

3.2 Chorioallantoic Membrane Assay

Fertilized chicken embryos were maintained at 37°C in an incubator for three days (72 hours), upon receipt. Prior to incubation, dirt, feathers, and excrement were carefully removed from the egg shells by dry wiping with hand paper towels. The relative humidity of the incubator should be 86-100%. The eggs should be stored so that the base lies horizontally. The incubator rotates the eggs once every hour.

3.2.1 In ovo chorioallantoic membrane assay.

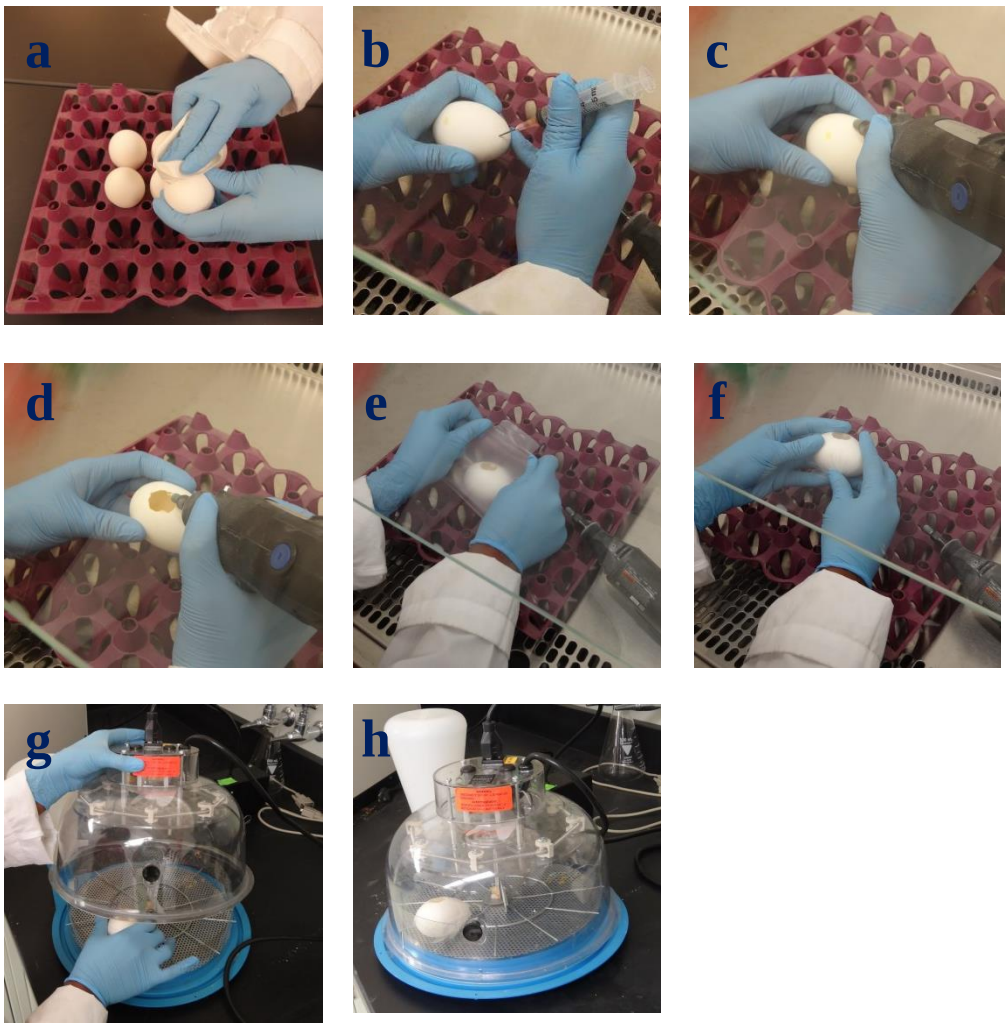


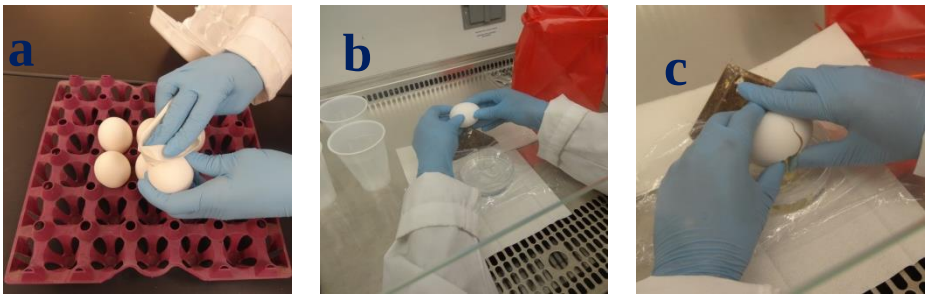
Figure 8. Methodology of the In ovo chick Chorioallantic Membrane Assay

Residual moisture is removed from the surface of the egg, once it is removed from the incubator, Figure 8a. On day 3 of development, a 1" x 1" piece of parafilm is placed left of the base to protect the area where the albumin will be drawn out. Using a 10cc syringe, with an 18 gauge needle, a small hole is drilled into the egg shell with the needle, Figure 8b. The needle is driven into the egg at a 45° angle towards the bottom of the egg. The needle is then used to draw 3-4 mL of albumen. Using a Dremel, a 3" x 3" square or circle is cut into the top surface of the

egg, Figure 8c and d. Parafilm then used to cover the opening, Figure 8e and f. The embryo is then returned to the desktop incubator to allow for further development of the chorioallantoic membrane, Figure 8g and h. On embryonic day 5 photographic images are taken to track the developmental changes that occur. After the embryos are photographed, a research specimen is placed on the chorioallantoic membrane with forceps, preferably in an area that is slightly vascularized, at a branching point. Once the implant has been placed, photographic images are again taken to note any immediate changes that occur at the implantation site. The embryo is then covered with parafilm and returned to the incubator. Photographic images are taken during the course of development.

The magnesium AZ31 rods were used in in ovo experimentation. The implant was in place 3 days and photographic images were taken every 24 hours. The embryo was further visualized using Nanotom X-ray computed topography to analyze the corrosion product development.

3.2.2 Ex ovo chorioallantoic membrane assay. Any residual moisture on the surface of the egg is wiped with a dry papertowel, as shown in Figure 9a. On embryonic day 3, the developing embryo's shell is cracked an opened into a plastic sling resting atop a water filled petri dish. A demonstrative representative of this is shown in Figure 9, images b, c, and d.



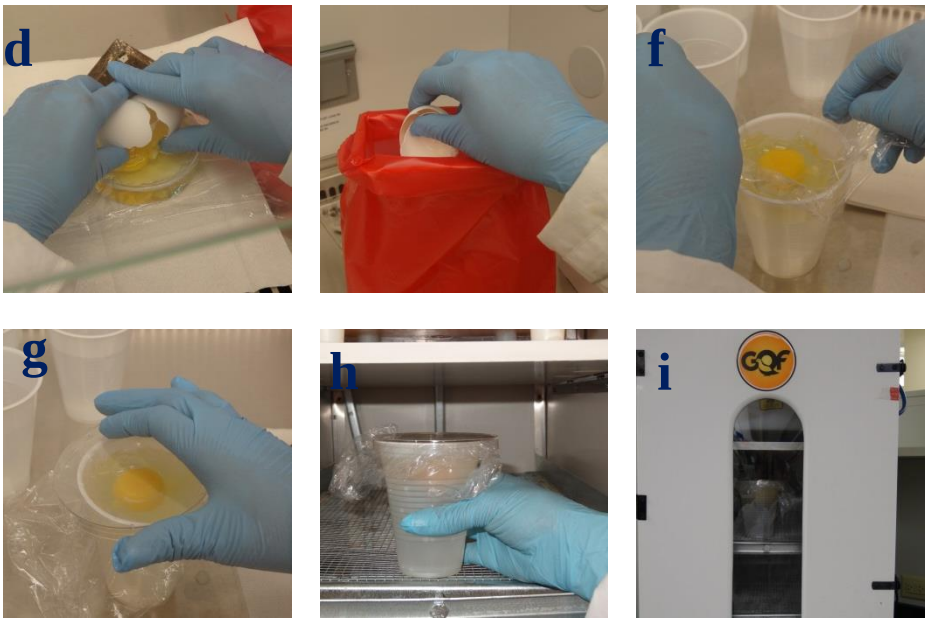


Figure 9. Methodology of the Ex ovo Chick Chorioallantoic Membrane Assay

The eggshell is discarded in a hazardous waste bag, Figure 9e. The sling is placed inside of a cup of 9 milliliters of water that has been warmed to 37°C, displayed in Figure 9f. The sling is then covered with a sterilized petri dish lid, as shown in Figure 9g. The apparatus is then returned to the climate controlled incubator to continue development, displayed in Figure 9, images h and i. On embryonic day 5, the research specimen is placed on the chorioallantoic membrane after reference photographs have been taken of the membrane prior to implantation. Photographs were taken on a consistent basis for the duration of the experiment.

3.3 Immersion test

Magnesium AZ31 and Mg-5Zn-0.3Ca disk were placed in 10mL of Hank's simulated body fluid in 15 milliliter centrifuge tubes. The tubes were housed in a holder and placed on a horizontal rocker inside a 37°C incubator. Initial pH and the pH of the solutions were taken daily for 7 days. A sample was removed from solution daily to assess the impact of prolonged exposure to the solution to the magnesium alloys AZ31 and Mg-5Zn-0.3Ca. After the pH was

taken, Hank's simulated body fluid was removed and 10mL of 1M Hank's simulated body fluid was placed in the centrifuge tube. The tube was then returned to the rocker inside the incubator. The magnesium alloy samples removed daily for the duration of the experiment.

3.4 Data Analysis

3.4.1 Microscopy

3.4.1.1 Light. A standard laboratory light microscope with a removable lens that allows for camera attachment was used for sequential photographic images.

3.4.1.2 Scanning electron microscope and electron dispersive x-ray. The Hitachi SU8000 Field Emission Scanning Electron Microscope (SEM) was used for analysis of surface morphology of the magnesium based alloys following implantation. The samples were further characterized by EDX technology.

3.4.2 Camera. A Canon EOS-60D and Nikon D3100 were used to capture sequential photographic images for this study. The Canon EOS-60D was used in conjunction with the light microscope.

3.4.3 Nanotom. The General Electric Phoenix Nanotom was used for non-destructive 3D analysis and volume reconstruction of in ovo experimental specimens. Phoenix CT Acquisition and Phoenix CT Reconstruction software was used for image acquisition and reconstruction.

CHAPTER 4

Results

4.1 Magnesium Wire.

4.1.1 Pure magnesium wire ex ovo experimental results. Pure magnesium wire began corrosion within twenty four hours of implantation, based upon change in appearance observed on photographic images displayed in Figure 10. The weight of the wire was conducive to angiogenic development of the embryo, but was not fully incorporated. With a dynamic model, it is imperative that the implant incorporate into the membrane. As the embryo shifts, the implant shifts also, therefore incorporation of the implant is vital to the experimentation, especially when changes are tracked via daily photographic images. Hallmark features of angiogenesis were observed in the ex ovo experiment, but the shifting of the embryo resulted in puncturing a blood vessel.

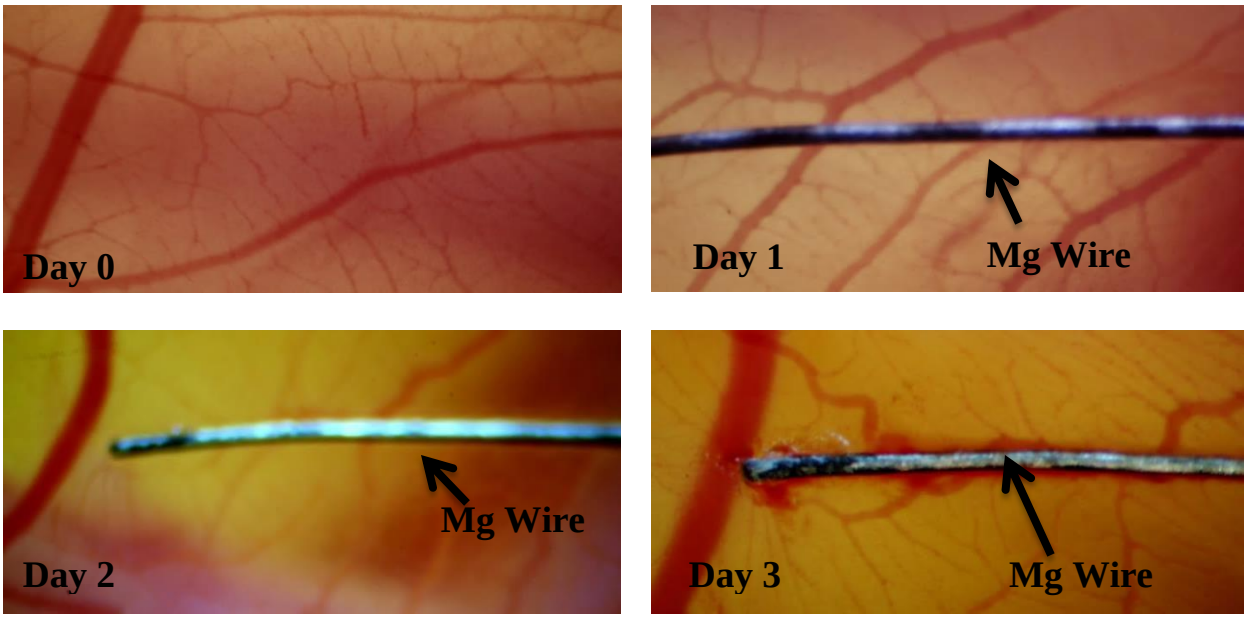


Figure 10. Pure Mg wire ex ovo experimental results.

Magnesium wire was suspended on polystyrene rings to provide a focal site for detection of angiogenic changes. The results displayed in Figure 11 indicate that the weight of the pure magnesium wire did not comprise the angiogenic development of the embryo. Corrosion of the magnesium was detectable within 24 hours of implantation. Within two days of implantation, blood vessel development was detected along the length of the wire implant as the pure magnesium degraded on the chorioallantoic membrane.

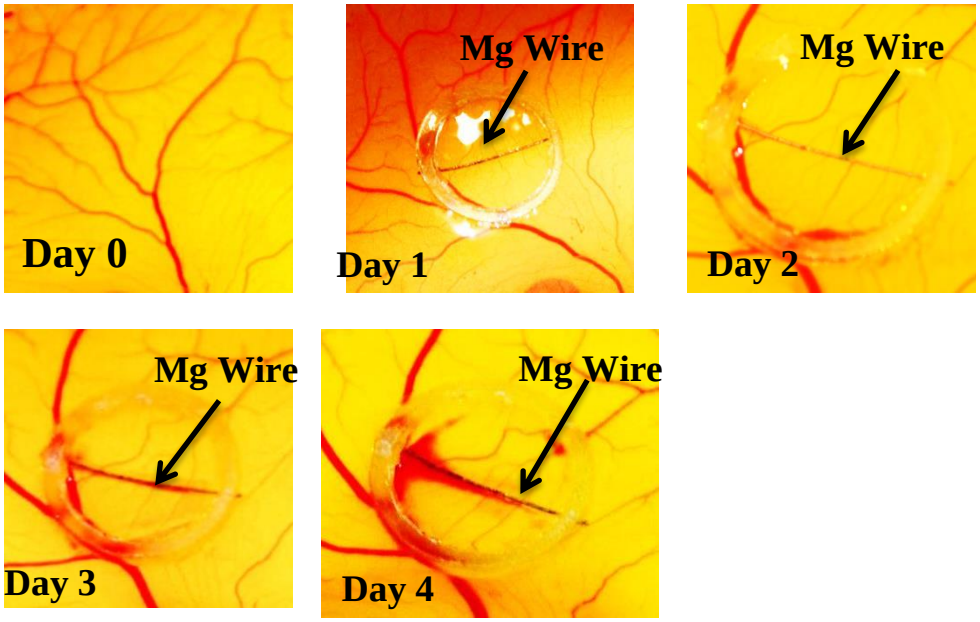


Figure 11. Pure Mg ex ovo results

In a subsequent ex ovo experimentation of pure magnesium wire placed on the surface of the chorioallantoic membrane, the magnesium wire was able to fully degrade on the surface of the chorioallantoic membrane within 8 days of implantation, as shown in Figure 12. The environment rich in proteins provided the ideal environment for corrosion to occur. The chorioallantoic membrane surface is composed of several proteoglycans, glycoproteins, and other adhesive proteins. Many intracellular proteins, similar to those found in the chorioallantoic membrane contain chloride channels. The ubiquitous circulating proteins required for vessel development and the innate chloride ions also found on the membrane allow for corrosion of the

implanted material. There were no notable changes in the number of blood vessels within the polystyrene ring, but the corroded magnesium was not deleterious to the development of the embryo.

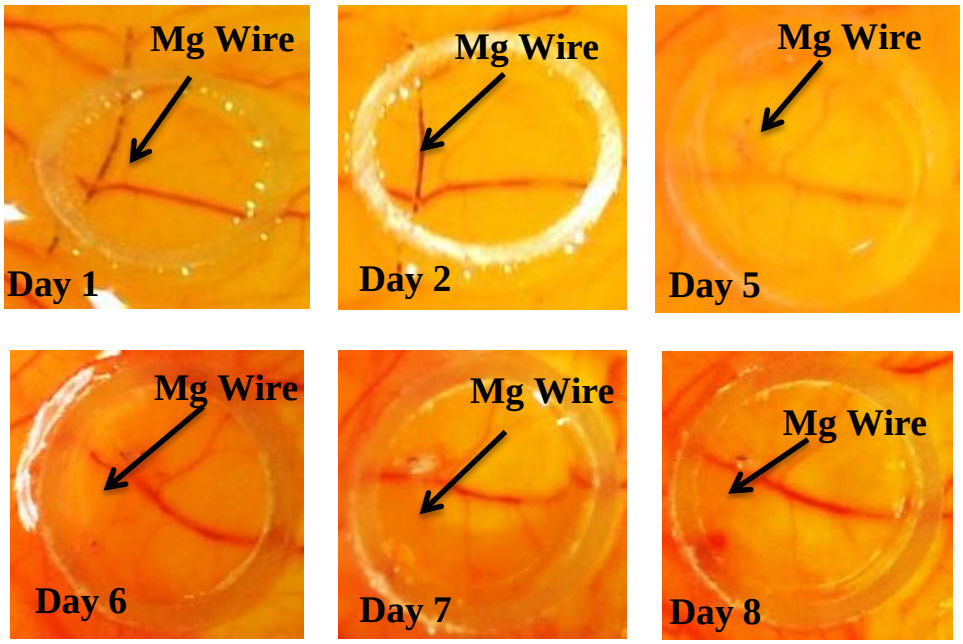


Figure 12. Pure Mg experimentation on ex ovo chorioallantoic membrane

4.1.2 Pure magnesium wire coated with vascular endothelial growth factor.

Magnesium wire was coated with PLGA and VEGF, as described in the materials and methodology section , and used in an ex ovo experiment, the results are shown in Figure 13. After day 3 of implantation, photographic evidence displays the formation of a blood vessel along the length of the implant. Degradation of the coated magnesium wire did not progress as quickly as observed in the uncoated magnesium wire, but more angiogenic development was observed. PLGA has been shown to reduce the rate of magnesium corrosion in previous studies. Vascular endothelial growth factor is responsible for recruiting endothelial cells to the area for angiogenic development. Serving as the positive control in this experiment, desired results were

achieved. Despite the appearance of saturated blood, the capillary structure was not disturbed and the endothelium was intact for developed blood vessels.

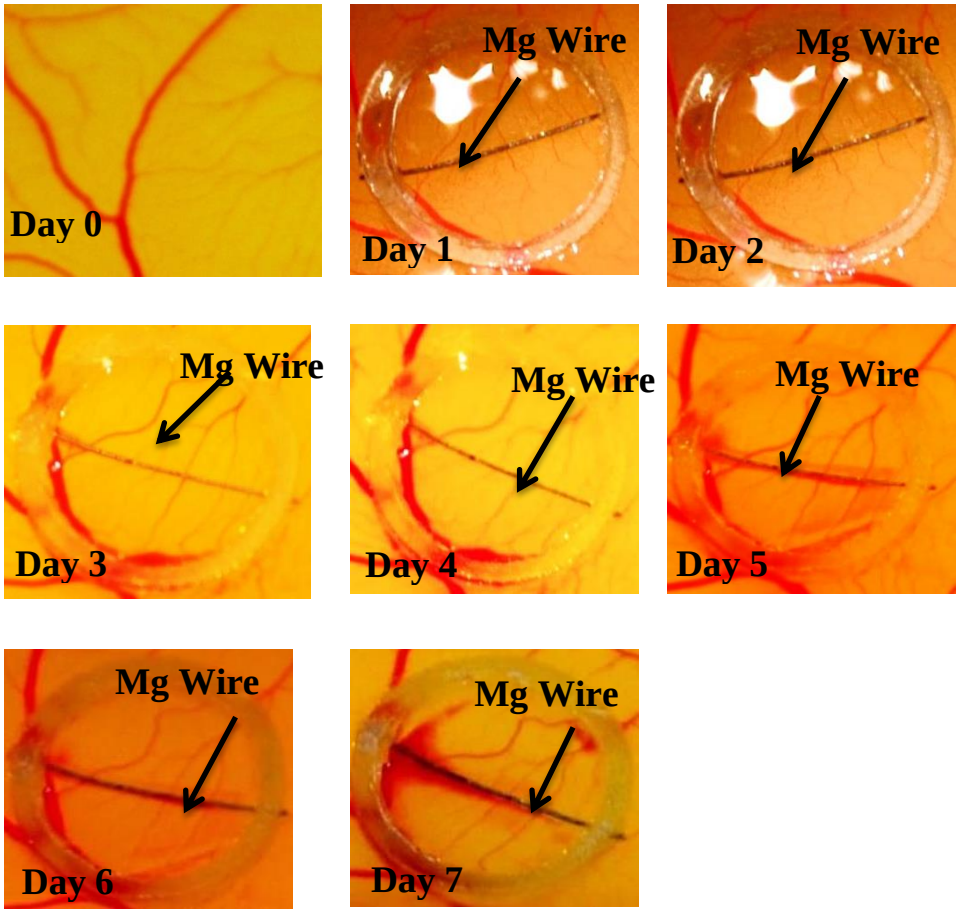


Figure 13. Pure magnesium wire coated with VEGF and PLGA ex ovo experimental results

4.2 Magnesium AZ31 Rod

4.2.1 In ovo experimental results. Magnesium alloy AZ31 was implanted onto the chorioallantoic membrane of an in ovo developing embryo. The implant remained on the chorioallantoic membrane for 3 days. Figure 14a depicts the buildup of corrosion productions that is a direct result of the alloy being placed on the chorioallantoic membrane.

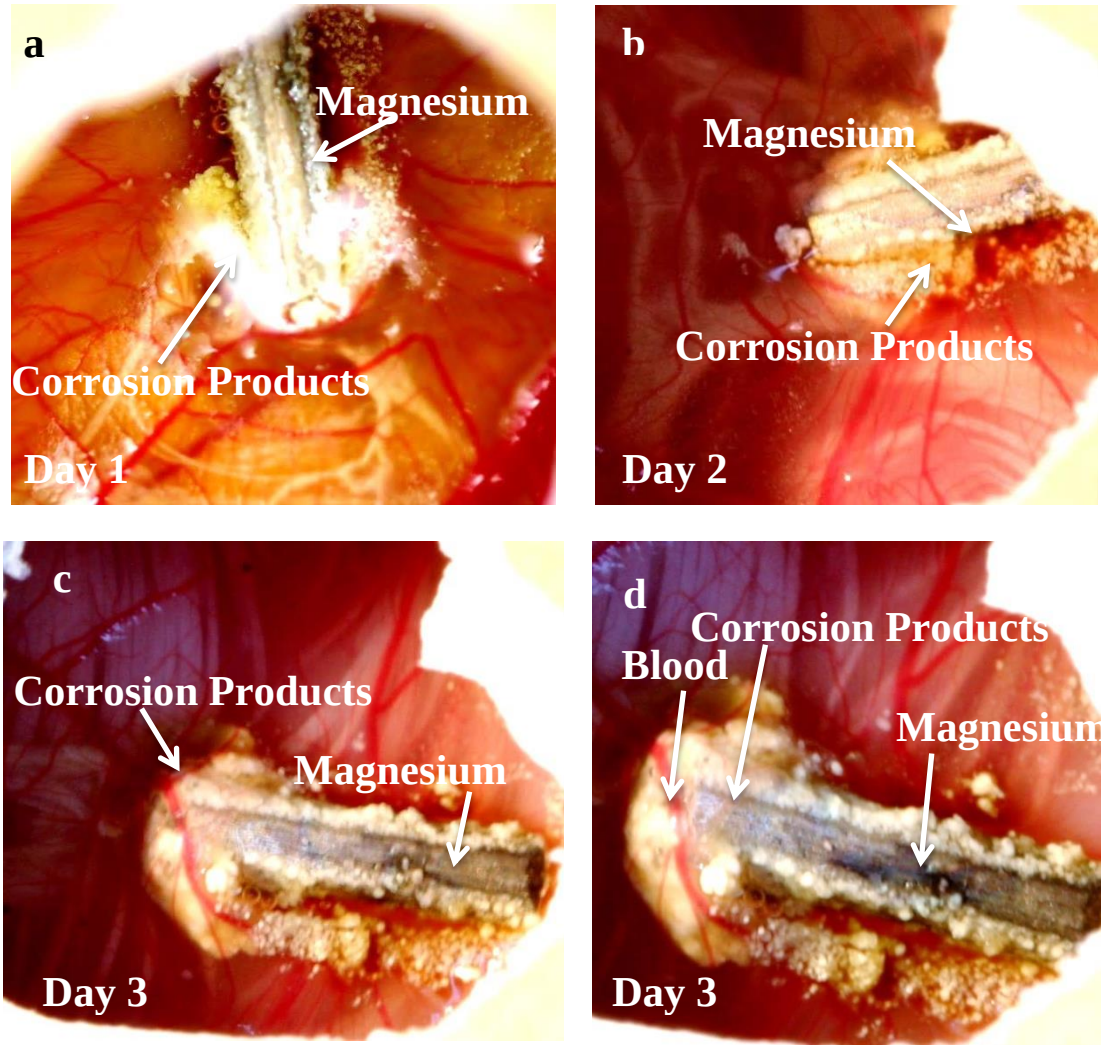


Figure 14. Magnesium AZ31 was implanted in ovo for 3 days.

Increased amounts of corrosion products are displayed in Figure 14b. Figure 14c and 14 clearly display blood vessel formation on the surface of the implanted material. The Magnesium AZ31 rod was integrated into the chorioallantoic membrane without eliciting a toxic response. The chorioallantoic membrane was able to grow over the implant. Corrosion products were further evaluated by Dr. Boyce Collins, utilizing the Nanotom X-ray computed topography technology. Blood vessel development occurred in the presence of the magnesium implant. Further characterization would be required to determine the influence that magnesium AZ31 had on the angiogenic development.

4.2.2 Nanotom ct x-ray reconstruction

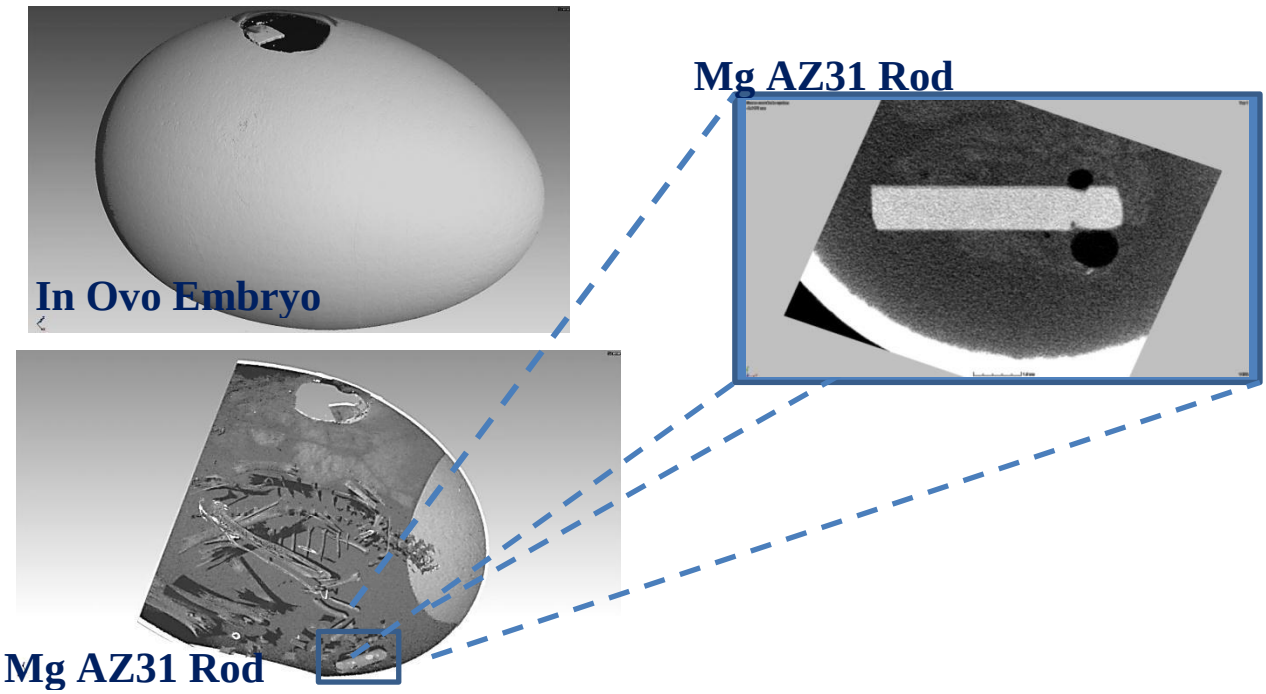


Figure 15. Nanotom X-Ray CT reconstruction of magnesium AZ31 in ovo experiment.

Figure 15 demonstrates the post experimentation analysis capabilities of the in ovo chick chorioallantoic membrane assay with Nanotom X-ray CT technology. The Nanotom is able to digitally remove the shell, revealing the contents of the fertilized egg, without disturbing the embryo. The developing embryo was deceased prior to x-ray exposure. The energy dispersed during image acquisition will euthanize the embryo. The Nanotom can be used in future research experiments to analyze the broad spectrum of effects the implanted material has on the embryo, as opposed to the localized analysis that results from use of sequential photographic images.

4.3 Magnesium AZ31 Disc.

4.3.1 Ex ovo experimental results.

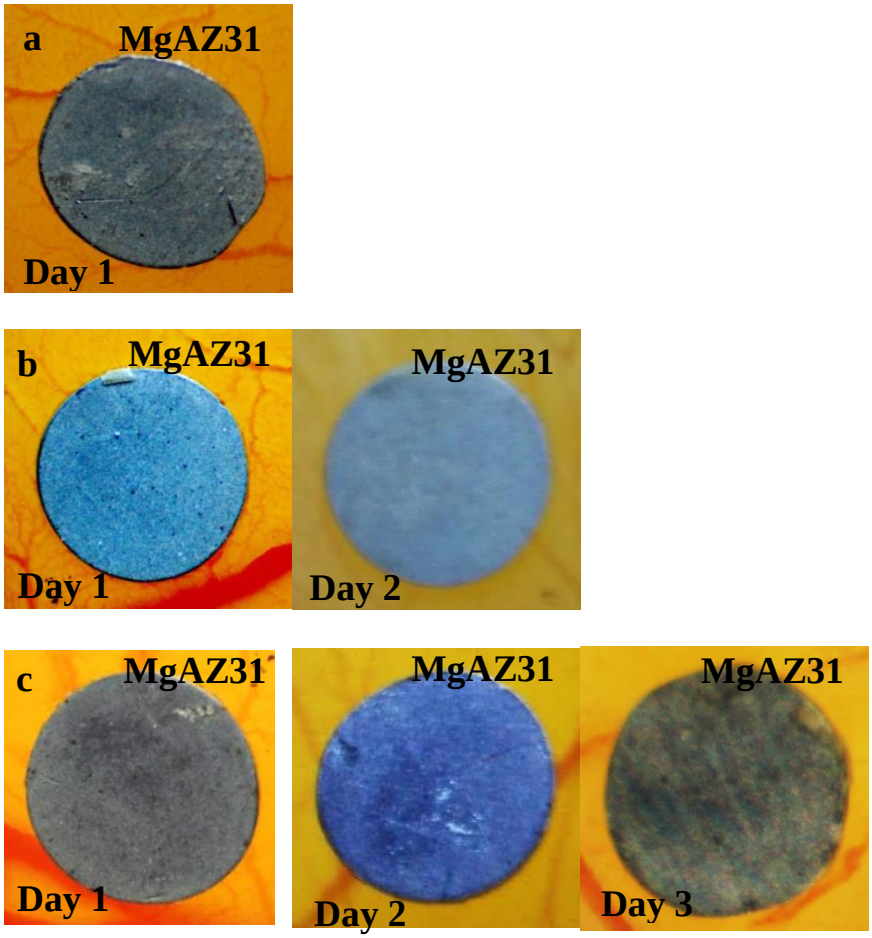


Figure 16. Magnesium AZ31 ex ovo experimental results

Figure 16 displays the photographic images for angiogenic changes that occurred for the experimentation of the interaction of Mg AZ31 disc with the ex ovo chick chorioallantoic membrane. Magnesium based alloy implants were removed from the surface after 1 day Figure 16a, 2 days Figure 16b, and three days Figure 16c. The diameter of the test material used in this experiment, 5mm, was too large to explicitly capture the angiogenic changes. Based on the data available, there were no notable angiogenic changes observed. Magnesium AZ31 has a very

slow corrosion rate that is not ideal for use with the chick chorioallantoic membrane assay research model.

4.3.2 SEM analysis of magnesium AZ31 ex ovo.

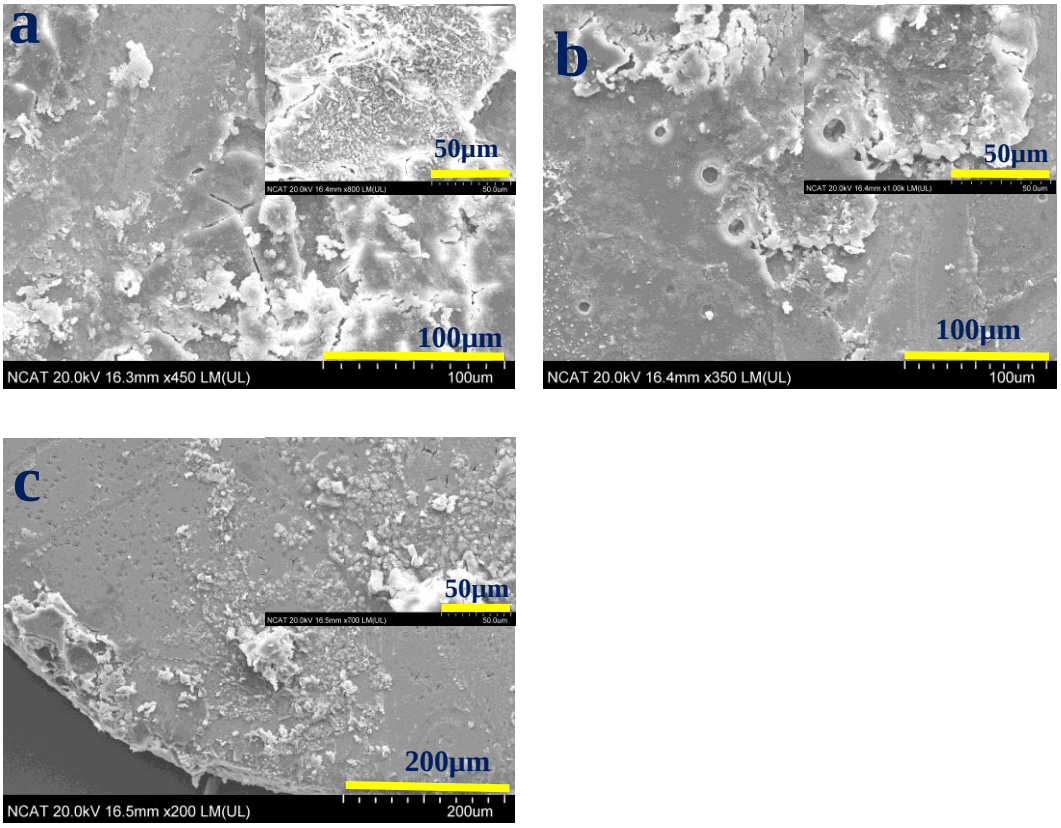


Figure 17. SEM analysis of magnesium AZ31 ex ovo

Once the implanted samples displayed in Figure 17 were removed from the surface of the chick chorioallantoic membrane assay after 1 day (Figure 17a), 2 days (Figure 17b), and 3 days (Figure 17c), they were analyzed on the HJitachi SU8000 Field Emission Scanning Electron Microscope. Analysis detected structural changes on the surface of the material that would indicate interaction between the chorioallantoic membrane and the magnesium alloy. Further characterization was conducted by EDX technology.

4.4 Mg-5Zn-0.3Ca

4.4.1 Ex ovo

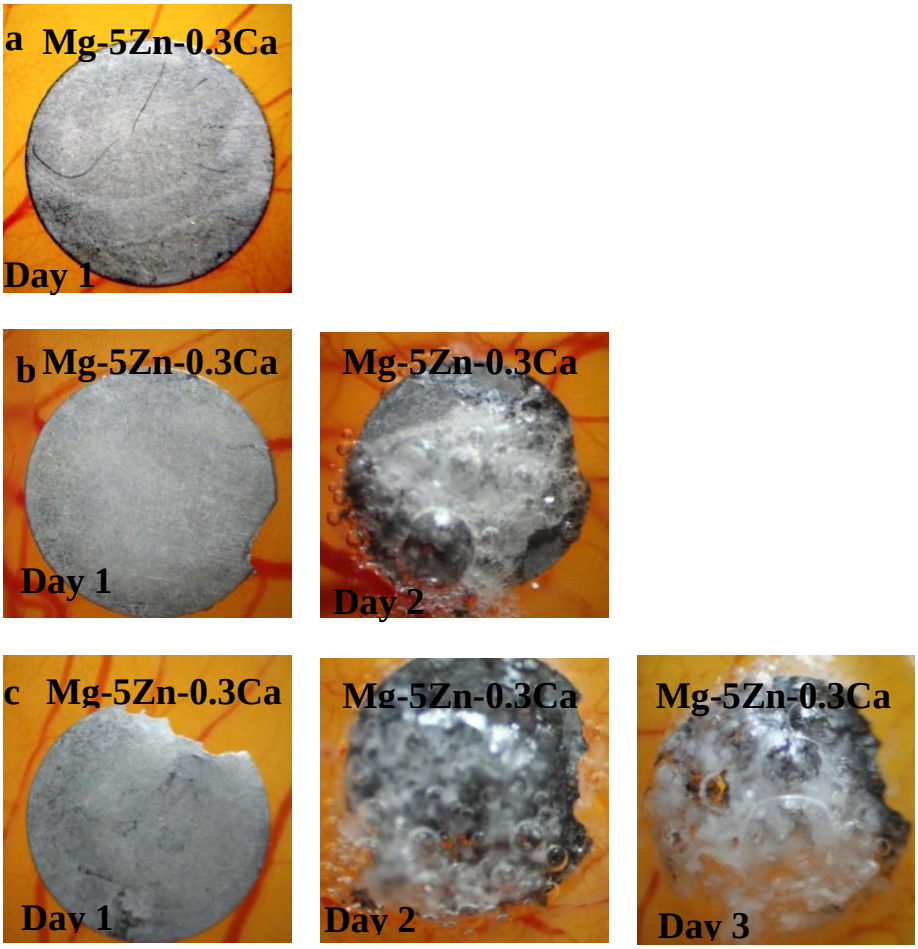


Figure 18. Mg-5Zn-0.3Ca ex ovo experimental results

The magnesium alloy containing zinc and calcium fabricated in the laboratory of Dr. Zhigang Xu was used to provide valuable information on the degradation properties of magnesium alloys. The quickly corrosive alloy is ideal for use in the chorioallantoic membrane assay. The alloy was cut into .25 micron x 2 mm and 5mm disc and utilized in an ex ovo experiment, as shown in Figure 18. A disc was removed each day for 3 days for analysis under scanning electron micrscopy and electron dispersive x-ray technologies. The highly corrosive magnesium alloy was not detrimental to the angiogenic development of the embryo. The interaction between the

proteins of the chorioallantoic membrane and the alloy caused an increase in the presence and formation of hydrogen gas, therefore making photographic detection of angiogenesis difficult to acquire.

4.4.2 SEM analysis of Mg-5Zn-0.3Ca ex ovo

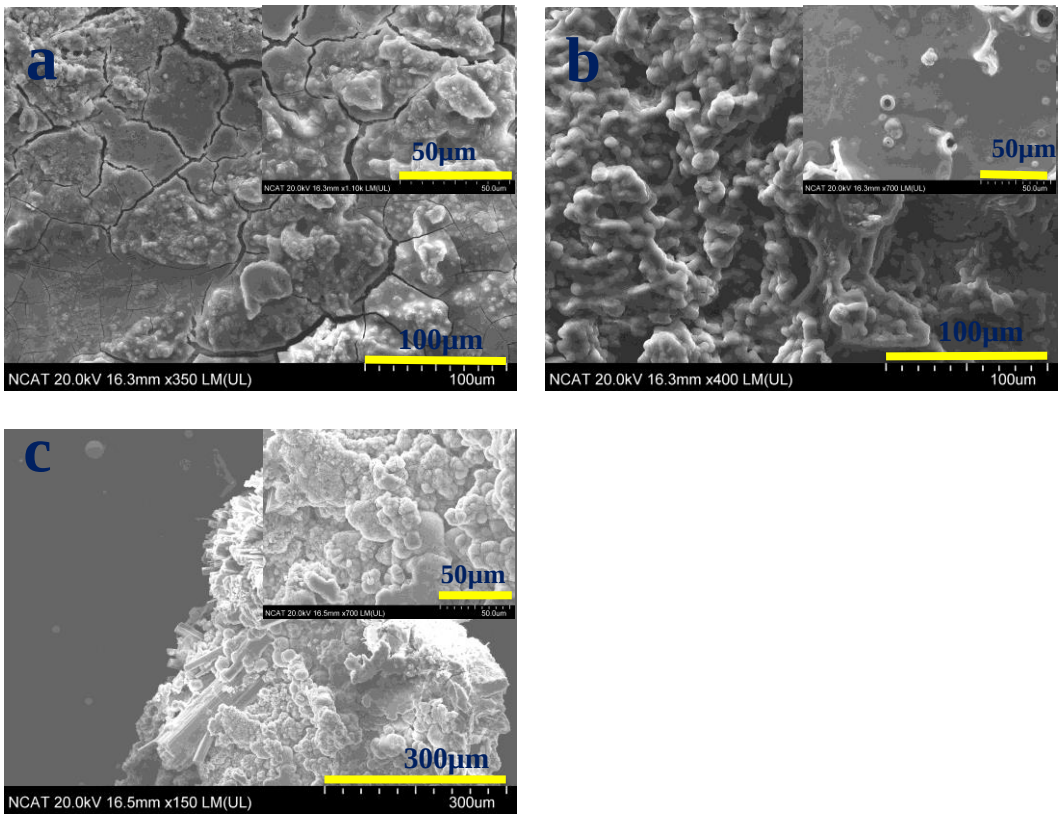


*Figure 19.*SEM analysis of Mg-5Zn-0.3Ca ex ovo

Following sequential photographs of the interaction between the magnesium alloy Mg-5Zn-0.3Ca, the implanted samples used in Figure 19 were further characterized via SEM to detect surface alterations that resulted from the material being exposed to the chorioallantoic membrane. The Mg-5Zn-0.3Ca implants were removed from the surface of the chorioallantoic membrane after 1 day, Figure 19a; 2 days, Figure 19b; and 3 days, Figure 19c. Unlike the formation of corrosion products as displayed in the SEM analysis of magnesium AZ31, the ex ovo experimentation of Mg-5Zn-0.3Ca resulted in material loss, or corrosion of the material,

with increased exposure to the surface of the chick chorioallantoic membrane. The material was further characterized by EDX technology.

4.5 EDX Analysis of Magnesium Based Alloys.

Table 6

Comparative data of the EDX detection analysis

Element	C	O	Mg	P	Cl	Ca	Zn	Al
MgAZ31 Day 1	43.88	32.26	5.18	3.05	0.31	0.00	0.00	0.55
MgAZ31 Day 2	47.19	8.08	20.25	9.16	0.52	0.87	0.00	9.91
MgAZ31 Day 3	76.14	6.91	5.98	7.07	1.34	0.01	0.01	0.04
Mg-5Zn-0.3Ca Day 1	0.00	59.43	6.90	14.17	1.84	17.65	0.00	0.00
Mg-5Zn-0.3Ca Day 2	0.00	57.24	2.79	16.73	1.68	21.54	0.00	0.00
Mg-5Zn-0.3Ca Day 3	0.00	38.92	4.32	24.76	1.59	27.92	2.48	0.00

The test materials used for the ex ovo experimentation of magnesium based alloys AZ31 and Mg-5Zn-0.3Ca were characterized using EDX technology to determine the significant elements present on surface of the material that was directly in contact with the chorioallantoic membrane and the Hank's SBF solution for the MgAZ31 and Mg-5Zn-0.3Ca, respectively, and displayed in Table 6. The data provided displays the trend of increased carbon detection on the surface of AZ31, with increased exposure to the membrane surface. Elemental carbon detection on the surface of the implant is indicative of incorporation of the material to the membrane. Protein adsorption is one of the key features of angiogenesis. Despite the fact that MgAZ31 does not produce significant corrosion products, the results garnered reveal that the test material is biocompatible with the environment. Subsequent histological testing could reveal the impact of the alloy on the membrane. Analysis of the prevalent atoms present on the surface of the Mg-

5Zn-0.3Ca implants displayed in Table 6 exhibit a trend of increase calcium phosphate production on the surface of the material with prolonged expsosure to Hank's SBF solution. The high chloride content environment produced by the components of the solution provides an ideal environment for magnesium corrosion. Mg-5Zn-0.3Ca is an alloy that is fabricated for the analysis of corrosion products. The increased oxygen production observed in the Mg-5Zn-0.3Ca results, in comparison to AZ31, is of no surprise and caused by the increased corrosion rate of Mg-5Zn-0.3Ca in comparison to AZ31.

4.6 Lead wire ex ovo experimental results

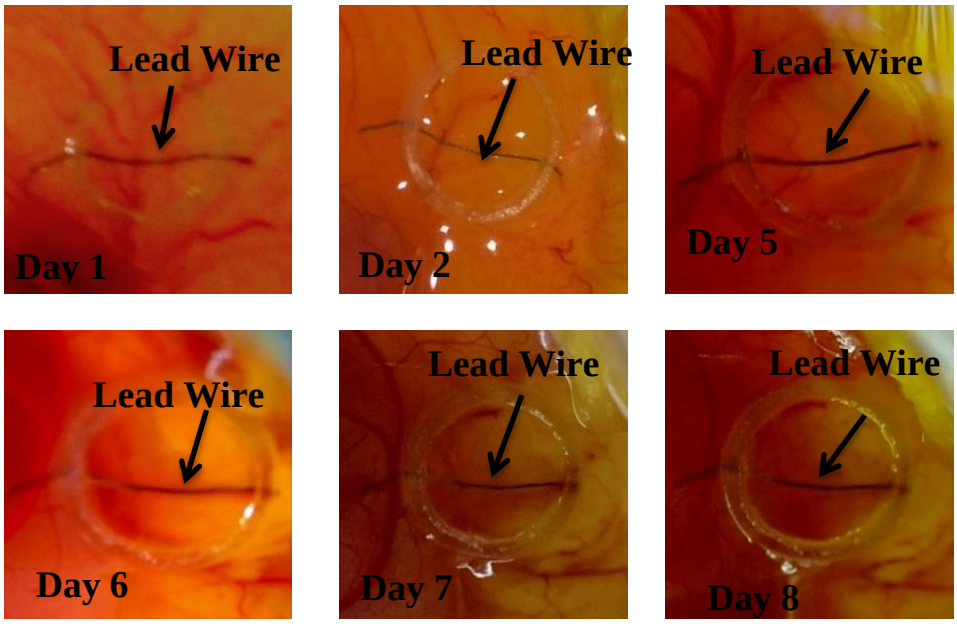


Figure 20. Lead wire ex ovo experimentation results

Lead wire (.125 microns x 2 mm) served as the negative control for the experiment, Figure 20. The small amount of lead present was not a toxic dose to the developing embryo, but by day 5 of implantation, there are visible changes in the location of the blood vessels. The presence of the lead wire caused blood vessels to become redirected away from the site of implantation. Desired results were achieved by the presence of the lead wire.

CHAPTER 5

Discussion and Future Research

Analysis of the results showed that the chicken chorioallantoic membrane assay is a viable method for understanding the relationship between magnesium degradation and angiogenesis. The results provided by the ex ovo or the in ovo method were shown to be useful. The in ovo method allows for further detection via Nanotom x-ray computed topography technology, but a method of differentiating blood vessels from other tissue types should be in place prior to beginning the experiment. The best results for the ex ovo method were garnered when using the hammock method of suspending the embryo contents in a plastic sling above water and placed in the incubator, rather than allowing the embryo to develop simply in a petri dish. The hammock method allows the embryo to move, as it would if it were in a shell, while developing. The petri dish method did not allow the embryo to rotate, but this was less of a hindrance when it is desired to have the implant remain at the same site for the course of the experiment. For short term experiments, those lasting a few hours, to no more and 1 day, the petri dish method may provide significant results, with embryonic developing being of no concern. The table top incubator used did not provide any different results from the cabinet incubator. The cabinet incubator was able to hold more specimens in comparison to the 15 embryos that could be held in the table top incubator. The humidity control was of ease with both incubators.

Implanting pure magnesium wire on the chorioallantoic membrane yield results that suggest magnesium wire is compatible with the chorioallantoic membrane, the embryo is able to incorporate the magnesium into the membrane, and the environment provided by the chorioallantoic membrane is suitable for degradation of the material. Coating the pure

magnesium wire with vascular endothelial growth factor encouraged blood vessel growth along the length of the wire and the PLGA slowed the rate of degradation of the wire. These results suggest that PLGA is capable of controlling the rate of degradation and VEGF encourages angiogenic development, both were desired results. In larger models, the key concepts may be pivotal in developing an implant that has sustained release of a drug or growth factor that is beneficial for healing, but allows the implant to remain in place for a desired period of time.

Magnesium alloy AZ31 was shown to provide more valuable results when thin disk were used, as opposed to rods or thicker disc, in the ex ovo experiments. Detection of the disc and wire was difficult when imploring Nanotom CT X-rays for post implantation data analysis. It is imperative that the sample size be proportional to the membrane, to not disturb the membrane, become dislodged during transport into and out of the incubator, or sink to the bottom of the embryonic tissue and yield no interaction with the chorioallantoic membrane, but the yolk. This was observed when the rods were used versus very small disk. The use of magnesium AZ31 in various shapes suggest that it is beneficial to utilize research samples that corrode within 5 days to assess the impact the material and the degradation by products have on angiogenesis. The use of commercially available AZ31, initially, limited the amount of data that could be collected during the duration of the experiment. More experimentation with the magnesium alloy AZ31 would not have proven to be beneficial due to the lack of buildup of degradative byproducts, as also seen in the immersion test. There was minimal interaction and impact with the use of AZ31. Calcium phosphate and hydrogen gas production were observed. Magnesium AZ31 is biocompatible with the chorioallantoic membrane, as evident with the proteins that were able to attach directly on the surface of the implant. The slow degradation rate of AZ31 makes it a less than desirable material to use in this research environment. The Engineering Research Center

for Revolutionizing Metallic Biomaterials fabricated magnesium zinc calcium alloy provided the most desirable corrosion results of all the research samples used. The alloy was used as a disk in ex ovo experiments and in the immersion test. The results of the *in vivo* and *in vitro* yield similar results, suggesting that the material is biocompatible with the chorioallantoic membrane, the material is capable of being incorporated into the membrane, and the degradation products of the alloy are not detrimental to the membrane. In the ex ovo experiment, the alloy began interacting with the membrane immediately. Hydrogen gas formed as soon as the implant was placed on the membrane. Hydrogen gas production continued and was visible for the duration of the experiment. Within 3 days of implantation, visible signs of material loss were captured by the photographic images, and this had no deleterious effects on embryonic development. The only drawback to the use of the Mg-5Zn-0.3Ca alloy was the diameter of the implant. The five millimeter diameter disk was too large and caused the production of a large amount of hydrogen, inhibiting photographic capture of the blood vessels immediately surrounding the implantation site. A ring of .125mm thickness, with a 5 mm diameter would be a reasonable alternative. It would provide fast degradation, but allows for detection of blood vessel changes at the same time.

The results of implantation of the positive and negative controls, titanium and lead, respectively, confirmed that implants should be in proportion to the membrane. A small amount of lead present on the membrane was not deleterious to embryonic development, whereas a large titanium rod was too large in proportion to the membrane to allow development. The aluminum chloride, calcium chloride, magnesium chloride, and zinc chloride solutions did not provide any viable results. This may be attributed to the experimentation methods used or weakened membranes of the embryos prior to implantation. The magnesium chloride solution at 1

millimole concentration was the only salt solution that was able to survive 24 hours of implantation. The blood vessels directly surrounding the filter paper used remained intact while those distal to the implantation site were no longer viable. Based on the experimentations that were conducted, these results suggest that the embryo had a weak membrane prior to implantation.

Previous knowledge of embryo development would have improved the likelihood of achieving desired results, as more time could have been spent on adequate sample preparation. It would be more desirable to have a consistent source for providing embryos of similar quality. The donated eggs were greatly appreciated, but understanding acquiring embryos from a company that profits from developed chicks versus eggs implies that the best available eggs will be allowed to fully developed and those that do not fare as well will be donated for research. Many cull eggs were used for experimentation in comparison to high quality eggs that may have been available from egg distributors. The cull egg membranes were typically weaker. Future research on the difference between egg sources may prove beneficial in progressing with this research model. Developing a protocol requires many trials and errors. Of the thousands of eggs that were assessed during the course of thesis research, the number that provided viable research results was in the teens. For future research, I would suggest that the eggs are acquired from the same vendor each time to ensure that factor of the experiment is controlled every time. I would suggest the use of implants that are proportional to the size of the embryo.

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