

ABSTRACT

Title of Document: ZEOLITE-LOADED ALGINATE-CHITOSAN
HYDROGEL BEADS AS A TOPICAL
HEMOSTAT

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Hemorrhage is the leading cause of preventable death after a traumatic injury. Commercial hemostatic agents exist, but have various disadvantages including high cost, short shelf-lives, or secondary tissue damage. Polymer hydrogels provide a promising platform for the use of both biological and mechanical mechanisms to accelerate natural hemostasis and control hemorrhage. The goal of this work was to develop hydrogel particles composed of chitosan and alginate and loaded with zeolite in order to stop blood loss by targeting multiple hemostatic mechanisms. Several

particle compositions were synthesized and then characterized through swelling studies, particle sizing, Scanning Electron Microscopy (SEM), and Fourier Transform Infrared Spectroscopy (FTIR). The *in vitro* interactions of the particles were evaluated through coagulation, degradation, platelet aggregation, and cytotoxicity studies. The results indicate that 4% alginate, 1% chitosan, 4% zeolite-loaded hydrogel beads can significantly reduce time to coagulation and increase platelet aggregation *in vitro*. Future research can look into the efficacy of these particles *in vivo*.

ZEOLITE-LOADED ALGINATE-CHITOSAN
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By

Team CLOT (Catalyzing Localized Onset of Thrombosis)

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2015

Preface

The Gemstone Program is a four-year multidisciplinary honors program at the University of Maryland that allows students the unique opportunity to design and conduct their own research projects with the guidance of a university faculty mentor. The mission of the Gemstone Program is to “develop students' research skills in the context of multidisciplinary team research projects of importance to society; develop students' ability to work effectively in teams; provide students' leadership opportunities through peer mentoring, teaching and service throughout their four years in Gemstone; provide students' with a close-knit community that supports them in their Gemstone and other commitments and activities at the University of Maryland.”

We are “Team CLOT: Catalyzing Localized Onset of Thrombosis,” a team of twelve multidisciplinary undergraduates who share an interest in healthcare, biomaterials research, and biomedical sciences. Our two team mentors are Mr. Adam Behrens and Dr. Peter Kofinas, from the Fischell Department of Bioengineering at the University of Maryland. Adam is a PhD candidate and Peter is a faculty member and associate dean who leads a laboratory with projects including sprayable nanofiber surgical sealants, elastic nanoconductors, point-of-care polymeric diagnostic sensors, colorimetric biosensors, and flexible solid batteries.

The projects created by Gemstone teams focus on the roles of science and technology in society. The program incorporates all aspects of basic to applied translational research: conception, experimental design, data analysis, and publication. Invaluable skills in teamwork, team dynamics, communication, and presentation are developed through the process. By the completion of the program, Gemstone students are capable of conceiving an applied research project that addresses an important societal issue and is feasible with respect to time, costs, ethics, and access to resources. They are prepared to design and complete experiments paying special attention to applicability. Additionally, Gemstone students develop skills in data and statistical analysis techniques. They author a formal thesis and develop proficient skills in technical writing and reviewing. Finally, they formally present their research throughout the remaining three years in academic and educational settings, culminating in a final thesis defense to a panel of experts in their field. This manuscript is the formal thesis and fulfills a portion of the requirements for the Gemstone honors citation.

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We are also extraordinarily grateful to Ms. Svetla Baykoucheva, our team librarian, and the entire current Gemstone staff – Dr. Frank Coale, Dr. Kristan Skendall, Ms. Vickie Hill, Ms. Leah Kreimer Tobin, Ms. Faith Rusk, Ms. Jessica Lee – as well as all former staff members and graduate assistants for their tireless efforts to promote our personal, social, and academic growth over our college career.

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Chapter 1: Introduction

1.1 PROBLEM DESCRIPTION AND MOTIVATION

Traumatic injury is the leading cause of death in the United States for people under age 44, with traumatic hemorrhage* causing 35% of pre-hospital deaths and 40% of deaths within 24 hours of injury (Kauvar et al., 2006). This makes it one of the largest contributors to loss of productive years of life. Hemorrhage is also a primary cause of death on the battlefield, contributing to the 90% of military deaths that occur before medical care is reached. More than half of these deaths occur prior to the victim receiving hospital care (Kauvar et al., 2006). These deaths are most prevalent in the emergency medical or military settings. For example, in Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF), 15% of battle wounds occurred in locations where direct pressure could not stop the bleeding (Kelly et al., 2008). A reduction in the time in which traumatic injuries causing major blood loss receive treatment would save lives and increase the productivity of our communities.

1.1.1 Blood as a Functional Tissue

Blood is a multi-component functional tissue that is produced in bone marrow and circulates throughout the human body. Erythrocytes*, or red blood cells, comprise approximately 40-50% of blood volume and play a key role in oxygen-

carbon dioxide gas exchange. Plasma, the major component of blood, makes up about 55% of blood volume and contains extracellular fluid, electrolytes, functional proteins, and clotting factors. Platelets* and leukocytes, or white blood cells, make up about 1% of blood volume and have important protective functions.

The major functions of blood tissue are transport, regulation, and protection. Oxygen, vital nutrients, and circulating hormones are transported to body cells while carbon dioxide and various wastes are carried away. Body pH, interstitial fluid pressure, and body temperature are maintained by blood's pH buffering capacity, fluid-electrolyte balance, and circulation of heat, respectively. Blood provides the body immunity against foreign materials and infections, and contains the biomolecules* necessary to stop fatal hemorrhage (Marieb, 2012).

1.1.2 Blood Loss and Traumatic Injury

The body reacts to hemorrhage, defined as potentially fatal blood loss due to blood vessel damage, by undergoing a natural process called hemostasis*. The damaged blood vessel cells activate platelets to begin forming the initial stages of a clot. Proteins known as coagulation factors act together with the platelets to form a fibrous net that prevents further blood loss. With hemorrhage under control, the body is able to repair the injury, and begin clot deconstruction through a process known as fibrinolysis*. The process of coagulation* is essential for preventing blood loss.

Traumatic injury or a defect in this mechanism can lead to uncontrollable blood loss that may result in death.

1.2 CURRENT TREATMENT

Typically, first responders will attempt to control bleeding with various tools. The standard of care is to use gauze with applied pressure to provide a physical barrier against blood loss and reduce blood flow to the injury site (Naimer and Chemla, 2000). Tourniquets are used when bleeding cannot be stopped by gauze and pressure alone, and work by constricting blood flow to large regions of the body (Kragh et al., 2012). Unfortunately, this method can only be used to stop blood loss from extremities and can result in minor to extensive tissue death.

Alternatively, materials exist that rapidly stop blood loss by accelerating the body's natural blood clotting mechanisms called hemostatic* agents. These materials can help stop bleeding in both extremity injuries and non-compressible injuries to the torso, head, and neck. In the past decade, hemostatic research has increased exponentially. Commercial products have taken the form of biologically active gels for surgical use and synthetic or biologically active powders and foams for military and emergency medical use (Achneck et al., 2010).

Modern hemostatic products are designed to form better physical barriers to bleeding, utilize natural hemostatic pathways in a biologically active system, or combine both of these methods. Glues are used to hold tissue together, physically restricting blood flow. Treated or hemostatic impregnated bandages provide both a barrier and often induce biological activation of a strong clotting mechanism. Other products solely utilize biological mechanisms, often consisting of concentrated natural clotting factors (Seyednejad et al., 2008).

1.3 AN IDEAL SOLUTION

Although there are commercial hemostatic agents available on the market, they each have their own drawbacks, such as high cost of production and short shelf life. Additionally, some of these products have potentially adverse secondary effects: exothermic reactions that cause burns; diffusion into the bloodstream, which can cause undesired clotting; and allergic reactions to components (Seyednejad et al., 2008). Some products are also ineffective in treating high pressure hemorrhage and are therefore not useful for some of the most deadly traumatic injuries.

An ideal hemostatic agent to treat traumatic injury would address uncontrollable hemorrhage without the drawbacks of current products. The agent would quickly induce coagulation and create a structurally stable clot as a physical barrier against blood loss. Ideally, the agent would be inexpensive, easy to use, and

have a long shelf life. To minimize risk to the patient, the agent would limit harmful chemical reactions on and within the body.

Hemostatic hydrogel* particles have the potential to offer an inexpensive, effective, and easily accessible alternative. Our research focuses on the synthesis, characterization, and *in vitro* assessment of topically applied hydrogel particles that augment hemostasis by promoting the formation of a robust hemostatic plug and forming a physical barrier to blood loss through electrostatic interactions and swelling. The hydrogel particles synthesized and studied in this project follow a simple mechanism, use accessible materials, and are easy to store and apply, making them appropriate for both civilian and military traumatic injuries.

Chapter 2: Literature Review

2.1 BACKGROUND

2.1.1 Hemostasis

The currently accepted model of hemostasis is composed of two phases fundamental to platelet aggregation and blood coagulation: primary* and secondary hemostasis*. Primary hemostasis is the formation of an initial platelet plug. Platelets activate within minutes of vascular* tissue damage and induce additional platelets to aggregate at the site of injury (Broos et al., 2011). The first event occurs when the tissue is damaged and free platelets tether to the subendothelial matrix*, a process mediated by adhesive macromolecules (Broos et al., 2011). Shear stress*, thrombin*, activated platelet granules*, and polyphosphates* such as adenosine diphosphate (ADP)*, then trigger the activation of integrin* receptors on the surface of platelets and induce a conformational change (Morrissey, 2012). Platelets release chemicals such as ADP, thrombin, and epinephrine, which amplify and sustain additional platelet adhesion (Beer et al., 1992). As platelet aggregation increases at the injury site, the primary platelet plug forms (Davie et al., 1991). This is an example of a positive feedback system (Figure 1) and sets the stage for secondary hemostasis, the formation of fibrin* via the coagulation cascade (Davie et al., 1991).

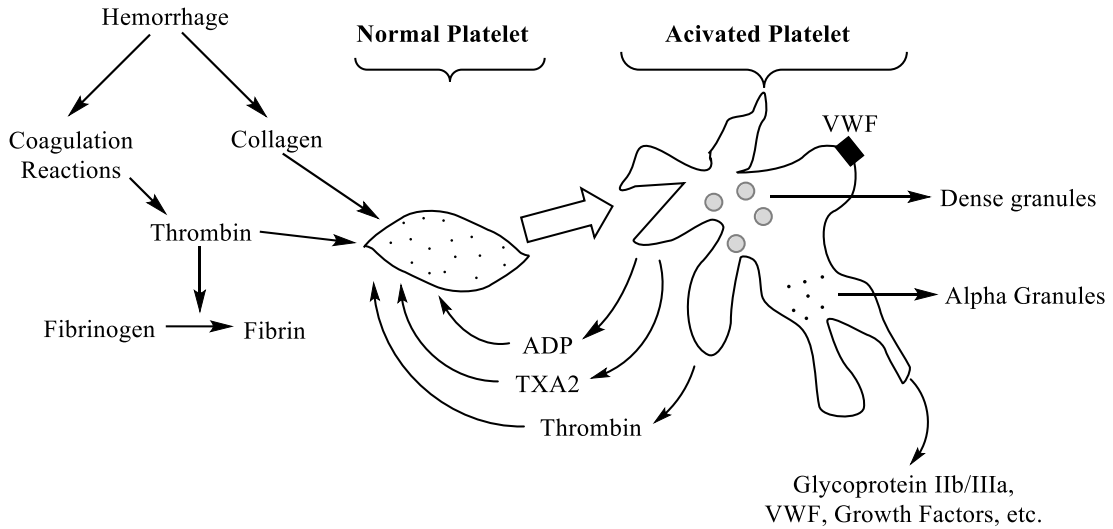


Figure 1: Platelet activation. Illustration of platelet activation and the factors involved. Adapted from “Platelet Activation”

Secondary hemostasis results in the formation of a fibrin clot that strengthens the platelet plug formed during primary hemostasis. This formation consists of two principal mechanisms: first the extrinsic (tissue factor) pathway, which is followed by the intrinsic (contact activation) pathway (Mackman et al., 2007). Together, they create the coagulation cascade (Figure 2).

Coagulation Cascade

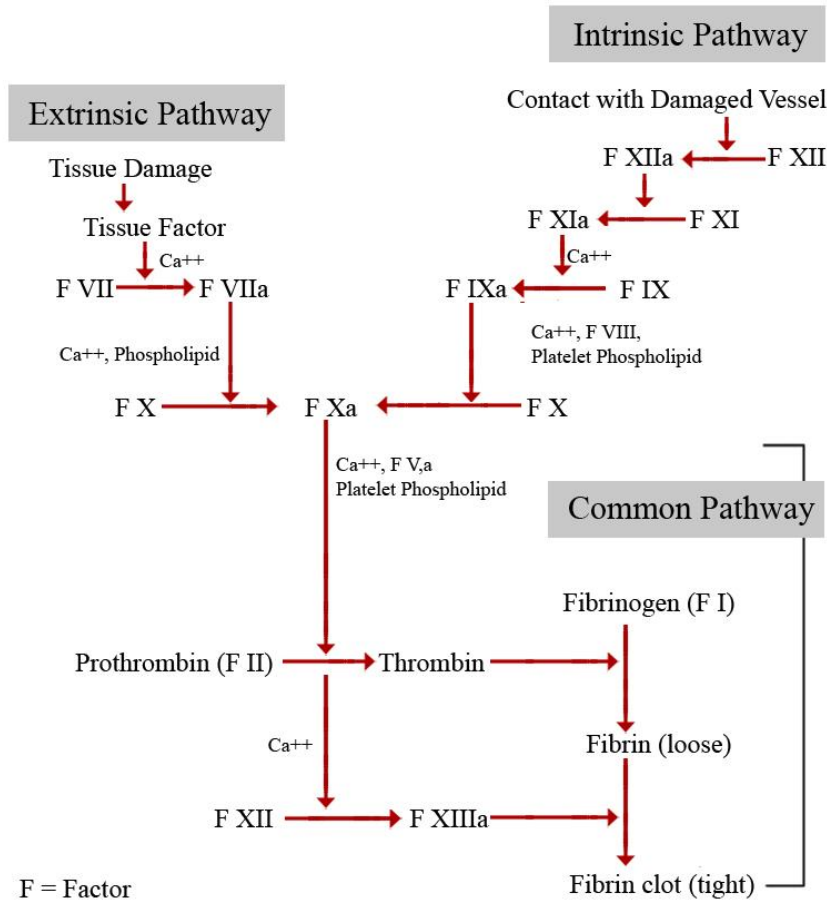


Figure 2: The coagulation cascade. Illustration of the extrinsic and intrinsic pathways and their convergence into the common pathway.

The extrinsic pathway begins with vessel damage in which the exposed subendothelial matrix releases tissue factor (factor III, FIII). Tissue factor binds to coagulation factor VII (FVII). Once FVII is bound, it becomes active (FVIIa). FVIIa uses calcium as a cofactor to activate factor X (FX) into factor Xa (FXa). The activation of FX to FXa marks the beginning of the common pathway between the contact activation and tissue factor pathways.

The intrinsic pathway amplifies hemostatic response, primarily through the activation of factor XII (FXII). Active FXII cleaves and activates downstream factors that lead to additional active FX at the junction of the two pathways. At this point, the FXa binds with cofactor FVa and initiates the conversion of prothrombin* (FII) to thrombin (FIIa) (Gailani and Renné, 2007). Thrombin cleaves fibrinogen* to form insoluble fibrin monomers (Mackman et al., 2007). Fibrin cross-links* with the primary platelet plug, effectively forming an insoluble clot at the site of injury.

As the injury is healed, the clot is degraded in a process called fibrinolysis. First, plasminogen, an inactive proenzyme*, is converted to its active form, plasmin, by tissue plasminogen activator (t-PA) and urokinase* (uPA). Activated plasmin cleaves the cross-linked fibrin clot and activates matrix metalloproteinases* to degrade the extracellular matrix (Collen, 1999). This is further promoted by platelet derived growth factor (PDGF), which aids in healing and wound repair (Fredriksson et al., 2004). t-PA is released into the bloodstream at a slow rate by damaged vascular endothelium. When released, it activates fibrin-bound plasminogen and cleaves plasminogen at a certain sequence, causing a conformational change that promotes enzymatic activity (Cesarman-Maus and Hajjar, 2005). Activated plasmin is then able to carry out fibrinolysis. As a result, there is equilibrium between clot formation and degradation, and the propagation of fibrin and the activation of plasminogen.

2.1.2 Academic Approaches

Recent academic studies have been conducted to improve current hemostatic agents and explore new mechanisms. Both biological and mechanical approaches have been investigated. Biological methods directly initiate primary or secondary hemostasis. Biological approaches must consider platelet activation, coagulation, and biocompatibility* to work effectively. Platelet activation and coagulation are functions that can be exploited to induce hemostasis through the acceleration of existing biological pathways. Biocompatibility is essential for any hemostatic agent; however, excess inflammation, toxicity, and morbidity could deem an approach unusable. Mechanical approaches employ adhesives, sealants, matrices, and dressings to act as physical barriers to blood loss. Biocompatibility and hemostatic efficacy are major factors in mechanical agents as well. As research has evolved, platforms have incorporated multiple hemostatic mechanisms. Approaches that function by multiple mechanisms are categorized based on their primary mechanism.

2.2 BIOLOGICALLY ACTIVE MECHANISMS

Biologically active agents directly target primary and/or secondary hemostasis. Materials are biologically derived, synthetic, or composites of the two. Three major types of biologically active agents have been identified: naturally occurring polysaccharides* and proteins, intravenous hemostats, and aluminosilicate minerals. Polysaccharides and proteins are isolated and modified to target specific

steps in the hemostatic pathways. They are generally incorporated into topical agents for surgical or military use. Common polysaccharides include chitosan* and cellulose. The major proteins used are collagen, gelatin, keratin, and myeloperoxidase. Intravenous hemostats are used to enhance platelet activation and aggregation by mimicking the action of natural platelets and signaling proteins. They offer the major advantage of allowing treatment of internal or distal hemorrhage without direct application to the wound site. Cell derived platelets, synthetic platelets, and antifibrinolytics are the primary intravenous approaches and are often functionalized and modified with coagulation proteins. Finally, aluminosilicate minerals offer the advantages of rapid water absorbance and electrostatic interactions to promote hemostasis. This section is separated into hemostatic agents that augment either primary hemostasis or secondary hemostasis.

Primary Hemostasis

2.2.1 Polysaccharides

Naturally derived polysaccharides offer high biocompatibility, high biodegradability*, and low toxicity byproducts (Slaughter et al., 2009). Many natural polymers are derived from the mammalian extracellular matrix, while others, like hyaluronic acid, are obtained from animal tissues. Marine life provides another source of natural polymers, such as the heavily studied polysaccharide chitosan (Peppas et al., 2006).

CHITOSAN

Chitosan is a derivative of chitin, which is an inexpensively harvested polysaccharide found in crustacean shells and cell walls of fungi. It is highly biocompatible and has wound healing properties due to its hydration abilities and antibacterial activity (Jayakumar et al., 2011). The structure of chitosan (Figure 3), characterized by amine groups that become deprotonated above the pH range of 6.2 – 7.0, is the main contributor to its functional properties (de Alvarenga, 2011).

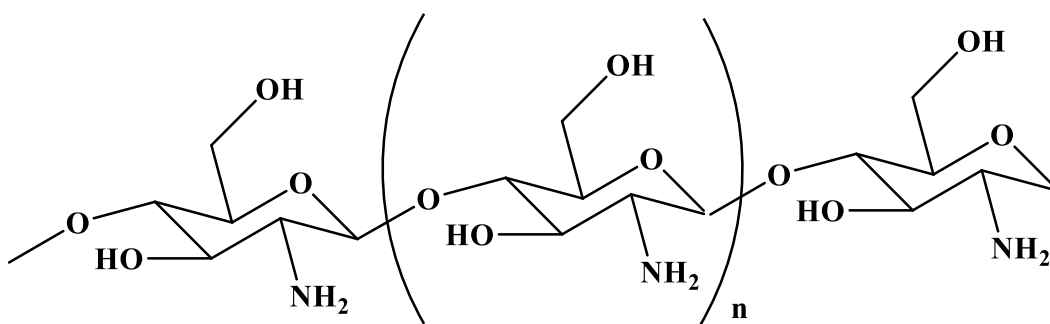


Figure 3: Molecular structure of chitosan. Chitosan monomers can combine to form polysaccharide chains of various lengths. Each repeat unit contains an amine (NH_2) functional group which has a pKa of 6.5. At physiological pH of 7.4, more than 99% of the amine groups are protonated and the molecule has an overall positive charge.

Naturally insoluble in water, blood, and inorganic solvents, chitosan can be dissolved in aqueous solutions with a pH below 6.2, such as acetic acid. The amine groups become protonated, creating an overall positive charge and allowing solvent-solute polar interactions to take place. This cationic character allows chitosan to

adhere to wound sites and aid in blood coagulation as most biological surfaces and blood proteins have a net negative charge (Mercy et al., 2012). Chitosan has been shown to form a blood coagulum in defibrinated*, deheparanized*, and whole blood (Malette et al., 1983). Okamoto et al. demonstrated chitosan's positive effects on platelet aggregation, the release of PDGF, and transforming growth factor β 1. All of these function in wound healing (Okamoto et al., 2003). Chitosan also increases platelet adhesion, in part by increasing intracellular calcium ion concentrations and significantly increasing expression of glycoprotein* Iib/IIIa (GPIIb/IIIa) on platelet membrane surfaces, which is highly relevant in cell-to-cell interactions (Chou et al., 2003). The GPIIb/IIIa integrin complex is the calcium-dependent fibrinogen receptor that is part of the common platelet aggregation pathway (Phillips et al., 1991). When GPIIb/IIIa is blocked, platelet aggregation and blood coagulation is directly inhibited (Sheu et al., 1992). The cationic nature of chitosan can also attract glycosaminoglycans*, which in turn attract growth factors that enhance cell growth and proliferation (Jayakumar et al., 2011). Chitosan absorbs the water present in blood and becomes an adherent to exposed cellular material through electrostatic, hydrophobic, and van der Waals interactions as well as hydrogen bonding.

Chitin is deacetylated to form chitosan. Deacetylation* (DA) controls the solubility of chitosan in water and influences its physiochemical and biological properties (Yuan et al., 2011). After deacetylation, a chitosan polymer made up of 50% deacetylated units (D-glucosamine) and 50% acetylated units (N-acetyl-D-

glucosamine) transforms the naturally insoluble chitosan to a substance that is soluble in blood and water and has a pKa of approximately 7.5 (Whang et al., 2005). However, random asymmetrical order of acetylated and deacetylated units inhibits crystallization and reduces the tensile strength of the material (Knaul et al., 1999). Yang et al. evaluated the hemostatic effects of degree of deacetylation and molecular weight (MW) of chitosan and compared the hemostatic mechanisms of solid-state chitosan, chitosan in acetic acid physiological saline solution, and carboxymethyl chitosan in physiological saline solution. Low DA chitosan in acetic acid physiological saline solution showed the most significant increase in erythrocyte* aggregation and deformation, which are important in hemostasis, while solid-state chitosan could not initiate these processes. Low DA solid-state chitosan induced coagulation through high platelet absorption. In both cases, low DA chitosan (MW 105 – 106 Daltons) showed higher hemostatic ability than medium or high (Yang et al., 2008).

Biocompatibility is an essential characteristic for a hemostatic agent. Chitosan hydrogels are nontoxic when applied and are naturally degraded by lysosomal* pathways in mammalian cells into naturally metabolized aminosugars*. Cellular immune responses and antibody responses measured using lymphocyte* proliferation assays and immunoglobulin* G assays, respectively, further demonstrated negligible chitosan-specific binding reactions (VandeVord et al., 2002).

Solutions, powders, and hydrogels are the three most common forms of chitosan used for hemostatic purposes. In one study, lingual incisions receiving 2 mg/mL chitosan (MW 8×10^5 – 1.5×10^6 Daltons) solution showed a 43% improvement in bleeding time when compared with lingual incisions receiving the control solution without chitosan in a heparinized animal model. Using scanning electron microscopy (SEM), chitosan was shown to alter morphology and increase affinity between erythrocytes, suggesting that chitosan influences erythrocyte affinity and aggregation (Klokkevold et al., 1999). When whole blood and its components were tested, chitosan solution concentrations above or equal to 800 $\mu\text{g/mL}$ stopped circulatory blood flow completely due to the strong interactions of chitosan with erythrocytes (Klokkevold et al., 1997). Thus, the solution form of free chitosan is not practical in a clinical setting due to the harmful thrombosis* that may take place. Alternatively, powders and hydrogels can be applied in a concentrated fashion. In *in vitro* and *in vivo* studies, chitosan powders induced a strong aggregatory effect on platelets without causing a significant hemolysis* or distal thrombus* event (Whang et al., 2005). Chitosan hydrogels have also produced positive results *in vitro* and *in vivo*. The most effective results in hemostatic applications of chitosan hydrogels were demonstrated when compared to chitosan solutions and powders. For instance, application of a chitosan hydrogel on rat tail amputations significantly reduced bleeding. Additionally, the hydrogel significantly reduced the size of the wounds and accelerated the early phase of healing (Ishihara et al., 2002).

New chitosan based hemostats are in development. Chitosan dressings have been loaded with different compounds to enhance chitosan's hemostatic properties. ChitO2-Clot is a product composed of a fibrous chitosan mat that contains perfluorocarbon* oxygen carriers. First, ChitO2-Clot absorbs blood and swells. This helps form a clot that fills the wound site and applies pressure to decrease bleeding. Like most other chitosan based hemostats, ChitO2-Clot activates primary hemostasis by activating platelets and causing erythrocyte aggregation. The perfluorocarbons supplement chitosan's native properties by increasing the amount of oxygen available at the wound site, expediting the healing process. The chitosan in ChitO2-Clot is metabolized by the body and degraded into aminosugars, while the perfluorocarbons are released from the body through the lungs (Ulsh et al., 2013). Sung et al. has developed a chitosan dressing loaded with minocycline* to increase healing time (Sung et al., 2010), while Ong et al. has created a chitosan dressing loaded with antimicrobial silver, and procoagulant* polyphosphates (Ong et al., 2008).

In situ forming hydrogels offer another platform. Mechanical strength, in vivo dissolution properties, and cytotoxicity* are especially important considerations. Lih et al. developed an in situ forming chitosan-poly(ethylene glycol)-tyramine (CPT) hydrogel. The hydrogel was enzymatically cross-linked in situ using peroxidase and hydrogen peroxide to create an adhesive agent with variable mechanical strength and adhesiveness. The results from a hemorrhaging liver mouse model demonstrated hemostatic capability and healing efficacy. Adhesive strength was 3- to 20-times

stronger than fibrin glue, a commercially used sealant. The hydrogel has hemostatic properties due to the presence of chitosan, and the polyethylene glycol (PEG)-tyramine component enhances the water solubility of chitosan (Lih et al., 2012). While chitosan is naturally insoluble, the PEG permits the formation of the chitosan hydrogels via cross-linking.

Recently, Gu et al. fabricated via electrospinning* a pure chitosan nanofiber mat with highly controlled pore size and thickness. Ultrasonification was used to control pore architecture. This technique uses sound waves at ultrasonic frequencies (>20 kHz) to influence material structure. Maximum porosity, maximum exposure of chitosan to blood, and improvement in water absorptivity rate by more than 90% was achieved. These factors resulted in a chitosan nanofiber mat with hemostatic efficacy greater than commercially available chitosan hemostats. Additionally, the mat architecture improved dermal fibroblast* proliferation, which may assist in wound healing. This biomaterial demonstrates a significant way to improve upon existing platforms (Gu et al., 2013).

Chitosan is an example of a fundamental naturally-occurring polymer that has been used extensively in the design of hemostatic agents. Its unique electrostatic properties at physiological pH and its variable physical and mechanical properties enable integration into an array of biomaterial platforms.

CELLULOSE

Cellulose is a polysaccharide that is the basic structural component of the cell wall in plants. Cellulose has been applied in otolaryngology*, in biliary*, and in plastic surgery to heal burn wounds and other topical wounds (Lamphier and Goldberg, 1961). Active water-insoluble form of cellulose, oxidized regenerated cellulose (ORC), is created when alpha-cellulose is oxidized to polyanhydroglucuronic acid, with anhydroglucopyranose as its fundamental unit (Miller et al., 1961). ORC is often complexed with other molecules to increase viability and effectiveness as a hemostat. It functions in hemostasis by both mechanical and chemical mechanisms, including swelling, physical wound blockage, and surface interactions with proteins and platelets (Schonauer et al., 2004). ORC has a high affinity for hemoglobin and reacts to form the acid haematin when applied to a wound site. Acid haematin results in a black discoloration and shortly thereafter, bleeding ceases. The gel that forms from the haematin acid formation acts as a matrix for fibrin formation, causes vasoconstriction*, and aids in the local deposition of fibrin (Miller et al., 1961).

One product that utilizes oxidized regenerated cellulose is Surgicel, which has been used for over 50 years. In one particular study, Surgicel fibrillar efficacy was tested in spinal surgery. It can be cut to size, conforms well to most shapes, is easily manipulated, and does not stick to instruments (Sabel and Stummer, 2004). Its multilayer design facilitates placement of the hemostat; however, it is important to

remove the Surgicel fibrillar after considerable hemostasis has occurred, as it firmly adheres to the clot and can break apart dried sections if left on for too long. This is one of ORC's main concerns, which fuel the debate of its overall efficacy.

One of the negative aspects of ORC is its pH of approximately 3.1. It can damage nervous tissue at this pH and ORC's hemostatic property has also been suggested to be relatively poor in most applications (Wu et al., 2012). Fortunately, a basic salt of a weak acid can be used to neutralize ORC. In an ORC-based hemostat, ORC was fabricated into a single layer and immersed into a solution of NO₂ dissolved into CCl₄; this solution was stirred at 19.5 °C for 40 hours (Wu, et al., 2012). After a series of freeze dryings and washings in NaOH/ethanol solutions, an ORC-sodium (ORC-Na) complex was formed. The resultant ORC-Na hemostat arrested bleeding from a hepatic injury in rabbits. The ORC-Na material had the shortest time to hemostasis and the least blood loss when compared to other compositions and the commercial product Surgicel (Wu et al., 2012). The ORC-Na material is biodegradable via beta-elimination* and susceptible to macrophage* digestion via hydrolytic enzymes (Domb et al., 1998). The ORC-Na material is a promising hemostat with strong hemostatic efficiency, high biodegradability, and broad applications.

Microporous polysaccharide hemospheres (MPH) is a cellulose-based hemostatic agent. MPH concentrates platelets and coagulation proteins, and enhances

natural hemostatic plug formation (Murat et al., 2004). MPH products primarily facilitate coagulation by absorbing water through osmotic action to concentrate serum proteins, platelets, and other elements on the surfaces on wounds (Antisdel et al., 2008). In a laparoscopic trocar* injury to the spleen in female pigs, MPH proved to be a powerful hemostatic agent and had the ability to achieve hemostasis (Humphreys et al., 2008).

2.2.2 Proteins

Naturally occurring proteins are commonly incorporated into hemostatic agents for either their direct or passive roles in hemostasis. Those used to influence primary hemostasis typically offer a scaffold for platelet binding and aggregation, as well as facilitation of platelet activation reactions. They also absorb fluid, leading to subsequent local cell and protein concentration. They may be combined with other platforms in order to create a more robust hemostatic agent. Drawbacks associated with these are typically short shelf lives, high costs, and risk of viral transmission.

COLLAGEN

Collagen is the most abundant protein in mammals and is a primary component of the extracellular matrix of animal cells. Collagens are critical platelet activators with roles in platelet adhesion, anchoring, signaling and direct activation. Several membrane glycoproteins have been identified as the major platelet receptors

for collagen (Kehrel, 1995) and others have been shown to interact with von Willebrand factor* and subsequently assist in collagen-platelet interactions (Clemetson and Clemetson, 2001). One physical method of achieving hemostasis is through the use of microfibrillar collagen, a bovine-based partially-insoluble acid salt (Ribalta et al., 2004). This agent adheres tightly to the wound and blocks injured vessels, providing a surface for platelets to bond with and facilitating platelet release reactions (Schonauer et al., 2004). While microfibrillar collagen has been shown to reduce time to hemostasis (Qerimi et al., 2013), it carries a risk of infection as well as interference with bone healing (Schonauer et al., 2004). Collagen has been further incorporated into various commercially available hemostatic sponges, matrices, and bandages (Achneck et al., 2010).

GELATIN

Gelatin, derived from denatured collagen, has also been shown to act as a physical barrier to blood flow both by swelling and by bonding with the surface of a wound (Imani et al., 2013). Many absorbable gelatin sponge hemostats include thrombin to achieve hemostasis. Gelfoam Plus, manufactured by The Baxter Healthcare Corporation, has a primarily physical mechanism. However, the incorporation of thrombin enhances the hemostatic capabilities of the sponge by influencing the coagulation cascade (*Gelfoam Plus [Hemostasis Kit] Instructions For Use*, 2009). Gelatin's rough surface morphology and high porosity allow platelets and cells to adhere and fluid to be absorbed (Hajosch et al., 2010). One of the drawbacks

of gelatin is that it has been found to be a cause of mass lesions (Tomizawa, 2005). This occurs due to embolization, or blocking of blood vessels, when applied to intravascular tissue (*Gelfoam Plus [Hemostasis Kit] Instructions For Use*, 2009). Similarly, microfibrillar collagen hemostats like Avitene are a subgroup of gelatin-based hemostats. Applied topically, the hemostat utilizes a large surface area mechanism to entrap and activate platelet clotting factors (Kubo et al., 1984). Once activated, the platelets adhere to the fibrous mass, initiating platelet plug formation (UM BWMC, n.d.).

KERATIN

Hemostatic mechanisms of keratin-based hydrogels have recently been characterized. Keratin is a protein typically derived from mammalian hair that has intrinsic biocompatibility, biodegradability, and mechanical durability. It has a high cysteine* residue content and therefore forms tough and durable structures due to intermolecular disulfide bond formation. It also is well tolerated by the body and has been shown to induce cell adhesion (Burnett et al., 2013). A simple physically cross-linked keratin gel showed hydrophilicity, adhesion to bleeding tissue, fluid absorbance, cell infiltration and attachment, and granulose tissue formation (Aboushwareb et al., 2009). When applied to a liver injury in New Zealand white rabbits, the hemostatic hydrogel proved to be just as or even more effective in stopping blood loss than the commercial hemostats QuickClot and HemCon.

However, the mechanism was not well characterized at the time (Aboushwareb et al., 2009).

Recently, Van Dyke's group has extensively investigated the mechanisms behind keratin-based hydrogel biomaterials. Specifically, keratin has been found to bind to cells by self-forming leucine-aspartic acid-valine (LDV) and glutamic acid-aspartic acid-serine (EDS) binding residues (Rouse and Van Dyke, 2010). The presence of keratin on the injury site instigates thrombus formation and forms a physical seal on the wound site that acts as a porous scaffold. The scaffold enables cellular infiltration and granulose tissue formation (Rouse and Van Dyke, 2010). Keratins significantly decrease plasma clotting times, even with the influences of clotting disorders. When its influence on fibrin polymerization* was studied, it was demonstrated that keratin increased fibril assembly in the lateral direction (Rahmany et al., 2013). Keratin is a readily available material with promising potential in tissue engineering and wound treatment. However, there are currently no keratin biomaterials in clinical use, limiting what is known about their efficacy in humans (Rouse and Van Dyke, 2010).

MYELOPEROXIDASE

Platelet activators have been previously derived from concentrated proteins naturally found in the body. One such natural protein is von Willebrand factor (Mackman et al., 2007). Recent efforts have been more limited, as platelet

aggregation is nearly useless without thrombin activation to create the matrix that captures aggregated platelets. However, some efforts have been pursued, such as research into myeloperoxidase. Myeloperoxidase is released by neutrophils* during an inflammation response and its introduction to the blood has shown to play a role in platelet activation. This results in fast activation without improving aggregation, suggesting that this protein modifies platelets and causes them to interact with coagulation factors. However, these platelets are damaged in the process and are no longer viable. This is not an anticipated issue for wound applications, as platelets only need to remain viable long enough to clot (Zarbock et al., 2007).

2.2.3 Biologically Derived Intravenous Agents

Intravenously-administered hemostatic agents have drawn increasing interest. Research has focused on cell derived platelets, synthetic platelets with coagulation enhancing modifications, and antifibrinolytics. These approaches offer therapeutic benefit without requiring direct access to the injury site, and can be used prophylactically.

Platelet transfusions are extremely important in hematological treatments today. They are used for the treatment of certain bleeding complications in patients, including hematologic or oncologic clotting disorders, chemo/radiotherapy-induced myelosuppression*, trauma, and surgery (Modery-Pawlowski et al., 2013). There are

two main categories of platelets with clinical applications. Biologically derived platelets are created using natural biological components, while synthetic platelets are created using synthesized materials. Biologically derived platelets were once commonly used, but have several disadvantages including supply shortages (“Blood Facts and Statistics,”), limited shelf lives, biological/pathological contamination, adverse immune responses, and unforeseen expenses (Lee and Blajchman, 2001).

FREEZE-DRIED PLATELETS

Freeze-dried, or lyophilized, platelets are extracted from either humans or animals and freeze-dried using a lyophilizer. Vacuum lyophilization removes water by sublimation and retains platelet morphology (Bode and Fischer, 2007). Freeze-dried platelets promote primary hemostasis by mimicking the natural platelet activation mechanism in which platelets begin to aggregate to form a primary platelet plug when exposed to the damaged endothelial surface. In addition, these activated platelets recruit neutrophils and monocytes* to the injury site by displaying P-selectin* on their surface. This then directs the assembly of activated clotting factors followed by fibrin formation and induced fibrinolysis (Mohanty, 2009).

There are numerous advantages to using freeze-dried platelets compared to fresh platelets. Fresh platelets can only be stored for about 5 days, whereas lyophilized platelets can be stored for years at room temperature. The freeze-drying procedure sterilizes platelets, reducing the concern for microbial contamination

associated with fresh platelets. Fresh platelets become less effective and lose some hemostatic function during storage. Lyophilized platelets, on the other hand, retain all properties necessary for hemostasis regardless of storage duration (Hawksworth et al., 2009).

One example of lyophilized platelets called thrombosomes were investigated by Fitzpatrick et al. Prior to and during freeze-drying, Group O leuko-reduced whole-blood derived platelets are stabilized with trehalose and polysucrose. Trehalose and polysucrose are natural lyoprotectants that protect freeze-dried platelets by increasing their solute concentration. Stabilizing the whole-blood platelets with lyoprotectants leaves them in a form very similar to natural platelets in terms of size and activity. They were shown to maintain glycoprotein GPIIb/IIIa expression, although GPIb expression and Annexin V binding were reduced. Hemostatic functions were maintained and consistent. This platform offers the benefits of platelet transfusions without adverse effects even at high doses (Fitzpatrick et al., 2013). They do not need to be kept on constant dry ice, which is a limitation to frozen platelet products in a hospital environment. Stored in a freeze-dried form, they are reconstituted with sterile water, and then can be delivered onsite in an IV in a few minutes (Lashof-Sullivan et al., 2013). Thrombosomes adhere to damaged vascular tissue matrices, promote assembly of a complex on aggregated platelets, and stabilize clot formation due to thrombin generation (Fitzpatrick et al., 2013). Overall, they have demonstrated the conservation of important characteristics for clot formation, primary adhesion of

platelets, aggregation, thrombin production, fibrinogen binding, and ultimately wound closure (Fitzpatrick et al., 2013). They stay in circulation for the same amount of time as a normal platelet transfusion without any adverse effects (Lashof-Sullivan et al., 2013). However, an uninvestigated disadvantage to thrombosomes is the potential immune response to biologically derived materials.

FIBRINOGEN-DERIVED RGD PEPTIDES

Coller et al. investigated modified red blood cells as an alternative to fresh platelet usage in thrombocytopenic* patients. Thromboerythrocytes are erythrocytes with a specific fibrinogen-derived arginine-glycine-aspartic (RGD) peptide bound to the surface amino groups. Specifically, the RGD containing peptide of the thromboerythrocytes interacts with GPIIb/IIIa receptors on activated platelets to form aggregates similarly to fibrinogen through covalent bond formation. It was shown that thromboerythrocytes interact selectively with activated platelets that were stimulated by ADP, epinephrine, and thrombin. The thromboerythrocytes aid platelet aggregation by tethering together platelets and erythrocytes into large aggregates of cells and proteins (Coller et al., 1992). Thromboerythrocytes also have been shown to induce platelet activation. This can cause potential problems when platelets are activated in areas without injury, resulting in blood clots. This can be avoided in some cases when the synthetic platelets only interact with activated platelets. Another possible mechanism of thromboerythrocytes involves ADP-induced platelet activation by the ADP rich erythrocytes. It is possible that leaked ADP from

thromboerythrocytes further influences the formation of a stable hemostatic plug (Coller et al., 1992). The initial in vitro results suggest therapeutic benefit for patients with platelet deficiencies. Further investigation into the hemostatic capabilities of this platform are necessary.

FIBRINOGEN COATED ALBUMIN MICROPARTICLES

Another platelet substitute, synthocytes are particles made with human albumin* microcapsules and coated with fibrinogen, a blood protein with an RGD sequence (Modery-Pawlowski et al., 2013). The mechanism of synthocytes involves an interaction between natural platelets and the fibrinogen-coated albumin microcapsules (Levi et al., 1999). Specifically, synthocytes have shown to induce platelet adhesion to the endothelial cell matrix and improve the impaired formation of platelet aggregates found in platelet poor blood (Levi et al., 1999). Synthocytes significantly reduce bleeding times as demonstrated in a rabbit ear injury model. (Levi et al., 1999). These particles are larger than many particle formations (3.5-4.5 micron diameter) and did not show evidence of potentially deadly distal thrombotic events in a rabbit venous model. Similar fibrinogen-coated albumin microparticles called thrombospheres developed by Yen had an average diameter one-third the size of synthocytes. They showed similar hemostatic efficacy for a longer duration in a thrombocytopenic rabbit model. Distal thrombotic events were not observed and lung function was not affected by the smaller sized particles (Yen, 1995).

2.2.4 Synthetic Platelets (Intravenous)

Synthetic platelets are a promising alternative platform to natural platelet products and their modified forms. They have been functionalized with coagulation proteins and peptides and manipulated to mimic the action of natural platelets. They have several advantages that include longer shelf-life, reduced risk of biological contamination or infection, sterilization, and reproducible quality. For these reasons, the evolution of platelets used for blood transfusions has evolved from biologically derived platelets, towards platelet-mimetic synthetic hemostats. Disadvantages involve biocompatibility, allergenic responses to synthetic materials, and cost of production.

LIPOSOMES

Certain liposomes can be used as a synthetic platelet substitutes, specifically those coated with dodecapeptide (H12), a specific ligand* binding sequence for the GPIIb/IIIa complex derived from one of the chains in fibrinogen (Okamura et al., 2009). They are significantly smaller than biologically derived particles, about 260 nm in diameter (Lashof-Sullivan et al., 2013). Capable of drug encapsulation, liposomes can be loaded with ADP or other platelet activating biomolecules to directly augment primary hemostasis (Okamura et al., 2009). Their mechanism of action is to interact with activated platelet surfaces through the GPIIb/IIIa receptors to improve platelet accumulation at sites of hemostasis (Takeoka et al., 2003). This occurs because the H12 coating has fibrinogen sequences that are recognized by the

GPIIb/IIIa receptor on activated platelets and the released procoagulant molecules induce platelet activation. This material is a promising synthetic that directly augments primary hemostasis; however, cost of production will be a challenge.

PLGA NANOPARTICLES

Poly(lactic-co-glycolic acid) (PLGA) is a biocompatible nanoparticle in which degradation can be easily controlled by varying PL:PG ratio. Functionalization using proteins and peptides with known hemostatic properties allows these materials to be highly optimizable for hemostatic applications. Recently, nanoparticles have been made with a biodegradable PLGA polymer core and RGD peptide functionalization spaced with polyethylene glycol (Bertram et al., 2009). They are small in size and can encapsulate drugs. Variable degradation directly influences drug release rate. The nanoparticles bind with activated platelets and increase their rate of aggregation (Bertram et al., 2009). They significantly reduce bleeding time based on rat femoral artery models and are advantageous as an early intervention following trauma (Lashof-Sullivan et al., 2013).

2.2.5 Aluminosilicates

Current approaches that have sparked recent interest in the field of hemostatics use aluminosilicates to induce coagulation. Aluminosilicates are derived from minerals made of aluminum oxide, Al_2O_3 , and silicon oxide, SiO_2 .

Aluminosilicates are formed from these derivatives, with Al^{3+} ions replacing some of the Si^{4+} ions. This creates an imbalance of charge and in many cases, positive ions such as Na^+ , K^+ , and Ca^{2+} are added to the framework to neutralize the material (Pinkas, 2005).

Two major aluminosilicates used in hemostatics include zeolite* and kaolinite. Zeolite is a hydrated microporous aluminosilicate; the structure (Figure 4) consists of AlO_4 and SiO_4 tetrahedrals (Pinkas, 2005).

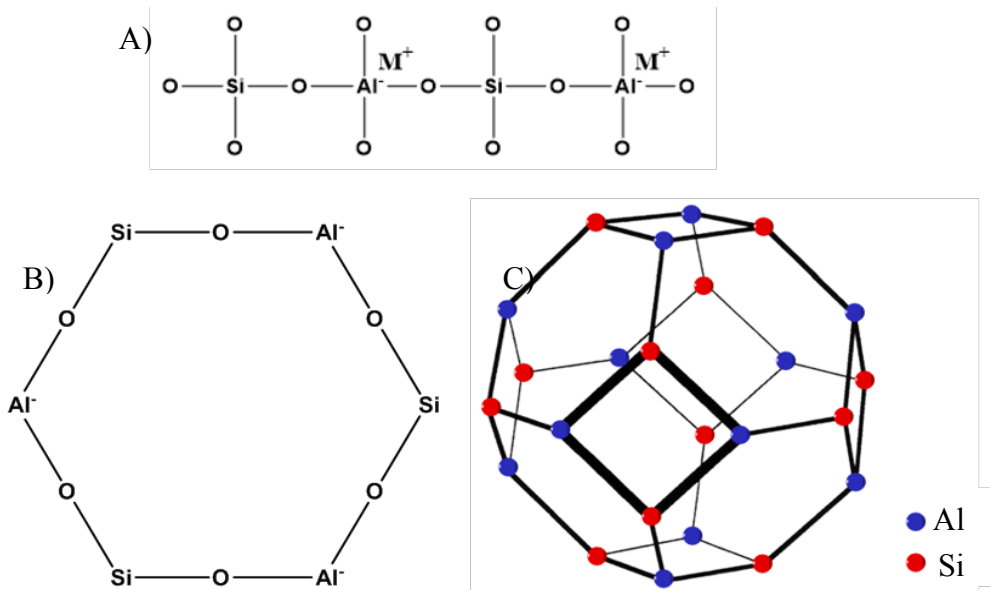


Figure 4: Atomic structure of zeolite. A) Depicts how zeolite interacts with cross-linking cations. M^+ represents elements such as Ca^{2+} , Mg^{2+} , K^+ , Na^+ , etc. B) Illustrates the hexagonal shaped planes that constitute the framework of the natural porous formation of zeolite depicted in figure 4C. C) The natural porous structure that zeolite assumes is known as a truncated octahedron with hexagonal faces.

These Al^{3+} and Si^{4+} ions form crystal structured cages with a negatively charged framework that is capable of trapping many positively charged ions (Modery-Pawlowski et al., 2013). Kaolinite is a layered aluminum silicate mineral with the kaolinite group $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$. Rocks rich in this mineral are referred to as kaolin (Modery-Pawlowski et al., 2013).

These two aluminosilicate minerals are increasingly common because they have recently proved effective in inducing blood coagulation via several mechanisms. First, when these aluminosilicates are loaded with oxygen or metallic ions, polar surfaces are created due to electronegativity differences. When in contact with blood, the most negatively-charged metal oxides have proven to reduce clot time and increase overall clot strength by activating the intrinsic pathway of the coagulation cascade (Ostomel et al., 2007). Additionally, the microporous structure, high surface area, and charge of aluminosilicates allow for high absorption of water. This absorption concentrates clotting factors and platelets, accelerating coagulation (Arnaud et al., 2008). Commercially used aluminosilicate hemostatic platforms have made use of these properties.

Initially, the major use of aluminosilicates was through modified gauzes infused with zeolite granules. The most popular commercial agent that has used this approach is QuikClot. QuikClot is a gauze loaded with these zeolite particles which,

when compressed to a wound, rapidly absorbed blood and held water molecules via its porous structure. Concentrating the blood led to a decreased clotting time (Arnaud et al., 2008). However, this approach had several disadvantages. An exothermic reaction was very common upon application, causing severe burns. This is a consequence of hydrogen bond formations between zeolite, water molecules and wounds, a process which may reach 70°C (Arnaud et al., 2008). Additionally, the zeolite granule itself adhered to the tissue, making it especially difficult to be removed from deep wounds (Li et al., 2013). Many products, including QuikClot, have moved towards using kaolin instead of zeolite to alleviate these issues (Trabattoni et al., 2011). Kaolin increases coagulation by activating factor XII in the coagulation cascade without the exothermic reaction of zeolite. Kaolin has also been used as a binder coupled with zeolite to reduce adverse side effects while maximizing the mechanisms of the two aluminosilicates (Zhang et al., 2011). Recently applications have loaded zeolite into hydrogel particles with the hopes of combining both biological and mechanical processes of coagulation. Employing the similar intrinsic hemostatic effects, nano-porous bioglass is also being examined as a means of obtaining hemostasis through water absorption. The presence of nanopores increases the surface area, leading to greater water absorption. A benefit of nanoporous bioglass is that it does not cause exothermic reactions (Hu et al., 2012).

Current research includes attempting to reduce the heat of hydration of the reaction to avoid any exothermic reaction by replacing calcium ions in the zeolite

framework with cations of lower standard free energy (Li et al., 2013). Moreover, hydrating zeolite by preloading it with water is another modification aiding in this decrease of an exothermic reaction. These modifications may decrease the harmful effects of zeolite without compromising its efficacy. Kaolin applications are also becoming more common due to the issues that arise from zeolite (Li et al., 2013).

2.2.6 Antifibrinolytics (Intravenous)

Antifibrinolytics are drugs used to inhibit the breakdown of the fibrin clot, or fibrinolysis. The proteolytic enzyme that is primarily responsible for the degradation of the fibrin clot is called plasmin. To function, plasmin activator molecules are required. Antifibrinolytic drugs inhibit plasminogen activators in order to inactivate the fibrinolysis system (Ortmann et al., 2013). This protein mechanism is desirable when there is an imbalance between the coagulation cascade and fibrinolysis. In some individuals, fibrinolysis occurs too readily, making it hard for a stable platelet plug to be formed. In others, coagulation is weak, meaning that all possible fibrin is necessary to form a secure clot. Hemorrhage can occur if fibrin is cleaved under these conditions (Prentice, 1980). Treatments with antifibrinolytics seek to prevent this. In 2010, research done by CRASH-2 coordinators in the UK studied the effect of tranexamic acid, a known antifibrinolytic lysine analogue, on patients in hospitals with greater risk of bleeding. They found that tranexamic acid was able to considerably reduce the risk of death due to bleeding in high-risk patients (CRASH-2

trial collaborators et al., 2010). Other effective agents include aminocaproic acid, another lysine analogue, and aprotinin, a protease inhibitor. These hemostats function by similar inhibitory mechanisms as described above (Mannucci and Levi, 2007). Although effective and widely used in a few specific surgical settings, antifibrinolytics are not useful in all situations, such as traumatic injury or unexpected blood loss in surgery.

2.2.7 Coagulation Proteins

Many natural platelet and factor-related activators have been produced to address hemostasis. The advantages of these types of activators are numerous – they rapidly induce clotting in a localized area, utilize natural pathways resulting in strong clots and are often good methods even for hemophiliacs. However, they have the complications of often being expensive with a short shelf-life. They may also have unwanted thrombogenic effects at distal locations.

Some studies have worked to control or alter bloodstream protein concentrations. These were originally aimed for use in hemophiliac patients. Increasing Factor VIIa was shown to result in quicker thrombin generation, working to convert prothrombin to thrombin at the wound site (Figure 5) (Kjalke et al., 2001). This has been shown to result in increased efficacy in halting traumatic hemorrhage in several wound types, most commonly in joint and muscle bleeds (Young et al.,

2012). In a clinical setting, intravenous FVIIa has been used to treat severe trauma. It has been shown that introducing FVIIa improves the 24-hour odds of survival; however, due to unwanted thrombotic events following treatment there was no significant difference in long-term survival rates (Nascimento et al., 2011). One novel delivery method that allows for safe use in non-hemophilic patients is through nanoparticles, in this case liposomes. These FVIIa nanoparticles were created by attaching FVIIa to the outer membrane of the PEG-liposomes. Liposomes can increase the shelf life of FVIIa and also help to deliver the protein to the targeted site. Since liposomes help to reduce degradation time for FVIIa, the required effective dose for treatment is also reduced, leading to fewer complications and lower costs (Ilinskaya and Dobrovolskaia, 2013a). However, there are drawbacks that make using nanoparticles potentially fatal. Risk of distal thrombotic events is not entirely eliminated, as the nanoparticles still disperse once in the body. Further, when factors are bound onto nanoparticles, the factors could become inactivated or immediately activated; this can lead to inaction or unwanted coagulation, respectively (Ilinskaya and Dobrovolskaia, 2013b).

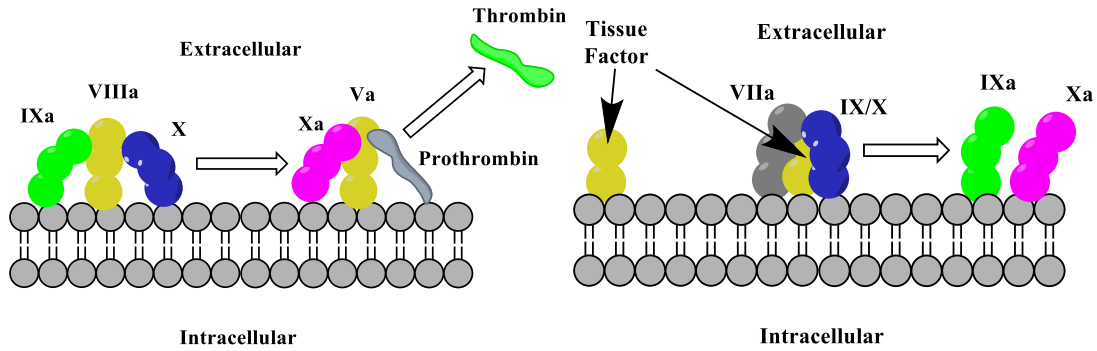


Figure 5: rFVIIa-TF complex formation and thrombin activation. Tissue factors are recruited from circulation and interact with clotting factors to initiate the cascade. Adapted from Karim, 2006 (Karim Brohi, 2006)

Similarly to the FVIIa studies, increasing prothrombin concentrations has also been shown to improve clot strength (Allen et al., 2006). A flaw to each of these studies is that increasing the concentration of a naturally produced protein in vivo could cause complications in non-hemophilic patients. Increasing any of these concentrations can lead to coagulation in more than just the localized wound site as the entire body will be experiencing the change in concentration, with the exception to the targeted PEG-liposomes. In addition, these proteins are highly expensive due to production and purification cost.

Direct topical protein application, such as fibrin sealants and dressings, has proven to be more effective for localized hemostasis. They work by both creating a physical barrier and activating coagulation directly at the application site. Fibrin

sealants utilize fibrinogen, factor XIII, thrombin, calcium, and sometimes fibronectin. Thrombin suspended in calcium is the catalyst, causing fibrinogen to cleave and form fibrin, which can then interact with factor XIII to cross-link into a sturdy clot. Fibronectin is a glycoprotein from the extracellular matrix that plays the role of the primary sealant in this platform. This process mimics the last two stages of the coagulation cascade (Canonico, 2003). These sealants have been shown to become active at the graft site in contact with blood and induce rapid coagulation (Agarwal et al., 2012; Hisagi et al., 2010). Although bovine fibrin can be used, it has disadvantages including a negative immune response by the host as a result of the production of antibodies. Human fibrin, obtained primarily from blood donations, carries a risk for viral transmission. The use of fibrin as a sealant proves to be expensive and therefore inaccessible to a wide range of individuals.

Thrombin has been used separately from fibrinogen to induce coagulation as well. Delivery of thrombin through a sponge is the main method for application used in a surgical setting, but this requires fresh thrombin solution to soak the sponge in (Lew and Weaver, 2008). One method for direct application was the use of poly(D,L-lactide-co-glycolide) 50:50 microspheres to deliver thrombin. The resorbable thrombin-loaded microspheres were delivered by a collagenous sponge and were shown to locally control thrombin release rate at the site of the wound (Smeets et al., 2011). Thrombin introduced with tannic acid in a layered film has also been used as a direct topical application of the protein. Since attaching thrombin to another material

can reduce its effectiveness, Shukla et al. utilized tannic acid in layers to bind to thrombin with hydrogen bonding. This also improved the delivery of thrombin, as the film nearly disintegrates when it comes in contact with blood, releasing large localized amounts of thrombin at once. In comparison to a control sponge without the thrombin film, the time to clot in vivo was reduced by almost half (Shukla et al., 2012). However, thrombin in the can still degrade over time, especially since tannic acid is slightly more acidic* than the biological pH. Furthermore, the overall sponge application method is flawed in that thrombin can 'leak' from the sponge into the bloodstream, leading to embolization (Lew and Weaver, 2008).

Protein	Application Method	Mechanism of Action	Products
Recombinant Factor VIIa (Novo Nordisk Inc., 2006)	Intravenous bolus injection	rFVIIa complexes with tissue factor to activate downstream factors in the coagulation cascade and ultimately accelerate conversion of prothrombin to thrombin	NovoSeven
Fibrin sealants (Spotnitz, 2014)	A fibrinogen/FXIII solution is combined <i>in situ</i> with a thrombin/calcium solution using a dual-syringe	Thrombin suspended in calcium solution catalyzes the cleavage of fibrinogen to form fibrin, which then combines with FXIII to form cross-linked fibrin	Tisseel, Evicel, Crosseal
Thrombin (Achneck et al., 2010)	Direct topical application to wound site using an absorbable gelatin sponge	Directly augments the coagulation cascade by converting fibrinogen to fibrin	Evithrom (human), Thrombin-JMI (bovine), Evithrom (pooled human), Recothrom (recombinant)
Albumin (Kennell et al., n.d.)	A dual-syringe allows a bovine serum albumin solution to mix <i>in situ</i> with a glutaraldehyde solution	Bovine serum albumin is covalently cross-linked by glutaraldehyde to form a mechanical seal	Bioglue

Table 1. Commercially available blood protein-based hemostatic agents.

2.3 MECHANICAL MECHANISMS

Hemostatic agents based on mechanical mechanisms primarily create physical barriers to blood loss and/or matrices in which platelets and biomolecules may accumulate to augment hemostasis and improve wound healing. General adhesives and sealants such as albumin and glutaraldehyde, polyethylene glycol, polyurethane, and cyanoacrylates are physically or chemically cross-linked in the wound site to stop blood loss. Physical matrices and dressings include bone wax, gelatin, self-assembling peptides, and silica-based xerogel materials. Finally, hydrogel polymers offer a versatile alternative with functionalization and modification potential.

2.3.1 General Adhesives and Sealants

Adhesives and sealants are materials that can attach to the surface of a tissue and form a physical barrier. They have important implications in medicine, as they serve as an alternative to sutures and staples and can prevent fatal hemorrhage in surgery. An ideal adhesive/sealant has the properties of high tensile strength, high biodegradability and biocompatibility, high efficacy, and low cost.

ALBUMIN

Albumin is a popular base for adhesives and sealants that utilizes a mixture of purified bovine serum albumin (BSA) derived from blood plasma and glutaraldehyde. Glutaraldehyde chemically cross-links amine groups present on BSA with

extracellular matrix proteins on the target tissue by forming covalent bonds between the tissue and the adhesive (Raanani et al., 2004). The fragile tissue is reinforced with the formation of a covalent bond between the tissue and the glue. Primarily, it allows for the formation of a physical seal in order to facilitate hemostasis and physically block blood loss. This strengthening of the tissue also allows for the subsequent use of sutures in sensitive areas, such as the circulatory system (Coselli et al., 2003). This adhesive is primarily used in cardiac and vascular surgery and is commercially available in the form of BioGlue; however, glutaraldehyde is a toxic chemical and its release from the glue into the body is of concern (Fürst and Banerjee, 2005).

In a prospective randomized clinical study by Coselli et al., the two-component mixture of BSA and glutaraldehyde in the form of BioGlue was tested in anastomotic* repair procedures. It functioned successfully in all but one out of 151 patients, reducing blood loss and operating times (Coselli et al., 2003). Long-term toxicity concerns need to be further evaluated because the covalent interaction between the glue and tissue may cause tissue necrosis (Chao and Torchiana, 2003). Further studies have shown that the use of an albumin based adhesive causes postoperative complications, such as inflammation and bacterial growth (Khan et al., 2011; Klimo et al., 2007). Another disadvantage of this adhesive is that it may provide an ideal environment for bacterial growth, leading to infection. It may also elicit an antigenic response in the body because of the use of bovine septum albumin (Lodi et al., 2012).

POLYETHYLENE GLYCOL

Polyethylene glycol (PEG) derivatives are used in a class of hydrogel sealants that create functional barriers that prevent liquid and air leakage from the wound (Lodi et al., 2012). These polymers contain carboxylic acid groups, which interact with the protein rich groups on the aminated tissue surface. They are primarily used in ophthalmology and cardiovascular surgery. Recent reviews of traditional wound repair techniques have shown that intraoperative pulmonary air leakage is present in over 75% of patients receiving elective pulmonary resection. PEG based hydrogels eliminate liquid leakage and increases the time to the last recorded air leak. Additionally, follow-up studies have demonstrated high biocompatibility and complete reabsorption after 30 days of application.

Coseal is a commercially used wound sealant that uses polyethylene glycol (PEG) hydrogels to stop blood and fluid loss and prevent cell in-growth. High-molecular weight PEG derivatives in two separate syringes are combined as they are applied, and cross-link to seal the wound (Figure 6). As stated above, Coseal is fairly effective; however, it has been shown to have some issues with continued swelling of the product (Achneck et al., 2010). Current research attempts include combining PEG with a silk fibroin in order to stabilize and strengthen the adhesive. When compared to Coseal, the silk-PEG sealant showed decreased tissue swelling and prolonged degradation (Serban et al., 2011).

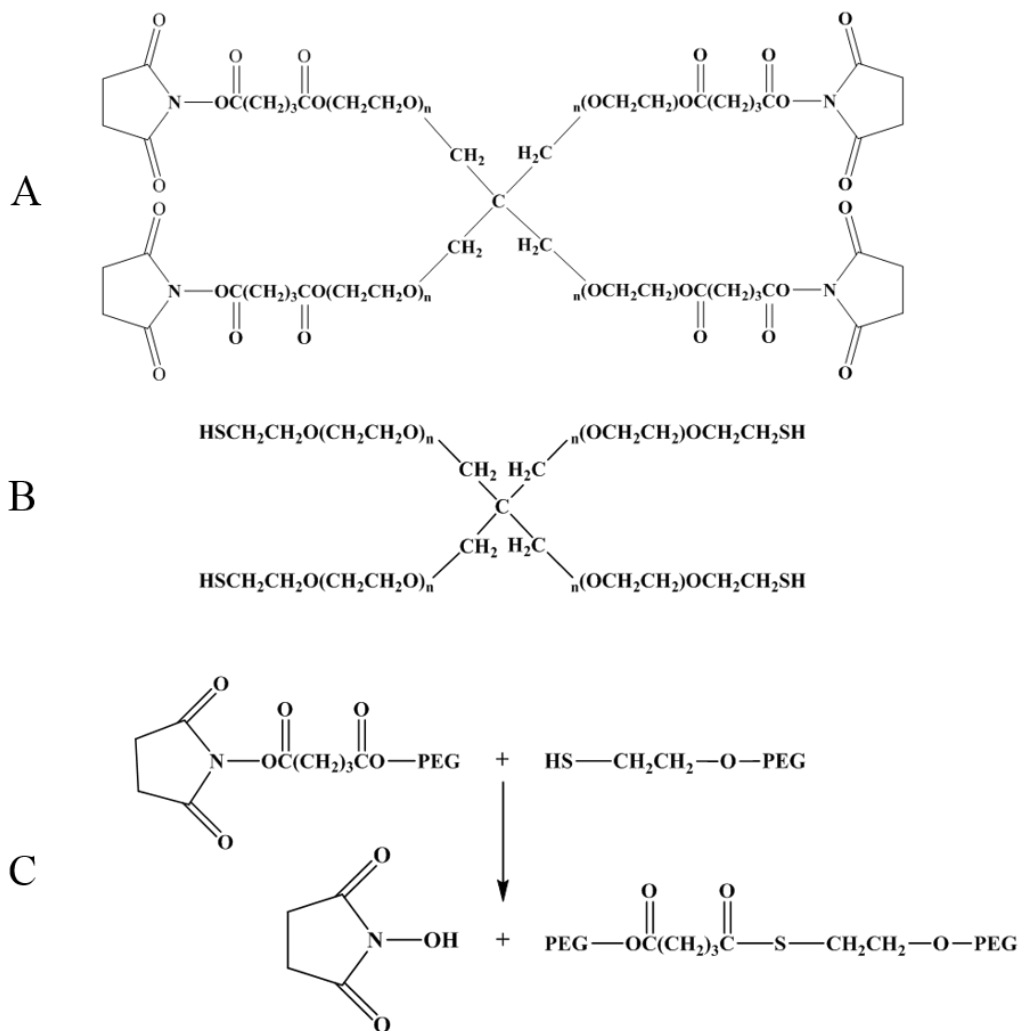


Figure 6: Coseal polymer components and cross-linking. A) The structure of 4S-PEG. B) The structure of 4T-PEG. C) 4S-PEG and 4T-PEG are cross-linked to form the sealant. The thio-ester and carboxy-ester bonds provide flexibility to the created sealant. Adapted from Wallace, 2001 (Wallace et al., 2001).

POLYURETHANE

Polyurethanes are polymers that can be combined with other compounds to form wound sealants and tissue adhesives. They are most often used to make foams or other viscoelastic* materials. Polyurethane Nanosan (R)-Sorb is a commercial hemostat that stopped severe blood loss in a high pressure swine femoral artery wound by rapidly absorbing fluid, concentrating cells and clotting factors in the blood, and physically blocking the wound while increasing mean arterial pressure and decreasing shock index, as compared to a commercial chitosan dressing and standard cotton gauze (Burnett et al., 2013).

In a gel with chitosan, polyurethane can be used as a tissue adhesive to block the wound by utilizing properties of both chitosan and polyurethane. Chitosan forms pores on the surface of the urethane pre-polymer, which acts as an anchor for cells to grow on and regenerate (Habib et al., 2011). A key feature of this adhesive is that it has a lower surface energy than the skin and blood clot that it is designed to adhere to. This makes the adhesion thermodynamically favorable, and increases the efficacy of this material.

Qiu et al., developed a block poly(ester-urethane) based on a bacterial copolyester* and a biodegradable polycaprolactone.* Through platelet counts, SEM, and plasma recalcification times, it was shown that the polymer promotes significant platelet adhesion. Hydrophobicity and rough surface morphology were the properties

that most contributed to this adhesion. Hydrophobicity can be controlled by varying the urethane linkage content, with decreased content leading to more hydrophobicity. This material is noncytotoxic and has strong potential in hemostatic and wound healing applications (Qiu et al., 2013).

2.3.2 Cyanoacrylates

Cyanoacrylates are another class of synthetic adhesives and sealants. They consist of an acrylic resin that rapidly polymerizes in water, forming long, strong chains capable of inducing hemostasis (Lumsden and Heyman, 2006). When these molecules come into contact with water, they undergo an anionic polymerization reaction, in which they link up in chains until they form a durable mesh, physically blocking the wound (Lodi et al., 2012). 2-octyl-cyanoacrylate is a tissue adhesive that assists natural closure of open wounds, usually traumatic lacerations. Marketed as Dermabond, this glue is in clinical use, but has been shown to fail under some high tensile conditions (Shapiro et al., 2001). Cyanoacrylates are cost effective and contain antimicrobial properties, which decrease the risk of infection. In recent clinical trials examining the efficacy and biocompatibility of various sealants, studies have shown that cyanoacrylates biodegrade in a slow and safe manner; however, their mechanism involves an exothermic reaction that may result in tissue necrosis. Therefore, their uses should be limited to topical, clean, and dry wounds (Spotnitz and Burks, 2012). A recent study used micro-textured patches coated with a thin layer of cyanoacrylate

glue as adhesives. This improved the biocompatibility of the cyanoacrylate patches by reducing the direct interaction between the cyanoacrylate and the tissue (Pereira et al., 2014).

2.3.3 Bone Wax

One common substance that is used as a physical barrier to bleeding is bone wax, a combination of Vaseline and beeswax, which is used during surgery to prevent blood flow from damaged vessels in the bone (Schonauer et al., 2004). The wax-like material is spread on the bleeding edge of the bone, blocking the holes and causing immediate bone hemostasis. This approach was examined by Vestergaard et al. who found that bone wax could lead to setbacks in bone healing. Some of the other drawbacks of bone wax include allergic reactions (Schonauer et al., 2004) and increased risk of infection (Gibbs et al., 2004; Vestergaard et al., 2010).

2.3.4 Self-Assembling Peptides

Self-assembling peptides are another physical matrix that can aid in hemostasis. Ellis-Behnke et al. examined a self-assembling peptide that could create a barrier or matrix leading to rapid hemostasis. Some of the benefits of this approach include biodegradability as well as an absence of biological contaminants that may lead to infection (Ellis-Behnke et al., 2006). Ruan et al. made use of amphiphilic* peptides that assemble into a nano-web in which hydrophobic drugs can be stored and

released. The nano-web alone demonstrated rapid hemostatic abilities that far surpassed standard gauze and chitosan bandages (Ruan et al., 2009). Self-assembling peptides offer an alternative to traditional physical barriers and have drug-encapsulating and functionalization capabilities, which make them likely candidates for future research.

2.3.5 Hydrogels

Polymer hydrogels offer a promising platform for hemostatic agents. They are easily synthesized from biocompatible materials and have wide possibilities for both surface and bulk functionalization and modification. Synthetic copolymers, micelles, and *in situ* forming sol-to-gel hydrogels are three recent advances with hemostatic application.

SYNTHETIC HYDROGELS

Synthetic copolymers can be combined in a hydrogel, or a highly absorbent network of polymer chains, to yield greater effectiveness. A research team in Italy combined the three biocompatible synthetic polymers – polyvinyl pyrrolidone (PVP), agar, and polyethylene glycol – to form a hydrogel (Coppa et al., 2012). They mixed the three polymers together with slight heat and sterilized them with gamma irradiation. Applied to dorsal incisions on a mouse model, the PVP-hydrogel adhered firmly to the dermal layer and muscular fascia. Even up to 45 and 90 days after

treatment, the PVP-hydrogel remained intact and did not biodegrade. The PVP hydrogel aided in wound repair and did not cause adverse histological reactions (Coppa et al., 2012). The PVP-hydrogel was biocompatible and aided in wound healing, but remained adhered to the tissue. Ideally, the hydrogel should either degrade or separate from the tissue after its beneficial influence on tissue repair. More research is required to determine if prolonged exposure to a PVP-hydrogel yields harmful effects.

Polymeric micelles have also been used to create hemostatic hydrogels. In particular, this kind of hydrogel may be formed by utilizing aldehyde-terminated poly(ethylene glycol) and poly(D,L-lactide) (PEG-PLA) block polymers. Schiff bases bond together amino and aldehyde groups on the surface of the polymeric micelles (Murakami et al., 2007). The hydrogel was formed *in vitro* after mixing the polymeric micelle solution with the polyallylamine solution. The resulting hydrogel was reported to adhere to tissue and it is known that PEG aids in cell membrane adhesion (Harris, 1992). PLA has also been associated with tissue adherence through its innate characteristic of making biomaterials stable and partially charged (Stolnik et al., 1994). Additionally, the hydrogel is non-cytotoxic and biocompatible because both PEG and PLA are biocompatible (Murakami et al., 2007). The same hydrogel was tested in the liver injury of a mouse model and it was determined that the hydrogel had significant potential as a local hemostat (Murakami et al., 2009).

Another form of hemostatic hydrogel is an *in situ* forming hydrogel. In this case, the hydrogel's components are combined using a specific method and order, and the hydrogel forms in the wound site. A specific *in situ* forming hydrogel of interest in one containing moieties of poly(ethyleneglycol) (PEG), N-hydroxysuccinimide ester (NHS), and poly(allylamine hydrochloride) (PAA). NHS is used to help stabilize and activate the hydrogel by binding with acidic residues, allowing formation of the hydrogel in an alkaline environment, favorable to that of physiological pH. PAA is a cationic electrolyte and has been associated with facilitating platelet aggregation (Doshi et al., 2012). Among the three, PAA formed the hydrogel matrix and as a result, its concentration among the three had the greatest effect on the swelling property of the hydrogel (Peng and Shek, 2009). Separately, the three polymers reduced clot strength as observed through thromboelastography, but in combination as a hydrogel, they enhanced procoagulant effects (Peng and Shek, 2009). The rapid gelation of this hydrogel and its swelling properties characterize it as a possible hemostat, but it requires animal injury models for further study.

POLYSACCHARIDE-BASED HYDROGELS

A group of natural hydrogels that has been studied incorporates diester cross-linked polyglucan hydrogels. These hydrogels utilize amylose, dextran, and pullulan succinates and glutarates in different combinations and structures to yield a variety of hydrogels (Manning and Stark, 1977). These hydrogels are cross-linked using the free acid of mono-ester succinates or glutarates with unreacted hydroxyl functional groups

of adjacent polyglucan molecules. This cross-linking enables separate molecules that would normally be soluble to adapt unique properties. Some qualities that polyglucan hydrogels possess are adherence to wounds, bioabsorption, lack of irritation, and lack of toxic tissue effects (Manning and Stark, 1977). Specifically, dextran decreases time to clot by increasing the fibrin polymerization rate (Rampling et al., 1976). However, polyglucan hydrogels have not seen much traction among research due to a few prevailing issues. These hydrogels, although they do adhere to tissue, fail to do so efficiently for bleeding tissue, greatly reducing their efficacy (Manning and Stark, 1977). They also are only effective in their acidic form, which actually delays healing and induces an inflammatory response. Polyglucan hydrogels also have been shown to lack uniform composition, resulting in a leftover residue after application, causing tissue inflammation (Manning and Stark, 1977). Polyglucan hydrogels are not ideal for hemostatic purposes, but they were a step in the right direction early on in hemostatic hydrogel research.

Dextran-based biomaterials have also demonstrated hemostatic applications. Oxidized dextran can be polymerized *in situ* with amine-containing polymers, such as polyallylamine, oligochitosan, and glycol chitosan to create a surgical sealant with adhesive and swelling properties that vary with the composition of the system (Peng and Shek, 2009). Recently, Mahdavi et al. modified the surface topography of a poly(glycerol-co-sebacate acrylate) elastomer substrate to mimic the nanostructure of highly adhesive gecko feet. A thin layer of oxidized dextran was applied to this

polymer material and *in vitro* and *in vivo* studies demonstrated significant increases in adhesive ability. This platform offers a biodegradable and biocompatible adhesive for wound sealant applications (Mahdavi et al., 2008).

ALGINATE

Alginate* is another natural polysaccharide derived from brown sea algae and is biodegradable into biocompatible structures under normal physiological conditions. It can be converted into a sponge-like hydrogel which provides a moist environment that promotes healing and epidermal regeneration (Kaneda et al., 2008). This beneficial process can be explained by how the cross-linked alginate interacts with blood to produce a hemostatic effect. When alginate is cross-linked with calcium chloride (CaCl_2), alginate becomes calcium alginate (Figure 7).

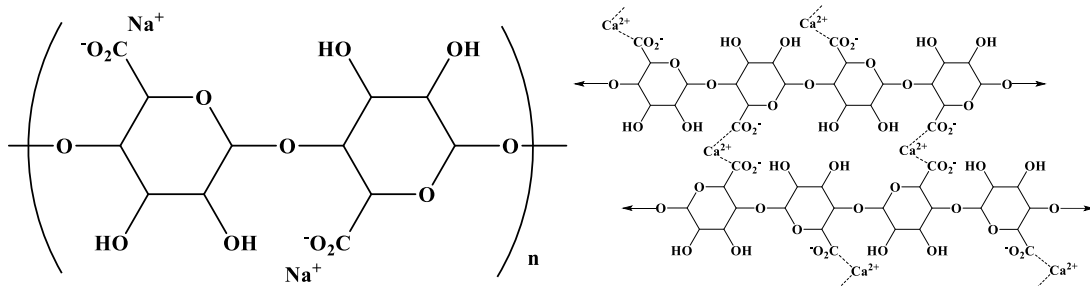


Figure 7: Cross-linking alginate. Sodium alginate (left) (Waldman et al., 1998).

Alginate polymer beginning to cross-link in CaCl_2 solution.

When introduced to blood, the calcium ions dissociate from the calcium alginate and enter the bloodstream where they aid in secondary hemostasis. The porous alginate matrix permits fluid absorbency and swelling which locally concentrates blood proteins and creates a physical barrier to blood loss. The introduction of calcium alginate decreased coagulation time from 35.1 ± 0.3 seconds to 25.7 ± 1.2 seconds (Lee et al., 2012). When used to create gels, naturally occurring polysaccharides such as alginate can be highly porous and biocompatible platforms that can be combined with other materials to create a multi-mechanistic agent.

2.4 CONCLUSION

Research on the clotting mechanism suggests that an ideal hemostat would target both primary and secondary hemostatic mechanisms. Current agents for military, surgical, and emergency use have not been greatly modified or improved in several decades. The most common problems with current hemostats are weak adhesion to tissue, tissue damage from exothermic reactions, material swelling, low absorbance, and therefore platelet aggregation, and high cost (Achneck et al., 2010). An ideal hemostat should offer safe and effective hemorrhage control without any adverse effects. It must also be easily manufactured and stored under various conditions. Recent academic approaches have begun to target both primary and secondary hemostasis and provide physical means to achieve hemostasis. A loaded polymer hydrogel follows this trend in research. Polymer hydrogels created from

naturally-derived chitosan and alginate are promising candidates for producing successful hemostatic agents because they are inexpensive biocompatible polymers with known hemostatic properties and can be loaded with aluminosilicates* or procoagulants to directly promote secondary hemostasis. Naturally-derived gels are more biocompatible than chemically formed hydrogels and are also degradable by the body. Application of this knowledge can potentially lead to more advanced methods of hemorrhage control.

Chapter 3: Research Strategy

Research on the clotting mechanism suggests that an ideal hemostat would target both primary and secondary hemostasis mechanisms. Current agents for military, surgical, and emergency could be improved upon by targeting both primary and secondary hemostasis. In order to target both levels of hemostasis a material must be able to both act as a physical barrier and chemically interact with blood proteins or platelets. A loaded hydrogel satisfies both of these requirements. Common problems with current hemostatics are weak adhesion to tissue, tissue damage from exothermic reactions, non-absorption, and high cost (Achneck et al., 2010). Polymer hydrogels created from chitosan and alginate are promising candidates for producing successful hemostatic agents because they are inexpensive and can be loaded with additional hemostatic materials (González-Rodríguez et al., 2002). Studies have shown that naturally-derived gels are more biocompatible than chemically formed hydrogels and are able to degrade in the body. Application of this knowledge will lead to more advanced methods of hemorrhage control involving polymer hydrogels and existing procoagulants.

Hydrogels possess many characteristics that make them ideal for hemostatic use (Peng and Shek, 2009; Pourjavadi et al., 2009), the first being water absorbance. This characteristic enables the hydrogel to absorb the water present in blood and

swell under physiological conditions. Swelling aids in blood coagulation because a reduction of water at the wound site will concentrate clotting factors, accelerating fibrin formation and platelet activation (Rosa and Casquilho, 2012). In addition, when a hydrogel swells, it takes up more volume than the dry hydrogel. As a result, the swollen hydrogel has increased surface area contact with the wound site and serves as a mechanical barrier (Rosa and Casquilho, 2012). Some hydrogels show promise in both active and non-active pathways. For instance, alginate and chitosan have shown to accelerate hemostasis when combined in a hydrogel (Hattori et al., 2010). Finally, the porosity of a hydrogel allows it to be loaded with procoagulants in order to target other hemostatic mechanisms. Another characteristic of hydrogels is that they may be loaded with procoagulants.

Naturally derived polymer hydrogels are preferred because of their high biocompatibility, biodegradability, and low toxicity byproducts (Slaughter et al., 2009). Alginate is a natural polysaccharide derived from brown sea algae and is biodegradable under normal physiological conditions. It can be converted into a sponge-like gel which provides a moist environment, promoting healing and epidermal regeneration (Kaneda et al., 2008). This beneficial process occurs because the cross-linked alginate interacts with blood to produce a hemostatic effect. Chitosan is another polysaccharide that produces hemostatic effects, although it interacts differently with the blood in order to promote hemostasis, through positively charged amine groups. The positively charged chitosan may then interact with many different

types of negatively charged cell surfaces. Therefore, chitosan is able to strongly adhere to tissue at wound sites via electrostatic interactions.

This project is focused on synthesizing and analyzing polymer hydrogel particles composed of chitosan and alginate, and loaded with the procoagulant zeolite. Chitosan and alginate were chosen because they are both biocompatible polysaccharides and have demonstrated hemostatic properties (Lee et al., 2012). Zeolite is water-absorbent and will help concentrate clotting factors and accelerate fibrin formation (Jiang et al., 2010; Ostomel et al., 2007). The size of the particles will be controlled in order to avoid being passed into the bloodstream.

In order to investigate and answer the research question, two major aims have been identified. The first aim is to synthesize and characterize the polymer hydrogel particles. The second aim is to assess the effect of the hydrogel microparticles *in vitro*.

Chapter 4: Methodology

4.1 AIM 1: SYNTHESIS AND CHARACTERIZATION

The first aim of this project was to synthesize and characterize the chemical, mechanical, and physical properties of zeolite-loaded hydrogel particles composed of alginate and chitosan. To achieve this, the following studies were completed: synthesis optimization, particle size analysis, Fourier Transform Infrared Spectroscopy analysis of particle chemical composition, swelling, and Scanning Electron Microscopy.

4.1.1 Hydrogel Particle Synthesis

Hydrogel beads were prepared using a previously reported calcium induced gelation with slight modification from a previous procedure (Bajpai and Tankhiwale, 2006). Briefly, a 10 mL plastic syringe with a 22.5 gauge needle was filled with 10 mL of alginate solution with or without zeolite. An automated KDS100 Infusion Pump was positioned vertically with the inverted alginate-loaded syringe positioned 12 cm over a 150 mL glass beaker. The glass beaker contained 80 mL of a solution containing 1% (wt/vol) chitosan (ACROS Organics, 10% max water, 2% max Ash), 1% (wt/vol) CaCl₂, and 1% (wt/vol) acetic acid. The syringe pump flow rate was set to 20 mL/hr for the alginate solution and the chitosan/CaCl₂ solution was stirred with

a magnetic stir bar at 300 rpm. Once the beads were formed, 50 mL of excess chitosan solution was decanted and the beads were placed in plastic 50 mL Falcon tubes and rotated on a Labquake rotator for 1 to 3 days. Beads were then vacuum filtrated to remove the remaining chitosan solution and washed with distilled water before being lyophilized in a Labconco FreeZone Triad Benchtop Freeze Dry System. The beads were pre-frozen at -80°C using liquid nitrogen and then freeze dried in the chamber at -55°C under a 0.02 mBar vacuum.

Different compositions of hydrogel beads were synthesized from varying alginate and zeolite solutions. Unloaded beads were made with 4-6% (wt/vol) alginate (Sigma Aldrich) solutions and dropped into the chitosan solution. Additionally, alginate beads without chitosan, and without chitosan or acid were synthesized. Zeolite loaded beads were formed by mixing zeolite- Na^+ powder (Fisher Scientific) into the prepared alginate solution, with the amount based on a 1:1 or 2:1 mass ratio of zeolite to alginate. The 1:1 or 2:1 zeolite-alginate solution was then dropped into the chitosan solution, and the rest of the procedure was repeated. Synthesized bead compositions include 4-6% alginate, 1% chitosan, lyophilized beads as well as 4% alginate, 1% chitosan, 4% zeolite, lyophilized beads, and 4% alginate, 1% chitosan, 8% zeolite, lyophilized beads. From this point forward, these compositions will be referred to as 4-6% unloaded, 1:1 zeolite-loaded, and 2:1 zeolite-loaded respectively. All beads were lyophilized unless otherwise noted.

4.1.2 Particle Sizing

4% unloaded and 1:1 zeolite-loaded beads were photographed using a microscope. A scale was added to the photographs and ImageJ was used to calculate the size of the beads in micrometers. Images of approximately 25-30 beads of each composition were taken and sorted into 100 micrometer ranges.

4.1.3 Fourier Transform Infrared Spectroscopy

A Fourier transform infrared (FTIR) spectrum was obtained for each bead composition. The spectrum was obtained on a Thermo Nicolet NEXUS 670 FTIR using a range of 500-1400 cm^{-1} .

4.1.4 Swelling

200 mg of 4% unloaded beads were measured and added into pre-weighed 5 mL glass vials. Each trial was conducted in triplicate. 2 mL of 1X phosphate buffered saline (PBS) was added to each of the three vials. The vials were immediately placed on a vortex mixer for 10 seconds to break up aggregates, transferred to a Labquake rotator, and rotated until a designated time point of 5, 10, 30, 60, 120, or 180 minutes was reached. The excess PBS was then removed, the final weight of the vial was

recorded, and the final bead weight was calculated. The change in bead mass was computed, and the percent change in mass was calculated using the equation below.

$$\% \text{ Change in Mass} = \left(\frac{\text{Final Total Weight} - \text{Initial Total Weight}}{\text{Initial Total Weight}} \right) * 100$$

This procedure was repeated with 5% unloaded, 6% unloaded and zeolite alone. Of the varying compositions, 4% unloaded had the highest rate of swelling, so they were then loaded with a 1:1 or 2:1 mass ratio of zeolite to alginate. These beads were tested at time points of 5, 10, 30, and 60 minutes using the above procedure.

4.1.5 Scanning Electron Microscopy

Scanning electron microscopy (SEM) imaging was conducted using various compositions of the previously described hydrogel beads. Beads were cut in half using a razor blade. The beads were transferred to carbon tape with their core facing up. Each piece of tape was placed into a dish for carbon coating. After carbon coating, images were taken with a Hitachi SU-70 Schottky field emission gun scanning electron microscope. The microscope was used with accelerating voltages ranging from 5 to 10 kV and a working distance of approximately 16.0 mm, in conjunction with a Bruker's silicon drift detector (SDD). This procedure was used for 4% unloaded, desiccated; 4% unloaded; 1:1 zeolite-loaded.

Pore size was quantified using ImageJ with the following procedure. Pore size was quantified using ImageJ with the following procedure. Each image was divided into four quadrants to account for variable brightness, and the scale in ImageJ was set to match that of the SEM image scale bar. Images were analyzed one quadrant at a time. To begin, the image type was changed to an 8-bit image, and the background correction plugin was used with its default settings. Next, the image was sharpened and then made into a binary image. The image was then inverted, such that the dark spots corresponded to the location of pores. Finally, pore area was determined using the particle analysis tool. Pores smaller than $0.002 \mu\text{m}^2$ were neglected as noise. A sample image analysis can be found in Figure A-1 in the Appendix.

4.2 AIM 2: IN VITRO ASSESSMENT OF MATERIAL PROPERTIES

The second aim was to predict hydrogel particle interactions with blood and the human body through in vitro studies of blood coagulation, degradation, and cytotoxicity.

4.2.1 Blood Coagulation

5 mL glass vials containing varying bead or material compositions were tested in triplicate. 150 mg of 1:1 zeolite-loaded beads were added to each of the three vials. These beads were used as the standard volume for the other bead and material compositions tested. Thus, each composition volume was measured by equating the

volume of the 150 mg of 1:1 zeolite-loaded beads with all other compositions. 1 mL of room temperature 20% citrated whole sheep blood (Hemostat) was added to each glass vial, followed by 56.2 microliters of 0.2 M CaCl_2 . Immediately after addition of the CaCl_2 , each vial was capped and examined for coagulation. If coagulation did not occur, each vial was set on a Labquake rotator for 1 minute. After a minute, each vial was removed from the rotator and examined again for coagulation. This procedure was repeated for each vial until coagulation was induced, in which the blood ceased to flow in the vial. This procedure was completed for all bead compositions as well as pure alginate, pure chitosan, pure zeolite- Na^+ , and calcium exchanged zeolite (1:1 and 2:1 zeolite- Ca^{2+}).

4.2.2 Zeolite Exchange

In order to exchange the Na^+ in the zeolite powder with Ca^{2+} , 3.5 g CaCl_2 was mixed with 3.5 mL of acetic acid, 6.38 g zeolite, and 506.5 mL of deionized water. This ratio reflects the aforementioned zeolite-alginate solution. Another exchange was completed by mixing the same amount of CaCl_2 , acetic acid, and water, with 12.76 g zeolite. This was used to replicate the earlier mentioned 2:1 zeolite-alginate solution. After creating these two solutions, each mixture was vacuum filtered to produce a zeolite paste. This paste was put into a vacuum oven at 70 °C for 24 hours and removed in powder form.

4.2.3 Blood SEM

100 mg of 4% unloaded beads were placed into a 5 mL glass vial. 1 mL of fresh human whole blood was added to the vial. The blood was collected in three 2.7 mL citrate tubes at the University of Maryland Health Center in concordance with IRB [400316-3] “Zeolite Loaded Chitosan and Alginate Microparticles Designed for Hemostatic Use.” After the vial was filled with blood, it was set on the Labquake rotator. After 5 minutes, the sample was removed from the vial and placed in a sample cassette to be washed. The sample was rinsed four times with PBS and then fixed in 25 mL of 0.025 M glutaraldehyde for an hour. The sample was then washed four more times in PBS to remove glutaraldehyde, with the first two washes allowing the sample to sit in the PBS for 2 minutes each. A series of ethanol solutions of increasing concentration were applied to the washed sample. The series consisted of 25%, 50%, 70%, 95% and 100% ethanol. The sample was dried in a desiccator and then followed the same protocol as described in Scanning Electron Microscopy. This procedure was repeated using 4% unloaded without chitosan and 1:1 zeolite-loaded beads. Samples were then imaged through SEM as described previously.

4.2.4 Degradation

Degradation studies were conducted over a 7 day time period. 100 mg of unloaded beads were measured and added into pre-weighed 5 mL glass vials. A 5000 microliter pipette was filled with 5 mL of 1X PBS. The PBS was added to each of the

three vials for the 7 day time point, and incubated at 37^o. After each subsequent 48 hours, a pipette was used to exchange the PBS from the vials in order to provide ample solution for degradation. Each vial was reweighed after 7 days to obtain a swelled weight of the beads. After weighing, each glass vial was covered with a Kim wipe and placed in a desiccator to dry. After 7 additional days, the desiccated beads were once again weighed to obtain a dried weight. Each trial was conducted in triplicate. This process was repeated using 1:1 zeolite-loaded beads.

4.2.5 Cytotoxicity

The sizes of the 4% unloaded, 1:1 zeolite-loaded, and 2:1 zeolite-loaded beads were calculated using dimensional analysis on ImageJ software. Based on the bead sizes, bead confluency calculations were conducted to determine the amount of beads to place in each well of a 24 well plate to obtain a 10-15% confluency.

The human fibroblast L929 cells were plated in media (Dilbecco's Modified Eagle's Medium) onto 24 well plates and returned to an incubator (37 °C) to grow for 48 hours. At 48 hours, 4% unloaded, 1:1 zeolite-loaded, and 2:1 zeolite-loaded beads were sterilized under UV light and added in triplicate to the previously plated cells. Media with cells served as a live control and cells treated with 70% dilution of methanol, for 30 minutes, served as a cytotoxic control. At 72 hours, a MTS

Proliferation Assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay) read at 490nm and a Live/Dead Cell Assay (Live/Dead Cell Imaging Kit, Life Technologies) were completed.

Chapter 5: Results

5.1 AIM 1: SYNTHESIS AND CHARACTERIZATION

Relevant statistically significant data is marked with “***”. Further details about statistical testing can be found in the Appendix.

5.1.1 Particle Sizing

A size histogram was created to compare the distribution of 4% unloaded and 1:1 zeolite-loaded bead sizes (Figure 8). The average 4% unloaded bead size was 1536 ± 96 micrometers and the average 1:1 zeolite-loaded bead size was 1693 ± 85 micrometers. The highest frequency for the 4% unloaded beads is in the 1500-1599 micrometer range and the highest frequency for the 1:1 zeolite-loaded beads is in the 1700-1799 micrometer range. The frequency is the same for the number of beads in the 1600-1699 micrometer range for both 1:1 zeolite-loaded and 4% unloaded compositions. However, the overall frequency for 4% unloaded beads is greatest below 1600 micrometers and the overall frequency for 1:1 zeolite-loaded beads is greatest above 1600 micrometers.

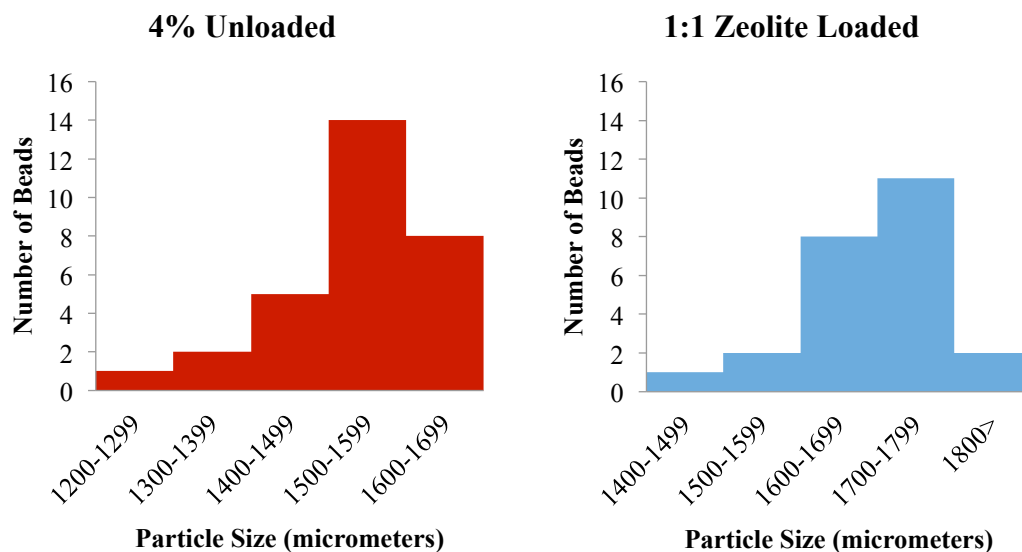


Figure 8: Particle sizing. A) Number of 4% unloaded beads by composition in each 100 micrometer size range based on image analysis (left). B) Number of 1:1 zeolite loaded beads by composition in each 100 micrometer size range based on image analysis (right).

5.1.2 Fourier Transform Infrared Spectroscopy

FTIR spectroscopy was used to confirm the chemical composition of the 1:1 zeolite-loaded bead. As seen in Figure 11, the hydrogel displays absorbance peaks of N-H and O-H (broad band at $\sim 3359\text{ cm}^{-1}$), O-H stretching ($\sim 2927\text{ cm}^{-1}$), C=O ($\sim 1602\text{ cm}^{-1}$), COOH ($\sim 1425\text{ cm}^{-1}$), SiO₄ tetrahedral stretching ($\sim 1033\text{ cm}^{-1}$).

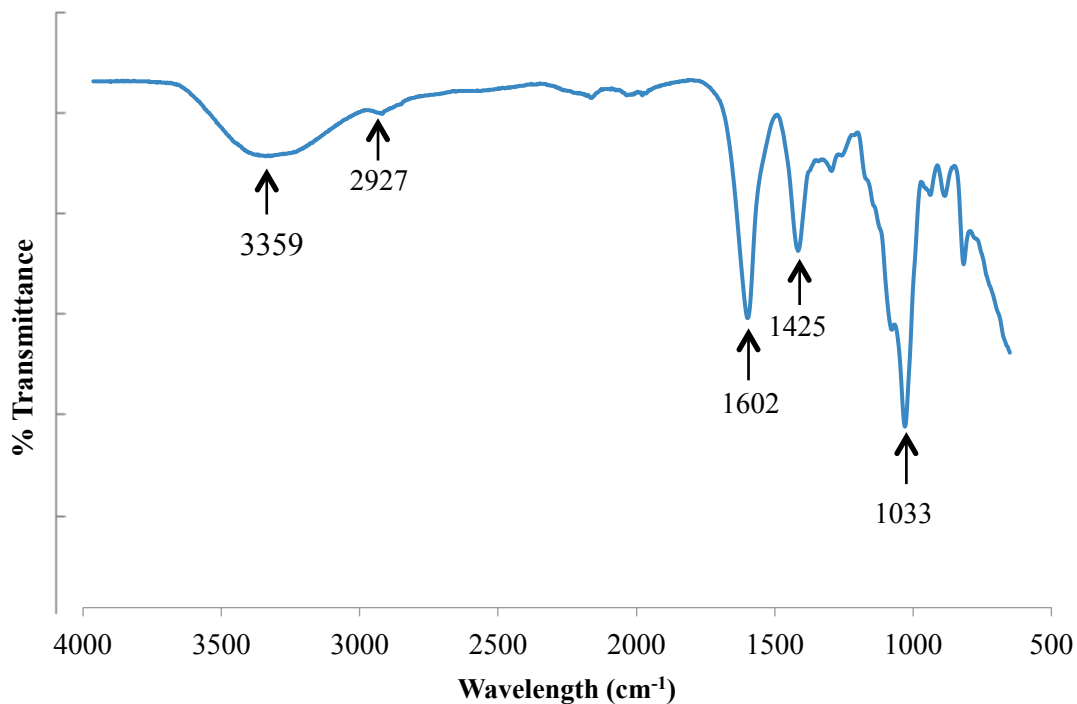


Figure 9: FTIR spectroscopy. Data for 1:1 zeolite-loaded beads.

5.1.3 Swelling

To study the effect of different bead compositions on swelling, the average percent change in mass was plotted over one hour. As shown in Figure 10, the 4% unloaded beads showed the greatest average change in mass, followed by 5% unloaded, and then 6% unloaded. Although not displayed on the graph, it was also observed that the increase in mass began to plateau after one hour.

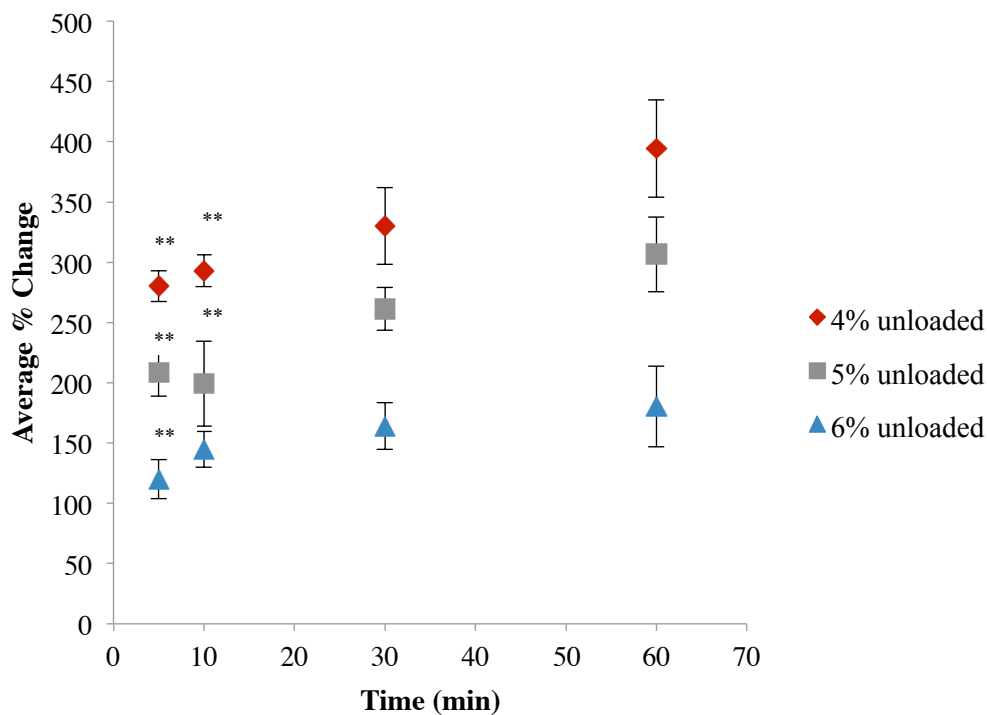


Figure 10: Swelling of unloaded beads. Comparison of the average change in mass of unloaded beads over one hour. Error bars were calculated with standard error, with N ranging from 3 to 6.

The bead composition with the greatest increase in mass, 4% unloaded, was then compared to two compositions of zeolite-loaded beads. As shown in Figure 11, the 1:1 zeolite-loaded beads showed the greatest increase in mass, followed by the 4% unloaded, and then by the 2:1 zeolite-loaded.

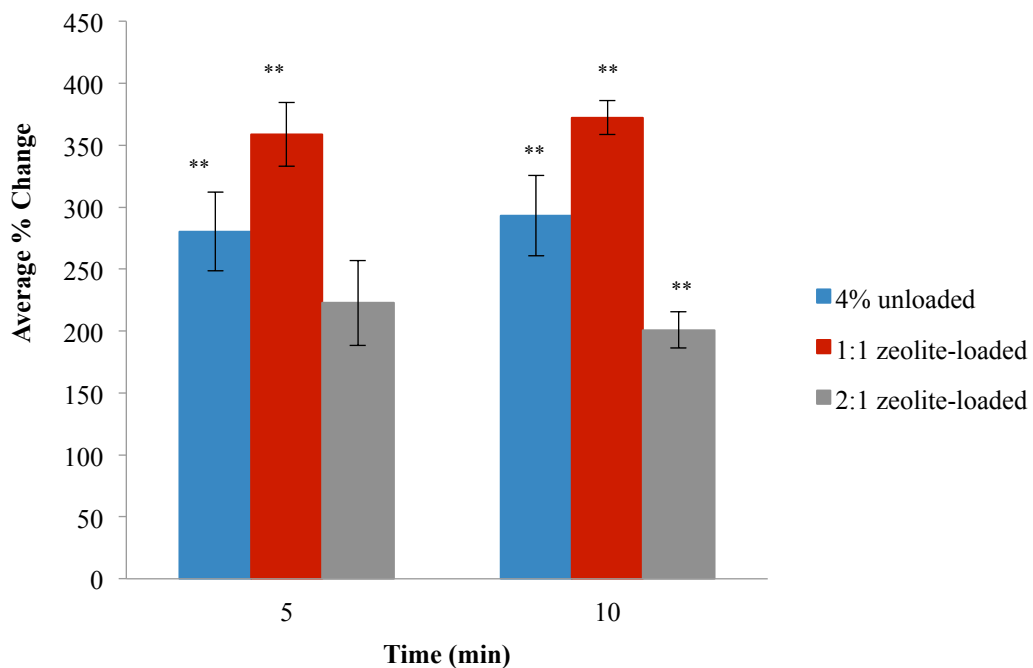


Figure 11: Swelling of loaded beads. Comparison of swelling of unloaded and zeolite-loaded beads at two time points. Error bars were calculated using standard error, with an N of 3.

5.1.4 Scanning Electron Microscopy

SEM images were taken for four bead compositions: 4% unloaded, desiccated; 4% unloaded; 1:1 zeolite-loaded; and 2:1 zeolite-loaded (Figure 12 and 13).

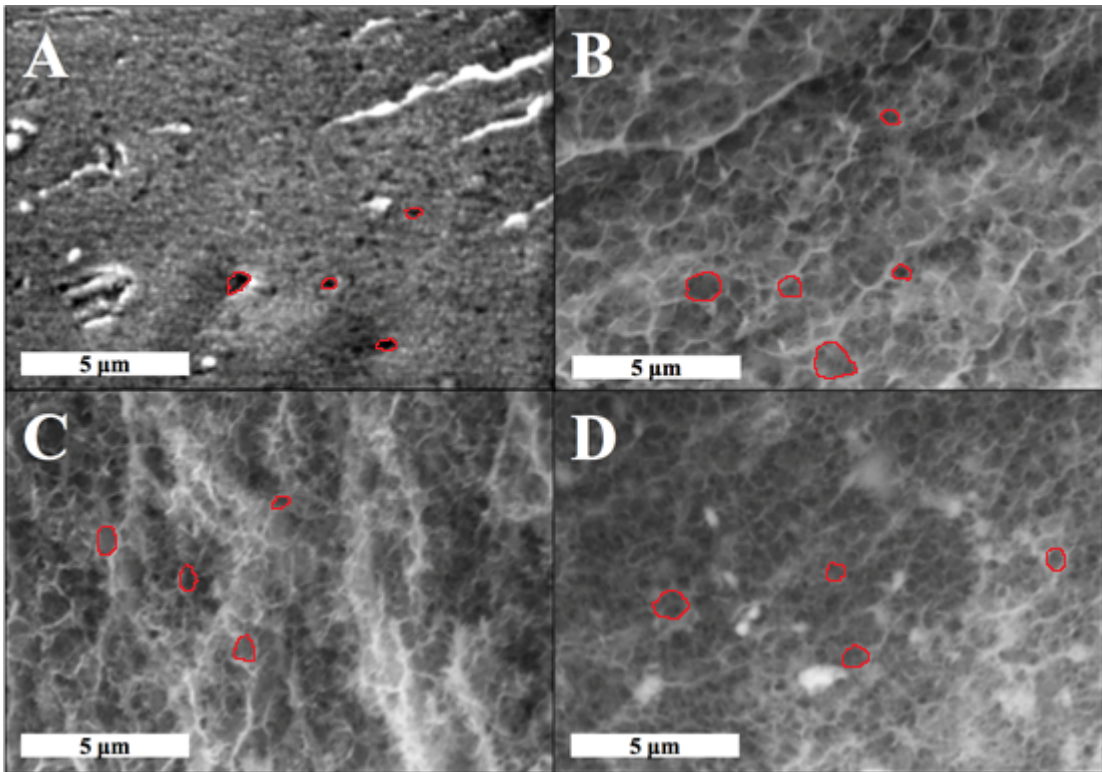


Figure 12: SEM imaging of pores. An SEM image of a A) 4% unloaded, desiccated bead; displaying small pore size, B) 4% unloaded bead, displaying relatively large pore size, C) 1:1 zeolite-loaded bead, displaying a relatively large pore size and, D) 2:1 zeolite-loaded bead, displaying moderate pore size. Example pores have been outlined in red.

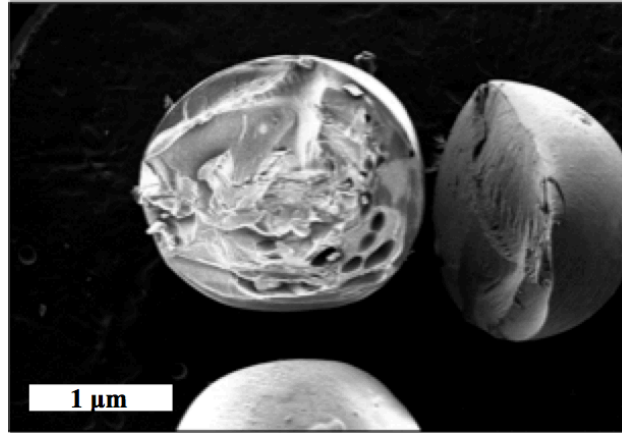


Figure 13: SEM image of a 1:1 zeolite-loaded bead.

Average pore size was quantified using ImageJ, and the results are presented in Figure 14. 4% unloaded desiccated beads displayed the smallest pore size, followed by 2:1 zeolite-loaded beads. 4% unloaded beads and 1:1 zeolite-loaded beads each displayed a larger pore size than 2:1 zeolite-loaded beads.

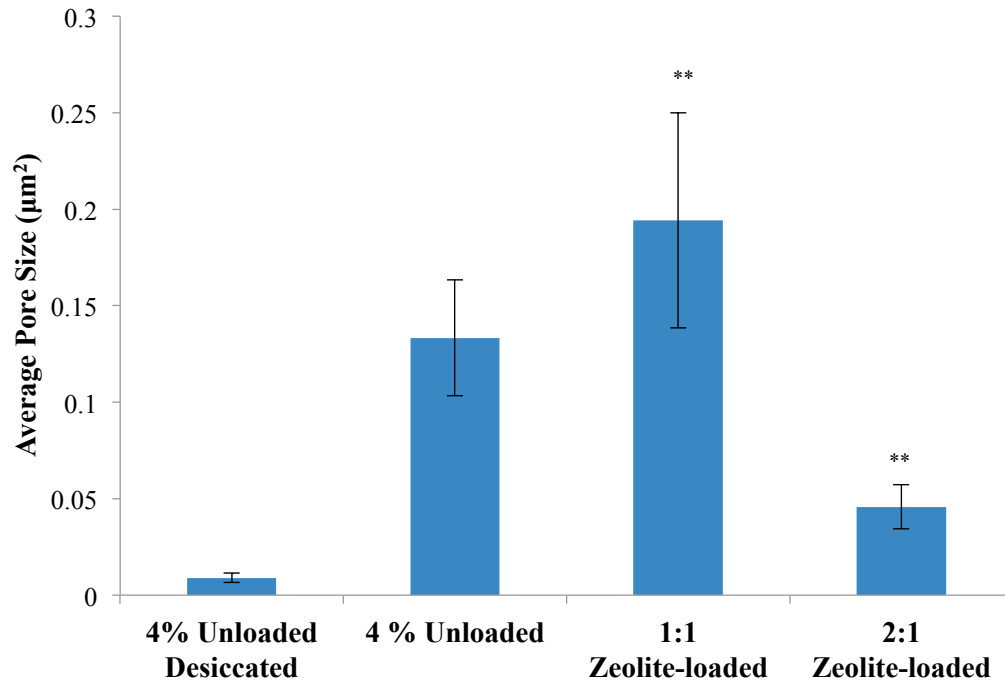


Figure 14: Pore sizing. Average pore size of various bead compositions based on image analysis of SEM images. Error bars were calculated using standard error with N ranging from 85 to 225.

5.2 AIM 2: IN VITRO ASSESSMENT

5.2.1 Blood Coagulation

A blood coagulation study was conducted to compare different bead compositions and their effects on inducing blood coagulation *in vitro*.

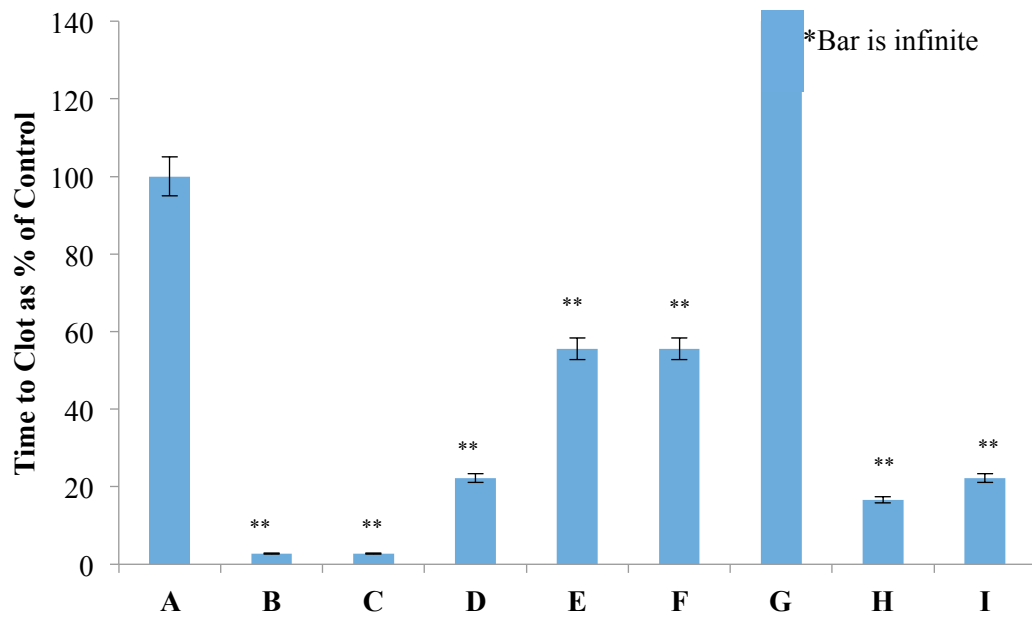


Figure 15: Blood coagulation. Comparison of coagulation times as a percentage of the control of whole blood for five different bead compositions and three different zeolite compositions. A) Control. B) 1:1 zeolite-loaded beads. C) 2:1 zeolite-loaded

beads. D) 4% unloaded beads. E) Beads without chitosan. F) Beads without acid. G) Pure zeolite- Na^+ . H) 1:1 Ca^{2+} exchanged zeolite. I) 2:1 Ca^{2+} exchanged zeolite. Composition G never reached coagulation while all other compositions reached coagulation more rapidly than the control. Error bars represent standard deviation.

As seen in Figure 15, the control of recalcified whole sheep blood reached coagulation at an average of 9 minutes. The 1:1 zeolite-loaded and 2:1 zeolite-loaded beads induced coagulation in under 30 seconds. In comparison, the 4% unloaded beads induced coagulation in under 2 minutes. The 4% unloaded without chitosan reached full coagulation in under 5 minutes and the 4% unloaded without chitosan or acid beads reached full coagulation in under 5 minutes. The vial containing pure zeolite- Na^+ did not induce coagulation, while the 1:1 zeolite-exchanged and 2:1 zeolite-exchanged zeolite- Ca^{2+} reached coagulation in under 1.5 and 2 minutes, respectively.

5.2.2 Blood SEM

After interacting with fresh blood, SEM images were taken of three bead compositions: 4% unloaded without chitosan, 4% unloaded, and 1:1 zeolite-loaded. The following images illustrate the erythrocyte aggregation differences between the different compositions of beads tested. The 1:1 zeolite-loaded bead composition resulted in the greatest erythrocyte aggregation.

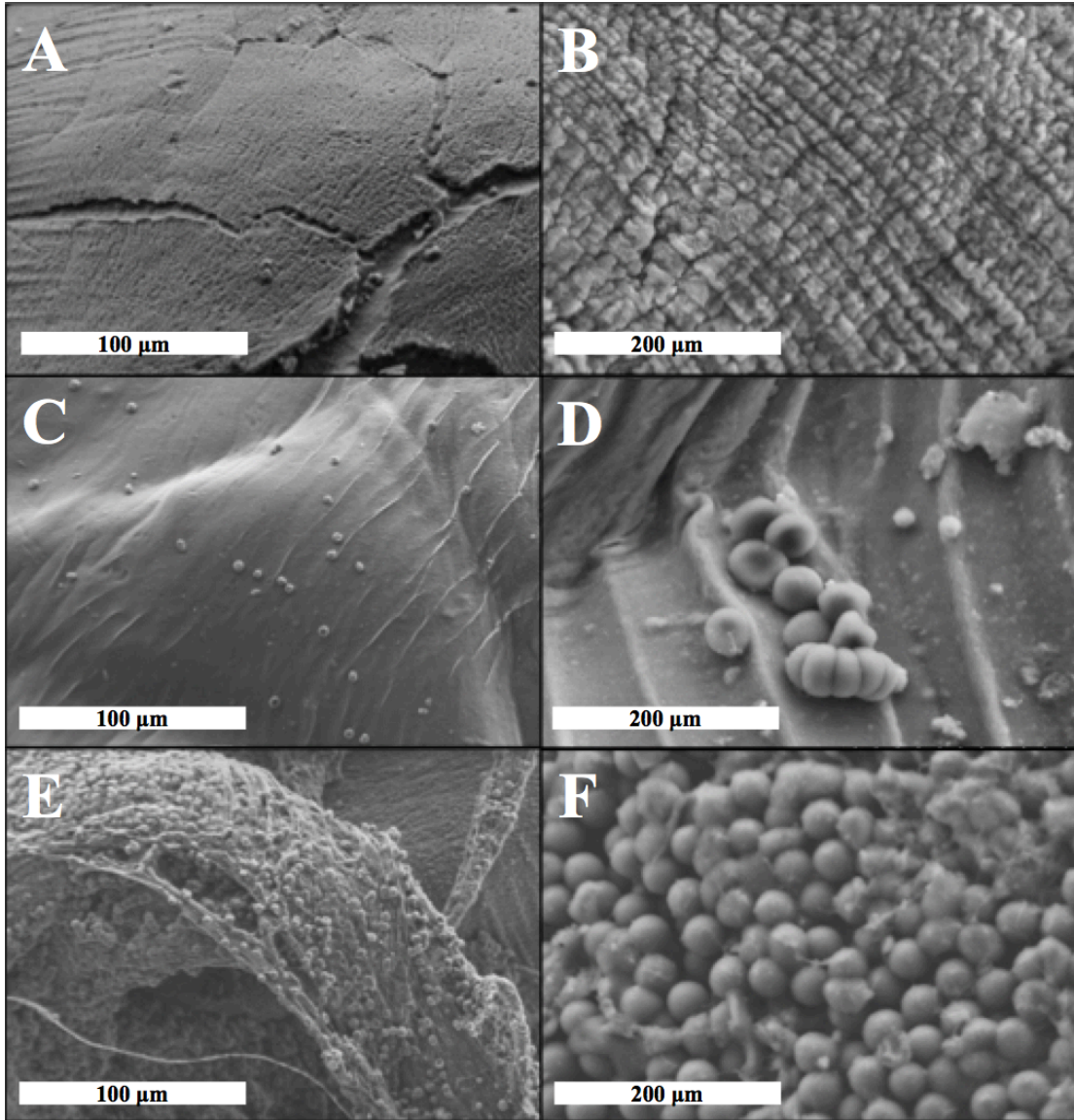


Figure 16: SEM imaging of erythrocyte adhesion. A) An SEM image of a 4% unloaded without chitosan bead, showing minimal cell adhesion. B) An SEM image of a 4% unloaded without chitosan bead, showing a striated surface, but no cell adhesion. C) An SEM image of a 4% unloaded bead, showing a smooth surface and sparse amounts of erythrocyte adhesion. D) An SEM image of a 4% unloaded bead, showing a small cluster of aggregated erythrocytes adhering to the bead's surface. E)

A SEM image of a 1:1 zeolite-loaded bead, showing a blanket of erythrocytes covering the surface of the bead along with the presence of fibrous structures. F) An SEM image of a 1:1 zeolite-loaded bead, showing a layer of erythrocytes covering the bead's surface.

5.2.3 Degradation

Incubation of both unloaded and 1:1 zeolite-loaded beads in 37°C conditions for 7 days yielded an average loss of mass of 12.76% and 68.36%, respectively. Average change of mass was obtained by subtracting the final desiccated mass of each set of beads from the initial lyophilized mass before the addition of 1X PBS.

	Average Percent Change in Mass	Standard Deviation
Unloaded	12.76	3.66
1:1 Zeolite-Loaded	68.36	2.56

Table 2: Average percent change in mass for unloaded and 1:1 zeolite-loaded beads.

5.3.4 Cytotoxicity

Fluorescent microscopy was used to visualize the Live/Dead Cell Assay which showed dead cells as red and live cells as green (Figure 17). The dead control showed the greatest amount of dead cells followed by the 2:1 zeolite-loaded, 4% unloaded, and 1:1 zeolite-loaded, and live control. The 1:1 zeolite-loaded and 4% unloaded bead well plates both demonstrated greater cell viability than the live control. The cell viability of the 1:1 zeolite-loaded beads had 118.9% of the metabolic activity of the live control and the metabolic activity of the 4% unloaded beads had 110.3% of the metabolic activity of the live control. The 2:1 zeolite-loaded beads and plain zeolite well plates had lower metabolic activity than the live control (80.4% and 23.4% respectively), but not as low as the dead control (-4.85%) (Figure 18).

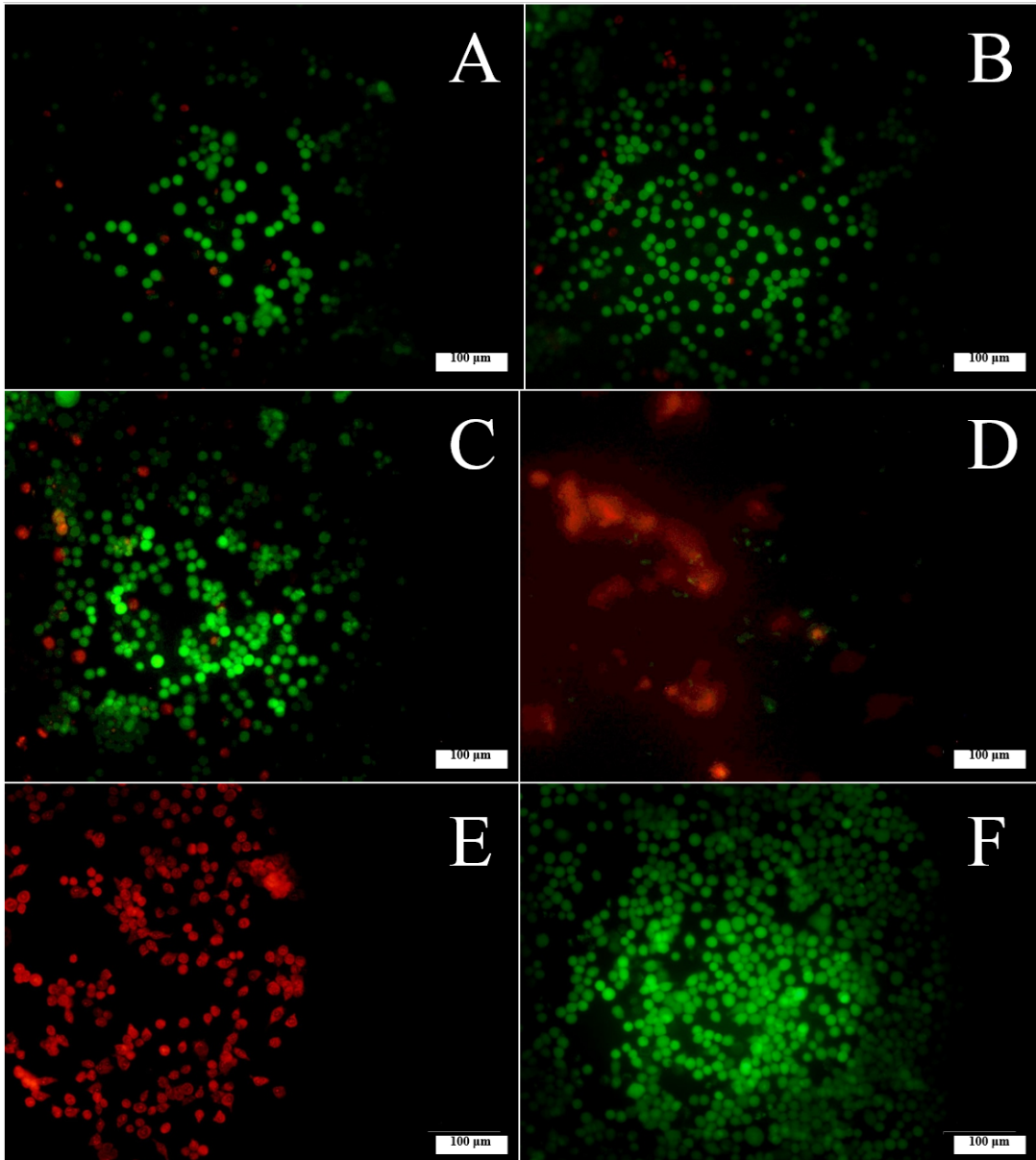


Figure 17: Live/Dead Cell Assay of human fibroblast L929 cells. Dead cells fluoresced red against live cells, which fluoresced green. Cells exposed to A) 1:1 zeolite-loaded beads, B) 2:1 zeolite-loaded beads, C) 4% unloaded beads, D) plain zeolite, E) dead control and, F) live control.

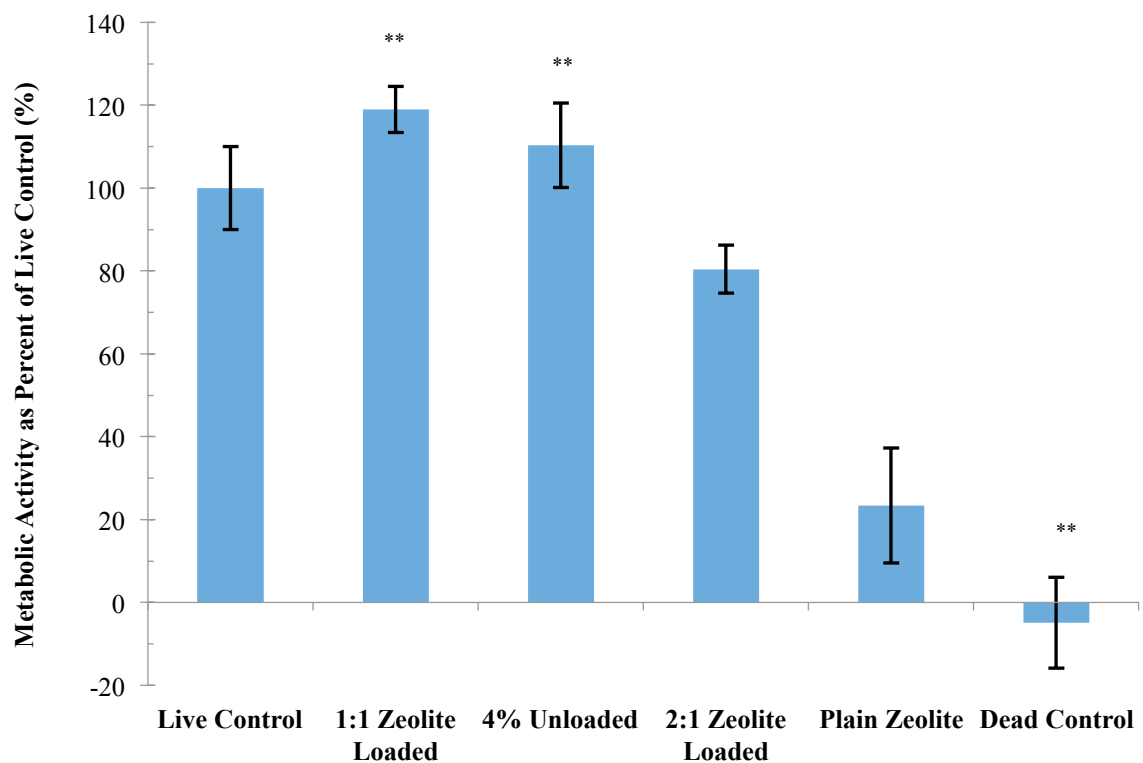


Figure 18: Metabolic activity as percent of live control by material. The percentage of cellular metabolic activity compared to live control as determined by MTS assay. Error bars were calculated using standard error with a sample size of N=3.

Chapter 6: Discussion

6.1 AIM 1: SYNTHESIS AND CHARACTERIZATION

The process of synthesizing the hemostatic hydrogels involved deciding which biomaterials to use, how to integrate them together, how to create replicable beads, and ensuring their structure was maintained through the drying process. Material selection was dependent on biocompatibility, biodegradation, and the ability to induce hemostasis. Chitosan was chosen as a constituent because it has hemostatic characters and is a polysaccharide that can be broken down by the body (Jayakumar et al., 2011) (Figure 3). Exploration of chitosan's wound healing capabilities has shown its hydration abilities, antibacterial activity, and the role of its amine group (Jayakumar et al., 2011). The amine group on chitosan plays a key role in synthesis because it becomes protonated* at a pH under 6.2 and gains a positive charge (de Alvarenga, 2011). The amine group gives chitosan cationic character, allowing it to adhere to wound sites and tissue surfaces with a negative charge (Mercy et al., 2012). This cationic character is also able to interact with other functional groups on molecules, such as the negatively charged carboxyl group on alginate. Alginate was incorporated into the hydrogel because, like chitosan, it is a polysaccharide with characteristics that contribute to hemostasis. Alginate has a unique ability to cross-link with itself in the presence of a divalent cation, such as calcium (Waldman et al., 1998) (Figure 7). This cross-linking property enables alginate to form a porous

matrix, which takes the form of a sphere when an alginate solution is dropped into a cross-linker. Alginate is water absorbent, which causes the hydrogel to swell and serve as a physical barrier to blood loss (Lee et al., 2012). As a result, alginate serves as the framework for the hydrogel itself.

Other biomaterials with hemostatic nature can be incorporated into the alginate sphere to create a multi-mechanistic hemostat. Zeolite was incorporated into the hydrogel for its hemostatic properties and ability to absorb water. As an aluminosilicate, zeolite has a negatively charged framework (Figure 4). This structure enables zeolite to trap many positively charged ions, playing a role in absorbing water (Modery-Pawlowski et al., 2013). This absorption concentrates clotting factors and platelets, accelerating coagulation (Arnaud et al., 2008). Additionally, zeolite helps activate the intrinsic pathway through its negatively charged ions (Ostomel et al., 2006). Together, these three biomaterials contribute to the structure and hemostatic character of the hydrogel. However, they must be combined in a particular way to achieve a desired hydrogel that will induce coagulation.

Combining the three biomaterials of interest, chitosan, alginate, and zeolite, was primarily based on literature. However, synthesis was based on the empirical method of trial and error. With each step, optimization and reproducibility played a key role in determining appropriate modifications to the procedure. A slightly acidic solution of chitosan, acetic acid, and calcium chloride was prepared and placed below

a mechanical syringe pump. The slightly acidic solution allowed chitosan's amine group to become protonated and soluble. The alginate solution was dropped into the chitosan solution via a mechanical syringe pump.

Calcium is used as a cross-linker, rather than aluminum or any other cation, because calcium is a common ion found in the bloodstream. Additionally, its presence aids in the activation of clotting cofactors (Mackman et al., 2007). Throughout the synthesis process, it was essential to produce a near identical procedure each time beads were dropped so reproducibility could be established. In doing so, near identical beads were created for each batch and studies across batches could be compared with one another to draw clear interpretations of the data. This is the main reason why a mechanical syringe pump was used rather than dropping the beads by hand.

Bead compositions were selected based on prior research (Bajpai and Tankhiwale, 2006). The concentration of alginate was initially varied between 4 and 6%. The effect of alginate concentration on swelling was analyzed, and 4% unloaded had the greatest swelling of the unloaded beads (Figure 10). The decrease in swelling with an increase in alginate concentration is most likely caused by the increase in cross-linking. At higher concentrations, a greater amount of cross-linking can occur, therefore allowing for less expansion due to water intake. The 4% beads were chosen for further study.

Zeolite-loaded beads were then synthesized, with zeolite concentrations of 4% and 8%. When zeolite was added, the 1:1 zeolite-loaded beads had the greatest swelling (Figure 11). The addition of zeolite increased swelling even further, because zeolite's porous structure allows it to absorb water. This effect is clear in the 4% unloaded versus the 1:1 zeolite-loaded beads. However, the addition of twice the amount of zeolite in the 2:1 zeolite-loaded beads resulted in less swelling than the 4% unloaded beads. This is likely because the addition of zeolite causes the bead to become denser. The zeolite takes up more room in the bead, so when the bead begins to swell, there is not as much room for water to enter. The addition of some zeolite does increase swelling, as seen in the 1:1 zeolite-loaded beads. However, there seems to be a threshold level of zeolite, after which the bead cannot take in as much water as it did before its addition. The combination of the porous structure and high swelling in the 1:1 zeolite-loaded beads made them desirable for further characterization. FTIR was used to confirm bead composition (Figure 9). The broad absorbance peaks of N-H and O-H at $\sim 3359\text{ cm}^{-1}$ indicate the polyelectrolyte complexes formed between chitosan and alginate. The O-H stretching at $\sim 2927\text{ cm}^{-1}$ and C=O $\sim 1602\text{ cm}^{-1}$ correspond to chitosan and the $\sim 1425\text{ cm}^{-1}$ peak is due to the COOH group in alginate. The $\sim 1033\text{ cm}^{-1}$ peak indicates the SiO_4 tetrahedral structure of zeolite.

After forming the hydrogels, they had to be dried before being stored. Early batches were dried using a desiccator, but lyophilization soon became the preferred

method to dry the hydrogels after obtaining results from a swelling study. Lyophilization removes the water from the hydrogels through sublimation. By drying the beads through lyophilization, voids are left behind in the structure where water used to be. This method works by creating thermodynamic instability and promoting phase separation (Annabi, N. et al., 2011). SEM was used to compare desiccated and lyophilized beads (Figure 12). These images show that the desiccated beads have very small or indistinguishable pores, while the lyophilized beads exhibit a more well-defined pore structure. Quantitative analysis of the SEM images confirmed these observations, with desiccated beads having significantly smaller average pore sizes than lyophilized beads (Figure 14). The differences in pore size observed in each case reflect the differences between the chosen drying methods, with lyophilization preserving the porous structure of the beads. The addition of zeolite was also found to significantly impact the pore size, with the 2:1 zeolite-loaded beads showing smaller pore size than the unloaded and 1:1 zeolite-loaded beads (Figure 12 and 13).

6.2 AIM 2: IN VITRO ASSESSMENT

The different bead compositions were compared using a time to coagulation study (Figure 15). Each vial composition was compared to the initial control and its time to coagulation. The 1:1 zeolite-loaded beads and 2:1 zeolite-loaded beads took the shortest time to induce coagulation. This is most likely due to the combination of the electrostatic properties of chitosan and the porosity induced by zeolite inclusion.

Chitosan's positive charge interacts with the negatively charged cell membranes of erythrocytes, allowing for rapid clot formation (Baldrick, 2010). Moreover, zeolite's ability to absorb water through its porous structure concentrates clotting factors and accelerates fibrin formation and platelet activation. These benefits of both chitosan and zeolite explain why these beads induced coagulation so quickly. As each above component was removed in the order listed, the beads lost an important mechanism required for a more efficient time to coagulation. The pure zeolite- Na^+ failed to induce coagulation due to zeolite's ability to exchange ions, therefore absorbing the free calcium ions originally introduced for recalcification of the blood. The Na^+ ions originally in its structure were exchanged with the Ca^{2+} ions present in the recalcified blood. This exchange removed all of the Ca^{2+} ions present within the blood, making it impossible for the vial to coagulate. However, the 1:1 zeolite-loaded and 2:1 exchanged zeolite- Ca^{2+} -loaded were given the opportunity to exchange their original Na^+ ions with Ca^{2+} ions before being incorporated into the vial, and thus there were still Ca^{2+} ions present in the recalcified blood, allowing both vials to reach full coagulation. This suggests that the 1:1 zeolite-loaded or 2:1 zeolite-loaded beads were also able to reach full coagulation because during the bead synthesis phase, the pure zeolite- Na^+ was able to exchange its Na^+ ions with the Ca^{2+} ions provided by the original chitosan solution containing CaCl_2 . Thus, this allowed for the zeolite-loaded beads to use multiple mechanisms to reach full coagulation in under 30 seconds, as compared to a control of 9 minutes.

SEM imaging was also used to visualize different bead compositions interacting with fresh blood (Figure 16). A lesser amount of erythrocyte aggregation was observed in the bead compositions without zeolite. Particularly, the 4% unloaded bead attracted the fewest number of erythrocytes. There is an observable increase in the amount of erythrocyte adhesion present with the bead composition containing both chitosan and alginate. This is likely due to the positive charge of protonated chitosan, which allows chitosan to interact with biological surfaces and blood proteins that are negatively charged. Furthermore, the bead composition loaded with zeolite, in addition to alginate and chitosan, shows a substantial increase in erythrocyte aggregation. Zeolite naturally forms a truncated octahedron with charged aluminum ions dispersed on the outside of its frame (Modery-Pawlowski et al., 2013). The spatial properties and dispersed charges of zeolite provide a porous platform upon which water can bind. When water binds to the inner space of zeolite, the components of the solution become concentrated (Arnaud et al., 2008). In the case of coagulation, the clotting factors and cells within the blood become concentrated. When the blood is concentrated, erythrocytes are brought into closer interaction with the beads where they can react with the Al^{3+} and Si^{4+} ions on zeolite, and positively charged amine group on chitosan. Negatively charged metal oxides have been shown to reduce clot time by activating the intrinsic pathway of the coagulation cascade (Ostomel et al., 2007). As a result, these processes substantiate the increase in erythrocyte aggregation observed with the zeolite loaded bead composition. Moreover, the zeolite

loaded bead composition shows evidence of the attraction of fibrous structures, presumably the initiation of platelet aggregation.

In order to assess the cell toxicity of the bead compositions, the cell viability of L929 human fibroblast cells was determined using an MTS Proliferation Assay (Figure 18) and Live/Dead Cell Assay (Figure 17). Before performing these assays, the beads were measured in order to perform confluency calculations. The percentage of cellular metabolic activity compared to live control was determined by the MTS Proliferation Assay. Specifically, the MTS Proliferation Assay showed that both the 4% unloaded beads and 1:1 zeolite-loaded beads showed significantly greater cell viability than the live control (110.8% and 119.6%). In addition, the plain zeolite and the 2:1 zeolite-loaded beads had significantly lower cell viability than the live control (24.7 % and 90.0%). The Live/Dead Cell Assay was used as a tool to visualize dead cells, which fluoresced red, against live cells, which fluoresced green. This assay showed that the dead control killed the most cells followed by the 2:1 zeolite-loaded, 4% unloaded, 1:1 zeolite-loaded, and live control. This data directly correlated with the results of the MTS Proliferation Assay. Overall, the cytotoxicity experiment showed that although Zeolite has shown to have toxic side effects, if used in certain amounts alongside Alginate and Chitosan, it can be used as an effective and safe hemostatic agent.

Although the beads are intended to be taken out of the body after inducing hemostasis, it is necessary to gauge the degradation time in order to determine the effects if any beads are left behind. Ideally, degradation should occur eventually, however not before the beads induce their desired outcome of hemostasis. Incubation at 37°C at pH = 7.4 (1X PBS) was chosen in order to mimic physiological conditions. According to the results, there was a significantly greater change in percent mass in the 1:1 zeolite-loaded beads compared to the unloaded beads. This disparity is likely due the porous structures contributed by the addition of zeolite, making the bead degrade easier over time. The appearance of the beads following desiccation provides a further explanation for this disparity, as the 1:1 zeolite-loaded beads were individually separated and round in shape, whereas the unloaded beads were irregular in shape and clumped together. The tendency for the unloaded beads to clump together during the drying process may have also contributed to the significantly lower percent change in mass when compared to the 1:1 zeolite-loaded beads since large portions of the beads may have been shielded from the 1X PBS by decreasing the overall surface area. These results provide additional justification for using 1:1 zeolite-loaded beads, as they are able to degrade under physiological conditions in a timely manner.

Chapter 7: Conclusion

7.1 GENERAL SUMMARY

This project demonstrates the potential of utilizing a polymer hydrogel material made of naturally occurring polysaccharides and loaded with an aluminosilicate procoagulant to induce rapid coagulation of blood. Specifically, hydrogel particles made of chitosan and alginate and loaded with zeolite were synthesized and their chemical, mechanical, and physical properties were characterized. *In vitro* studies were conducted to evaluate the behavior of the particles in blood and their biocompatibility. The goals of these studies were to demonstrate hemostatic efficacy through multiple mechanisms using cheap, biocompatible, and biologically inactive materials.

Zeolite-loaded chitosan-alginate particles were synthesized reproducibly with a narrow size distribution between 1699 to 1799 μm . The chemical composition was confirmed using FTIR. The 1:1 zeolite-loaded particles swelled to 350% of their original mass, which allows for concentration of clotting factors and physical blockage of damaged blood vessels. This mechanical behavior can be explained by the porous nature of the particles. Pore sizes were measured to be 0.19 μm^2 . The

introduction of zeolite also increased maximum swelling capabilities by 75%, as compared to the unloaded particles.

When introduced to blood in a sheep whole blood coagulation study, the 1:1 zeolite-loaded particles induced blood coagulation in under 30 seconds, as compared to 9 minutes for an untreated sample of whole blood. SEM imaging of beads exposed to human whole blood revealed high erythrocyte aggregation on the surface. When chitosan was removed from the bead composition no aggregation occurred, which supports the cell adhesive characteristics of chitosan. Degradation studies showed that 1:1 zeolite-loaded beads degraded significantly more than unloaded beads due to various factors including both increased porosity and surface area. Cell viability was assessed in L929 mouse fibroblasts using an MTS proliferation assay and the 1:1 zeolite-loaded particles induced equal and even greater metabolic activity as compared to a live control. When the amount of zeolite in the beads was increased, cell metabolism fell and when zeolite alone was used cell metabolism reached a minimum. Zeolite has been highly effective as a hemostatic agent in the form of QuikClot but induced secondary cell death. The toxicity study suggests zeolite may be safe to use in moderate amounts.

The material developed in this study offers a potential platform for a safe, cheap, accessible, and effective hemostatic agent for use in treatment of traumatic hemorrhage. It also demonstrates the plausibility of incorporating multiple

mechanisms of action into a hemostatic agent. Further research should be completed to better understand the material mechanisms, the behavior in living physiological systems, and the viability of the material as a commercial product.

7.2 CHALLENGES AND LIMITATIONS

There were many challenges during our research process. One of the greatest obstacles was arranging schedules to conduct tests with long duration requirements. We maintained flexibility and communication with our schedules to ensure the experiments were conducted on a necessary time scale. Subgroups were formed based on availability and interest for the task at hand. Another obstacle was finding additional lab equipment and funding to complete our experiments. We collaborated with other campus laboratories to obtain SEM results, perform lyophilization, and complete cytotoxicity.

Another challenge we encountered was having the ability to replicate results. By standardizing protocols, we created procedures for bead production, bead storage prior to lyophilization, and calcium exchange during the blood coagulation experiment. With each of these examples, we overcame inconsistent or nonreplicable results through trial and error of new protocols. In this fashion, we modified each protocol of each experiment until acceptable and replicable results were obtained.

7.3 RECOMMENDATIONS FOR FUTURE WORK

7.3.1 Understanding Material Mechanisms

The material's specific mechanism of hemostasis can be better understood, rather than inferred, through the identification and quantification of the material's impact on specific primary and secondary hemostatic pathways. Platelet activation studies can help verify the role of chitosan's positively charged amine group on platelet aggregation and adhesion. Alginate's role in calcium release and secondary hemostasis can also be quantified and better described. To assess the material's impact on secondary hemostasis, it is important to measure the concentrations of specific clotting factors and their activated forms as they change in response to the introduction of the particles. This could be completed using ELISA assays, for example. Further tests on blood samples with sequestration of individual coagulation cascade proteins would also help indicate exactly which step(s) in the cascade is accelerated by the hemostat. In order to further study the bead composition, ninhydrin staining will be used to characterize the relative location of the chitosan within the bead. The ninhydrin solution will react with the primary amines in the chitosan, resulting in a purple-red stained bead where chitosan is present.

A cytotoxicity study was completed in this project; however, additional *in vitro* studies with different cell lines could further support the claim that the particles are safe, prior to *in vivo* assessment.

It is also important to quantify clot strength in order to quantify the hemostatic efficacy of the hydrogel particles beyond simply blood coagulation rates. Dynamic Mechanical Analysis (DMA) was investigated as a potentially suitable method to measure the strength of polymer-blood clot disk-shaped composites. Preliminary data for quantification of the strength of a recalcified blood control is provided below (Figure 19). Three samples were tested until 5% strain was achieved, with a compressive force applied at a frequency of 1 Hz. The modulus of elasticity was found from the linear portion of the stress strain curve between 0 and 1% strain, giving an average value of 28.2 Pascals with a standard deviation of 7.53 Pa.

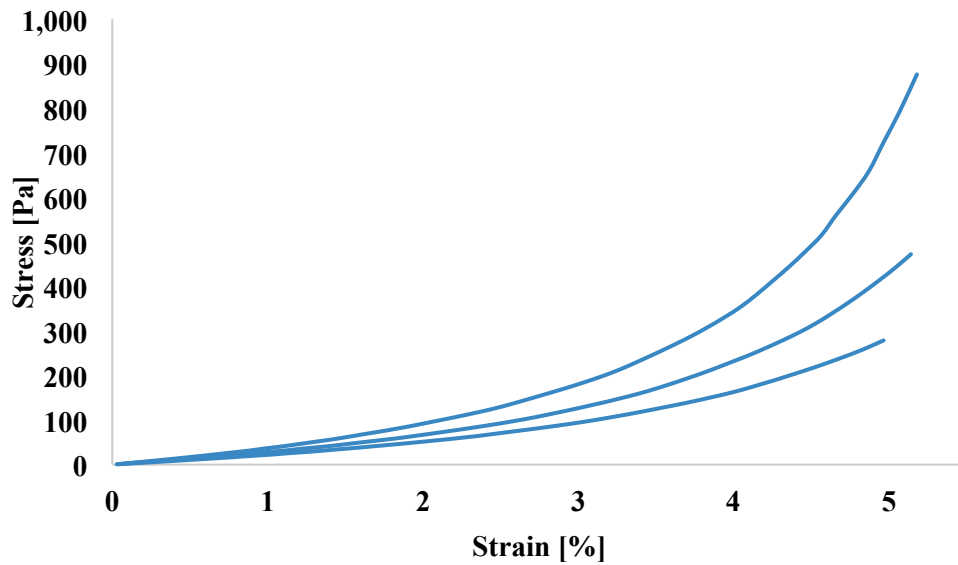


Figure 19: Stress-strain curve for three DMA trials of blood clot control.

Future work could include DMA testing of polymer-blood samples containing a homogeneous distribution of polymer beads. The polymer and blood clot composites should have a greater modulus of elasticity than a blood control, displaying a combination of the mechanical properties of the polymer and the blood. When compared to other clotting products, such as QuikClot, a relative clot strength can be determined.

It is expected that the results of these mechanical characterization and in vitro studies of the composite zeolite-loaded alginate chitosan particles would correlate with the individually described hemostatic abilities of each material component. The separate hemostatic properties of zeolite, chitosan, and alginate should clearly contribute to the overall properties of the material.

7.3.2 Behavior in Living Physiological Environments

The efficacy of the biomaterial in a living physiological environment is necessary and should be investigated through animal models. Rat models are cheapest and most commonly used for initial in vivo tests. Different injury model, such as liver punctures or external lacerations, would provide a physiologically relevant environment for the biomaterial to interact with natural biomolecules. Coagulation rates could confirm the in vitro studies conducted in this project and comparisons with commercially available products. Histological staining of the animal tissue post-

application of the biomaterial could identify the potential immunological responses to a foreign body. Similar degradation studies could be conducted to determine the potential of particle breakdown and dislocation of hydrogel particle pieces, which could result in distal thrombi events. After initial small animal studies are completed, large animal models should be considered prior to commercialization steps are made.

7.3.3 Assessment of Commercial Viability

If the additional data suggested above is gathered and if it supports the hemostatic efficacy and biocompatibility of the hydrogel particles, the commercial viability of the biomaterial should be investigated. Scalability is necessary to ensure that full-scale production is achievable. The synthesis method described in this project would need to be scaled or modified in order to produce enough product to market. A consumer audience would also need to be identified and profiled. This would be aided by understanding the laws and standards of practice in a given jurisdiction and incorporating this information into a marketing strategy. For example, the State of Maryland recently approved the use of “hemostatic impregnated dressings [...] by all providers for hemorrhage control” in its protocol updates effective July 1, 2014 (“The Maryland Medical Protocols for Emergency Medical Services Providers,” 2014). This policy underscores the current need for and use of hemostatic agents, such as the hydrogel particles investigated in this project, for the rapid control of traumatic bleeding.

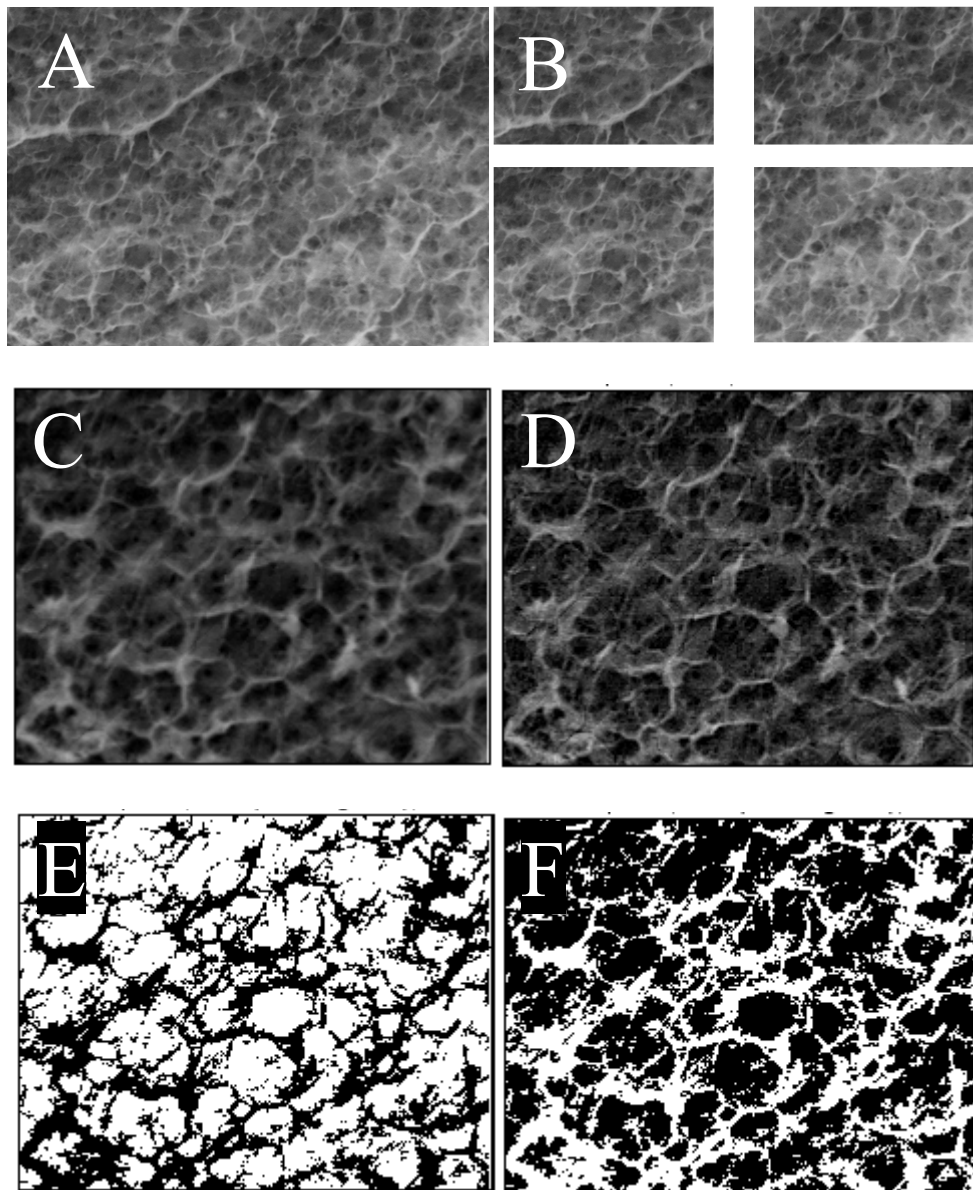
7.4 FINAL WORDS

Team CLOT formed in spring 2012 around the idea to synthesize hydrogel particles for hemostatic (blood clotting) applications in surgical, military, and emergency medical settings. Our team has since experienced every aspect of the research process, from conception of a feasible project and experimental design to data analysis and presentation. Through a comprehensive literature review and peer-driven discussions, our team learned about the biological mechanisms of hemostasis and current approaches of hemostatic agents. Then, we focused on how different polymers interact with platelets and blood proteins to affect clot formation and wound healing. Applying our knowledge of hemostasis, blood-biomaterial interactions, and polymer science, we designed and synthesized novel hydrogel particles from naturally occurring polymers with known hemostatic qualities. We characterized them by composition, size, degradation, swelling, porosity, biocompatibility, and time-to-clot formation. We also present our findings at the Biomedical Engineering Society (BMES) annual conference in October, 2014. We have learned to work closely with individuals with very different educational and cultural backgrounds and we have overcome many obstacles associated with biomedical research, such as narrowing our research topic, acquiring funding through grants, and obtaining IRB approval for experimentation. Most importantly, we set a goal that would take three years to reach and then relied on one another to achieve it.

Appendices

APPENDIX A: SEM IMAGE ANALYSIS

A sample SEM analysis using ImageJ is shown below.



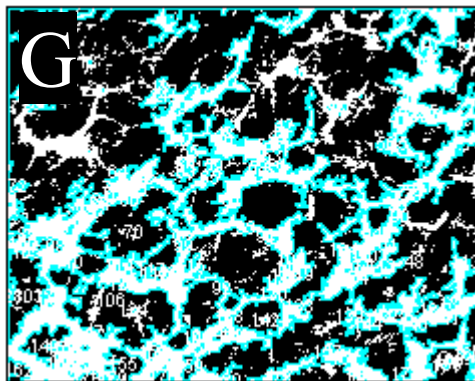


Figure A-1: Sample SEM Image Analysis. A) A sample SEM image prior to analysis B) The sample SEM image divided into four quadrants C) Lower left quadrant of the SEM image after background correction D) Lower left quadrant after sharpening E) Lower left quadrant after conversion to binary image F) Lower left quadrant after image inversion G) Lower left quadrant after particle analysis.

APPENDIX B: STATISTICAL TESTING

Statistical significance was determined using a two-tailed T-test with a significance (α) value of 0.05 or less.

Comparison	Significance	P-Value
4% Unloaded and 1:1 Zeolite-loaded	Significant	5.52735×10^{-8}

Table A-1: T-test results for Particle Sizing

Time Point	Comparison	Significance	P-Value
5 minute	4% Unloaded and 5% Unloaded	Significant	0.013684
	4% Unloaded and 6% Unloaded	Significant	1.64×10^{-5}
	4% Unloaded and 1:1 Zeolite-loaded	Significant	0.010538
	4% Unloaded and 2:1 Zeolite-loaded	Not Significant	0.071353
10 minute	4% Unloaded and 5% Unloaded	Significant	0.046835
	4% Unloaded and 6% Unloaded	Significant	2.25×10^{-5}
	4% Unloaded and 1:1 Zeolite-loaded	Significant	0.001336
	4% Unloaded and 2:1 Zeolite-loaded	Significant	0.000596
30 minute	4% Unloaded and 5% Unloaded	Not Significant	0.094991
	4% Unloaded and 6% Unloaded	Significant	0.002131
	4% Unloaded and 1:1 Zeolite-loaded	Not Significant	0.998576
	4% Unloaded and 2:1 Zeolite-loaded	Significant	0.009013
60 minute	4% Unloaded and 5% Unloaded	Not Significant	0.119352
	4% Unloaded and 6% Unloaded	Significant	0.002255
	4% Unloaded and 1:1 Zeolite-loaded	Significant	0.029962
	4% Unloaded and 2:1 Zeolite-loaded	Significant	0.004057

Table A-2: T-test results for Swelling Studies

Comparison	Significance	P-Value
4% Unloaded and 4% Unloaded Desiccated	Significant	5.2506×10^{-5}
4% Unloaded and 1:1 Zeolite-loaded	Not Significant	0.00971558
4% Unloaded and 2:1 Zeolite-loaded	Significant	0.00684478
1:1 Zeolite-loaded and 2:1 Zeolite-loaded	Significant	0.00971558

Table A-3: T-test results for pore sizing

Comparison	Significance	P-Value
A and B	Significant	5.45×10^{-5}
A and C	Significant	5.66×10^{-5}
A and D	Significant	2.67×10^{-7}

A and E	Significant	0.039726
A and F	Significant	0.002483
A and H	Significant	1.22*10 ⁻⁷
A and I	Significant	2.67*10 ⁻⁷
B and C	Not Significant	0.698489
B and D	Significant	0.002209
B and E	Significant	0.001413
B and F	Significant	0.001465
B and H	Significant	0.002784
B and I	Significant	0.002209
H and I	Significant	0.007895

Table A-4: T-test results for Coagulation studies

Comparison	Significance	P-Value
4% Unloaded and Live Control	Not Significant	0.53815
2:1 Zeolite-loaded and Live Control	Not Significant	0.21432
1:1 Zeolite-loaded and Live Control	Not Significant	0.22282
Pure Zeolite and Live Control	Significant	0.014403
Dead Control and Live Control	Significant	0.002851
4% Unloaded and Dead Control	Significant	0.002058
2:1 Zeolite-loaded and Dead Control	Significant	0.007928
1:1 Zeolite-loaded and Dead Control	Significant	0.002659
Pure Zeolite and Dead Control	Not Significant	0.214091
1:1 Zeolite-loaded and 2:1 Zeolite-loaded	Significant	0.011429

Table A-5: T-test results for Cytotoxicity studies

Glossary

(* indicates “See Glossary”)

Acidic: A substance able to donate protons, releasing and exchanging them upon dissolution in water, effectively lowering the pH below neutral 7 (Alberts, et. al, 2008)

Adenosine diphosphate: a nucleotide produced by ATP hydrolysis composed of adenosine and two phosphate groups, used to store energy (Alberts, et. al, 2008)

Alginate: a polysaccharide salt of alginic acid (Dictionary.com, 2012)

Aluminosilicate: a combination of silicate and aluminate, containing alkali-metal or alkaline-earth metal ions (Dictionary.com, 2012; Merriam-Webster.com, 2012)

Biocompatibility: being compatible with an organ or organism by not causing any harmful reactions or effects (Merriam-Webster.com, 2012)

Biomolecule: an organic molecule that can be found in a living organism (Merriam-Webster.com, 2012)

Biodegradable: the tendency to break apart and become absorbed by the environment (Dash et al., 2011)

Chitosan: a polysaccharide that is derived from Chitin (Dictionary.com, 2012)

Coagulation: the process by which blood clots form (Dictionary.com, 2012)

Cross-link: a connection between neighboring chains that connect to form a large molecule such as a polymer (Merriam-Webster.com, 2012)

Cytotoxic: toxic to cells (Merriam-Webster.com, 2012)

Endothelial matrix: matrix of flattened cells that form an inner lining for all blood and lymphatic vessels (Alberts, et. al, 2008)

Erythrocyte: red blood cell (Alberts, et. al, 2008)

Fibrin: an insoluble protein that is the result of coagulation (Dictionary.com, 2012)

Fibrinogen: a protein that occurs in blood and results in fibrin formation during coagulation. (Dictionary.com, 2012)

Fibrinolytic: see “Fibrinolysis”

Fibrinolysis: the disintegration of fibrin, often caused by enzymes (Dictionary.com, 2012)

Hemorrhage: profuse bleeding from a ruptured blood vessel, often difficult to control (Dictionary.com, 2012)

Hemostasis: the process by which blood loss from a damaged vessel is stopped (Dictionary.com, 2012)

Hemostatic: an agent that induces and/or augments hemostasis* (Dictionary.com, 2012)

Hydrogel: a gel whose liquid constituent is water (Dictionary.com, 2012)

Integrin: a complex protein that can act as a receptor for various proteins, aiding in clot formation (Merriam-Webster.com, 2012)

Ligand: any type of molecule which binds to a specific receptor site on a protein or other molecule (Alberts, et. al, 2008)

Platelet: a small colorless building block of blood that aids in coagulation by sticking to other platelets and to damaged epithelial cells (Merriam-Webster.com, 2012)

Platelet granules: made up the platelets and are secreted during the process of hemostasis; contain ADP and ATP, calcium, serotonin, enzymes, or growth factors, depending on the type of granule (Harrison & Cramer, 1993)

Polymerization: the process in which monomer molecules combine to form a larger polymer molecule (Dictionary.com, 2012)

Polyphosphate: a form of phosphate polymer consisting of a series of condensed phosphoric acids containing more than one atom of phosphorus (Dictionary.com, 2012)

Polysaccharide: a carbohydrate that is made up of two or more monosaccharides (Merriam-Webster.com, 2012)

Primary hemostasis: platelet formation at the site of an injury (Broos, 2011)

Procoagulant: an agent that promotes blood coagulation (Dictionary.com, 2012)

Protonation: to acquire an additional proton (Merriam-Webster.com, 2012)

Secondary hemostasis: the response of coagulation factors in a complex cascade to form fibrin strands, which strengthen the platelet plug (Davie, 1991)

Subendothelial Matrix: matrix of cells that form an outer lining due to endothelial cell retraction (Broos, 2011)

Thrombin: an enzyme of the blood plasma that catalyzes the conversion of fibrinogen to fibrin, the last step of the blood clotting process (Dictionary.com, 2012)

Thrombus: a clot composed of fibrin (Dictionary.com, 2012)

Thrombosis: formation of a blood clot inside of a blood vessel that obstructs blood flow (Merriam-Webster.com, 2012)

Vascular: having to do with vessels that convey bodily fluids such as blood (Dictionary.com, 2012; Merriam-Webster.com, 2012)

Vasoconstriction: the narrowing of the blood vessels resulting from contraction of the muscular wall of the vessels (Dictionary.com, 2012)

Viscoelastic: exhibiting both viscous and elastic behavior when deformed (Dictionary.com, 2012)

Zeolite: any of various natural or synthesized silicates of similar structure used especially in water softening (Merriam-Webster.com, 2012)

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