

ABSTRACT

Title of Document: POLYMERIC MATERIALS FOR
HEMOSTATIC AND SURGICAL SEALANT
APPLICATIONS

Adam Michael Behrens, Ph.D., 2015

Directed By: Professor Peter Kofinas, Fischell Dept. of
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Commercial hemostatic agents and surgical sealants do not meet the current clinical need. The available options suffer from a variety of shortcomings including high costs, short shelf lives, difficult preparations, and concerns over safety. This work aims to utilize synthetic polymers to develop alternative approaches that have the potential to improve outcomes from traumatic injuries and surgeries while minimizing risk and cost.

The first aspect of this research focuses on the development of hemostatic hydrogel particles. These spherical hydrogels with a narrow size distribution were synthesized via inverse suspension polymerization. A cationic monomer was utilized in the hydrogel formulation to facilitate rapid swelling, leading to the formation a physical barrier to blood loss. Coagulation studies demonstrated the ability to cause localized aggregation through charge interactions with erythrocytes while reducing clotting

activity in the bulk. This mechanism allows the hydrogel to quickly block blood flow and may mitigate thrombotic complications at distal sites. Hemostatic efficacy was exhibited by decreases in both the time to hemostasis and mass of blood loss in rat liver puncture and tail amputation injury models when compared to compression with gauze alone.

The second aspect of this research focuses on the development of a synthetic surgical sealant. This work is centered on the investigation of a polymer fiber mat deposition method called solution blow spinning. This fabrication technique allows for the rapid *in situ* generation of polymer fibers, offering the ability to conformally deposit polymeric materials directly on the surgical site of interest. Solution blow spinning was utilized to deposit a body temperature responsive, biodegradable polymer blend. Above a critical temperature, the two phase fibrous polymer mat transitioned into a one phase polymer film. This transition resulted in plasticization and promoted polymer-substrate interaction, leading to increased adhesion. Sealant efficacy was demonstrated in a cecal intestinal anastomosis mouse model, where the polymer blend was used to supplement sutures. Both burst pressure and survival rate were significantly improved over the suture-only control.

POLYMERIC MATERIALS FOR HEMOSTATIC AND SURGICAL SEALANT
APPLICATIONS

By

Adam Michael Behrens

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Advisory Committee:
Professor Peter Kofinas, Chair
Professor Robert Briber
Professor John Fisher
Professor Kimberly Stroka
Professor Christopher Jewell

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Chapter 1: Background and Introduction

1.1 Significance

Uncontrollable bleeding poses significant fatality risks and costs in battlefield, emergency, and hospital settings. In the military, fifty percent of deaths are the result of exsanguination. Eighty percent of these deaths result from non-compressible injuries making it the leading cause of death in military settings.^{1, 2} It is imperative that hemorrhage is controlled immediately to decrease fatality rates as bleed outs can occur within 5-10 minutes.³ Improvements to treatment of penetrating and truncal injury is believed to have the greatest potential impact on decreasing killed in action (KIA) and died of wound (DOW) rates.⁴ In emergency civilian settings, hemorrhage accounts for one third of prehospital deaths, a number that has not decreased in the past 30 years.⁵ In the operating room, surgeries including cardiovascular, hepatic, orthopedic, and spinal procedures have a high incidence of severe blood loss requiring some sort of hemostatic intervention.⁶

Hemostasis is the body's multifaceted response to hemorrhage. In primary hemostasis, an initial platelet plug is formed. When vascular tissue is damaged, platelets activate and release chemical signals that induce aggregation and cause adherence to the subendothelial matrix. Activated surface receptors interact and protein bridges are created between the subendothelium and other activated platelets to form a robust initial hemostatic plug.⁷ Secondary hemostasis, or the coagulation cascade, is divided into two enzymatic pathways, the intrinsic (contact activation) and

extrinsic (tissue factor). These pathways converge and result in the formation of a fibrin clot that strengthens the primary platelet plug (Figure 1.1). The extrinsic pathway begins when trauma to vasculature exposes tissue factor to blood, activating coagulation factor VII (FVII). The resulting formation of the tissue factor-active FVII complex initiates and amplifies the coagulation cascade. The intrinsic pathway activates factor XII upon surface damage resulting in downstream proteolytic activation of other coagulation factors. The two pathways converge into the common pathway upon the activation of factor X (FX). FX cleaves prothrombin into thrombin, which in turn activates fibrinogen into fibrin, reinforcing the platelet plug.^{8,9} In cases of severe or uncontrolled blood loss, the body's natural clotting process is not able to facilitate hemostasis on its own.

The field of hemostatic materials research has lagged behind other medical advances making few major clinical developments. Compression with gauze is still prevalent practice for most injuries and the most common surgical techniques have been in use with minimal modifications for the last decade. The lack of major progress does not reflect a diminishing need. In the most recent comprehensive reviews of topical hemostatic agents,¹⁰ wound sealants,¹¹ surgical hemostats,¹² and hemostatic agents for military and first response applications,¹³ the need for future research and development on improved hemostatic agents and devices is emphasized. Their evaluations of hemostatic agents currently available for commercial use in surgery and traumatic hemorrhage control are categorized in Table 1.1.

An ideal hemostat is safe, effective, easily stored and used, cheap, and capable of regulatory approval.^{12, 14, 15} Many of the materials available at the commercial level fail to meet all of these qualifications.

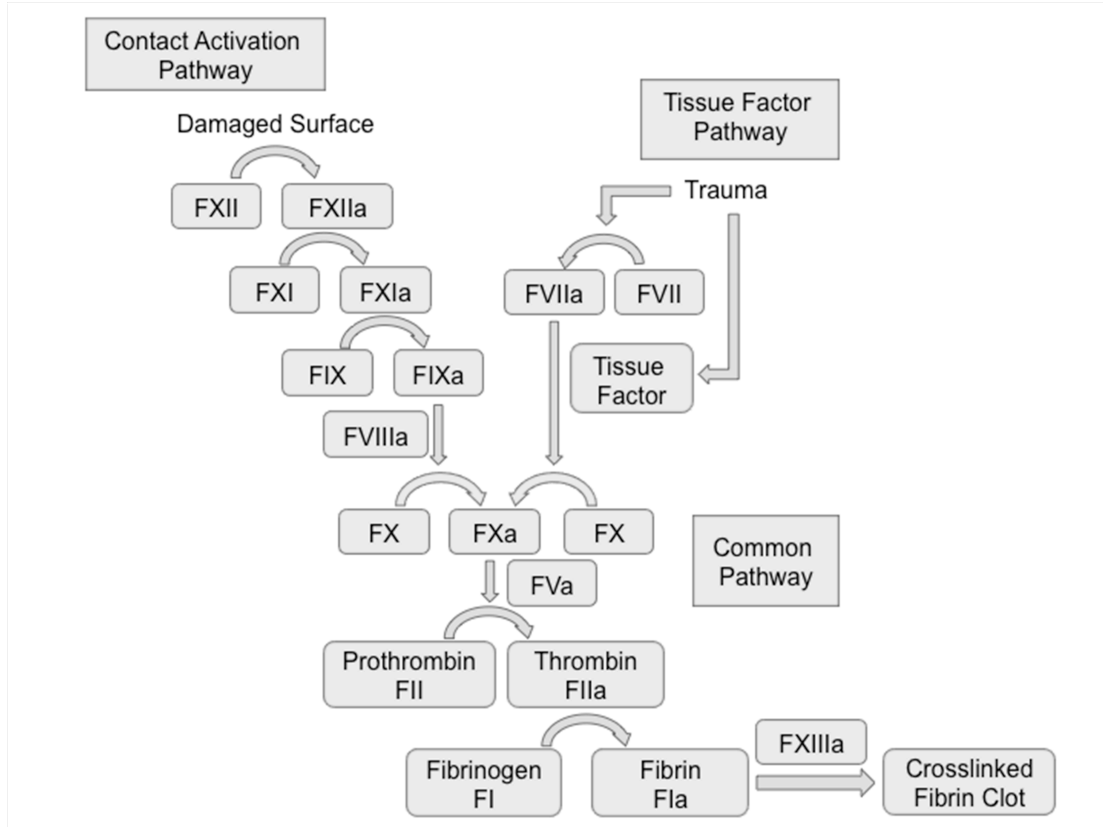


Figure 1.1. Schematic of the coagulation cascade. Activation of the contact activation and/or the tissue factor pathway causes successive proteolytic steps that result in the formation of a crosslinked fibrin clot. Each coagulation factor is represented with a Roman numeral, and active form with the letter a.

While common practice has not changed drastically, there has been significant investigation into new hemostatic approaches at the academic level. These strategies encompass the use of coagulation proteins, *in situ* forming gels, synthetic polymers, and artificial platelets, among others. This section will focus on material platforms, functionalization strategies, and their targeted hemostatic mechanisms. We have

identified three major classes for hemostatic approaches: biologically derived materials, synthetically derived materials, and intravenously administered hemostatic agents. The general class is first discussed, then specific approaches in detail, including the hemostatic mechanisms and the advancement of the method. The categorization of each approach is based on the primary platform; however, many recently developed agents use multiple materials or mechanisms and may tie into several different classes.

Table 1.1. Commercially Available Hemostatic Agents

Categories	Types	Pros	Cons
Physical and Absorbable ^{10, 12}	Bone wax, ostene, gelatin foams, sponges, and powders, oxidized cellulose, microfibrillar collagen, bovine and porcine collagen	Tamponades bone surface bleeding, absorbable, controls small vessel low pressure bleeding	May embolize, prevent bone fusion, reduce structural stability, possible interference with healing process
Biologically Active ^{10-12, 16, 17}	Pooled and recombinant thrombin, thrombin and gelatin, fibrin sealants, platelet gels, albumin and glutaraldehyde	Easily applied, rapid response, effective against mild to moderate bleeding, effective in heparinized patients, broad applications	Immunological response, viral infection, expensive cost per application, short shelf lives, adverse distal thrombotic events
Synthetic sealants ¹⁰⁻¹²	Cyanoacrylates, polyethylene glycol hydrogel	Waterproof barrier, replacement for sutures, full strength within minutes, arterial bleeding	Limited topical usages, dangerous if unreacted, difficult to apply to irregular wounds
Hemostatic Dressings ^{10,11,13}	Dry fibrin, chitin, chitosan, alginate, mineral zeolite, kaolin, smectite	Military and emergency response usage, can stop heavy arterial bleeding, long shelf-life, enhances normal compression treatment, typically inexpensive	High pressure wounds can expel powders, zeolite causes exothermic reaction, success related to responder training, inconsistent results from animal studies

1.2 Biologically Derived Materials

The prevalence of biologically derived hemostatic materials can be attributed to their clear mechanisms of action and efficiency. Naturally occurring proteins and polysaccharides that are commonly used may either have direct roles in hemostasis, such as coagulation factors, or other hemostatic attributes. The latter includes albumin, collagen, gelatin, polypeptides, keratin, chitosan, cellulose, and dextran. Chitosan, cellulose, and dextran are crustacean, plant, and bacteria derived, respectively (Figure 1.2). All of these compounds are isolated and manipulated to create effective and easily administered hemostatic agents.

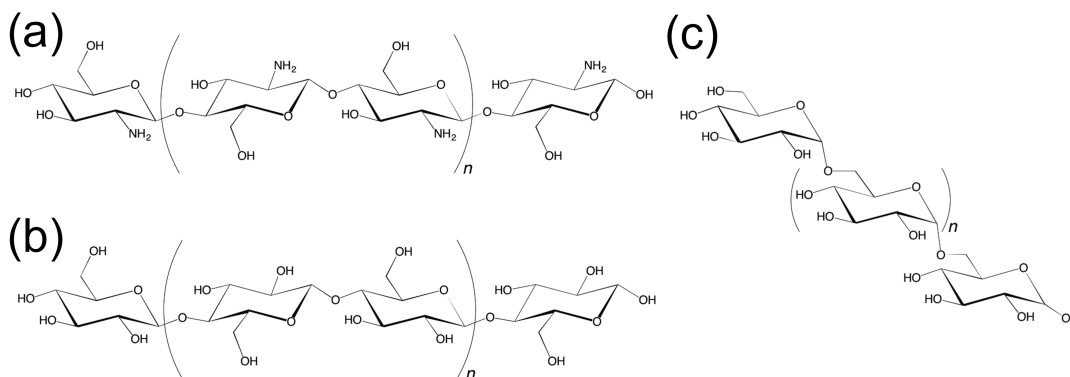


Figure 1.2. Structures of naturally derived polysaccharides: (A) chitosan, (B) cellulose, and (C) dextran.

Coagulation Proteins

Fibrinogen, fibrin, and thrombin have been widely investigated for hemostatic use given their direct role in secondary hemostasis and clot formation. In the final common cascade, thrombin reacts with fibrinogen to create fibrin monomers, which polymerize to form a clot structure. The derivation and concentration techniques for these compounds have changed over time in order to improve efficiency and reduce

risk of contamination with pathogens.¹⁶ Once isolated, fibrinogen and thrombin can be contained in pressure dressings for arterial hemorrhage control¹⁸ and turned into absorbable dry fibrin adhesive bandages.^{16, 19, 20} Fibrinogen has also been electrospun into 3D nanofiber structures²¹ and photochemically crosslinked using ruthenium and persulphate *in vivo* to produce a potentially non-cytotoxic wound sealant.^{22, 23}

Recently, novel delivery systems of these coagulation proteins have been investigated. Smeets et al. developed a sponge made of collagen that contains thrombin-loaded biodegradable microspheres for local thrombin release and postsurgical hemorrhage control.²⁴ Shukla et al. coated active coagulation proteins through layer by layer assembly onto a gelatin sponge with water absorbent properties.²⁵ The successes of these types of agents are largely due to the combination of multiple direct hemostatic mechanisms. None of these approaches have fully addressed the issues of short shelf lives, expensive costs per application, potential risk of viral contamination, adverse thrombotic events at distal sites, and significant manufacturing hurdles.^{16, 17}

Albumin

Albumin is a water-soluble protein derived from blood plasma, often bovine. It has grown in popularity as a commercially available hemostatic agent in the form of a surgical glue. BioGlue® is commonly used in cardiac surgery and forms a strong tissue sealant through chemical crosslinking of bovine serum albumin and

glutaraldehyde.²⁶ While initially effective and relatively affordable, there have been major concerns about glutaraldehyde release causing *in vitro* and *in vivo* toxicity.²⁷ Alternatively, Xie et al. demonstrated the use of concentrated albumin as a surgical glue to enhance the effects of argon beam coagulation in a liver injury model.²⁸ The method improved time to hemostasis and overall surgical outcome, but the method required access to an argon beam and does not mitigate the risk of mammalian disease transmission.

Collagen and Gelatin

Collagen is a compound present in the extracellular matrix of animal cells. Adherence of platelets to collagen fibrils or insoluble collagen microparticles in a vascular tissue wound is an early step in primary hemostasis.^{29, 30} Several proteins such as platelet glycoproteins and von Willebrand factor molecules have been shown to interact and bind with collagen types I and III.^{31, 32} Recombinant human collagen type III induces platelet aggregation, and in turn, hemostatic activity.³³ In lieu of its high cost, collagen's efficacy has led to its incorporation into commercially available hemostatic sponges, pads, bandages, and foams.¹⁰

Gelatin, derived from denatured collagen, has been used in a variety of hemostatic materials. Making use of the high porosity and surface roughness of chemically crosslinked gelatin, Hajosch et al. developed a highly blood absorbent sponge.³⁴ The hemostatic activity in an *in vitro* and *in vivo* model is attributed to the interaction and adhesion of platelets and coagulation proteins with the rough surface of the sponge. In

another study, gelatin, acting in place of a structural protein, was crosslinked *in situ* with calcium-independent microbial transglutaminase enzyme to form a surgical glue that was mimetic of a natural clot.³⁵

Polypeptide

The use of polypeptide hemostatics is attractive because of inherent biocompatibility and the possibility of self-assembly. A poly(L-glutamic acid)-gelatin glue was developed by Otani et al., but it required preheating and a crosslinking step with carbodiimide that may interact with surrounding tissue.^{36, 37} Ruan et al. have used self-assembling complementary amphiphilic peptides that require sonication before application.³⁸ These pre-application steps and the high cost of these approaches limit their potential for future use in a clinical environment.

Self-assembled polypeptides that undergo sol-gel transitions in ionic environments have also been investigated.^{39, 40} These strategies require no pre-application step and are able to immediately induce hemostasis in a variety of rat injury models. Although polypeptide based hemostatics are believed to be biodegradable, no long-term biocompatibility or degradation studies have been investigated for any of the discussed approaches. Additionally, cost and manufacturing issues are not addressed.

Keratin

Keratins are proteins that make up protective structures in vertebrates and are generally extracted from hair, but are also present in epidermal and skeletal tissues.⁴¹ Van Dyke's group has investigated the hemostatic potential of keratin-based materials, utilizing human hair derived hydrogels that cause significant red blood cell aggregation, adhere well to tissue, and have similar survivability rates in a lethal liver transection rabbit model as compared to commercial hemostatic agents Hemcon® and Quikclot®.⁴² Keratin's ability to foster cell adhesion as a ligand and hemostatic ability in a lethal liver transection swine model were also demonstrated.^{43, 44} Further investigation into keratin hydrogel hemostatic mechanisms revealed direct acceleration of the coagulation cascade, but a specific activation mechanism has not been specified leaving safety concerns.⁴⁵ Keratin hydrogels are expected to be biocompatible and biodegradable through macrophage phagocytosis.^{46, 47} However, neither has been reported for hemostatic application, and degradation timescales are not well characterized.

Chitosan

Chitosan, a polysaccharide, is the deacetylated form of chitin and is derived from the exoskeletons of crustaceans. Chitosan forms a coagulum in contact with whole blood arising from its polycationic structure and non-specific binding to cell membranes, leading to wide hemostatic material interest.⁴⁸ Furthermore, chitosan has been shown to be nontoxic and enzymatically degradable.⁴⁹ Benesch et al. found that acetylated chitosan was a strong coagulation activator but did not bind fibrinogen or other

plasma proteins as deacetylated chitosan did.⁵⁰ Yang et al. investigated the differences in chitosan molecular weight and degree of deacetylation in addition to comparing the hemostatic mechanism of solid-state chitosan, chitosan in an acetic acid physiological saline solution, and carboxymethyl chitosan in physiological saline solution.⁵¹ They concluded that solid-state chitosan aided in hemostasis through platelet adsorption, while chitosan in solution was able to cause erythrocyte aggregation. Additionally, they found that molecular weight and deacetylation had significant effects on hemostatic activity.

Whang et al have comprehensively reviewed non-modified chitosan in solution, filament composites, coatings, powder, films, and hydrogels of varying molecular weights, and degrees of deacetylation for hemostatic application.⁵² These material approaches have led to widespread military use in the form of HemCon® and ChitoFlex® dressings.⁵²⁻⁵⁴ While they have shown significant advantages over standard gauze, there have been reported limitations with major injury, and bandage variability.^{55, 56} Despite these shortcomings, chitosan is still a very promising candidate for hemostatic material development leading into investigation into its functionalization (Figure 1.3) and use in composite materials.

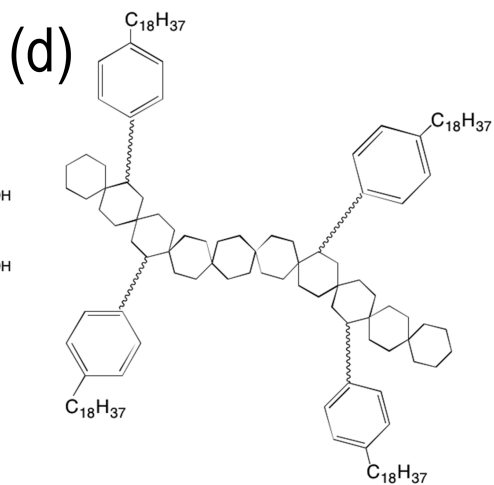
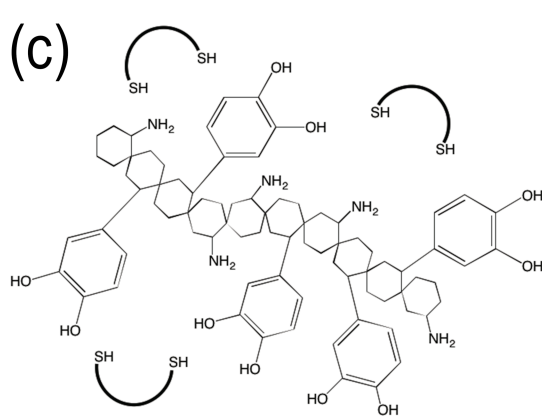
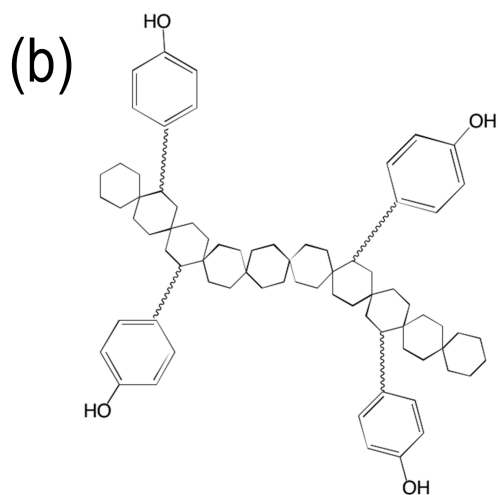
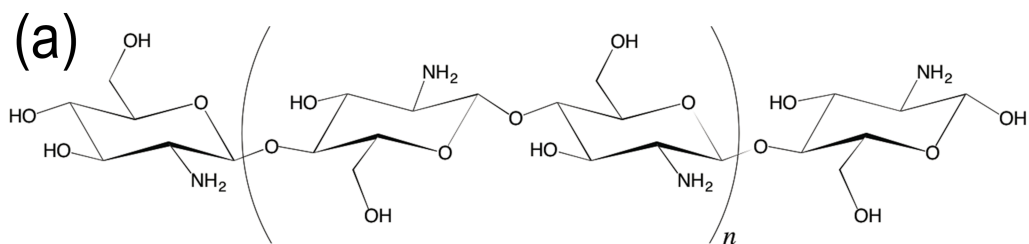


Figure 1.3. Structure of (A) chitosan, (B) PEG-tyramine functionalized chitosan,⁵⁷ (C) catechol-conjugated chitosan,⁵⁸ and (D) hydrophobically modified chitosan.⁵⁹

Ong et al. took steps to improve chitosan-based hemostatics by including polyphosphate and silver nanoparticles with aims to improve hemostatic and antimicrobial properties. Polyphosphate enhances fibrin clot structure, and acts as a procoagulant.⁶⁰⁻⁶² This approach however resulted in *in vitro* fibroblast toxicity,⁶³ and imparted substantial costs. Kumar et al. formed microporous chitosan hydrogel zinc oxide composite to improve platelet aggregation and blood absorbance, and to introduce antibacterial elements.⁶⁴ The material was only tested in wound healing models and increased zinc oxide content led to a decrease in cell viability over 24 hours *in vitro*.

Chitosan modifications have had commendable successes. Dowling et al. hydrophobically modified chitosan to create a “reversible” hemostatic agent with a proposed mechanism of hydrophobic anchoring in red blood cell membranes.⁵⁹ This mechanism was reversible through the introduction of α -cyclodextrin, which contains a hydrophobic pocket. The addition of aliphatic chains is a simple and cost effective addition that drastically improved femoral artery injury outcomes in both rat and swine femoral artery injury models. Chitosan functionalization was further investigated by Ryu et al. who utilized catechol and thiolated pluronic to form thermally sensitive composite hydrogels *in situ*.⁵⁸ This was accomplished by taking advantage of catechol-chitosan and catechol-thiolated pluronic covalent crosslinking upon oxidation. Lih et al. took a similar approach utilizing PEG-tyramine functionalized chitosan with horseradish peroxidase and hydrogen peroxide.⁵⁷ This creates a hydrogel through enzymatic crosslinking upon application, as horseradish

peroxidase catalyzes the conjugation of phenol and aniline derivatives *in situ*. These materials have great potential in minimizing cost, thrombotic complications, and disease transmission risk. Further evaluation of long term storage, especially pertaining to approaches that rely on oxidation or enzymatic reactions are needed to fully evaluate their potential.

Cellulose

Oxidized-cellulose, a water-insoluble derivative of cellulose and an important structural component of plant cells, has been widely used and reviewed for clinical applications.⁶⁵ It is believed to aid in hemostasis through a variety of mechanisms, including calcium and sodium ion interactions, acid induced small vessel contraction, and sealant properties.⁶⁶⁻⁶⁸ To improve on basic cellulose bandages, Wu et al. created a micro-scale gradient structure that utilized cellulose of varying hydrophilicities through different degrees of sodium carboxylate functionalization.⁶⁹ This enabled the material to adopt different hemostatic mechanisms at different stages and led to a two-week degradation profile *in vivo*. Humphreys et al. took a different approach by utilizing microporous cellulose microparticles that act as molecular sieves to concentrate coagulation proteins and showed efficacy on a spleen injury model.⁷⁰ Like chitosan, cellulose has great potential in minimizing cost, thrombotic complication, and disease transmission risks. These approaches should also offer the benefit of a long shelf life. Further investigation into using clinically relevant traumatic bleeding models will allow for the full utility of these approaches to be assessed.

Dextran

Another polysaccharide, dextran, and its derivatives have been recently incorporated into a wide variety of hemostatic materials. Peng et al. developed *in situ* forming hydrogels using oxidized dextran and a variety of primary amine containing polymers, including PAA. Utilizing the Schiff-base reaction (Figure 1.4), the two mixtures polymerize upon application.⁷¹ This material reduced clotting time and improved clot strength as measured using thrombelastography, but hemostatic efficacy was not evaluated *in vivo*.

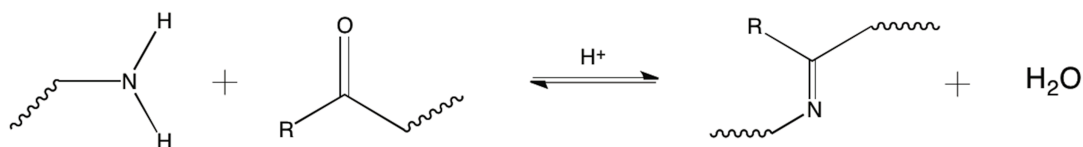


Figure 1.4. Schematic of the Schiff-base reaction utilized for gel formation or tissue adhesion.

Another adhesive material made of a biocompatible elastomer and modified with oxidized dextran was designed with dense nano-pillared morphology resembling gecko feet. This architecture is more explicitly discussed in the biologically inspired tissue adhesives sections. The oxidized dextran coating provides aldehyde functionality, enabling the material to covalently crosslink with the amine groups of proteins in tissue. Chemical crosslinking and optimization of the pillar array structure contributed to strong adhesive and sealant properties both *in vitro* and *in vivo*.⁷² Dextran based materials are promising because of their low cost, shelf stability, and ease of imparting aldehyde functionality. Further investigation into clinically relevant

in vivo injury models is necessary to evaluate the hemostatic potential of dextran-based materials.

1.3 Synthetically Derived Materials

Entirely synthetic materials in various polymeric and mineral forms can be effective hemostatic agents with adhesive, antimicrobial, biocompatible, adsorptive, and biodegradable properties. They can be categorized as biologically mimetic adhesives, *in situ* forming sealants, direct activators and aggregators, and aluminosilicates. Typically, the synthetic sealants and adhesives are not designed for high-pressure uncontrollable hemorrhage but are applicable in surgery, whereas the mineral-based aluminosilicates are designed for rapid arterial hemorrhage control in the field.

A major advantage to these synthetic polymer systems is the reduced infectious risk or allergic reaction associated with protein-containing products.^{71, 73} In a recent review of hemostatic surgical glues and sealants, Lodi et al. emphasizes the importance of synthetic hemostatic agents in surgery and urges research to focus on better understanding their mechanisms and applications.⁷⁴

Biologically Inspired Tissue Adhesives

Temporary tissue adhesives often model natural processes observed in biological systems, such as in geckos and marine mussels.^{72, 75, 76} Geckos have the ability to climb vertically and upside-down due to unique adhesive properties of their feet.⁷² A dense array of nanohairs provides high surface area and strong temporary adhesive

forces in a dry system. Mussels have a powerful ability to attach to wet surfaces through the secretion of a catechol functional amino acid that cause formation of chemical crosslinks through Michael addition with amine and sulfhydryl groups as well as through the Schiff-base reaction (Figure 1.5).⁷⁷⁻⁷⁹ Lee et al. combined these naturally occurring physical and chemical adhesive mechanisms to create a wet/dry biological adhesive with strong potential for medical applications. An organic scaffold composed of nano-pillars was coated with a polymer film based on the mussel adhesive protein.⁷⁵ The requirement of soft lithography techniques may diminish the advantages of this approach.

Mehdizadeh et al. designed and tested injectable citrate-based mussel-inspired bioadhesives (iCMBAs) from citric acid, poly(ethylene glycol) (PEG), and catechol-containing monomers.⁸⁰ The material promotes strong chemical crosslinking between oxidized catechol and primary amine groups in tissue. This synthetic bioadhesive demonstrated instant hemostasis in a rat dorsum incision wound and has potential as a surgical glue. More recently, Barrett et al. demonstrated the general mechanism for chemical crosslinking with controlled swelling and mechanical properties via thermosensitive oxidation of a catechol-modified amphiphilic block copolymer inspired by mussel adhesive proteins.⁸¹ For the catechol functionality to be clinically useful it must be oxidized quickly at the point of injury. This introduces storage issues as well as possible toxicity problems for biologic applications if reducing agents must be present during storage or oxidizing agents must be present for application.

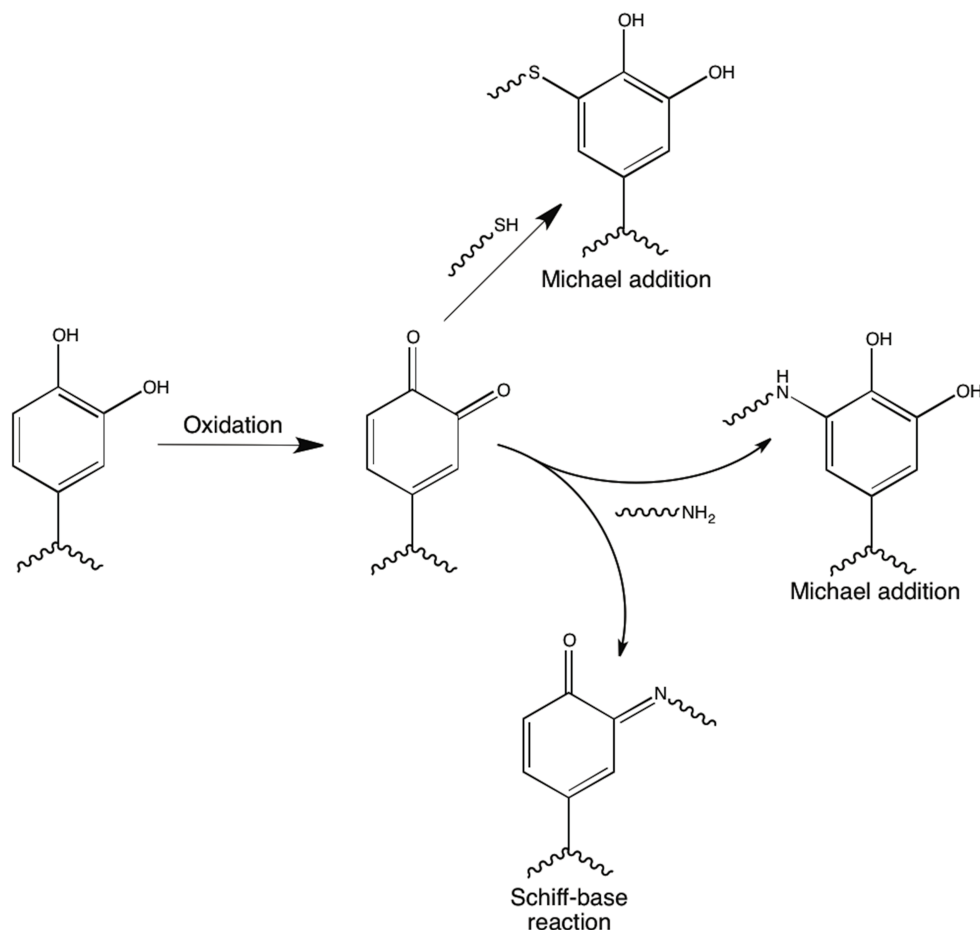


Figure 1.5. Schematic of catechol reactions with thiol and amine containing compounds.

In situ Forming

In situ forming synthetic tissue sealants are materials that transition from a liquid to a solid or gel state through physical or chemical crosslinking in a localized site.⁷¹ Physical crosslinks may be initiated through temperature-responsive gelation⁸² or ionic charge interactions.⁸³ Chemical crosslinking seen in hemostatic materials is achieved through chemical reactions such as photoinitiated polymerization,^{73, 84} the

Schiff-base reaction between primary amines and aldehyde groups,^{85, 86} and reactions between PAA and PEG derivatives.⁷¹

Photoinitiated polymerization can cause gel formation when light irradiation initiates radical activity and subsequent crosslinking between compounds.⁸⁷ PEG-lactide was used in the formation of a biodegradable occlusive barrier and tissue adherent with promising hemostatic qualities. A primer of eosin-PEG-lactide was brushed on a kidney surgical injury site, PEG-lactide macromer was added, and high intensity xenon light was used to initiate photopolymerization of the two networks *in situ*.⁷³ Nivasu et al. demonstrated another approach in which polyesterpolyols were synthesized from succinic acid and PEGs, acrylated, and photopolymerized using a long-wave UV light.⁸⁴ The resulting film has variable mechanical strength, swelling, degradation, tensile strength, and elasticity, which can be optimized by varying PEG content and adding reactive diluents. Cytotoxicity is of concern when choosing the photoinitiator but advances with photopolymerization has demonstrated its potential in biomaterials.⁸⁸ The requirement of a specialized light source increases the difficulty of application for these approaches making them less useful.

The Schiff-base reaction has also been used to chemically crosslink polymers *in situ*. Crosslinkable aldehyde terminated micelle hydrogels made of poly(ethylene glycol)-poly(DL-lactide) (PEG-PLA) mixed with polyallylamine (PAA) induced local hemostasis and tissue adhesion in mice liver models.^{86, 89} The Schiff-base reaction takes place in seconds as covalent bonds form between terminal aldehyde groups on

the micelle surface and amino groups in PAA. PAA was also shown to form a chemically crosslinked hydrogel *in situ* when mixed with multifunctional PEG.⁷¹ Using the Schiff-base reaction to crosslink multiple components typically requires a pre-mixing step or a dual nozzle set-up that diminishes ease of these approaches.

Polymer Activators and Aggregators

More recently, synthetic materials have been designed to target platelet activation and aggregation in primary hemostasis,^{90, 91} clotting factor activation,^{92, 93} and red blood cell aggregation to promote clot formation.^{94, 95}

Platelet activation and aggregation can be indicative of hemostatic potential. Ou et al. synthesized and characterized biodegradable and biocompatible poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) block poly(ester-urethane)s (PU3/4HB).⁹¹ Using SEM and the lactate dehydrogenase (LDH) assay, high platelet adhesive and activation properties of PU3/4HB were demonstrated. Several material properties were identified as key factors for influencing platelet adhesion. These include the degree of crystallinity, hydrophobicity, surface free energy, and charge. The presence of urethane linkages increased the degree of negative charge of the polymer, which is associated with increased platelet activation. Another class of polymers with hemostatic potential is polyacrylate-based polymers for their ease of manipulation and functionalization. Using a fluorescence high-throughput polymer microarray technique, several polyacrylate polymers were identified as platelet activators and aggregators.⁹⁶ Some were shown to selectively bind various proteins including von

Willebrand factor and fibrinogen while others bound proteins such as fibronectin, thereby allowing for interaction with platelets. Hemostatic activity of these polymers is attributed to cationic charge content. Higher availability of non-sterically hindered tertiary amines correlated to significant increases in platelet binding. These polymers have not been assessed for hemostatic efficacy in solution, powder, or gel form.

Electrostatic charge and polyelectrolyte complex (PEC) coatings are also common mechanisms by which many of these synthetic hemostats act. Electrospun amphiphilic and PEC-coated polylactic acid and 2-(Dimethylamino)-ethyl methacrylate (DMAEMA)-based amine containing materials increased red blood cell content and augmented the intrinsic and extrinsic coagulation pathways shown by a decrease in prothrombin and activated partial thromboplastin time in blood plasma.⁹⁴

⁹⁵ Electrostatic charge of the DMAEMA-based functional groups likely dictates the function of these materials. The effect of electrostatic charge on secondary hemostasis was also demonstrated by a primary amine containing synthetic polymer hydrogel that induced FVII activation *in vitro*.⁹² Similar cationic hydrogels exhibited high swelling and hemostatic ability *in vitro* and *in vivo*.^{97, 98} Synthetic polymers with mechanisms that directly target primary and secondary hemostasis are increasingly prevalent features of hemostatic agents. Concerns with these approaches are long-term immunological response, degradation, and toxicity.

Zeolite, Kaolin, and Silicates

Mineral zeolite, kaolin, and other related aluminum silicates have been used commercially since the United States military approved the first generation QuikClot® for field treatment in 2003.⁹⁹ These granular agents are composed of inactive metal oxides, salts, and mineral silicates and are highly porous. Their mechanism of action is thought to be a concentration of clotting factors and platelets in the wound site by rapidly adsorbing water, thereby aiding in clot formation.¹⁰⁰ Due to the mechanism of action, zeolite-based products such as first and second generation QuikClot® were shown to cause a severe exothermic reaction upon water adsorption with temperatures ranging from 44 to 95°C and averaging at 67.4°C.¹⁰¹⁻¹⁰⁸ Secondary tissue burns were also recorded in a series of case studies and these products were discontinued by 2008.¹⁰⁹ Kaolin, an aluminum silicate similar to zeolite but without notable associated exothermic reactions, replaced zeolite in recent commercial products in the form of an impregnated gauze (Combat Gauze®). Kaolin has been shown to activate the intrinsic clotting pathway and is not able to be left in the injury site.^{54, 100}

Biomaterials made with metals oxides have demonstrated hemostatic potential through contact-activated hemostasis due in part to what is known as the glass effect without the damaging exothermic reaction of mineral zeolite. This effect has been attributed to the highly electronegative character of inorganic metal oxides such as silicon dioxide, or silica.¹¹⁰ Inorganic mesoporous bioactive glass microspheres,¹¹¹ mesoporous silica spheres,¹¹² and mesocellular silicate foams,¹¹³ also show hemostatic

abilities by nature of their negative charge and highly absorptive pores. These porous biomaterials have been successfully loaded with calcium ions for release *in situ* to aid in hemostasis and dental or bone reconstruction,¹¹¹ silver exchanged for antibacterial properties,¹¹² and loaded with thrombin for its direct initiation of fibrin formation upon release.¹¹³ Metal oxide based spheres and foams do not induce exothermic reactions and can significantly decrease clotting times, but most of these materials have not yet been shown to be biocompatible or biodegradable.

Gels synthesized from naturally occurring polysaccharides known to be biocompatible can have high porosity and can be combined with other known hemostatic agents to make use of multiple hemostatic mechanisms. Dai et al. combined zeolite with chitosan in a complex synthesis of a xerogel and demonstrated contact activation, high water adsorption, and erythrocyte immobilization.¹¹⁴ *In vitro* cytotoxicity assays showed proliferation and no cell damage. *In vivo* efficacy was established in a rabbit lethal artery injury model. Similarly, zeolite composite hollow microspheres made with various biodegradable polymers including gelatin, chitosan, and alginate and loaded antibiotics were shown to have high water absorption and prolonged drug release.¹¹⁵ It is unclear if any of these particles can be left in the wound site or thrombotic complications are a significant risk.

1.4 Intravenously Administered

Intravenously administered hemostatic agents have gained significant interest due to their capacity to treat injury without direct access to the bleeding site. Clinical

approaches have focused on the use coagulation factors, antifibrinolytic agents, and lyophilized or frozen platelets. Platelet substitutes have become widely investigated alternatives to these treatment options on an academic level.

Recombinant Factor VIIa

Recombinant factor VIIa (rFVIIa), first used as a treatment for bleeding episodes in hemophiliacs, has showed success during clinical investigation for use in surgical and traumatic bleeding.¹¹⁶⁻¹¹⁹ rFVIIa is a coagulation factor that plays an integral role in the tissue factor (or extrinsic) pathway. Tissue factor is released at an injury site upon damage to the vascular endothelium and complexes with rFVIIa. This complex initiates the common coagulation cascade directly through activation of factor X, or indirectly through activation of factor IX. Since rFVIIa cannot lead to the activation of subsequent steps of the coagulation cascade without the presence of tissue factor, major thrombotic complications are believed to not be a serious threat.^{120, 121} However, complications including myocardial infarction and deep vein thrombosis have still been seen in a significant percentage of patients.¹²²

Antifibrinolytics

Antifibrinolytic approaches that directly inhibit plasmin or the binding of plasmin to fibrin have been used clinically.^{123, 124} Plasmin is a fibrinolytic enzyme that is the active form of plasminogen.¹²⁵ Aprotinin, aminocaproic acid, and tranexamic acid are pharmaceuticals that act as inhibitors, and have been shown to minimize blood

transfusion requirements in surgery.^{6, 126, 127} Aprotinin directly inhibits plasmin, kallikrein, and trypsin.¹²⁸ Aminocaproic acid and tranexamic acid act as lysine analogues, inhibiting binding of plasmin to fibrin.^{129, 130} The use of these therapies has been associated with myocardial infarction, stroke, and renal failure, but some of the reporting clinical studies have undergone scrutiny.^{6, 131, 132}

Platelets and Platelet Substitutes

Lyophilized, frozen, and fragments of platelets have all been used intravenously to varying efficacy.¹³³⁻¹³⁵ All platelet-derived products require virus inactivation and have variability and storage issues.^{136, 137} To remedy some of these problems platelet substitutes have been widely investigated (Table 1.2). Generally, platelet substitutes aim to augment primary hemostasis using injury-site specific targeting moieties to promote platelet aggregation and fortify platelet plug formation.

Table 1.2. Intravenous Hemostatic Agents

Platform	Targeting Moiety	Discussion
Red Blood Cell	Fibrinogen, ¹⁴¹ fibrinogen mimicking peptide ¹⁴²	Biologically derived, viral transmission risk, autologous red blood cells investigated, high cost, low stability
Albumin	Fibrinogen, ^{144,145} fibrinogen γ -chain dodecapeptide ¹⁴⁶	Crosslinked albumin particles, viral transmission risk, lower cost than red blood cell derived
Liposome	Red blood cell and platelet proteins, ¹⁴⁷ fibrinogen mimicking peptide, ¹⁴⁸ fibrinogen γ -chain dodecapeptide, ¹⁴⁹ fibrinogen mimicking peptide, collagen binding peptide and von Willebrand factor binding peptide ^{150,151}	Earlier approached still used biologically derived components, later iterations used multiple non-biologically derived targeting mechanisms, issues with cost and scalability
PLGA	Fibrinogen mimicking peptide, ¹⁵²⁻¹⁵⁴ fibrinogen γ -chain dodecapeptide ¹⁵⁵	Synthetic and degradable, non-spherical morphologies investigated, issues with cost and scalability

Early platelet substitute approaches that utilized blood derived components were first intended for developing treatments for thrombocytopenia. Fibrinogen has been added to a variety of delivery platforms to serve as a targeting moiety, as it binds to the glycoprotein IIb-IIIa receptors of activated platelets.

Autologous erythrocytes covalently bound to fibrinogen were used with aims of passive participation in platelet aggregation, significantly reducing the bleeding time of thrombocytopenic rats.¹³⁸ Using a fibrinogen mimicking peptide sequence as the targeting moiety on the surface of autologous red blood cells, Coller et al. removed the risks and difficulties associated with using human derived fibrinogen.¹³⁹ Beer et

al. also investigated the use of immobilized peptide chains to probe the glycoprotein IIb-IIIa receptor.¹⁴⁰

Levi et al. and Takeoka et al. used fibrinogen coated albumin microcapsules showing *in vitro* platelet interaction and administered them to chemotherapy-induced thrombocytopenic rabbits demonstrating significant reduction in bleeding.^{141, 142} Okamura et al. also used albumin particles, but instead utilized the dodecapeptide (H12) from the fibrinogen γ -chain, replacing fibrinogen as an activated platelet-targeting moiety and showed efficacy *in vitro* and in thrombocytopenic rats.¹⁴³

Rybak et al. utilized liposomes, using a variety of proteins derived from platelets and red blood cells, namely glycoprotein IIb-IIIa and fragmented RBC membrane.¹⁴⁴ These proteins were harvested and adsorbed to a heterogeneous liposome. They found that lipid content greatly affected hemostatic efficacy in thrombocytopenic rats. Similarly, surface-conjugated peptides to modulate binding without using human or animal derived components have been used.¹⁴⁵ Okamura et al. coupled this approach with the release of adenosine diphosphate (ADP), a platelet activator.¹⁴⁶ Further iterations of this approach utilized both von Willebrand factor and collagen adhesion promoting peptides motifs in concert with platelet aggregation promoting peptides on the surface of liposomes to increase surface interaction *in vitro*.^{147, 148}

The Lavik group investigated the use of functionalized poly(lactic-co-glycolic acid)-b-poly(L-lysine)-b-poly(ethylene glycol) nanoparticles that also utilize glycoprotein

IIb-IIIa receptor binding, and confirmed efficacy in surgical and blunt trauma models.^{149, 150} More recently, they have demonstrated precise control of ligand density, showing dramatic improvement in hemostatic ability.¹⁵¹ Okamura et al. have also constructed H12-poly(lactic-co-glycolic acid) nanosheets made of microparticle aggregates, and showed an increased adhesive rate over microparticles alone.¹⁵² While these approaches show promise and have the great advantage of not needing direct access to the site of injury, high production cost and limited scalability may limit clinical utility.

1.5 Conclusion

The lack of affordable, safe, and effective hemostatic materials has led to wide interest in the development of new approaches. Assessment of new technologies is difficult due to the wide variety of injury models utilized in hemostatic efficacy studies. Many studies forgo *in vivo* evaluation all together or use a single small animal injury model. While a rat liver needle-prick injury model could be useful as a proof of concept, the same animal should be utilized for injuries of varying severity (tail amputation, lung and liver resection). Assessing injury models in large animals such as sheep or swine more accurately predict material performance in humans.

A wide variety of approaches exist in the field, but only iterative improvements have been made on existing commercial options. While active systems can achieve impressive hemostatic results and have obvious mechanisms, they carry the risk of thrombotic complications and disease transmission. Passive participation in clot or

platelet aggregate formation, as exemplified in the recent advances in intravenous hemostatics, allows for the utilization of natural hemostatic process without this risk. Biologically inspired architectures and chemistries have also led to exciting advances. Only a handful of chemistries behind biocompatible wet-tissue adhesives have been explored. These wet-adhesive approaches deserve further investigation.

Academic strategies must thoughtfully consider the end goal of clinical translation. Complicated delivery methods, high cost, limited scalability, and even safety are often overlooked when developing new solutions. Collaborations with surgeons or other clinicians are encouraged to vet out non-clinically applicable technologies. One-step approaches, topically or intravenously administered, should be the chief focus of future research.

1.6 Introduction and Directions

The goal of this dissertation was to investigate a variety of synthetic polymeric materials and fabrication techniques for developing new hemostatics and surgical sealants. New hemostatic materials for traumatic injury are imperative, as trauma remains a leading cause of death worldwide. Many first response treatment approaches have not shown significant progress. Towards this application, a cationic monomer (N-(3-Aminopropyl)methacrylamide (APM)) was used to synthesize spherical hydrogel particles. These particles were designed to induce hemostasis by forming a physical barrier to blood loss through swelling. Additionally, the cationic character of the hydrogel particles led to the formation of a physical aggregate in the presence of blood cells due to charge interactions. The second focus of this dissertation was on the development of a surgical sealant. Conventional suturing and tissue stapling have defined limitations, but remain ubiquitous to many procedures. Few advances have been made in modern surgery with regards to minimizing the risks of these techniques. A method called solution blow spinning was used to directly deposit polymer fiber mats. On demand fabrication of conformal fiber mats/meshes allows for precise and site specific construction in minutes. This approach was combined with the use of a biodegradable polymer blend that exhibits a body temperature responsive transition that results in increased adhesion and sealant performance.

Chapter 2: Blood Aggregating Hydrogel Particles

2.1 Introduction

The body's natural response to injury is comprised of three stages: the formation of a platelet plug, an enzymatic cascade resulting in formation of fibrin, and the dissolution of the clot and healing of the wound site.¹⁵³⁻¹⁵⁵ These stages are referred to as primary hemostasis, secondary hemostasis, and fibrinolysis, respectively. The body's natural mechanisms are not able to control massive hemorrhaging caused by major trauma or surgery resulting in the requirement of hemostatic intervention. Depending on the type of injury and the capabilities at site of treatment, different hemostatic approaches are utilized.

In the operating room hemorrhage control is imperative as many procedures including cardiovascular, hepatic, orthopedic, and spinal have a high incidence of severe blood loss requiring use of a hemostatic.⁶ Storage and handling capabilities at hospitals allow for the use of costly and biologically active hemostatic agents. These hemostatic formulations typically include one or more coagulation cascade or hemostatically active proteins such as thrombin, fibrin, and collagen and are applied topically as a patch,^{156, 157} matrix,^{158, 159} or liquid.^{160, 161} Non-protein materials based on polyethylene glycol¹⁶² or oxidized cellulose^{68, 159} are also widely used. Although generally effective, issues including cost, storage, and handling illustrate the need for inexpensive hemostatics with comparable efficacy.¹⁰

Current approaches have investigated the use of topically applied self-assembling systems using polypeptides or hydrophobically modified chitosan to varying successes.^{39, 59} Most approaches require direct access to the injury site. To circumvent this, intravenous hemostatics such as recombinant factor VIIa (NovoSeven) have been investigated for clinical use.^{117, 163} Further exploration into the use of intravenous hemostatic agents that augment primary hemostasis by utilizing artificial platelets have shown promising results but may be difficult or costly to produce at large scale.

149, 164

This research focuses on investigating the use of topically applied cationic hydrogel particles as a hemostatic agent. We have synthesized and characterized hydrogel particles capable of aiding in rapid hemostasis by promoting the formation of a robust hemostatic plug and forming a physical barrier to blood loss through electrostatic interactions and swelling. *In vitro*, the particles locally form hydrogel/blood aggregates, while delaying coagulation in any excess blood present. This is potentially important as the material will not cause, and may even inhibit thrombotic events at distal sites while locally acting as a hemostatic through the interplay of multiple mechanisms. This work reports the synthesis and characterization of the particles including potentiometric titration to determine the extent of protonation, and temporal swelling behavior to investigate serum uptake. *In vitro* coagulation studies, *in vivo* efficacy studies on rat and ovine surgical models, and characterization via hematoxylin and eosin (H&E) and Carstairs' method staining of the incision site were then used to assess the bioactivity and efficacy of the material.

2.2 Materials and Methods

Materials

N-(3-Aminopropyl)methacrylamide hydrochloride (APM) was purchased from Polysciences (Warrington, PA). Ammonium persulfate (APS), inhibitor removers, N,N,N',N'-Tetramethylethylenediamine (TEMED), 1X Phosphate Buffered Saline (PBS), Poly(ethylene glycol) diacrylate average Mn 700 (PEGDA), and Tween 80 were purchased from Sigma-Aldrich (Milwaukee, WI). Human platelet poor plasma (pooled, sterile filtered, 4% (w/v) sodium citrate) was purchased from Vital Products (Boynton Beach, FL). Ovine Blood (4% (w/v) sodium citrate) was purchased from Hemostat Laboratories (Dixon, CA). Deionized water (DI water) was obtained using a Millipore Super-Q water system (Billerica, MA). Sodium hydroxide (NaOH) was purchased from Mallinckrodt Baker (Phillipsburg, NJ). Acetone, silicone oil, paraffin, and xylene were purchased from Fisher Scientific (Pittsburgh, PA). Plastic-capped glass vials were purchased from VWR Scientific (West Chester, PA). All materials were used as received except for PEGDA, which was passed through a column containing inhibitor removers before use.

Synthesis of Hydrogel Particles

APM hydrogel particles were synthesized via inverse suspension polymerization. 1584.2 mg of APM and 84.4 μ L of PEGDA were dissolved in 2.5 mL of DI water. This corresponds to 98.5% by mole APM and 1.5% PEGDA for a 3 M solution. The

solution was titrated to pH 7 by addition of 10 M NaOH. DI water was added to achieve a final volume of 3 mL, forming the prepolymer solution. 390 mg of APS was then dissolved in the prepolymer solution, then 500 μ L immediately added to vials containing 2 mL of 5% (v/v) Tween 80 in silicone oil, stirring at 2000 rpm by magnetic stirrer. 5 μ L of TEMED was added to each vial and allowed to react for 24 hours. The particles were washed with acetone until the supernatant was clear and isolated via vacuum filtration. The hydrogel particles were then washed in 350 mL of DI water for 72 hrs, changing the DI water every 24 hrs. This was done to ensure any unreacted monomer, initiator, crosslinker, or catalyst was removed. Finally, the hydrogels were washed again with 350 mL of acetone until they completely collapsed, vacuum filtered, and stored in a vacuum desiccator for up to 3 months. Dry hydrogel particles were imaged using an Axioskop 50 microscope and AxioCam color camera from Zeiss (Germany). 200 measurements were taken (n= 200).

Fourier Transform Infrared Spectrum

A Fourier transform infrared (FTIR) spectrum of the hydrogel particles was obtained on a Thermo Nicolet NEXUS 670 FTIR, range 500-4000 cm^{-1} , equipped with the attenuated total reflectance accessory for liquids and powders. Washed and dried hydrogel particles were ground by mortar and pestle to obtain a fine powder before the measurement.

Potentiometric Titration

Potentiometric titrations were conducted using a Fisher Scientific Accumet Excel XL15 pH/mV/Temperature Meter with a Fisher Scientific Accumet Glass Body Standard Size Combination Electrode (mercury-free) at room temperature. First, the APM polymer (poly(APM)) was prepared. In a glass vial, 1584.2 mg of APM was dissolved in 2.5 mL of DI water. 10 M NaOH was added drop wise until the solution reached a pH of 7. The volume of the solution was then raised to 3 mL with DI water. 30 μ L of TEMED and 390 mg of APS were dissolved into the solution. The solution was allowed to react at room temperature for 24 hours. The prepared polymer was dissolved in 100 mL of deionized water and stirred at 1000 rpm using a magnetic stir bar. During titration, 1.0 M NaOH was added in 400 μ L increments with a 30 s time interval between each addition until the pH of the solution exceeded 13.5.

Temporal Swelling

50 mg of dry hydrogel particles were portioned into pre-weighed glass vials, followed by the addition of 2 mL of DI water, human source platelet poor plasma, or 1X PBS, and placed on a bench top rotator. At time intervals of 1, 2.5, 5, 10, 30, 60, 90, 120, 150, and 180 minutes vials were removed from the rotator and drained of excess fluid by pipette before being weighed. All samples were replicated ($n = 9$). Swelling percent at each time point was calculated using the formula below.

$$SP = \frac{M_t - M_d}{M_d} \times 100$$

SP = Swelling Percent, M_t = mass of swollen particles at time t , and M_d = mass of dry particles. Water swollen particles at equilibrium were imaged. The imaged particles were measured and the swelling percentage based on volume was calculated using the formula below.

$$SP = \frac{V_f - V_d}{V_d} \times 100$$

SP = Swelling Percent, V_f = volume of swollen particles at equilibrium, and V_d = volume of dry particles. 200 measurements were taken ($n = 200$).

Coagulation Time

100 mg of dry hydrogel particles were portioned into glass or plastic vials. Refrigerated ovine blood was allowed to reach room temperature. 0.5 mL, 1 mL, and 2 mL of ovine blood were added to glass or polypropylene vials containing hydrogel particles. The same volumes of ovine blood added to glass or polypropylene vials served as the controls. Samples in glass vials were recalcified to a 10 mM CaCl_2 final concentration using a 0.2 M CaCl_2 stock. Samples in plastic vials were recalcified to a 20 mM CaCl_2 final concentration using a 0.2 M CaCl_2 stock. The difference in final calcium concentration was established to ensure consistent coagulation times amongst the control groups as determined by previous unpublished studies. Vials were then rotated for 1 minute, and set up vertically on the lab bench. Vials were inverted every

minute until the blood/hydrogel aggregate completely ceased to flow, and the time recorded. All experimental groups and controls were run in triplicate (n = 3).

Rat Liver Puncture and Tail Amputation

Surgeries were completed on 2 to 4 month old Sprague-Dawley rats (weight of 300-400 g). Anesthesia was induced with 4% isoflurane, and maintained with 2.5% isoflurane by mechanical ventilation. All animal experiments and care were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Maryland School of Medicine and were in accordance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1996). Two injury models were used on each animal, liver puncture and tail amputation. Liver injury was accomplished by a puncture of approximately 5 mm in depth with an 18 G needle. Tail amputation at 50% tail length was completed using surgical scissors. In the mass of blood loss experiments, pre-weighed gauze was applied for 5 minutes (n= 6 for each injury) with minimal pressure and the resulting mass immediately recorded. The experimental group consisted of application of 150 mg of hydrogel particles with minimal pressure from pre-weighed gauze for 5 minutes and resulting mass immediately recorded (n = 5 for each injury). In an analogous experiment, time to hemostasis was recorded after checking for bleeding in 1-minute intervals (n = 5 for each injury). At the conclusion of each experiment, rats were euthanized by exsanguination while under anesthesia.

Ovine Liver Laceration

Surgeries were completed on adult Dorsett hybrid sheep (weight of approximately 65 kg). Anesthesia was induced with sodium thiopental (10 mg/kg) and maintained by mechanical ventilation with 2% isoflurane mixed with oxygen. A Draeger anesthesia monitor (North American Draeger, Telford, Pa) was used during surgery. An incision approximately 5 cm in length and 1 cm in depth was made with a surgical scalpel on the right lobe of the sheep's liver, corresponding to a grade II liver laceration^{165, 166}. 1 g of dry hydrogel particles was poured onto the laceration, which was then compressed with gauze for 5 minutes. The gauze was then removed and the incision was inspected visually. The incision site was then washed with 50 mL of saline solution to remove free particles. Compression with gauze alone served as the control. Two incisions were performed on each animal's liver, one serving as an experimental group and the other as the control group. If bleeding was not stopped, the incision was cauterized immediately in order to not hamper the validity of the second incision. The first incision was alternated between the experimental and control group. Immediately following the surgery the liver was excised fixed in formalin for 24 hours, placed in a histological cassette, then dehydrated in graded ethanol solutions. Following fixation and dehydration, the sample was set in paraffin, cut into 5 mm sections and mounted on glass slides. The sections were then deparaffinized with xylene and rehydrated with a series of graded alcohol solutions. Hematoxylin and eosin and Carstairs' method staining were completed on the samples. Images were taken using an Axioskop 50 microscope and AxioCam color camera from Zeiss (Germany). Three animals were used in this study (n = 3). After the conclusion of

each study, sheep were euthanized according to the American Veterinary Association's (AVMA) Guidelines for Euthanasia of Animals. The sheep were administered a lethal dose of potassium chloride (2 mmol/kg) intravenously to stop the heart, followed by exsanguination while still under general anesthesia.

Statistical Analysis

All experimental groups and controls were performed in replicate as referred to in each specific methods section. Where suitable, data was analyzed using ANOVA single factor analysis to demonstrate differences between groups assuming a normal data distribution with a confidence of 95% ($p < 0.05$). Mean values and error bars are reported on each figure as well as relevant statistical relationships, aside from Figure 2.3, which presents the raw particle size distribution.

2.3 Results and Discussion

Hydrogel based materials have been widely investigated for hemostatic use due to their functional flexibility and the ability to easily impart adhesive and absorptive properties.¹⁶⁷⁻¹⁶⁹ This research utilizes cationic APM hydrogel particles absorptive an electrostatic properties. Hydrogel particles were synthesized via inverse suspension polymerization as described in Figure 2.1, with a narrow size distribution and spherical morphology (Figure 2.2). A size distribution of the dry hydrogel particles can be seen in Figure 2.3.

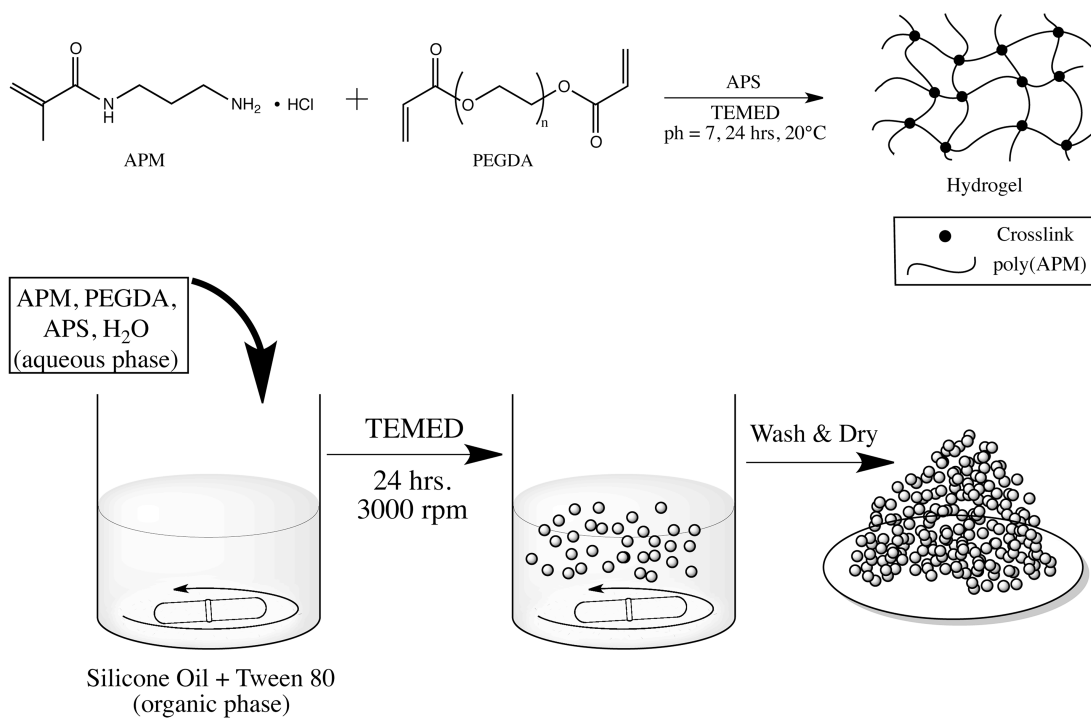


Figure 2.1. Synthetic scheme of hydrogel particles consisting of a copolymer of APM and PEGDA, showing chemical structures and the described inverse suspension polymerization.

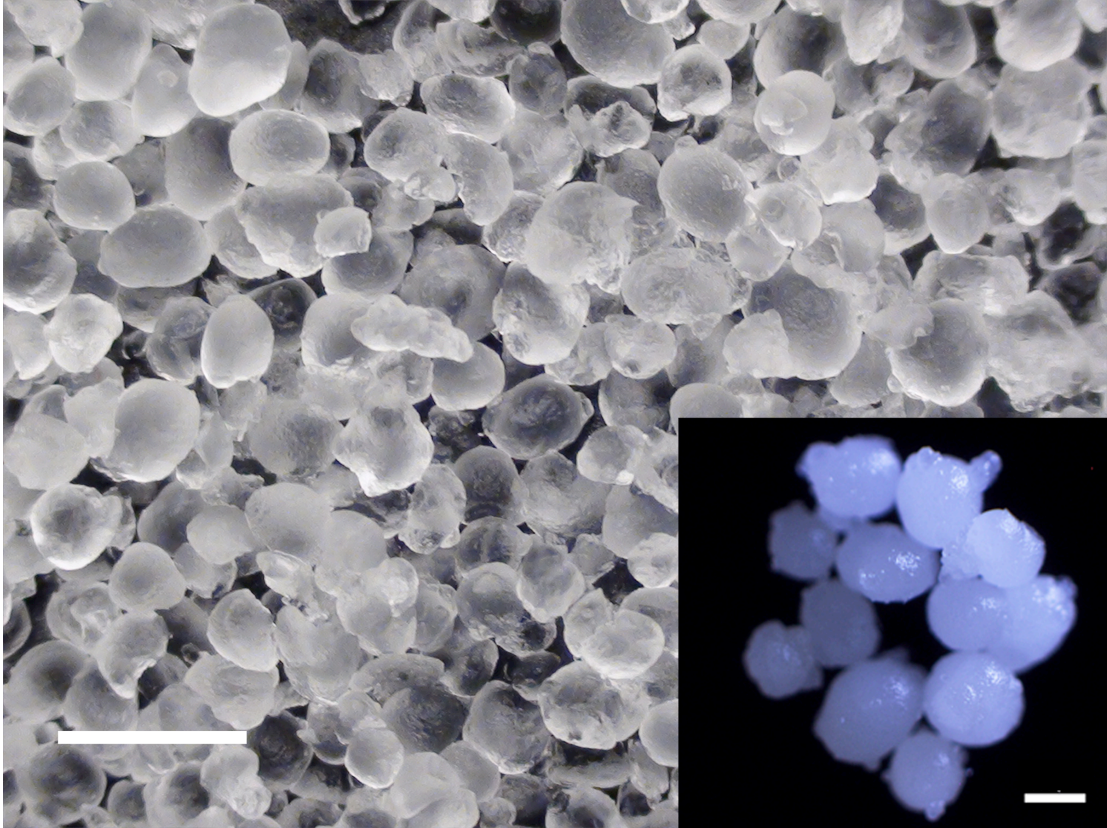


Figure 2.2. Photograph of APM hydrogel particles after swelling to equilibrium in DI water (scale bar represents 5 mm). Inset, optical micrograph of APM hydrogel particles after washing and drying procedures (scale bar represents 500 μm).

Particle diameter ranges from 450 μm to 1250 μm with an average value of 795 μm and a standard error of $\pm 9.97 \mu\text{m}$ ($n = 200$). The size of the particles ($\sim 800 \mu\text{m}$ dry, $\sim 2000 \mu\text{m}$ swollen) will help decrease the likelihood of migration into the circulatory system, avoiding thromboembolic complications. Sub-micron size particles have a high likelihood of systemic circulation that could cause thrombus formation at distal sites. Additionally, the hydrogel particles were stored dry up to 3 months in a vacuum desiccator with no evidence to suggest that they would be degraded or altered by typical environmental factors.

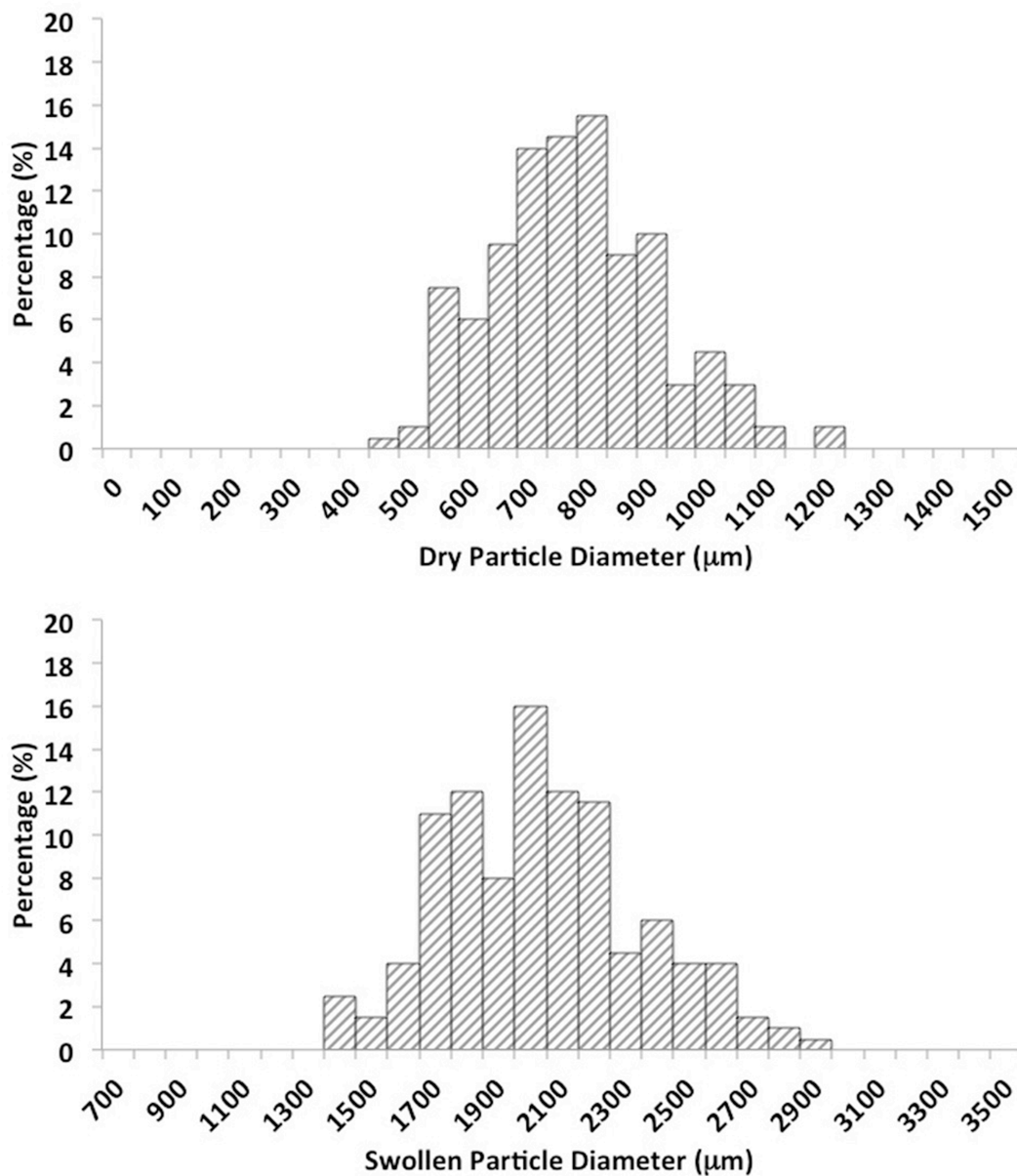


Figure 2.3. Size distribution of hydrogel particles by diameter. (Top) dry, (bottom) swollen to equilibrium in water (n = 200).

To confirm the chemical composition of the hydrogel FTIR spectroscopy was used. The hydrogel particles exhibit the characteristic absorbance peaks of N-H ($\sim 3329\text{ cm}^{-1}$), $-\text{CH}_3$ ($\sim 2960\text{ cm}^{-1}$), C=O ($\sim 1620\text{ cm}^{-1}$), and N-H ($\sim 1522\text{ cm}^{-1}$) as shown in the spectrum displayed in Figure 2.4. The absence of a peak at $\sim 1640\text{ cm}^{-1}$ corresponds to

the complete conversion of C=C during the polymerization process. This is consistent with hydrogels of similar composition.¹⁷⁰

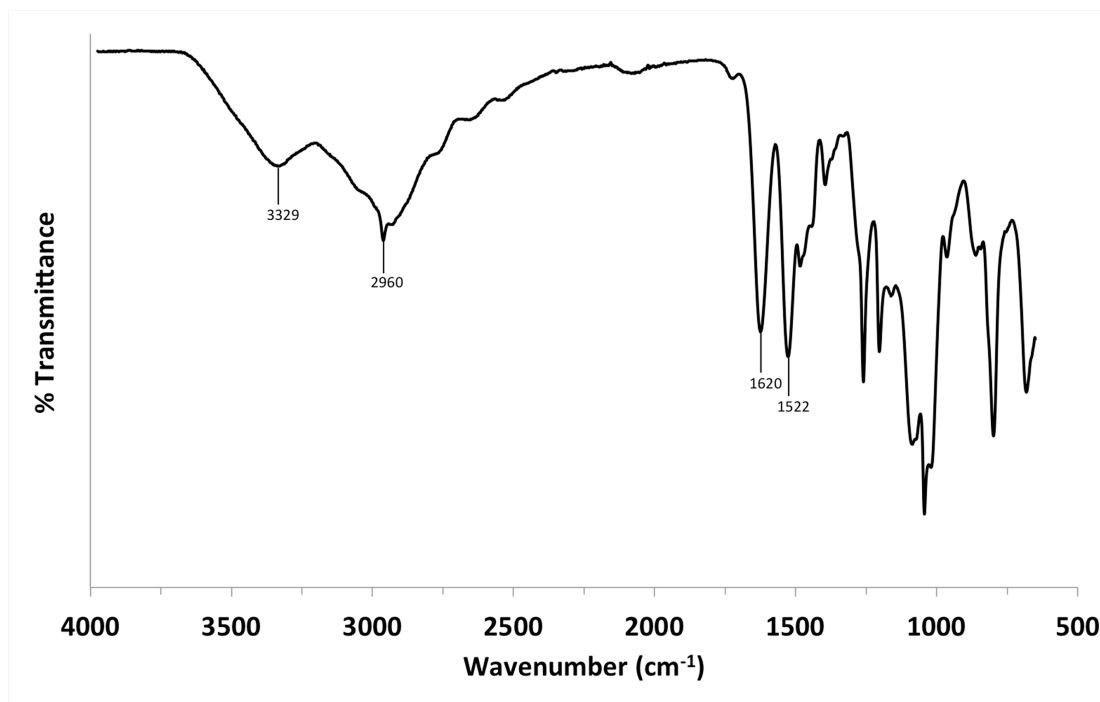


Figure 2.4. FTIR spectrum of hydrogel particles with characteristic peaks labeled.

The pKa of the polymer that makes up the majority of the hydrogel was determined to be 9.82 by potentiometric titration. The equivalence point was determined by finding the maximum of the first derivative of pH with respect to volume. The pKa was then determined by the pH at half of the equivalence point. At physiological pH of 7.4, this corresponds to greater than 99% protonation of the primary amine groups of poly(APM). Comparing the majority component of this hydrogel to chitosan, which has a pKa 5.5-7.8 depending on the degree of acetylation,¹⁷¹ chitosan displays significantly less cationic character than poly(APM) in physiological conditions. The high degree of poly(APM)'s positive charge coupled with a low degree of crosslinking (1.5% by mole) leads to rapid swelling behavior. This amount of

crosslinking was chosen as a balance between mechanical properties and swelling capacity previously investigated.^{92, 172} The material therefore has the propensity to locally concentrate coagulation factors, red blood cells, and platelets as it quickly absorbs water from serum. Additionally, the same behavior allows the particles to act as a physical barrier to blood loss.

Temporal swelling studies were conducted with DI water, 1X PBS (pH= 7.4), and human source platelet poor plasma over time scales pertinent to hemostasis. Water caused the hydrogel particles to swell greater than 1600% by mass at equilibrium. The PBS and plasma equilibrium percentages were smaller in magnitude at approximately 1000% by mass (Figure 2.5). On a time scale more imperative to hemostasis, hydrogels exposed to both DI water and PBS swelled to their maximum within 10 minutes, where as plasma swelled to approximately 80% of its maximal value in this time (Figure 2.5).

Equilibrium swelling in water was also measured by volume; the size distribution of the swollen particles can be seen in Figure 2.3 and an image of the swollen particles in Figure 2.2. Swollen particle diameter ranges from 1400 μm to 2900 μm with an average value of 2079 μm and a standard error of ± 22 μm . This corresponds to a mean swelling of 1687% by volume with an error of $\pm 0.27\%$ as determined through standard error propagation. The slight discrepancy between the equilibrium and maximum swelling percentage calculated by mass and volume is due to the assumption of equal density in the calculation by mass.

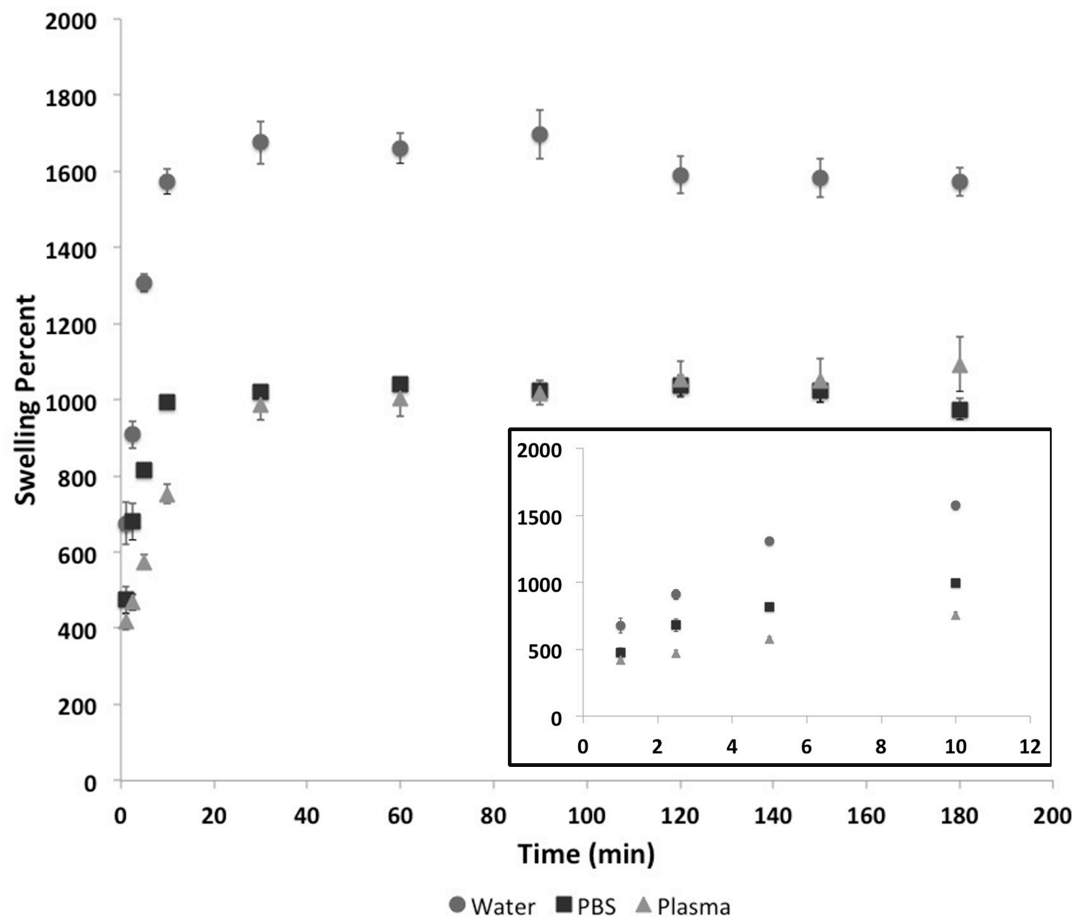


Figure 2.5. Temporal swelling of hydrogel particles in DI water, PBS, and plasma over 180 minutes, and (inset) over 10 minutes. Error reported as standard error (n= 9).

Hydrogel particles in PBS, and plasma, saw decrease in time to maximum swelling and the maximum swelling percentage as compared to water. This can be attributed to the buffering capacity and ionic strength of both PBS and plasma. Swelling in plasma is further retarded by the presence of proteins and other biomolecules that cause osmotic pressure differences and present the possibility for more charge shielding. Although there is a measurable decrease in swelling in the more physiologically relevant serum, the degree of swelling and charge interaction proved to be sufficient

in the subsequent animal modeling. These differences, however, deserve significant consideration when designing any biologically functional polymeric material.¹⁷³

Coagulation time was evaluated in glass and plastic vials to elucidate any direct effect on coagulation *in vitro*. In both cases, when the recalcified volume of blood was comparable to the swollen volume of particles, a coagulum occurred significantly faster than the control case (Figure 2.6) through the formation of a blood/hydrogel aggregate. However, when the volume of recalcified blood was significantly larger than the amount of hydrogel particles, a delay in the coagulation of the excess blood was observed. Even in the cases of delay, an aggregate was quickly formed between a fraction of the blood and the particles. Glass vials were used to determine if there was any significant difference when a coagulation activating surface was present, as glass is known to activate the contact activation pathway.¹⁷⁴ Polypropylene vials were also used to remove any activating surface effect. In both cases, coagulation time was dependent on the blood sample volume relative to the amount of hydrogel particles. In instances where hydrogel and blood sample volumes were comparable, immediate blood/hydrogel aggregate formation occurred. Excess blood sample volumes resulted in delay in coagulation, although local aggregate formation still persisted. This aggregate formation was not seen in the plasma swelling studies and therefore can be contributed to the presence of cellular bodies.

Although the inhibition mechanism was not directly investigated there have been reports of polyamine and cationic hemostatic inhibition through platelet and/or

polyphosphate interactions.^{175, 176} This aggregate/inhibition mechanism could offer a local hemostatic effect while possibly mitigating risks of thrombotic complications elsewhere in the body. Conversely, this could have negative ramifications for situations with multiple injuries or longer-term implications if a similar material was left implanted. Future studies could include a comprehensive investigation of the inhibition mechanism, and whether it is a local phenomenon or if it could have systemic effects on coagulation.

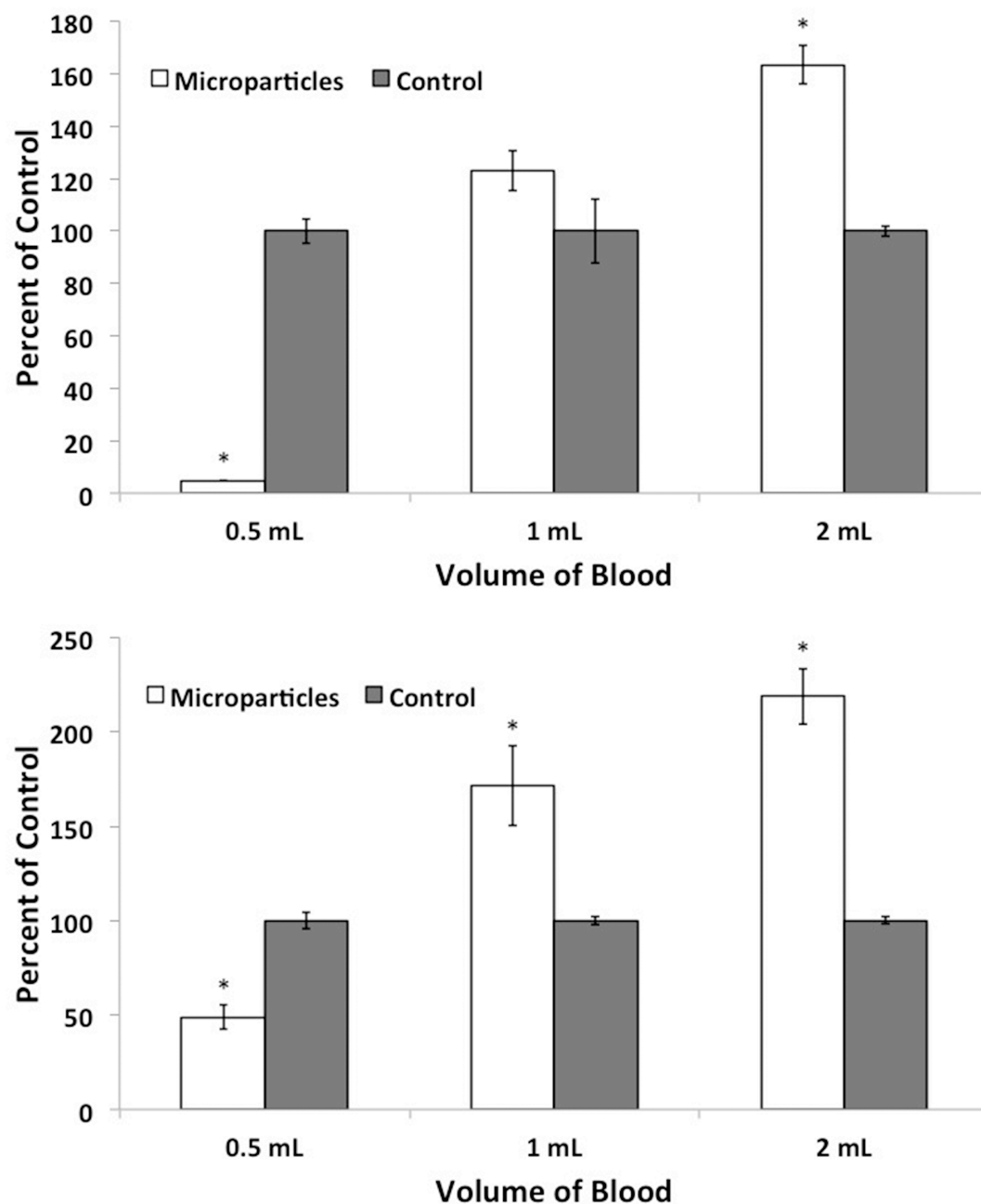


Figure 2.6. Coagulation time reported as percent of control. Top, clotting time in glass vials. Bottom, in plastic vials. The symbol (*) denotes statistical significance from relevant control ($p < 0.05$). Error reported as standard error ($n = 3$).

Initial efficacy studies were performed on liver puncture and tail amputation rat models. In both injury models a significant decrease in mass of blood loss was seen (Figure 2.7). The liver puncture and tail amputation models saw over a 300% and 500% reduction of blood loss, respectively, relative to the controls. Within the 5 minutes of application of the gauze control 4 of 6 liver punctures continued to bleed and 6 of 6 tail amputations. In comparison, after five minutes of particle application 0 of 5 liver punctures continued to bleed and 2 of 5 tail amputations. The hemostatic efficacy was also demonstrated in a time to hemostasis experiment where hydrogel application achieved hemostasis an average of over 1 minute faster in the liver puncture model and 12 minutes faster in the tail amputation model versus the gauze control (Figure 2.7). Any hydrogel in contact with a bleeding surface formed a visible aggregate, in some instances incomplete coverage with the hydrogel particles lead to a delay in time to hemostasis or an increase in mass of blood loss. This aggregation, also seen in the coagulation studies, forms a rapid sealant at the surface of the injury that allows hemostasis to be rapidly reached.

An ovine liver laceration model was the used to simulate significant surgical bleeding. This animal was chosen as a model to accurately replicate human vasculature size, blood pressure, and coagulation system.^{177, 178} Liver lacerations and subsequent blood loss presents major mortality risks due to the organ's extensive vascularization, the inability of the liver's structure to secure vasoconstriction, and ineffective implementation of suture and compression techniques on the organ.¹⁷⁹⁻¹⁸¹

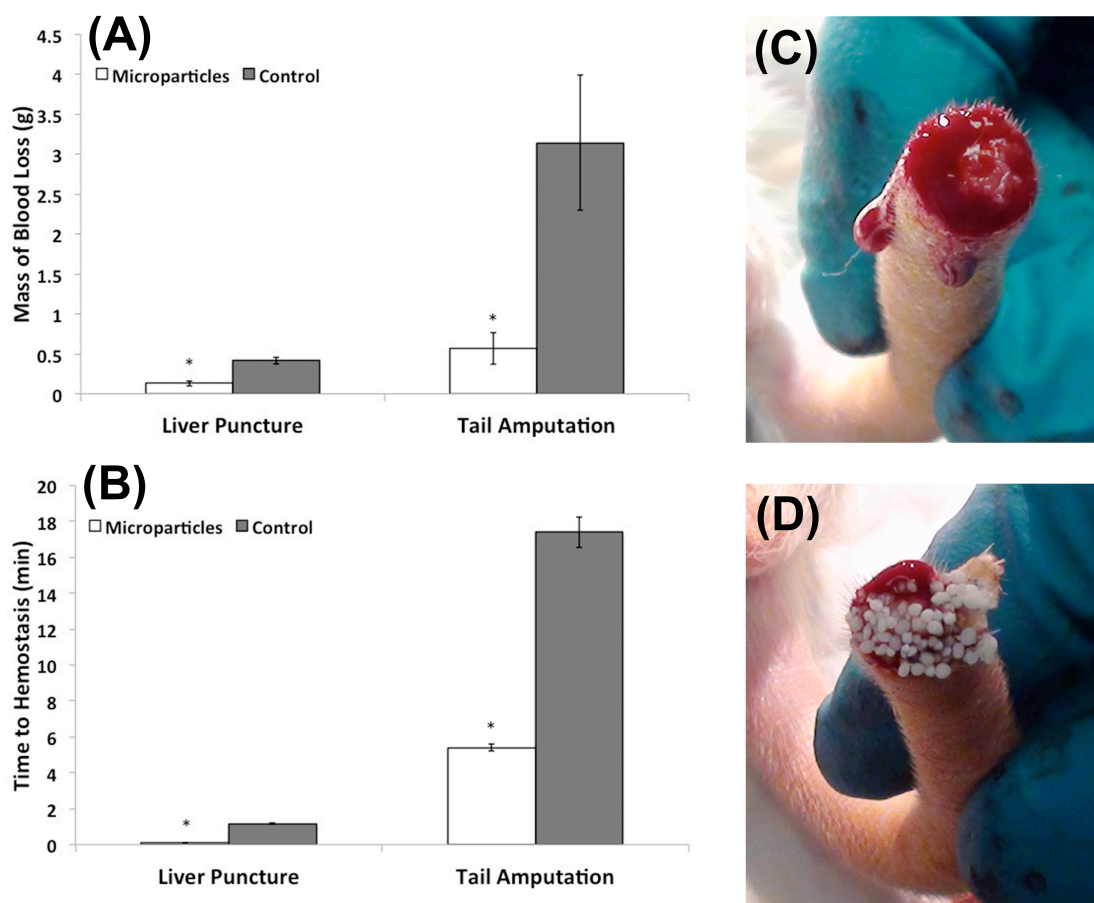


Figure 2.7. (A) Mass of blood loss study in Sprague-Dawley rat liver puncture and tail amputation models assessing the hemostatic ability of the hydrogel particles ($n = 5$ for each injury) versus a gauze control ($n = 6$ for each injury). (B) Time to hemostasis for the same injury models ($n = 5$ for each group). (C) Photograph of tail amputation after 5-minute application of gauze showing bleeding. (D) Photograph of tail amputation after 5-minute application of hydrogel particles showing bleeding as stopped. In A and B, the symbol (*) denotes statistical significance from relevant control ($p < 0.05$). Error reported as standard error.

A surgical scalpel was used to induce bleeding that would not be stopped by compression. Upon contact with blood the hydrogel particles swelled at a sufficient rate to halt blood loss and effectively formed a sealant through aggregation. After 5 minutes the gauze was removed and the injury site was inspected showing complete hemostasis (Figure 2.8). The organ was then washed with saline solution confirming

no blood loss was occurring. H&E staining and Carstairs' method staining of the incision site after saline irrigation showed significant cell accumulation (Figure 2.9). Pressure with gauze alone for 5 minutes resulted in no significant hemostatic effect and required cauterization to seal the injury site in all cases.

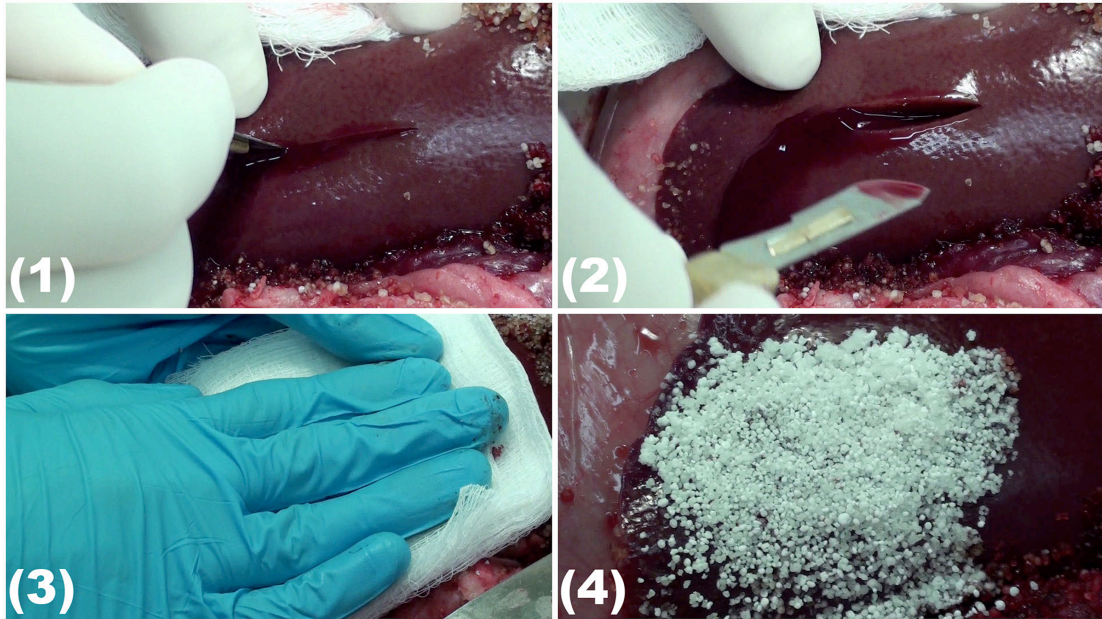


Figure 2.8. A series of photographs taken during the liver laceration sheep model. (1) Incision by scalpel, (2) significant bleeding, (3) hydrogel particles applied with pressure from gauze for 5 minutes, and (4) gauze removal showing complete hemostasis.

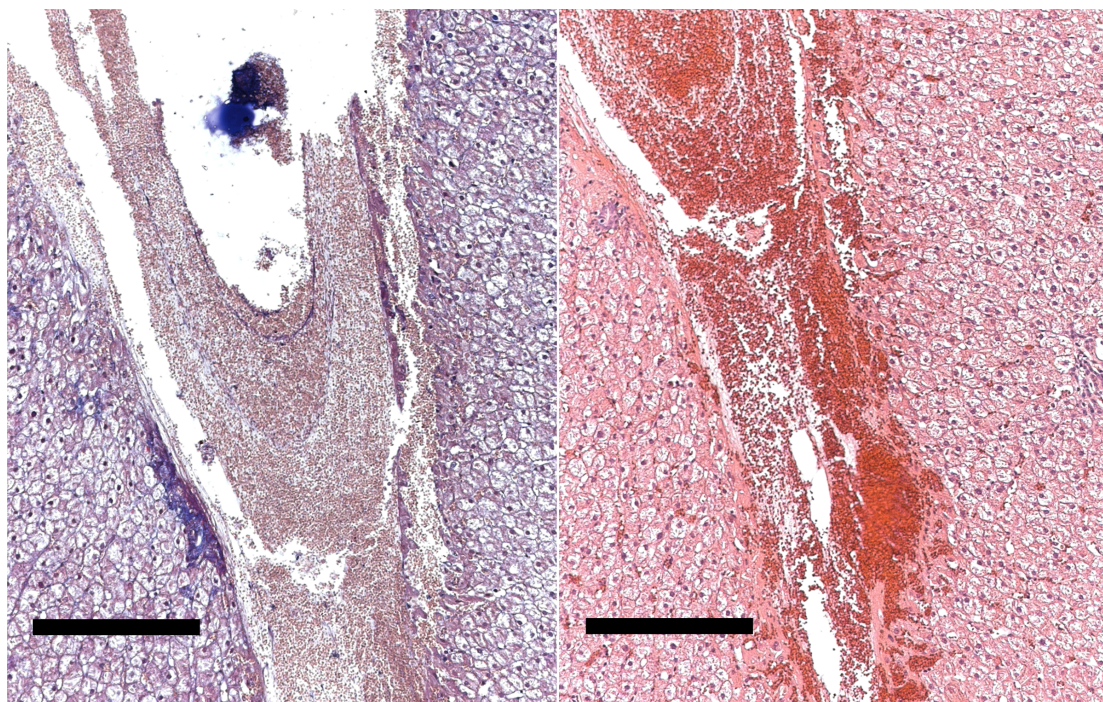


Figure 2.9. Carstairs' method stained (Left) and H&E stained (Right) micrographs, showing significant accumulation of red blood cells and platelets deep within the incision site (scale bars represent 200 μ m).

2.4 Conclusion

The present study demonstrates facile synthesis of APM hydrogel particles that show promise as an effective hemostatic agent. Potentiometric titration, temporal swelling experiments, and coagulation time experiments were used to predict the *in vivo* behavior of the hydrogel particles. Coupling high positive charge with a low crosslink density gives the hydrogel the ability to rapidly swell to over 1000% in size over time frames pertinent to hemostasis and cause localized aggregation, while retarding bulk blood coagulation. This mechanism allows the hydrogel to quickly block blood flow as demonstrated *in vivo*, and may mitigate risk of distal thrombosis due to coagulation activation. The inhibition/aggregation mechanism elicits further investigation to

assess the localization of the antithrombotic effect and to assess long-term implications of the inhibition. Hemostatic ability was exhibited in both decrease in time to hemostasis and mass of blood loss in multiple rat injury models as compared to a gauze control. Further validation of hemostatic efficacy on a physiological pertinent ovine liver laceration was shown. Subsequent staining of the ovine liver incision site showed significant hemostatic plug formation, alluding to the hydrogel's ability to promote accumulation of red blood cells and platelets through electrostatic interactions. In its current form the material would need to be removed after application but future iterations will introduce degradable chemistries and different initiation/synthesis schemes. APM hydrogel particles have potential as a hemostatic material with a practical hemostatic mechanism that could induce hemostasis while mitigating risks associated with many other approaches.

Chapter 3: In situ Deposition of PLGA Nanofibers via Solution Blow Spinning

3.1 Introduction

Nanofiber mats and scaffolds have a wide range of biomedical applications including drug delivery,¹⁸² wound dressings,¹⁸³ tissue engineering,¹⁸⁴ and enzyme immobilization.¹⁸⁵ Nanofibers are often generated by electrospinning, a process that utilizes an electric field applied to a drop of polymer melt or solution on the tip of a nozzle.¹⁸⁶ The droplet deforms forming a Taylor cone and a charged jet accelerates toward the target, generating nanofibers.¹⁸⁷ While electrospinning is a powerful and widely studied technique it requires specialized equipment, high voltages, and electrically conductive targets. It also suffers from a relatively low deposition rate. These restrictions prohibit the use of electrospinning for any *in situ* deposition of fibers in surgery or for conformal coverage of non-conductive targets without the use of polymer melts¹⁸⁸ or the assistance of air flow.¹⁸⁹

Solution blow spinning is a promising alternative that requires a simple apparatus, a concentrated polymer solution in a volatile solvent, and a high-pressure gas source.¹⁹⁰ Commercial airbrushes, typically used for painting, have successfully generated nanofibers through this technique.^{191, 192} Many polymer/solvent systems and deposition conditions have been investigated, but convincing control over fiber diameter has not been demonstrated.¹⁹³⁻¹⁹⁷

Applications that are pursued are largely analogous to electrospinning and include enzyme immobilization,¹⁹⁸ drug delivery,¹⁹⁹ and microfiltration.²⁰⁰ The reported examples of this technique cite the ease of use and rapid deposition rate as compared to electrospinning.^{190, 192, 193} Tutak et al. were the first to move towards using the method to generate conformal coatings for tissue engineering scaffolds, but no exploration into *in situ* deposition has yet been reported.¹⁹²

The possibility of direct deposition introduces a host of new applications and advantages over pre-formed nanofiber mats/meshes and scaffolds. On-demand fabrication of conformal nanofiber mats/meshes allows for precise and site specific construction. This could be exceedingly useful for reconstruction of tissue defects such as hernias, which treatment frequently uses preformed polymer mats with a high incidence of recurrent herniation and bowel obstruction.²⁰¹ The approach also holds great promise as a surgical sealant in place of or in addition to sutures in applications such as vascular, intestinal, or airway anastomosis. These procedures can be technically difficult, and when complications of leakage occur, have high morbidity.^{202, 203} Solution blow spinning could also be useful in areas requiring the use of a hemostatic material or sealant, especially when large areas are exposed and conventional suturing may not be possible as is the case with liver and lung resections.^{179, 204, 205}

Here we use solution blow spinning to fabricate conformal mats of poly(lactic-co-glycolic acid) (PLGA) *in situ* using a commercially available airbrush and

compressed CO₂. We investigate and optimize system parameters and characterize mechanical properties. Nanofiber mat degradation is monitored for morphologic and molecular weight changes *in vitro*. Biocompatibility of the direct deposition of nanofibers onto two cell lines is demonstrated *in vitro* and blood interaction is qualitatively assessed with scanning electron microscopy. Finally, a pilot animal study is used to illustrate the wide utility of this technique across multiple surgical applications.

3.2 Materials and Methods

Nanofiber Mat Fabrication

A commercially available airbrush (Master Airbrush G22-SET, 0.2 mm nozzle diameter, gravitational feed) was used as the solution blow spinning apparatus in all experiments. The airbrush was connected to pressurized CO₂ tank equipped with a regulator through a gas flow meter (McMaster-Carr panel mount flowmeter for air, 2-20 SCFH). The distance from nozzle to target was held at 10 cm for all experiments.

Optimization

Solutions of poly(lactic-co-glycolic acid) (PLGA, Lactel), were prepared at 10% (wt/vol) in acetone. All PLGA was composed of 50:50 lactic:glycolic acid with inherent viscosities (IV) of 0.64 or 0.93 dL/g in hexafluoroisopropanol and was used as purchased (herein referred to as 0.64 IV PLGA and 0.93 IV PLGA). Inherent viscosity is defined as the ratio of the natural logarithm of the relative viscosity to the

mass concentration of the polymer (IUPAC), and corresponds with polymer molecular weight ²⁰⁶. 10% solutions were loaded into the airbrush and blown at CO₂ flow rates of 10,13, and 15 SCFH onto glass coverslips for 5 seconds. Samples were then allowed to dry overnight in a vacuum desiccator, sputter coated with a 20 nm layer of silver (Electron Microscopy Sciences 150T S Turbo-Pumped Sputter Coater/Carbon Coater) and imaged by scanning electron microscopy (SEM, JEOL JSM-6390LV). Nanofiber diameter was measured using Image J (National Institutes of Health). Average fiber diameter and distribution were determined from 150 random measurements from 4 SEM micrographs.

Dynamic Mechanical Analysis

Samples on glass coverslips were prepared from 10% 0.93 IV PLGA and 10% 0.64 IV PLGA in acetone with 13 SCFH CO₂ for 60 seconds from a distance of 10 cm. The resulting mats were cut with razors to 8 mm height by 5 mm width, mounted on a Dynamic Mechanical Analyzer Q800 (TA Instruments) equipped with a film tension clamp and run from 0% strain to failure at 1%/min strain rate and a 0.001 N preload force. All samples were run in triplicate (n = 3).

Degradation

Nanofiber mats were prepared on glass coverslips from solution blow spinning 10% 0.93 IV PLGA in acetone at 13 SCFH for 30 seconds. Samples were then placed into six well plates, and submerged in 5 mL of 1X phosphate buffered saline (PBS) at 37

°C in a shaker incubator rotating at 50 rpm. The PBS was exchanged every 24 hours for 42 days. Experimental groups were removed from the incubator at time points of 0, 1, 7, 14, 21, 30, and 42 days. Once the PBS was removed, the samples were air dried overnight, and then stored in a vacuum desiccator. At each time point (n = 3), molecular weight distribution and polydispersity index (PDI) was determined by gel permeation chromatography (GPC) using a Waters 515 HPLC pump, in-line degasser, a Waters 2410 refractive index detector, and Agilent columns in series. Samples were dissolved at 1 mg/mL in tetrahydrofuran (THF). THF was also used as the eluent at a flow rate of 1 mL/min. Molecular weights are reported as poly(styrene) equivalent molecular weights, using a calibration curve constructed from EasiCal™ polystyrene standards (Agilent). Nanofiber mat morphology was also characterized with SEM after being sputter coated with a 20 nm layer of silver before imaging.

Cell Viability

L929 mouse fibroblast cells (L929, Lonza) were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Mediatech) containing L-glutamine, sodium pyruvate, phenol red, and supplemented with 10% fetal bovine serum (Hyclone), 25 mM HEPES buffer (Life Technologies), penicillin (100 units/mL), streptomycin (100 units/mL), and fungizone (250 ng/mL) (Lonza BioWhittaker). Human coronary artery endothelial cells (HCAEC, Lonza) were cultured in Clonetics Endothelial Cell Basal Medium-2 (EBM-2, Lonza). Each cell line was grown in a 12 well plate until 50 - 75% confluent. With 1 mL of media present, cells were sprayed for 5 seconds at a 13 SCFH CO₂ flow rate from 10 cm with CO₂ alone, acetone, and 10% 0.93 IV PLGA in

acetone. The well plates were then returned to an incubator for 24 hours. After 24 hours, cell viability was quantified using a CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS, Promega). Cell media was changed to 1 mL of the phenol free versions followed by the addition of 200 µl of MTS reagent. The well plates were returned to the incubator for 2.5 hours, 200 µL from each well was transferred to a 96 well plate and absorbance was read at 490 nm. Unsprayed cells served as the live control, a 30 minute incubation with 1 mL of methanol served as a dead control. All values are reported as percent of live control, each experiment was done in triplicate (n = 3).

Qualitative Blood Interaction

Human blood from healthy adult blood donors was obtained from the National Institutes of Health Blood Donor Research Program. The study protocol was approved by the US Food and Drug Administration (FDA) Research Involving Human Subjects Committee. Venous blood was drawn into polypropylene tubes containing anticoagulant citrate dextrose solution A (ACDA) at a ratio of ACDA:Blood of 1:6. A PLGA nanofiber mat (prepared on glass slides from solution blow spinning 10% 0.93 IV PLGA in acetone at 13 SCFH for 30 seconds) was placed into a 6 well plate, followed by the addition of 2 mL of the ACDA blood at room temperature. The well plate was placed into a shaker incubator at 50 rpm for 1 hour. The nanofiber mat was then washed twice with 1X sterile calcium and magnesium free Dulbecco's phosphate-buffered saline (Ca(-)Mg(-)DPBS) then fixed with a 2% glutaraldehyde solution (in Ca(-)Mg(-)DPBS) for 24 hours. The sample was then

washed twice with sterile PBS and dehydrated with a series of graded ethanol solutions and hexamethyldisilazane, then allowed to dry overnight. Finally, the nanofiber mat was sputter coated with a 20 nm layer of silver before imaging with SEM.

Pilot Animal Study

Surgery was completed on a single 4-6 week old Yorkshire piglet (weight 5-8 kg). All animal experiments and care were approved by the Institutional Animal Care and Use Committee (IACUC) of Children's National Medical Center and were in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1996). Anesthesia was induced with and maintained by mechanical ventilation with isoflurane. A midline laparotomy incision was made extending from the xiphoid to the pubis to expose the intra abdominal contents. Circumferential intestinal incision then took place followed by anastomosis with 4 hand-sewn sutures. 2 mL of 10% 0.93 IV PLGA in acetone at a CO₂ flow rate of 13 SCFH was sprayed around the site of anastomosis. After anastomosis, the liver was exposed and superficially lacerated (~20 mm long and 0.5 mm deep). This was followed by application of 2 mL of PLGA under the same deposition conditions. Subsequently, a median sternotomy was performed to allow access to the lungs. This was followed by a segmentectomy of the right lung and application of 2 mL of PLGA under the same deposition conditions. Ventilation was continued to determine if air leakage occurred at the cut lung surface. Finally, a defect was created in the diaphragm (~60 mm by 30 mm) to simulate a

diaphragmatic hernia. This was followed by application of 2 mL of PLGA under the same deposition conditions. At the conclusion of the study the animal was euthanized with an intravenous injection with Beuthanasia and death confirmed by monitoring.

Statistical Analysis

All experimental groups and controls were performed in replicate as referred to in each specific methods section. Where suitable, data was analyzed using ANOVA single factor analysis to evaluate differences between groups assuming a normal data distribution with a confidence of 95% ($p < 0.05$). Data is reported as mean values and error bars are reported as standard deviation aside from Figure 3.4, which presents the raw nanofiber diameter distribution.

3.3 Results and Discussion

PLGA nanofibers were fabricated via solution blow spinning using a commercial airbrush and compressed CO₂ (Figure 3.1). This technique allows for rapid conformal nanofiber deposition onto any substrate (Figure 3.1).

10% (wt/vol) PLGA of two different inherent viscosities, corresponding to higher and lower molecular weights, were investigated (0.93 IV and 0.64 IV PLGA) in acetone at 3 different gas flow rates (Figure 3.2). The best nanofiber morphology solution and deposition conditions were determined to be 10% (wt/vol) 0.93 IV PLGA in acetone with a 13 SCFH CO₂ gas flow rate (Figure 3.3). The resulting nanofiber mat had an

average nanofiber diameter of $474 \text{ nm} \pm 262 \text{ nm}$ and a median value of 377 nm (Figure 3.4). This range of nanofiber diameter is consistent with fibrin fiber diameter ($\sim 376 \text{ nm}$).²⁰⁷

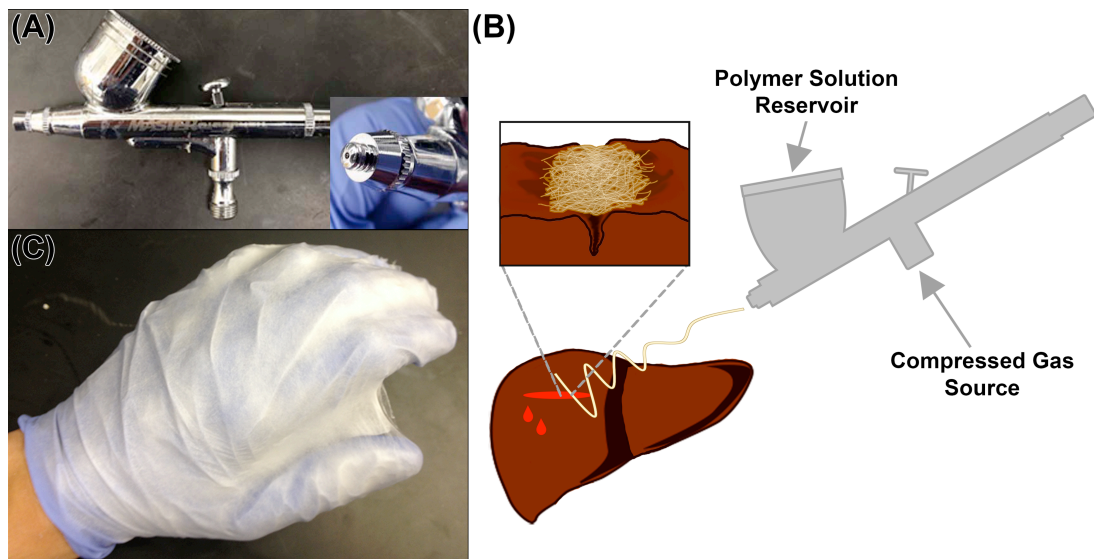


Figure 3.1 (A) The commercial airbrush used for nanofiber deposition. (B) Schematic representation of solution blow spinning procedure being applied to a liver injury. (C) A conformal nanofiber coating on a gloved hand.

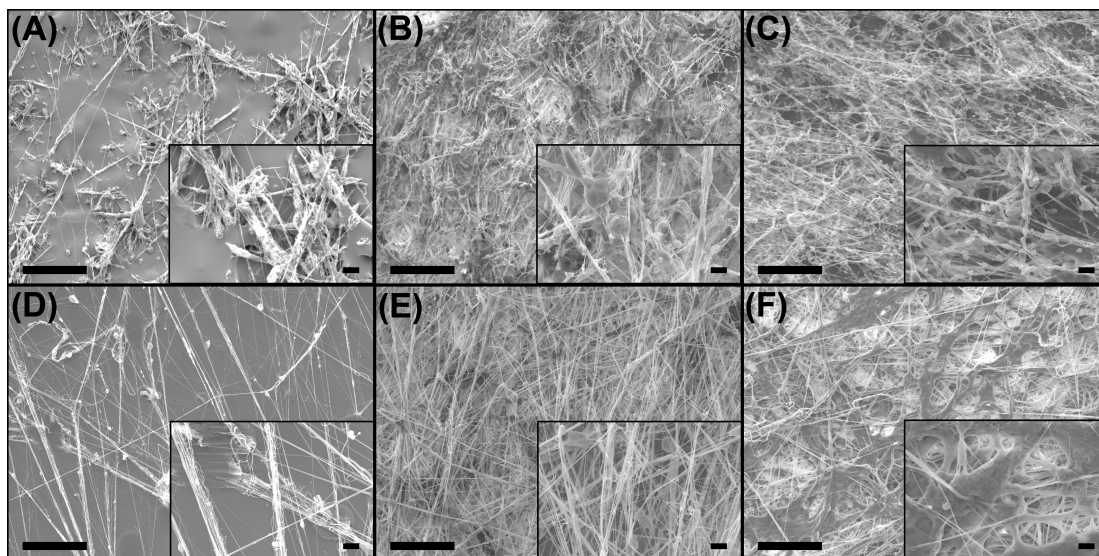


Figure 3.2. (A-F) SEM micrographs of solution blow spun 10% (wt/vol) PLGA in acetone. (A-C) Solutions of 0.64 IV PLGA, at CO₂ flow rates of 10, 13 and 15 SCFH. (D-F) Solutions of 0.93 IV PLGA, at CO₂ flow rates of 10, 13 and 15 SCFH. Solution and deposition conditions (e) resulted in the optimal fiber morphology. Scale bars represent 100 µm and 10 µm in the inset.

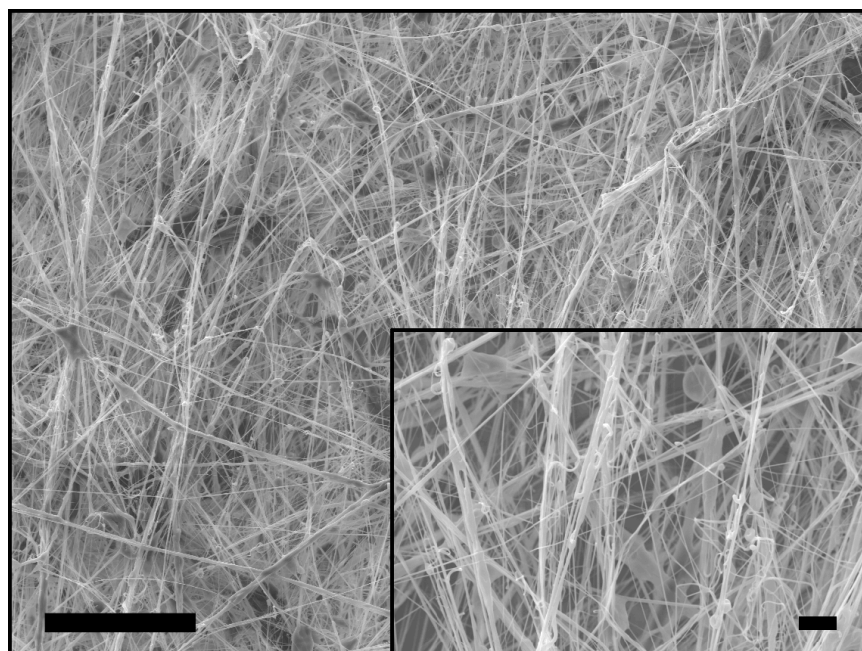


Figure 3.3. SEM of optimized solution and deposition conditions 10% (wt/vol) 0.93 IV PLGA in acetone at a CO₂ flow rate of 13 SCFH). Scale bars represent 100 µm and 10 µm in the inset.

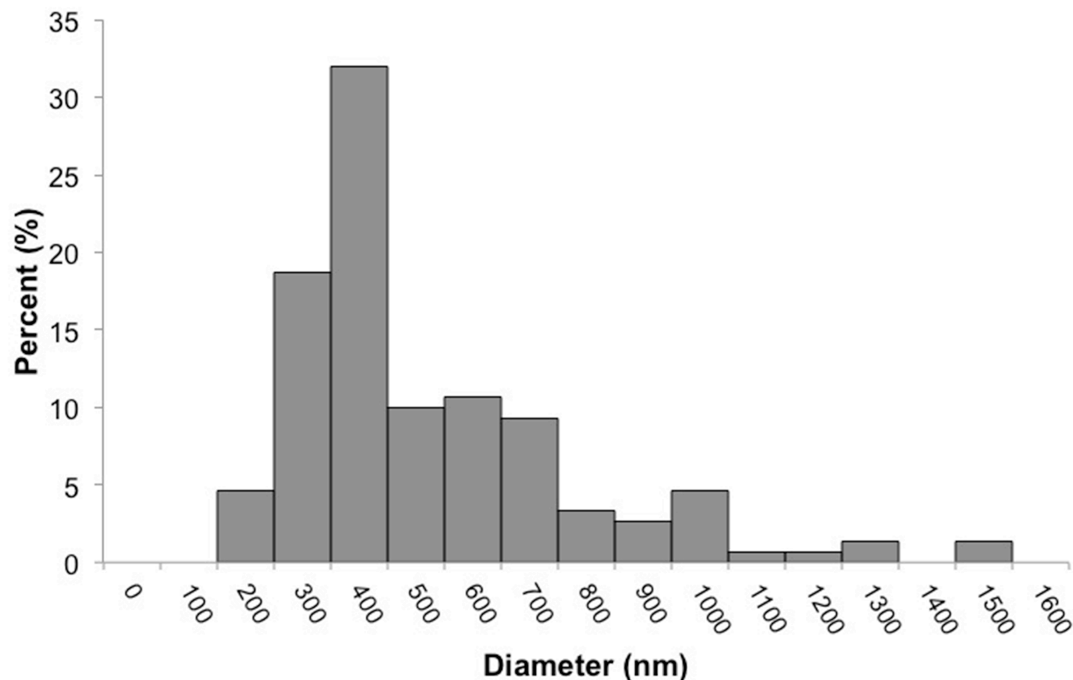


Figure 3.4. Size distribution of nanofiber diameter for optimal solution and deposition conditions (n = 150).

The ultimate strength was determined by the maximum of the stress-strain curve and the Young's modulus by the slope between 0-1% strain (Figure 3.5). The 0.93 IV PLGA had an average ultimate strength of 0.88 MPa $\pm$ 0.28 MPa and a Young's modulus of 0.33 MPa $\pm$ 0.06 MPa. The 0.64 IV PLGA had an average ultimate strength of 0.06 MPa $\pm$ 0.02 MPa and a Young's modulus of 0.01 MPa $\pm$ 0.01 MPa. The mechanical properties of the 0.93 IV PLGA nanofiber mats were significantly better than the same deposition conditions using the 0.64 IV PLGA. The Young's modulus of the optimal mat (0.33 MPa) was near that of fibrin (1-10 MPa) and many human tissues ($\sim$ 1 MPa).^{208, 209} These characteristics are well suited for *in vivo* use as tissue mimics in a variety of applications without the complications and costs associated with biologically derived materials.

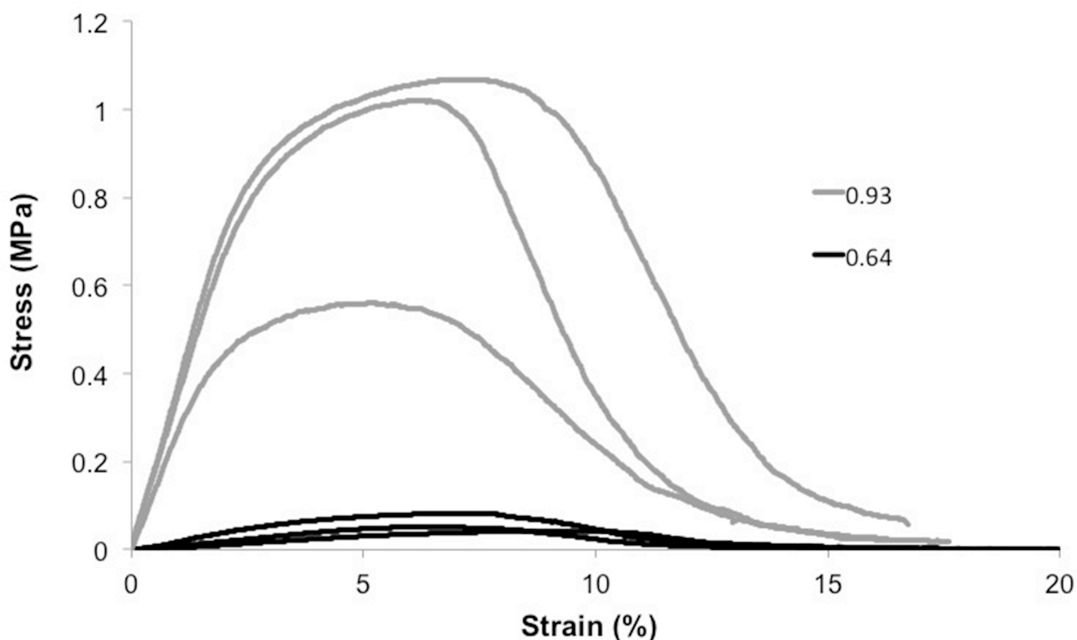


Figure 3.5. Stress strain curves of 0.93 IV PLGA and 0.64 IV PLGA nanofiber mats.

After identifying the optimal solution and deposition conditions, PLGA nanofiber degradation was evaluated over 42 days for morphologic and molecular weight changes (Figure 3.6). Nanofiber morphology changed noticeably over this time scale. As fibers degraded there was an increase in average diameter as a result of fibers fusing together, and a majority of the mat porosity was lost by day. As degradation continued (7-42 days), fibers welded into each other forming a more homogenous structure with evidence of obvious surface pitting and pore formation on day 30 and day 42.

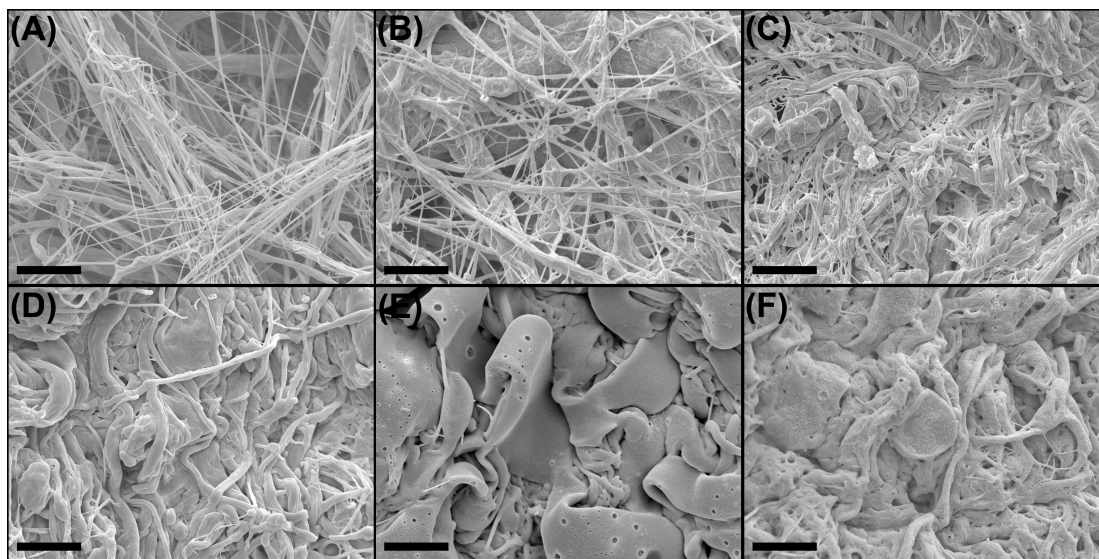


Figure 3.6. (A-F) SEM of PLGA nanofiber degradation at 0, 1, 7, 14, 30, and 42 days. Scale bars correspond to 20 μm . As fibers degraded there was an increase in average diameter as a result of fibers fusing together, and a majority of the mat porosity was lost by day 7 (C). As degradation continued (7-42 days), fibers welded into each other forming a more homogenous structure with evidence of surface pitting and pore formation beginning on day 30 and 42 (E-F).

During this same time frame, weight average molecular weight (M_w) and number average molecular weight (M_n) decreased in a linear manner from day 0 at $M_w = 123.9 \text{ kDa} \pm 9.4 \text{ kDa}$ and $M_n = 78.9 \text{ kDa} \pm 3.4 \text{ kDa}$ to day 42 at $M_w = 27.2 \text{ kDa} \pm 5.1 \text{ kDa}$ and $M_n = 12.5 \text{ kDa} \pm 2.6 \text{ kDa}$ (Figure 3.7). The polydispersity index (PDI) increased from 1.6 ± 0.1 to 2.2 ± 0.2 during this period (Figure 3.7). This linear decrease in molecular weight over a 42 day period, corresponds to a M_w decrease of $\sim 78\%$ and M_n decrease of $\sim 84\%$. A linear degradation profile is consistent with surface erosion in contrast to bulk erosion.²¹⁰ The high surface area/volume of the nanofibers presumably allows for quicker diffusion and neutralization of the acidic degradation species that typically cause autocatalytic degradation in bulk erosion.

This degradation profile can be altered by adjusting the ratio of lactic to glycolic acid in the copolymer.²¹¹

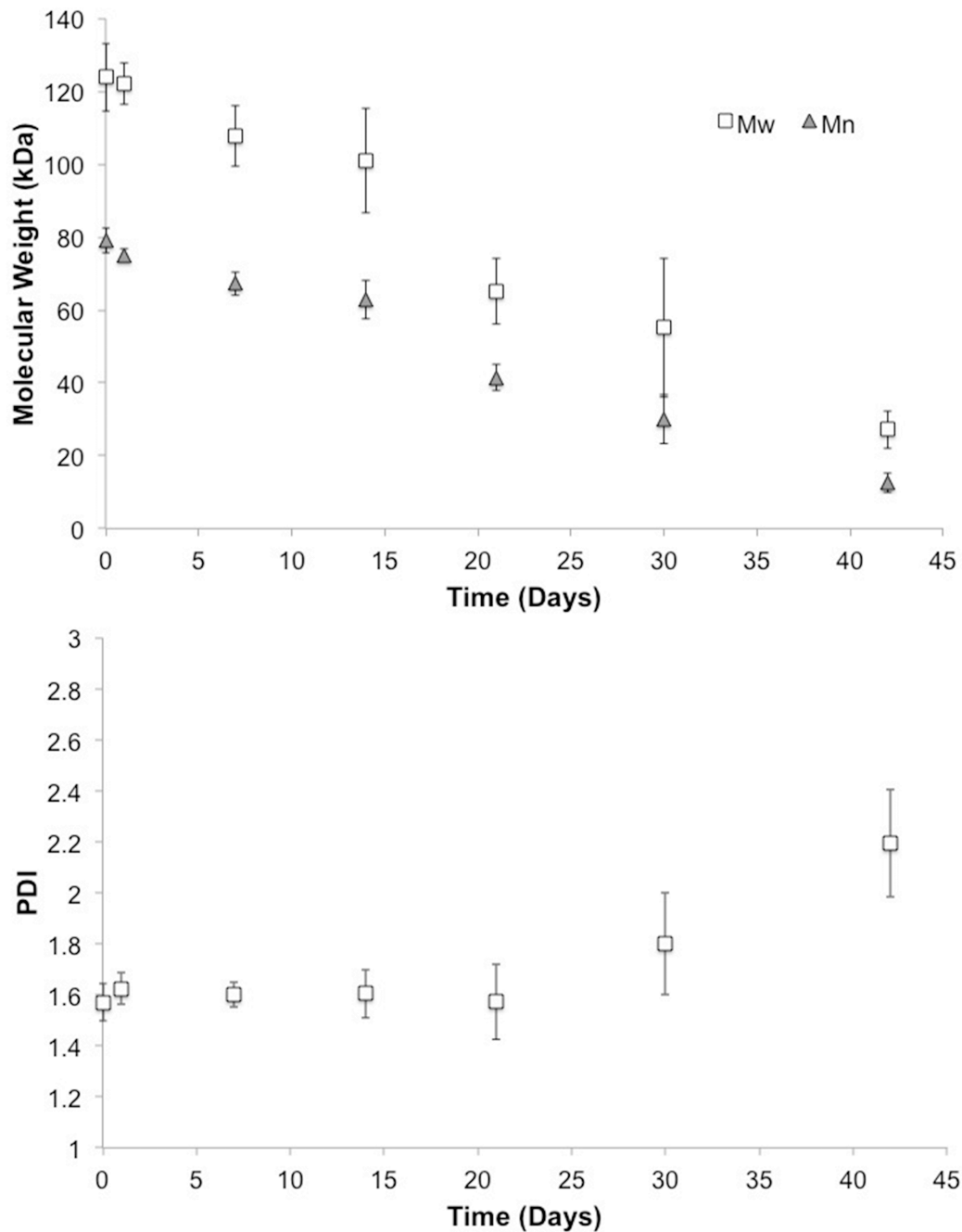


Figure 3.7. (Top) PLGA molecular weight over 42 days of nanofiber degradation ($n = 3$). Bottom, PDI change over the same timescale ($n = 3$). Error bars represent standard deviation.

Biocompatibility of solution blow spinning PLGA/acetone directly onto cells was quantitatively evaluated with an MTS cell viability assay. All experimental groups (CO₂, acetone, PLGA/acetone) showed no statistical difference in cell viability as compared to the live control group for both L929 mouse fibroblasts and human coronary arterial endothelial cells ($p < 0.05$, Figure 3.8).

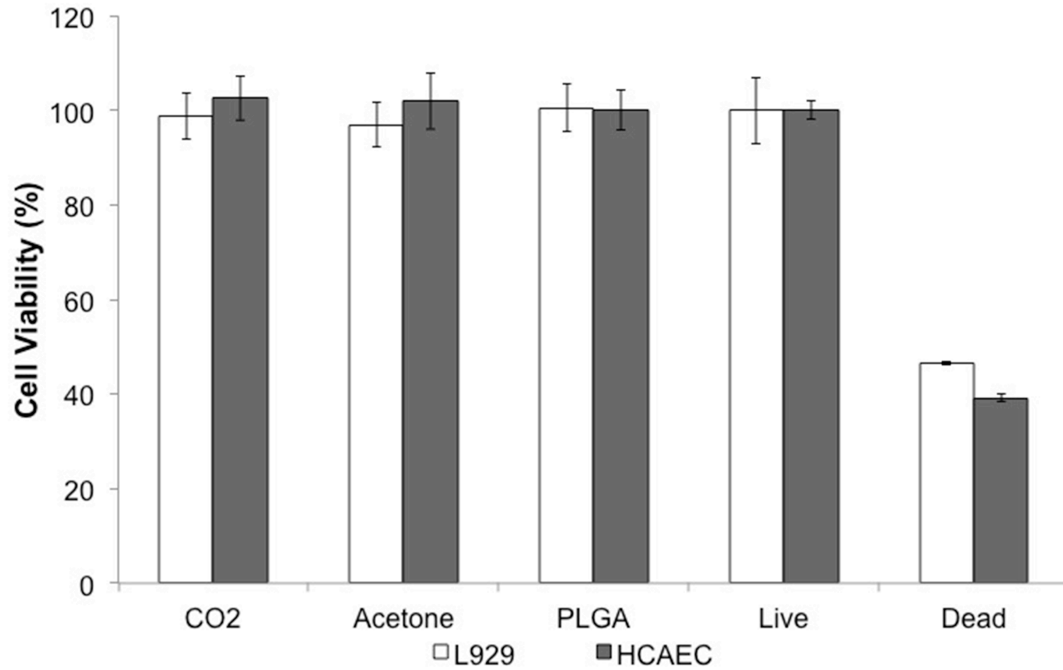


Figure 3.8. MTS cell viability assay of L929 mouse fibroblasts (L929) and human coronary atrial endothelial cells (HCAEC) showing no decrease in viability. Data reported as percent of live control ($n = 3$). Error bars reported as standard deviation.

Qualitative evaluation of blood interaction with nanofiber mats was completed using SEM (Figure 3.9). After an hour incubation and washing with PBS there was significant platelet and erythrocyte adsorption onto the nanofiber matrix.

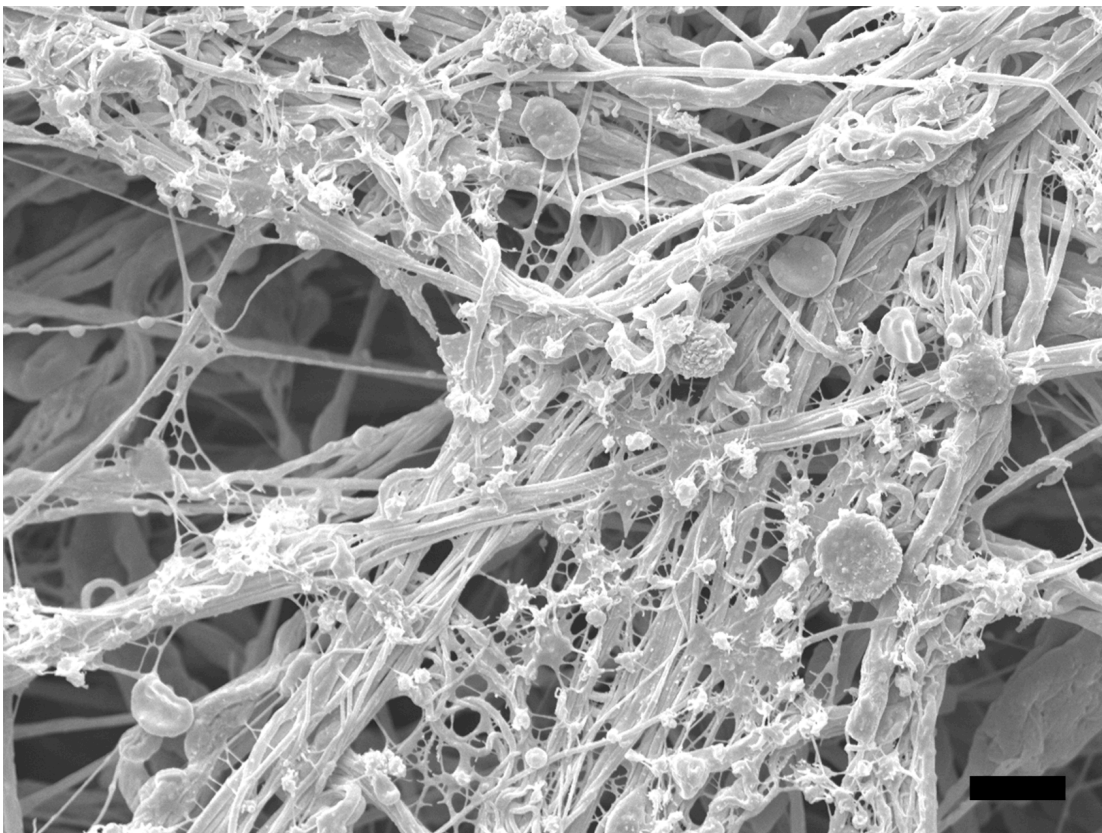


Figure 3.9. SEM of PLGA nanofibers incubated with citrated whole human blood. Scale bar represents 10 μm .

While solution blow spinning can be used with a variety of compressed gasses, CO_2 was chosen for its potential for *in situ* deposition. Other compressed gasses carry the risk of air embolism; CO_2 avoids this complication due to its solubility in blood.^{212, 213} Similarly, safety considerations must be given to the choice of polymer and solvent. Non-degradable polymers have the potential to cause harm if inhaled and most volatile solvents are too toxic to be considered for direct application. While the use of acetone may not be ideal, cell viability data from two cell lines suggests that the amount reaching the surface must be minimal (Figure 3.8). Additionally, for fibers to be generated and maintained, any solvent must evaporate from the system. At the highest gas flow rate tested (15 SCHF), nanofibers seemed to weld back together

(Figure 3.2 F). This can be attributed to the drop in temperature resulting from the compressed CO₂ release, leading to a decreased evaporation rate of acetone in the system. This led to the choice of the 13 SCHF flow rate for all subsequent experimentation (Figure 3.3).

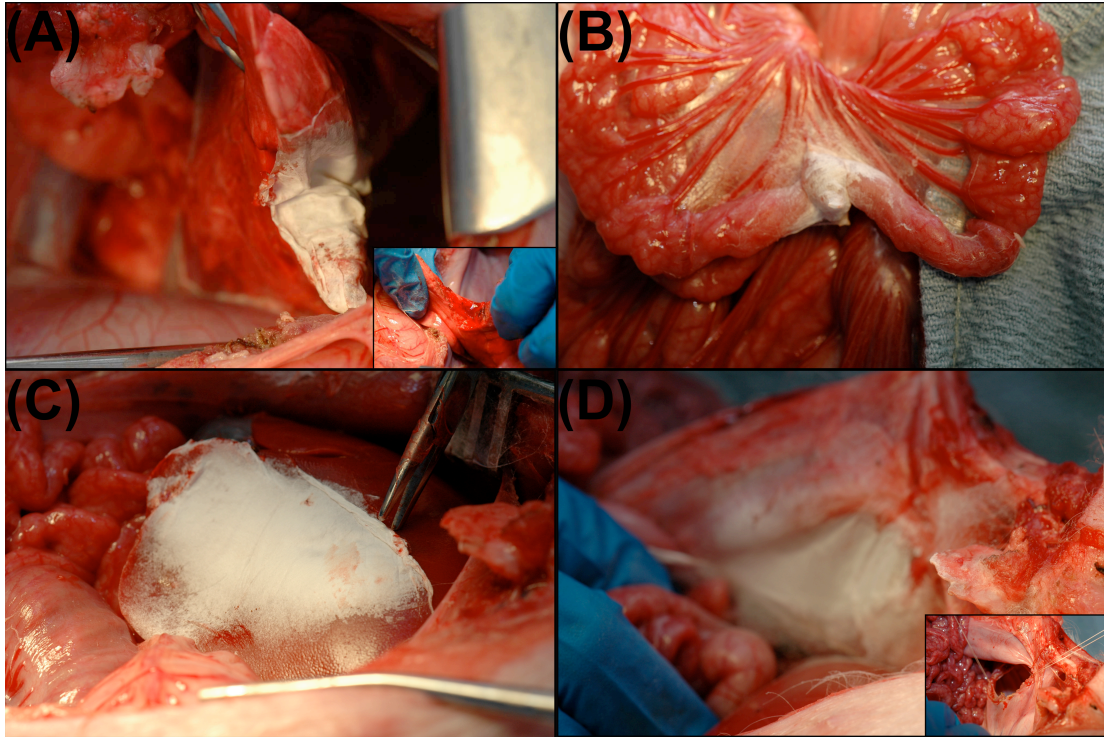


Figure 3.10. Direct deposition of conformal PLGA nanofiber mats. (A) Lung resection, (B) Intestinal anastomosis, (C) Liver injury, and (D) Diaphragmatic hernia.

To demonstrate possible future applications of solution blow spinning, PLGA nanofiber mats were directly deposited in multiple surgical models in a single piglet animal model. These include lung resection, intestinal anastomosis, superficial liver injury, and diaphragmatic hernia (Figure 3.10). In all cases, a conformal layer of nanofibers formed over the defect in less than 1 minute. Visual observation by a surgeon confirmed that PLGA nanofiber deposition stopped the liver bleeding and air leakage from the lung surface following segmentectomy.

3.4 Conclusion

The utilization of nanofiber mats and scaffolds has been widely investigated for a variety of surgical and tissue engineering applications. The current fabrication methods leave no realistic possibility of *in situ* deposition. Solution blow spinning offers an easily adaptable alternative that has the potential to generate on demand conformal nanofiber mats *in situ*. The present study demonstrates facile fabrication of PLGA nanofibers using only a commercial airbrush and compressed CO₂. The solution and deposition conditions were optimized and mechanical properties characterized. Nanofiber degradation was monitored over 42 days for molecular weight and morphology changes *in vitro*. Biocompatibility of nanofiber deposition was quantitatively assessed using a cell viability assay and blood interaction assessed qualitatively with SEM. A pilot animal study was then used to demonstrate some of the possible surgical applications including use as a surgical hemostatic, a surgical sealant, and for tissue reconstruction.

Chapter 4: Biodegradable Polymer Blend Based Surgical Sealant with Body Temperature Mediated Adhesion

4.1 Introduction

Surgical care suffers from a lack of materials that enable patient specific intervention. Conventional suturing and tissue stapling remain the preferred method of surgery associated wound closure irrespective of their defined limitations.^{214, 215} The development of practical and efficient surgical sealants has the propensity to enhance surgical competency, reduce the incidence of complications, and improve patient comfort.

Clinically available surgical sealants include fibrin, glutaraldehyde, cyanoacrylate, and poly(ethylene glycol) (PEG) hydrogel based materials. Fibrin glues are high in cost, complex to prepare for application, and carry the risk of disease transmission.²¹⁶ Glutaraldehyde-protein sealants are relatively low in cost, but suffer from toxicity issues that restrict their ultimate clinical potential.²¹⁷ Cyanoacrylate glues induce significant inflammatory responses and have been associated with tissue toxicity, limiting their use to external applications.^{218, 219} PEG hydrogel based sealants achieve adhesion with end group succinimidyl glutarate functionalization that covalently reacts with tissue.²²⁰ They are non-cytotoxic and do not involve the risk of disease transmission, but require multi-step preparations and can undergo excessive swelling after use that can cause tissue injury of neighboring anatomic structures like nerves or

even the tissue involved.¹⁴ These shortcomings have led to the investigation of new approaches for developing safe and effective surgical sealants.

Many current approaches utilize biomimetic strategies to promote tissue adhesion. This is accomplished through the use of reactive chemistries, components, or features found in a variety of aquatic animals^{221, 222} and reptiles.²²³ Aquatic animals rely on adhesive chemistries to adhere to wet surfaces. For instance, marine mussels use a catechol containing post-translationally modified amino acid (3,4-dihydroxyphenylalanine) to covalently bind to amines and thiols under oxidative conditions.²²⁴ This functionalization has been investigated for use in surgical sealants in the form *in situ* forming hydrogels^{225, 226} and coacervates.²²⁷ Sealants have also been designed to maximize interfacial adhesion by mimicking the high aspect ratio topographical features of gecko feet.^{223, 228} These micro- and nano-patterned surfaces have been used in combination with wet adhesive chemistries to improve utility in a surgically relevant environment.^{72, 75, 229}

An important consideration in the development of surgical sealants is the ease of use. Many approaches rely on a mixing and/or an initiation step that leads to a gelation or crosslinking event. Failure to optimize parameters such as cure time or solution viscosity can lead to poor performance due to clogging or inadequate sealant coverage. Shear-thinning hydrogels circumvent many of these problems by taking advantage of dynamic self-assembly.²³⁰ UV-curable sealants have also garnered wide interest.²³¹ A rapidly curing hydrophobic light-activated adhesive based on

poly(glycerol sebacate acrylate) recently demonstrated efficacy in cardiac procedures.²³² Another interesting strategy is the use of pre-formed polymer nanosheets.²³³ The nanometer-scale thickness of these films results in polymer-substrate interactions that promote adhesion. While promising, many of the aforementioned approaches are geometrically restricted by the area of both the sealant material and the application device.

Here we report a body temperature activated surgical sealant that utilizes the thermal behavior of a biodegradable polymer blend to promote adhesion. The polymer blend is deposited with an airbrush using a technique called solution blow spinning.¹⁹⁰ This method allows for a polymer fiber mat to be directly and rapidly fabricated onto any anatomical region of interest.²³⁴ After warming to a critical temperature (~ 31 °C), the fiber mat consisting of a poly(lactic-co-glycolic acid) (PLGA)/PEG blend undergoes a fiber mat to film transition. During this transition, the sealant increases in adhesive strength, and becomes transparent. While variations of PLGA and PEG have been incorporated into degradable sutures, surgical sealants, and hemostatic dressings, they only fill a structural or supportive role in these applications and do not typically take an active role in tissue adhesion on their own.

4.2 Materials and Methods

Polymer Deposition

A commercially available airbrush (Master Airbrush G22-SET, 0.2 mm nozzle diameter) was used to deposit all polymer fiber mats. The airbrush was connected to a

compressed CO₂ tank equipped with a regulator through a gas flow meter. Polymer solutions were composed of 10%(wt/v) PLGA (IV = 0.86 dL/g in hexafluoroisopropanol, Lactel) in combination with 0%-5%(wt/v) PEG (M_n = 950-1050 g/mol, Sigma Aldrich) in acetone.

Morphology Characterization

Fiber mat samples were prepared by solution blow spinning 50 or 500 µL of polymer blend solutions onto 1 inch cover slips. For scanning electron microscopy (SEM), samples were sputter coated with a gold before imaging (SEM, JEOL JSM-6390LV). Nanofiber diameter was measured using ImageJ (National Institutes of Health). Average fiber diameter and distribution were determined from 100 random measurements from 3 SEM micrographs. For optical microscopy, each sample was placed on a Mettler FP82HT hot stage controlled by a Mettler FP90 Central Processor and heated from room temperature to 37 °C at a rate of 1 °C per minute.

Differential Scanning Calorimetry

Approximately 10 mg samples of fiber mats were sealed in aluminum hermetic pans (TA Instruments) using a sample encapsulation press. DSC measurements were made on a TA Instruments DSC Q100. For modulated DSC measurements, samples were held isothermal at -50 °C for 5 minutes and then heated and cooled from -50 °C to 80 °C to -50 °C, at a rate of 3 °C per minute, ±0.20 °C amplitude, with a modulation period of 60 seconds for two continuous cycles. For standard DSC measurements,

samples were heated and cooled from -50 °C to 80 °C to -50 °C at a rate of 10 °C per minute for five continuous cycles.

Adhesion

Tensile adhesive strength measurements were made using an Instron E3000/R3703 mechanical tester. 1 mL samples were deposited onto the face of a 1 square-inch custom machined aluminum stand. The samples were then incubated for 10 minutes in compression with another 1 square inch aluminum face at either room temperature or 37 °C before testing. The crosshead speed was set to 5 mm per minute for all samples. Each sample type was replicated 5 times (n = 5).

Degradation

Polymer mats were prepared on 1 inch glass coverslips from solution blow spinning 1 mL polymer solutions. The samples were then placed into 6 well plates and submerged in 5 mL of 1X phosphate buffered saline (PBS). The plates were then put in a shaker incubator (37 °C, 50 rpm) and PBS was exchanged every 24 hours for 30 days. Samples were removed from the incubator at time points of 0, 1, 3, 7, 14 and 30 days. At these points, PBS was removed, the samples were air dried overnight and stored in a vacuum desiccator. At each time point (n = 3) PLGA molecular weight was measured. Molecular weight was determined by gel permeation chromatography (GPC) using a Waters Alliance Separations Module e2695, Waters 2414 Refractive Index Detector, and Waters HSPgel columns in series (HR MB-L and HR 3.0

columns, 6.0 mm I.D. X 15 cm). Both Columns and the RI detector were heated to 30 °C during all data acquisition. Molecular weights are reported as polystyrene relative molecular weights, as calculated from a 10-point calibration curve generated using Agilent EasiCal polystyrene standards. All data analysis was performed using Waters Empower 3 Chromatography Data software.

Cell Viability

Cell viability was assessed *in vitro* using both L929 mouse fibroblast cells (L929, Lonza) and Human coronary artery endothelial cells (HCAEC, Lonza). L929s were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM, Mediatech) containing L-glutamine, sodium pyruvate, phenol red, and supplemented with 10% fetal bovine serum (Hyclone). HCAECs were cultured in Clonetics Endothelial Cell Basal Medium-2 (EBM-2, Lonza). In the direct deposition studies, each cell line was grown in a 6 well plate until 50-75% confluent. With 3 mL of media present, cells were directly sprayed with 500 µL of either 10% PLGA/0% PEG, 10% PLGA/5% PEG, acetone alone, or CO₂ alone from the airbrush. These groups were compared to a live control receiving no spray and a dead control which was incubated in 70% methanol for 30 minutes. The cells were then returned to an incubator for 24 hours. After 24 hours cell media was replaced with 1 mL of the phenol free version of the aforementioned medias followed by the addition of 200 µL MTS reagent (CellTiter 96 AQueous One Solution Cell Proliferation Assay MTS, Promega). The well plates were returned to the incubator for 2.5 hours, 200 µL from each well was transferred to a 96 well plate and absorbance was read at 490 nm. In a separate study, 500 µL of

either 10% PLGA/0% PEG, 10% PLGA/5% PEG were deposited onto glass coverslips and placed into the bottom of a 12 well plate. Either L929s or HCAECs were then seeded on top of the materials (30,000 cells per well) and returned to the incubator for 24 hours. At 24 hours, cell viability was again assessed using an MTS assay and compared to the same controls. All values are reported as percent of live control, each experimental group was performed in triplicate (n = 3).

Cecal Intestinal Anastomosis Survival and Burst Pressure Studies

All animal experiments and care were approved by the Institutional Animal Care and Use Committee (IACUC) of Children's National Medical Center and were in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1996). 16-week old C57BL6/J mice were anesthetized using 100 mg/kg and 10mg/kg xylazine. An exploratory laparotomy incision was made, and the cecum was exposed. The cecum was transected, leaving the mesenteric sidewall intact in order to preserve the blood supply. The cecum was then re-anastomosed using 4 interrupted 7-0 vicryl sutures. 40 anastomoses were performed on 40 mice with 10 mice in each of 4 groups (n = 10). The groups included a control group that underwent the sutured anastomosis without any additional sealant and 3 experimental groups that underwent the sutured anastomosis and received additional sealant. These groups were supplemented with either solution blow spun 10% PLGA/5% PEG sprayed directly on the suture site, Coseal (PEG Hydrogel, Baxter International Inc.), or Tisseel (Fibrin glue, Baxter International Inc.). The commercial sealants were

prepared and applied to the anastomoses in accordance to the product insert directions. Following a set time of 2 minutes for all groups, the bowel was placed into the abdomen and the skin was closed using a 4-0 vicryl suture in a running fashion. All surgeries were performed by the same surgeon, who was blinded to the treatment groups. Loupes were used for magnification in all cases.

Each animal was weighed preoperatively, and weights were taken every other day until day of sacrifice. Animals were allowed to survive for 7 days and were sacrificed after necropsy and harvestation of the anastomotic bowel segment. Animals were monitored daily and were sacrificed prior to 7 days if they showed signs of extreme distress due to sepsis.

In the anastomotic burst pressure study, mice underwent the same procedure outlined above (n = 4 - 6 for each group). These mice were allowed to survive for one day, at which time they were re-anesthetized. After performing an exploratory laparotomy, the anastomotic segment was identified, and burst pressures were determined. A digital pressure monitor was attached to a three-way stopcock, allowing for a saline solution containing 0.05% methylene blue to be infused through the third port. After clamping the small bowel at the ileocecal junction, an angiocatheter with tubing attached to the three-way stopcock was placed into the distal aspect of the anastomosis in a water-tight fashion. Burst pressure was measured as the highest pressure reached prior to leakage of methylene blue solution from the anastomotic site.

Statistical Analysis

Data is expressed as mean $\pm$ standard error. Where appropriate, statistics were performed using SAS Version 9.3 (SAS Institute). ANOVA single variable analysis with Tukey's test for multiple comparisons was used. Relevant statistical relationships are reported on figures ($p < 0.05$).

4.3 Results and Discussion

Solution blow spinning is utilized for polymer fiber deposition. Rapidly coating non-uniform surfaces with polymer fiber mats has been demonstrated with this technique in non-medical²³⁵ and medical applications,^{192, 234, 236} including deposition *in situ*.²³⁴ This ability circumvents the limitations associated with pre-fabricating sealant materials without knowledge of specific procedural or anatomical details. Additionally, since the material reaches the tissue surface as a solid, there is no activation or gelation step. Fiber formation is accomplished when a combination of solution and deposition requirements are met. The major considerations are solution concentration with respect to polymer chain entanglement and solvent. The solvent must rapidly evaporate to facilitate the generation of the polymer fibers. Fibers will also not be formed if the solution concentration is below the polymer chain overlap condition.¹⁹¹ $\pm\pm$

Polymer Fiber mats were deposited from solutions containing 10%(wt/v) PLGA and 0%-5%(wt/v) PEG dissolved in acetone. These polymers are commonly investigated for a wide variety of biomedical applications such as drug delivery and tissue

engineering.²³⁷ This broad usage can be attributed to their biocompatibility and bioabsorbability.²³⁸ A commercially available airbrush with compressed CO₂ was used as the solution blow spinning apparatus in all cases without modification (Figure 4.1). Higher PEG content led to increased fiber diameter and less well define fiber morphology (Figure 4.2). Fiber Diameter ranged from $0.57 \pm 0.03 \mu\text{m}$ for the 10%/0% (PLGA/PEG) solution to $2.42 \pm 0.15 \mu\text{m}$ for the 10%/5% solution (Figure 4.3). A 0% PLGA/5% PEG solution was not able to generate fibers (Figure 4.4).

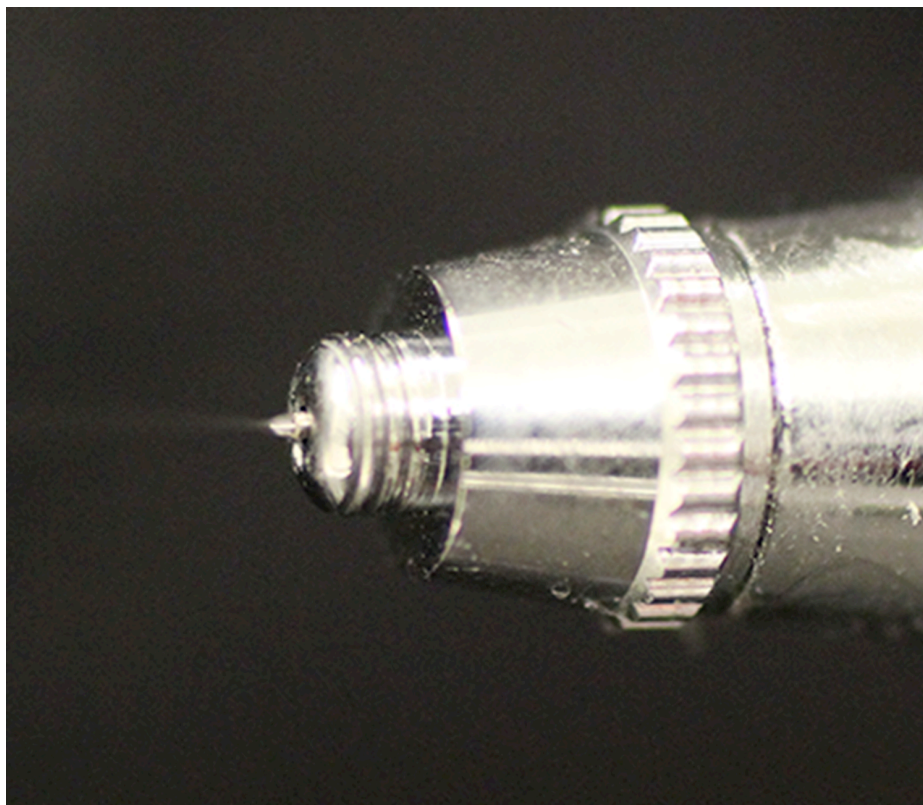


Figure 4.1. Nozzle of commercial airbrush while solution blow spinning 10% PLGA/5% PEG.

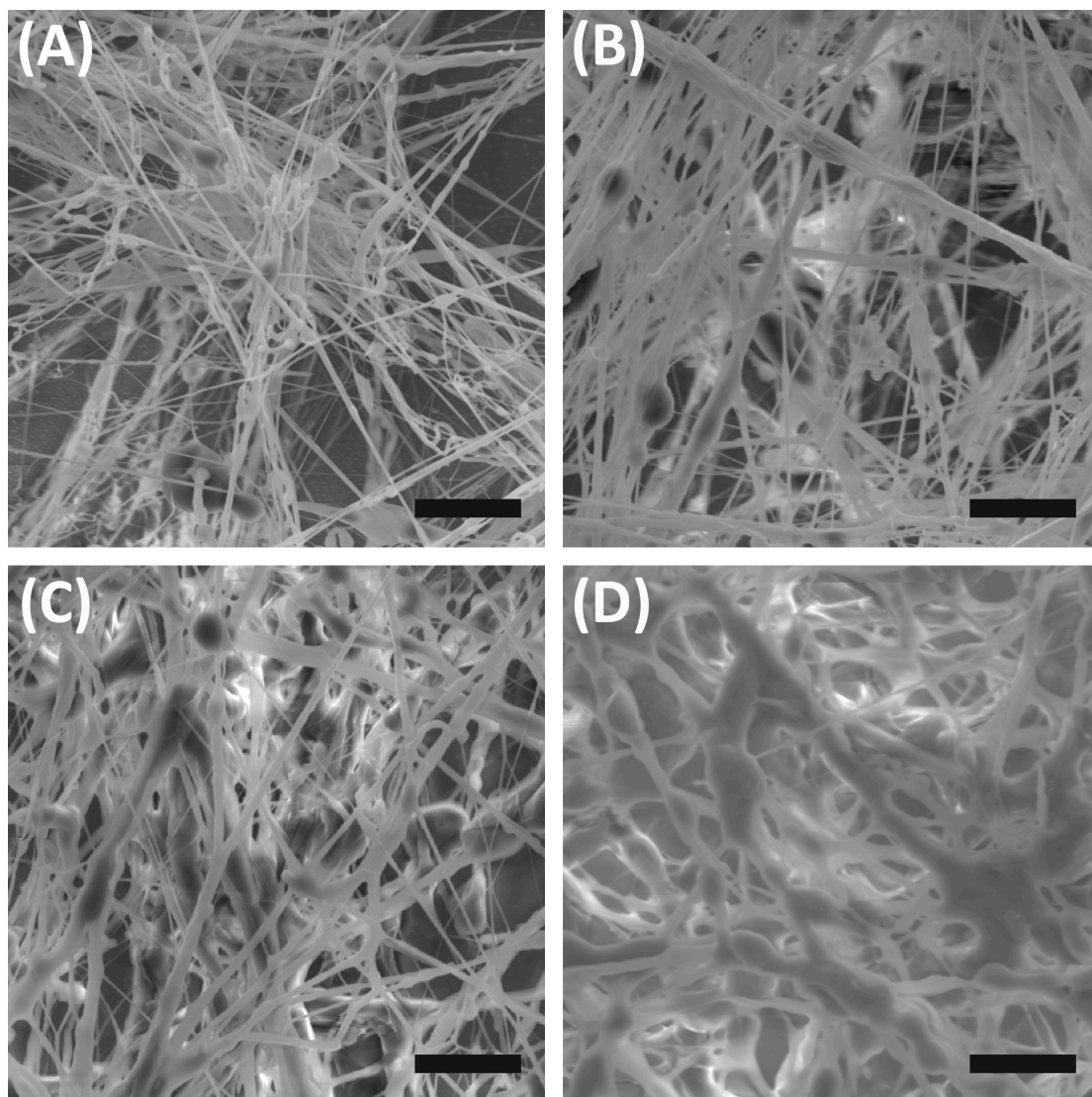


Figure 4.2. SEM images of solution blow spun PLGA/PEG blends showing changes in fiber mat morphology with increase PEG content. (A) 10% PLGA 0% PEG (wt/vol), (B) 10%/1%, (C) 10%/3%, and (D) 10%/5%. Scale bars represent 20 μm .

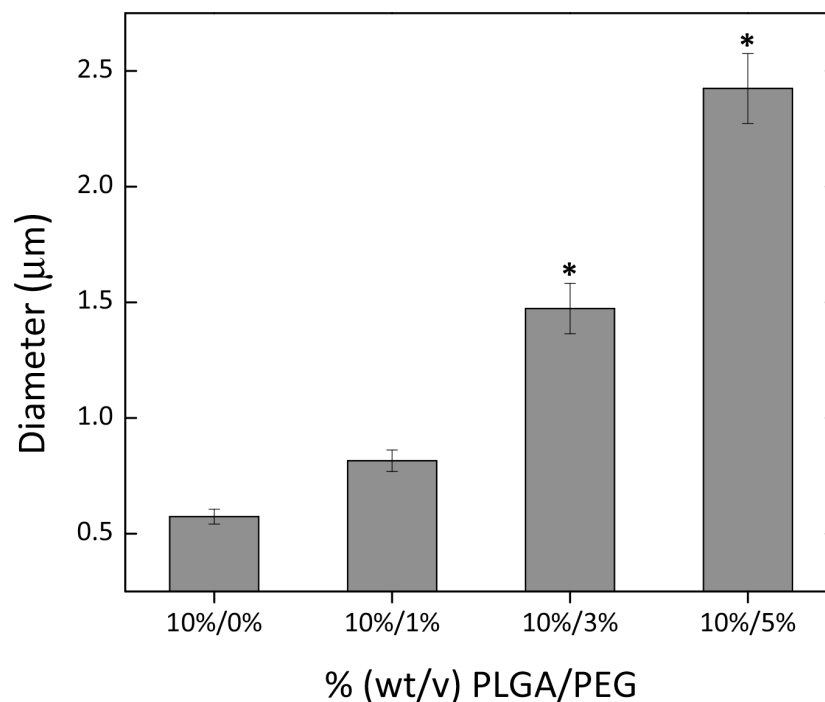


Figure 4.3. Average fiber diameter corresponding to polymer blend content. Fiber diameter increases with increasing PEG content. Error bars represent standard error.

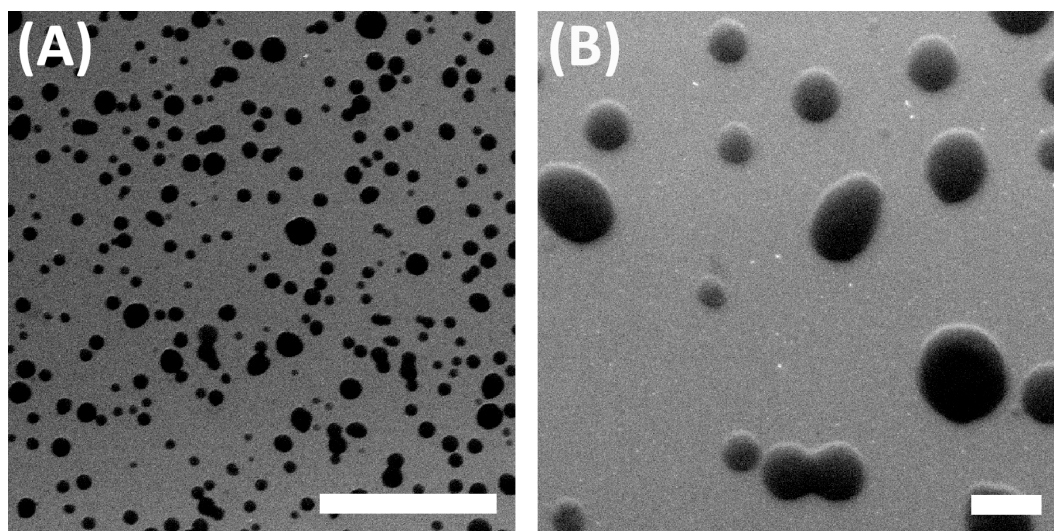


Figure 4.4. SEM images of samples deposited from solutions of 5% (wt/v) ~1kDa PEG in acetone. PEG at this molecular weight and concentration was unable to facilitate the generation of polymer fibers through the use of an airbrush. (A) Scale bar represents 100 μm and (B) scale bar represents 10 μm.

When deposited onto a warm substrate (over ~ 31 °C), the polymer fiber mats with higher PEG content (10%/3% and 10%/5%) undergo a melting transition between 31 °C and 32 °C (Figure 4.5). This is in agreement with the melting temperature of the crystalline domains of PEG alone (Figure 4.5, Table 4.1). Conveniently, melting occurs at a temperature that is below both topical and internal body temperatures. Thermal behavior in similar polymer blends has been previously exploited to create porous tissue engineering scaffolds^{239, 240} and injectable cell delivery systems.²⁴¹ Upon reaching the crystal melting temperature the fibers weld together and PLGA and PEG become miscible, creating a homogenous, transparent polymer film (Figure 4.6 and 4.7). During film formation the polymer blend becomes plasticized with a glass transition temperature (T_g) well below body temperature (Figure 4.8, Table 4.2), resulting in increased polymer-substrate interaction. After the initial melting event the recrystallization of PEG is suppressed in all cases except for the pure PEG sample (Figure 4.8). Correspondingly, adhesive strength drastically increases for the high PEG content blends after incubation at 37 °C (Figure 4.9). This is in contrast to the adhesive strength of PLGA alone at either room temperature or 37 °C and to the same blends at room temperature (Figure 4.9).

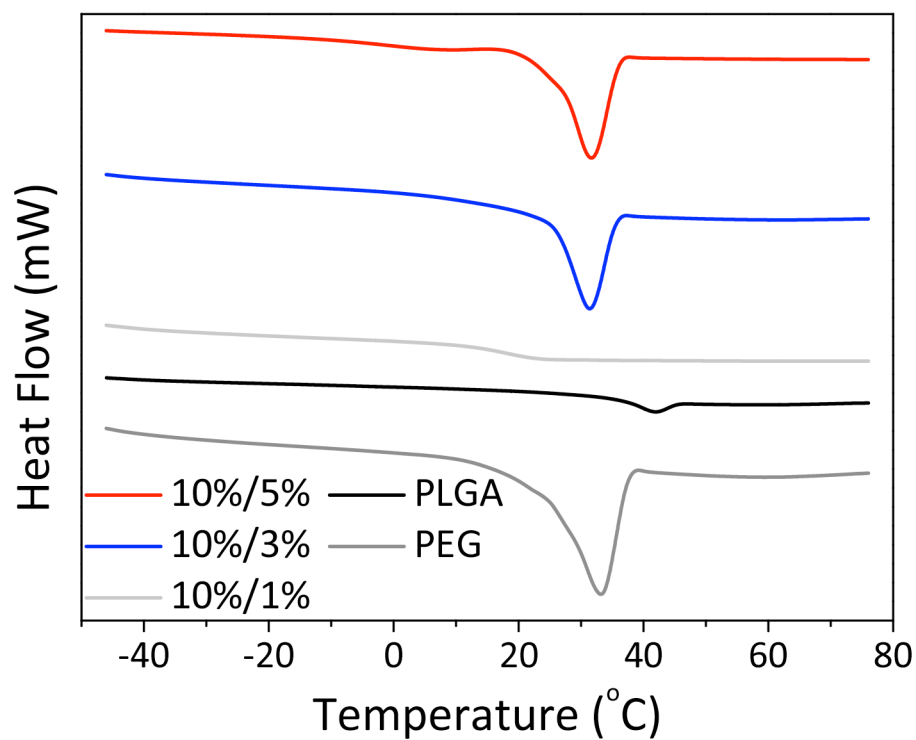


Figure 4.5. DSC of first heating cycle for various PLGA/PEG blends. High PEG content blends exhibited melting transitions.

Table 4.1. Melting Temperature (°C)

Sample	1 st Cycle	2 nd Cycle
10% PLGA/5% PEG	31.61	--
10% PLGA/3% PEG	31.40	--
10% PLGA/1% PEG	--	--
PLGA	--	--
PEG	33.19	30.98

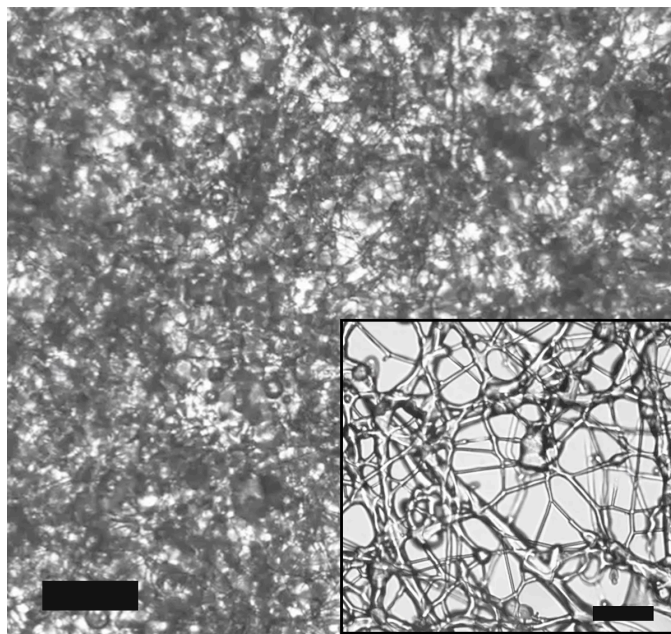


Figure 4.6. Optical micrograph of a 10%PLGA/5%PEG film showing fibrous morphology at room temperature. Scale bars represent 100 μm and 25 μm in the inset.

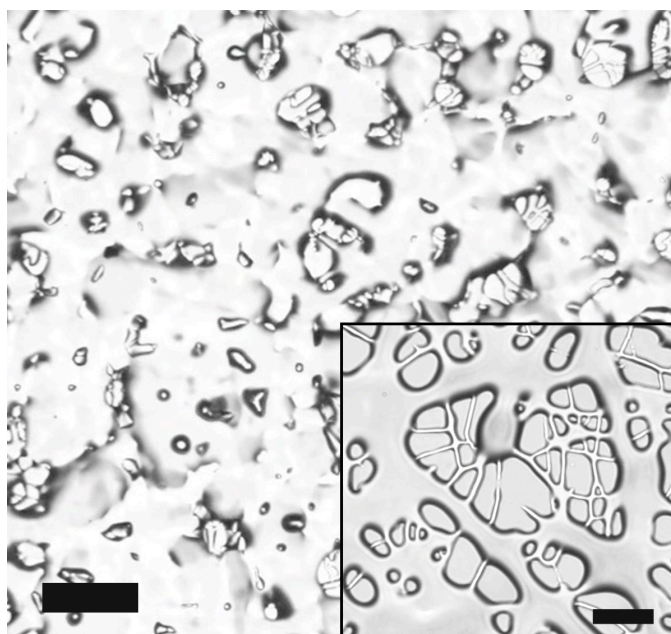


Figure 4.7. Optical micrograph of a 10%PLGA/5%PEG film showing the transition to film morphology at 37 °C. Scale bars represent 100 μm and 25 μm in the inset.

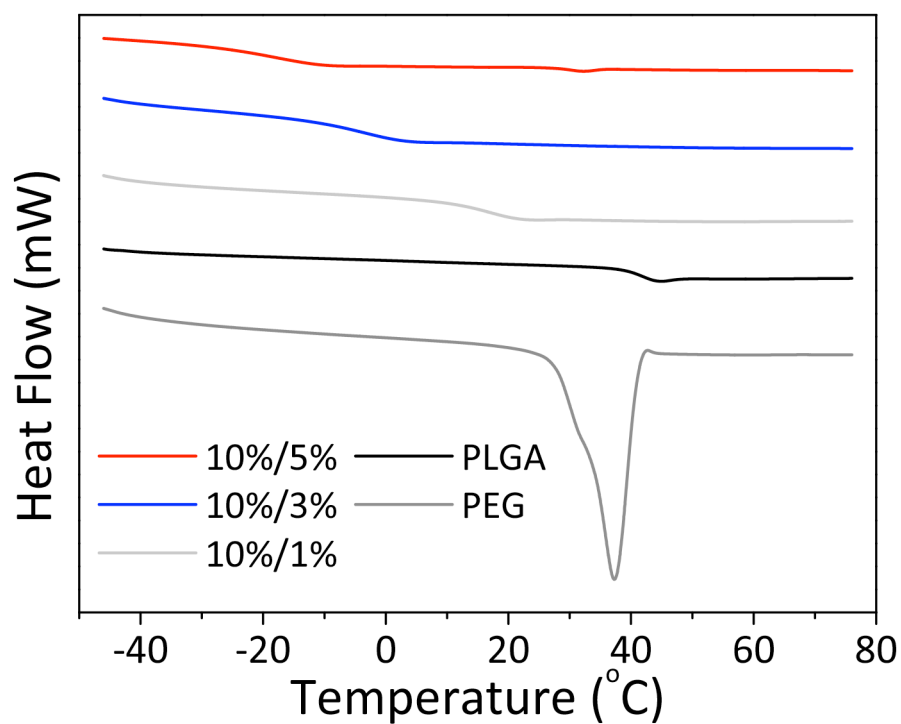


Figure 4.8. DSC of second heating cycle for various PLGA/PEG blends. Glass transitions are lower with increasing PEG content.

Table 4.4. 2nd Cycle T_g (°C)

Sample	Heating	Cooling
10% PLGA/5% PEG	-18.03	-19.96
10% PLGA/3% PEG	-3.60	-6.42
10% PLGA/1% PEG	18.29	17.45
PLGA	42.07	39.15
PEG	--	--

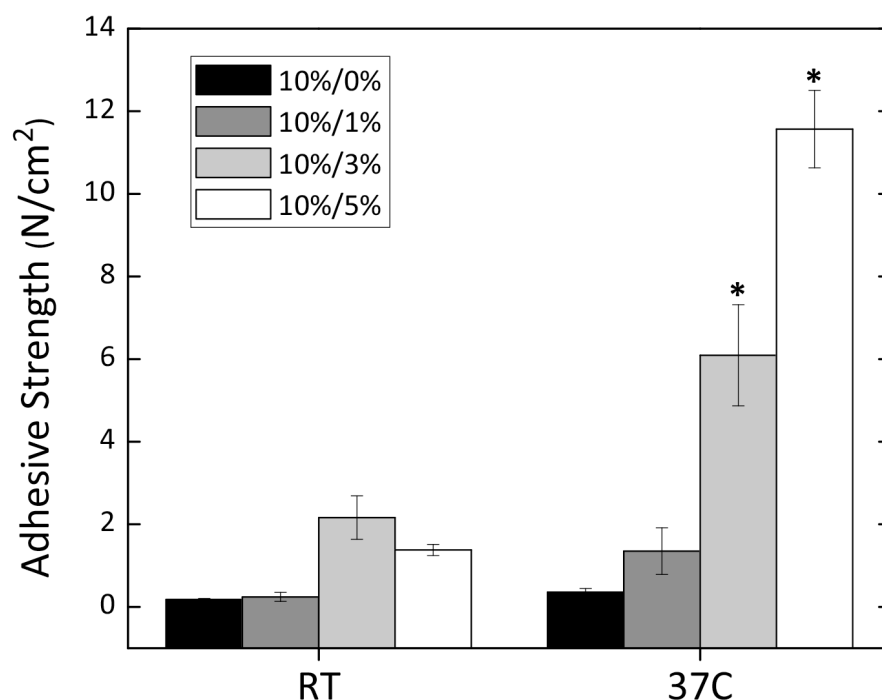


Figure 4.9. Adhesive strength measurement of various PLGA/PEG compositions, demonstrating the influence of PEG content and temperature on adhesive strength. Error bars represent standard error.

In vitro material degradation and biocompatibility studies were used to predict *in vivo* behavior. Degradation was monitored over a 30-day period. 10% PLGA/5% PEG was compared to 10%PLGA/0% PEG. PEG content did not have a drastic effect on PLGA molecular weight decrease over this time scale (Figure 4.10). PEG content after 30 days was also evident in the GPC chromatographs (Figure 4.11). The material also remained plasticized over this entire period (Figure 4.12). Cell viability was assessed for both the direct application of the material and cells seeded on top of the material via an MTS assay using both L929 mouse fibroblasts (L9292) and human coronary artery endothelial cells (HCAEC). Only moderate differences were seen all groups including the deposition of acetone alone when compared to the live control (Figure 4.13)

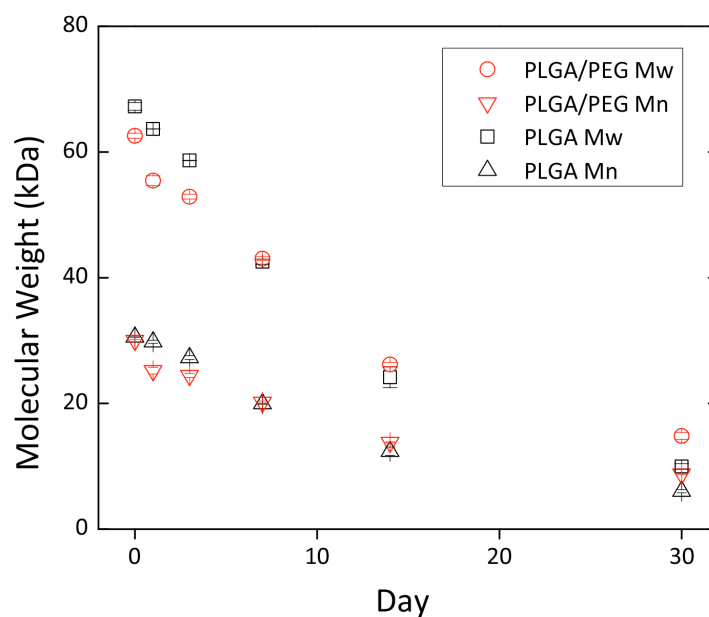


Figure 4.10. Number and weight average molecular weight corresponding to 10%(wt/v)/0%(wt/v) PLGA/PEG and 10%(wt/v)/5%(wt/v) PLGA/PEG solution blow spun samples. Incorporation of PEG did not significantly impact the degradation rate of PLGA over 30 days *in vitro*.

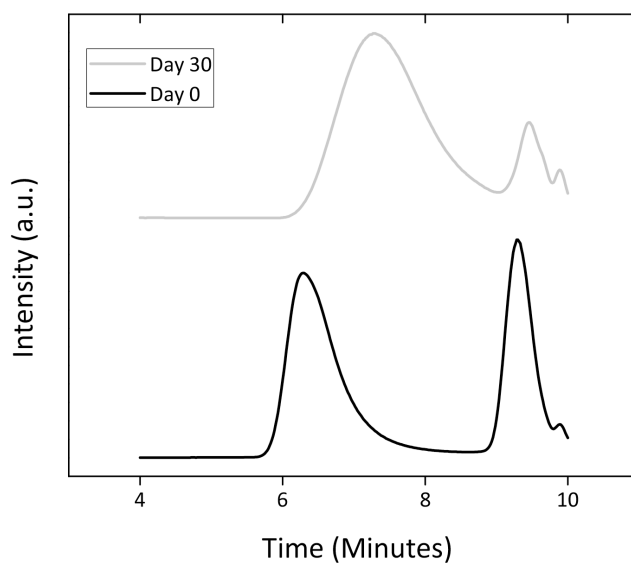


Figure 4.11. Gel permeation chromatograms of a 10%/5% sample at day 0 and day 30 of the *in vitro* degradation study, showing peaks representative of both PLGA and PEG at day 30.

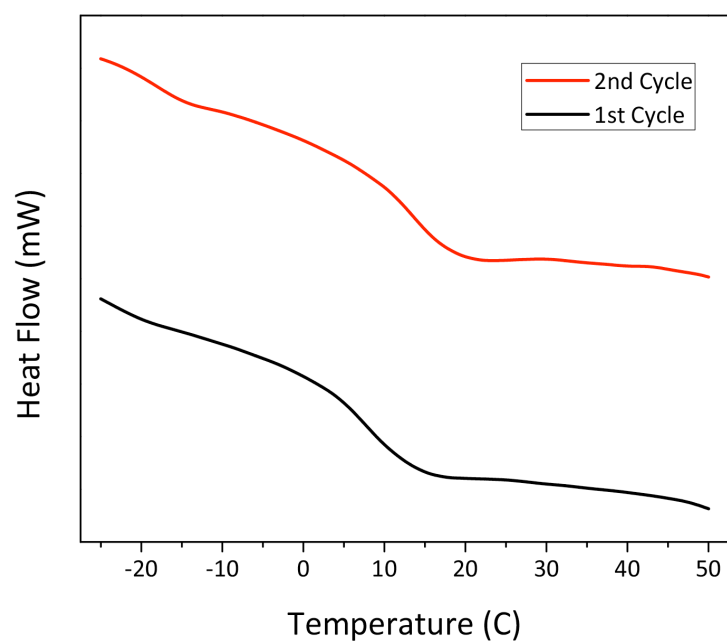


Figure 4.12. DSC of a 10%/5% sample at day 30, showing the persistence of the plasticization effect of PLGA over the full time period (T_g less than body temperature).

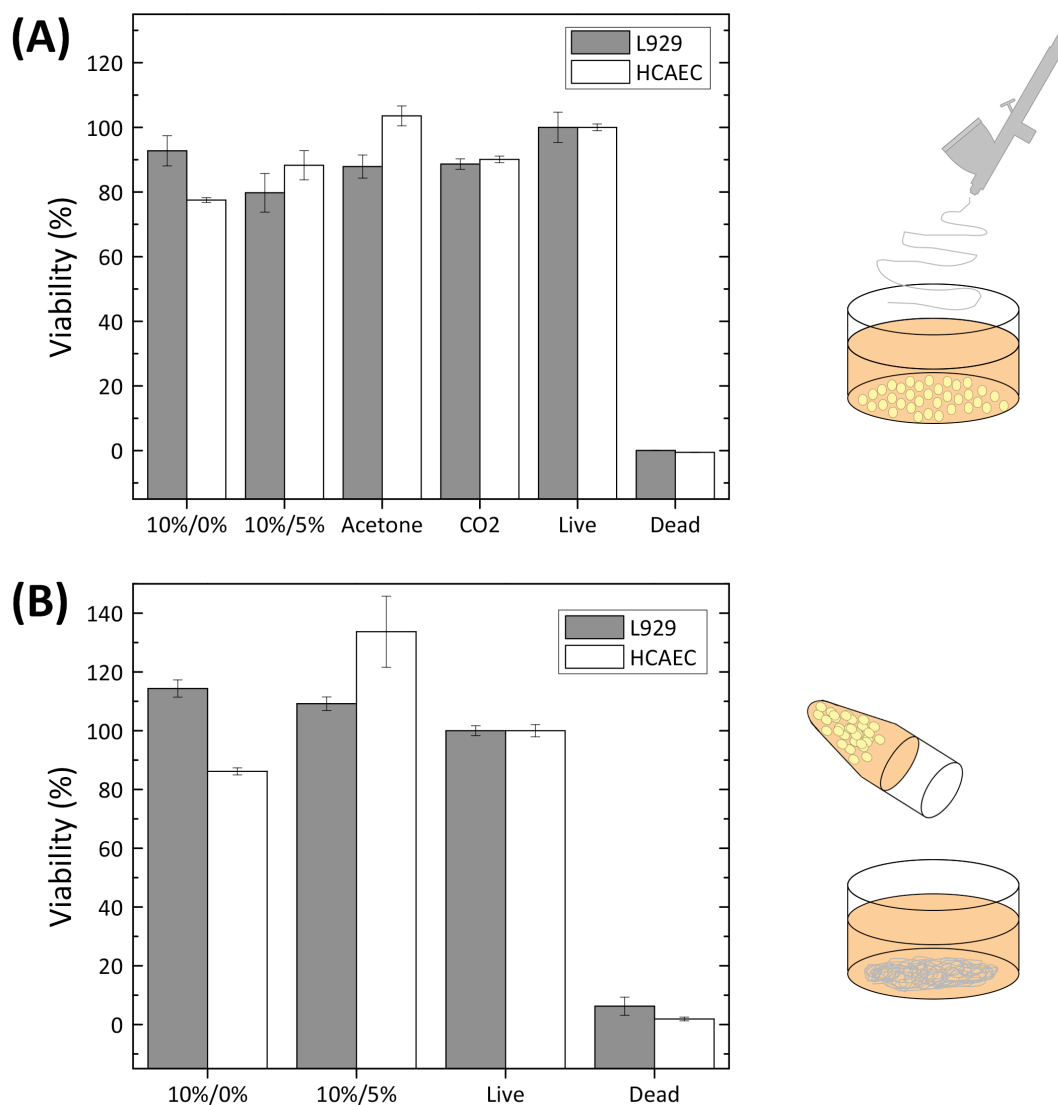


Figure 4.13. An MTS cell viability assay was used to assess the biocompatibility. A) direct deposition of 10%(wt/v)/0%(wt/v) PLGA/PEG, 10%(wt/v)/5%(wt/v) PLGA/PEG, acetone alone, and CO₂ alone onto pre-seeded L929 mouse fibroblasts (L929) and Human coronary artery endothelial cells (HCAEC). B) L929s and HCAECs seeded on top of pre-formed samples. Only moderate differences in cell viability was seen, including the direct deposition of acetone alone. Error bars represent standard error (n = 3).

A cecal intestinal anastomosis mouse model was used to assess the clinical potential of this material (Figure 4.14). This type of procedure can be technically challenging and suffers from high rates of morbidity and mortality when complications of leakage occur.²⁴² A high-risk model was adapted from other groups^{243, 244} in which a sub-clinical number of sutures leads to increased incidence of mortality. The solution blow spun PLGA/PEG sealant (10%/5%) was used to supplement the sutures (Figure 4.15 and 4.16). Performance was compared to a suture-only control, and the sutures supplemented with a commercially available fibrin glue or PEG hydrogel sealant. In an initial study, burst pressure at the site of anastomosis was measured at 24 hours (Figure 4.17). The PLGA/PEG sealant supplemented sutures were exceptionally resistant to leakage, reaching an average of 71.4 mmHg (Figure 4.18). The intestine complete filled with fluid, and significantly swelled before any minor leakage occurred (Figure 4.19). This is in contrast to the other 3 groups tested that had an average burst pressure that was over two times lower the solution blow spun PLGA/PEG sealant.

The same cecal intestinal anastomosis model was used in a 7-day survival study (Figure 4.20). As mentioned previously, the sub-clinical number of suture used in this model cause a high incidence of complication. Namely, bowl perforation, ischemia, obstruction, and sepsis. This is reflected in the suture-only control resulting in a 50% mortality rate over this 7-day time period, with all deaths occurring before day 3. The sutures supplemented with the PEG hydrogel sealant saw a similar mortality rate at 60%. One mouse from this group survived the 7 days but failed to thrive (Table 4.3).

This was characterized by weight loss and the observation of a walled off abscess during necropsy. The use of either the solution blow spun PLGA/PEG sealant or the fibrin glue resulted in a 10% mortality rate, representing a 40% improvement over the suture-only control.



Figure 4.14. Image of the 4-suture cecal intestinal anastomosis. A sub-clinical number of sutures was utilized to increase the incidence of complication.



Figure 4.15. Image of the PLGA/PEG sealant being deposited onto the sutured anastomosis. Intestine highlighted by black circle.



Figure 4.16. Image showing the thermal transition of the PLGA/PEG sealant in comparison to Figure 4.15.



Figure 4.17. Burst pressure experimental set up. An angiocatheter was used to supply fluid and was attached to a pressure monitor. Black circle indicates site of anastomosis.

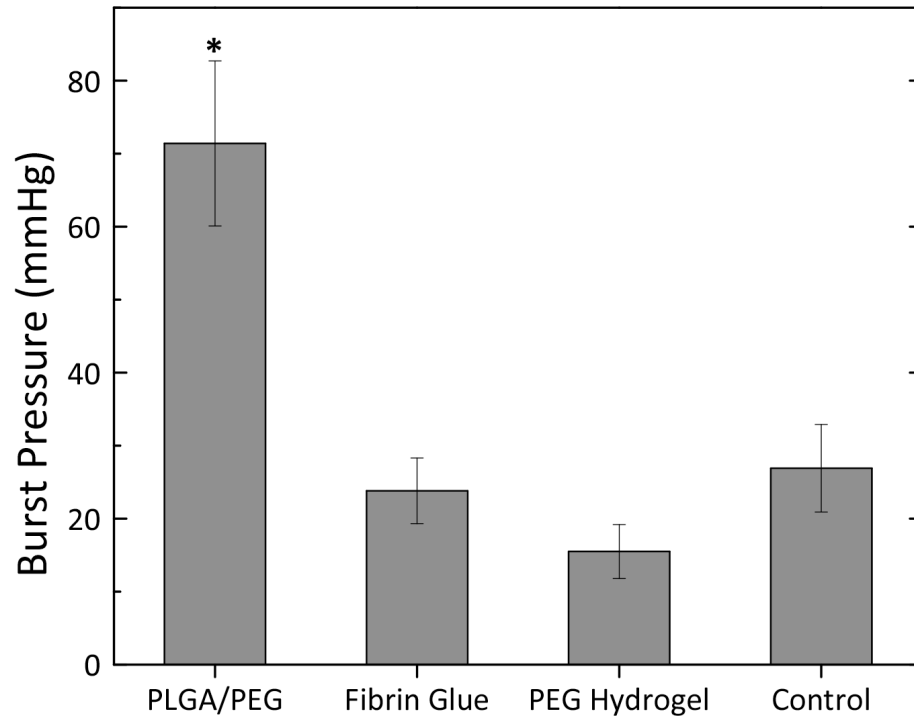


Figure 4.18. Burst Pressure study. At 24 hours, the bow spun PLGA/PEG sealant reinforced cecal anastomosis had an average burst pressure of 71.4 mmHg (n = 6), reinforced with the fibrin glue had an average burst pressure of 23.8 mmHg (n = 5), reinforced with the PEG hydrogel sealant had an average burst pressure 15.5 mmHg (n = 4), and the suture only control had an average burst pressure of 26.9 mmHg (n = 5). Error bars represent standard error.



Figure 4.19. Image of a PLGA/PEG supplemented anastomosis showing resistance to burst.

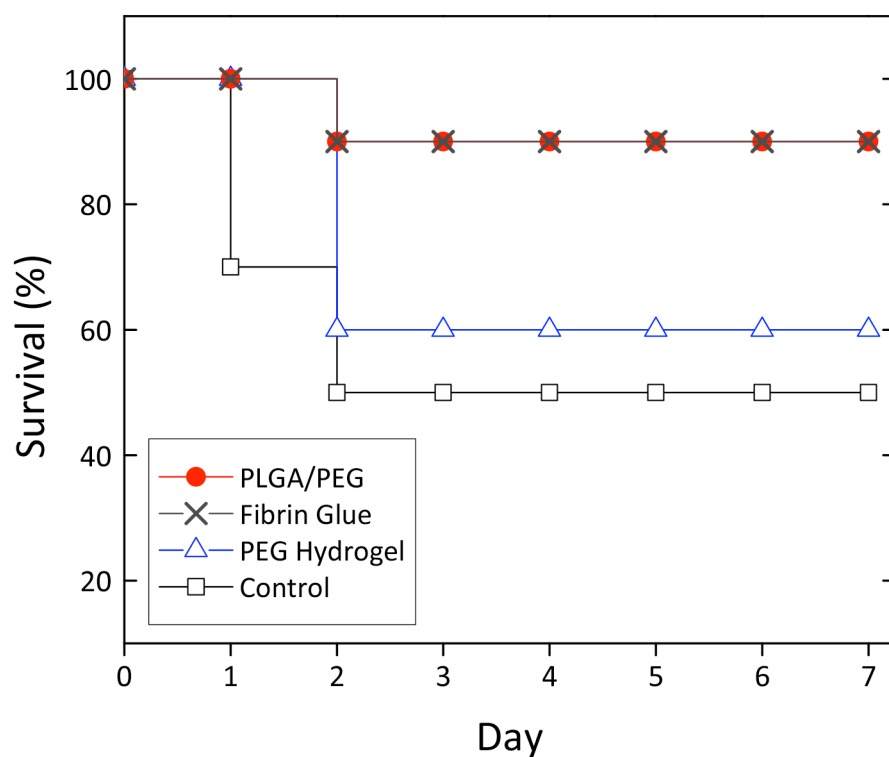


Figure 4.20. 7-day cecal anastomosis survival study. 50% of the suture-only control group died by day 3 due to complications such as leakage at the anastomosis ($n = 10$). In contrast, 10% of the 4 suture anastomosis supplemented with solution blow spun PLGA/PEG leaked, with 9 out of 10 mice surviving the 7 day study ($n = 10$).

Table 4.3. Cecal Intestinal Anastomosis Survival Data.

Animal	Days Survived	Initial Weight [g]	Final Weight [g]	Outcome
PLGA/PEG 1	7	20	19	Healthy
PLGA/PEG 2	7	19	19	Healthy
PLGA/PEG 3	7	20	19	Healthy
PLGA/PEG 4	7	19	18	Healthy
PLGA/PEG 5	7	21	20	Healthy
PLGA/PEG 6	7	20	17	Healthy
PLGA/PEG 7	2	20	19	Bowel perforation
PLGA/PEG 8	7	22	20	Healthy
PLGA/PEG 9	7	21	20	Healthy
PLGA/PEG 10	7	20	18	Healthy
Fibrin Glue 1	7	19	17	Healthy
Fibrin Glue 2	7	20	20	Healthy
Fibrin Glue 3	7	21	20	Healthy
Fibrin Glue 4	2	22	19	Bowel perforation
Fibrin Glue 5	7	18	17	Healthy
Fibrin Glue 6	7	19	18	Healthy
Fibrin Glue 7	7	20	18	Healthy
Fibrin Glue 8	7	18	19	Healthy
Fibrin Glue 9	7	19	17	Healthy
Fibrin Glue 10	7	20	19	Healthy
PEG Hydrogel 1	7	22	19	Healthy
PEG Hydrogel 2	7	23	20	Healthy
PEG Hydrogel 3	7	20	18	Healthy
PEG Hydrogel 4	7	20	13	Walled off abscess
PEG Hydrogel 5	2	20	18	Bowel perforation
PEG Hydrogel 6	2	20	18	Bowel perforation
PEG Hydrogel 7	7	22	19	Healthy
PEG Hydrogel 8	2	19	17	Bowel perforation
PEG Hydrogel 9	2	19	17	Bowel perforation
PEG Hydrogel 10	7	20	20	Healthy
Control 1	2	19	19	Bowel perforation
Control 2	7	19	18	Healthy
Control 3	7	21	20	Healthy
Control 4	1	19	18	Bowel perforation
Control 5	2	20	19	Bowel perforation
Control 6	1	21	19	Ischemia
Control 7	1	20	19	Ischemia
Control 8	7	20	17	Healthy
Control 9	7	20	19	Healthy
Control 10	7	20	28	Healthy

4.4 Conclusion

We have developed a body temperature responsive surgical sealant that is applied with a process that allows for conformal polymeric material deposition directly onto the tissue of interest. The surgical sealant was comprised of a polymer blend composed simply of a PLGA and PEG. The sealant transitioned from a fiber mat to film by taking advantage of the thermal behaviors of the polymer blend. This transition resulted in increased polymer substrate interaction. When utilized in a high-risk intestinal anastomosis model, the PLGA/PEG sealant was able to drastically improve the survival rate in comparison to the suture only control.

Chapter 5: Outlook and Future Directions

5.1 Outlook

In this work we have demonstrated the fabrication and application of materials for hemostatic and surgical sealant use. Both approaches warrant further investigation from a variety of standpoints. Discussion of new potential applications, material functionalizations, and apparatus design are included below.

5.2 Surgery

While gastro-intestinal tract anastomoses were the primary surgical models used in the fifth chapter, many other surgical procedures should ultimately be investigated. The current material properties may be more than adequate for other procedures such as lung resections, liver lacerations, liver segmental resections, femoral vessel injuries, and abdominal aortic anastomoses. Furthermore chemical functionalization or changes in polymer choice could lead to tailored material and adhesive properties for specific surgeries. Pilot animal models in which the PLGA/PEG sealant was used demonstrated viability as a hemostatic in both liver resection (Figure 5.1) and femoral vessel injury (Figure 5.2). These as well as the aforementioned procedure types should be investigated through clinical collaboration. All of the reported solution blow spinning contained in this work utilizes a commercial airbrush attached to a large CO₂ cylinder. It may be advantageous to design a small apparatus that operates on small CO₂ canisters from portability and usability perspectives. Further iterations of device design could consider accelerating solvent evaporation rate through heating or designing a vacuum/exhaust system for laparoscopic use.



Figure 5.1. The edge of the right lobe of the liver was sharply excised (A-B) and two mattress sutures were applied to slow blood loss. No hepatic vascular control was obtained prior to resection. The surface of the resection margin was sprayed with blow spun polymer. Complete vascular control at the resection margin was obtained and after 30 minutes of observation there was no evidence of bile leak or bile staining (C).

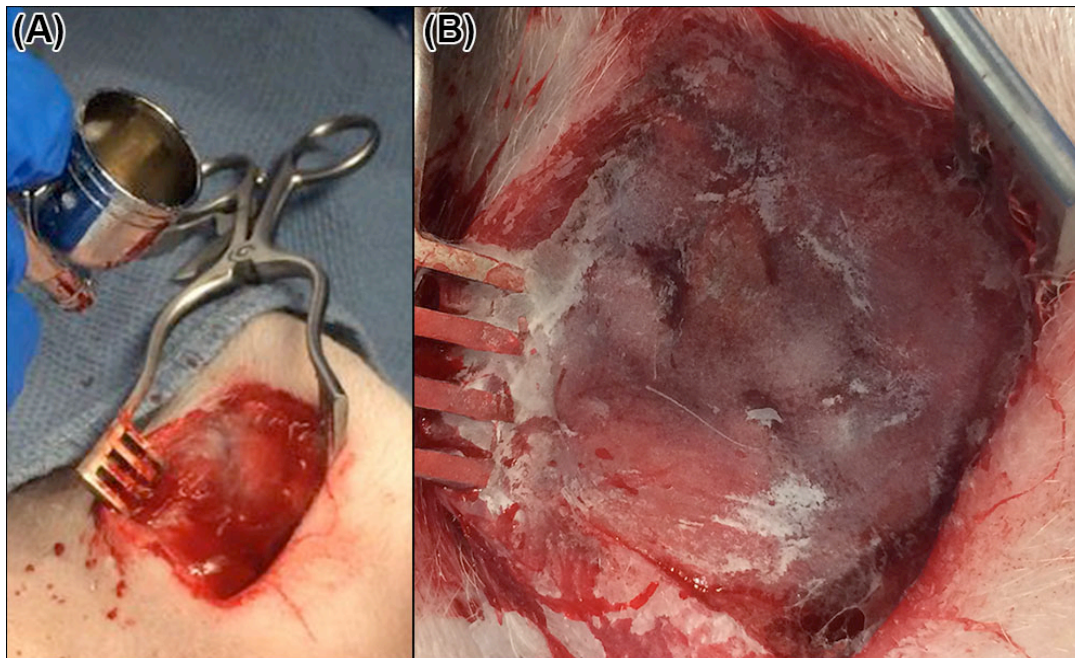


Figure 5.2. (A) Femoral vessel bleed induced by scalpel and start of application of PLGA/PEG blend via solution blow spinning. (B) Bleeding is controlled and a fully conformal polymer matrix is immediately deposited.

5.3 Wound Care

Another large opportunity for the use of solution blow spinning is in the treatment of large surface wounds, such as burns or diabetic ulcers. Solution blow spinning is well

suited for these applications as it can be used to rapidly generate conformal dressings. Furthermore, antibiotics and antimicrobials can be easily included in the deposition solution, allowing them to be encapsulated and released in a time dependent fashion. Silver nitrate is a prime candidate for initial investigation of antimicrobial activity. As long as the release candidate can be suspended in the solution blow spinning solvent it will have the ability to be deposited along with the polymer fiber construct. Aside from small molecule antibiotics and silver salts, nanoparticles could be incorporated in an analogous fashion. Depending on the material need, the polymers chosen could remain fibrous to facilitate tissue regeneration, have different release and degradation behaviors. In the case of the PLGA/PEG sealant discussed in chapter five, its opaque to transparent transition could allow for wound monitoring without the need for re-dressing the injury site.

5.4 Chemical Functionalization

Polymer functionalization chemistries that can improve adhesion of the polymer constructs formed by solution blow spinning should be investigated. It is important to investigate other potential enhancements for this biomaterial fabrication platform. It is not unrealistic to anticipate that certain adhesive properties will be best suited for certain clinical conditions. These characteristics may be demonstrated in differences between low-pressure tissue sealants (intestinal), intermediate pressure sealants (lung), and high-pressure hemostatic agents (vascular). Multiple functionalization strategies can be accomplished through either end group functionalization or aminolysis of PLGA.²⁴⁵ Similarly, PEG can be modified to have reactive end groups to improve adhesion.²⁴⁶ One potential approach is functionalization with oxidized

dextran (ODEX). ODEX has been investigated as a platform material for adhesive hydrogels and coatings, and is shown to be biocompatible.²⁴⁷⁻²⁴⁹ While it has advantageous wet adhesive properties, mechanical properties and solubility issues of ODEX restrict its utility in this application. The additive value of using ODEX-PLGA will allow for a balance of mechanical and adhesive strength. Functionalization can be accomplished via aminolysis and oxidation chemistry (Figure 5.3). Synthesis of ODEX-PLGA has also been previously reported using EDC chemistry.²⁵⁰

Similarly, PLGA can be modified with catechol functionality. Catechol groups mimic naturally occurring wet adhesives found in marine mussels,²⁵¹ and can be incorporated into PLGA through EDC chemistry or aminolysis (Figure 5.4). PEG end group modification with aldehydes (Figure 6.4) and N-hydroxysuccinimide (NHS) will also be explored to improve tissue adhesion.

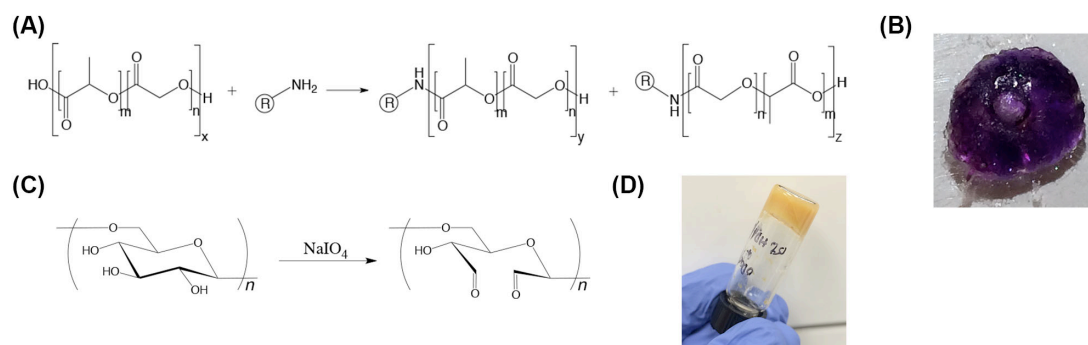


Figure 5.3. (A) Aminolysis reaction schematic for the functionalization of PLGA. (B) Ninhydrin stain (purple) showing the presence of primary amines after the reaction of PLGA with hexamethylene diamine. (C) Oxidation reaction schematic of dextran with sodium periodate. (D) Gel formation of oxidized dextran in the presence of a multifunctional primary amine-containing compound.

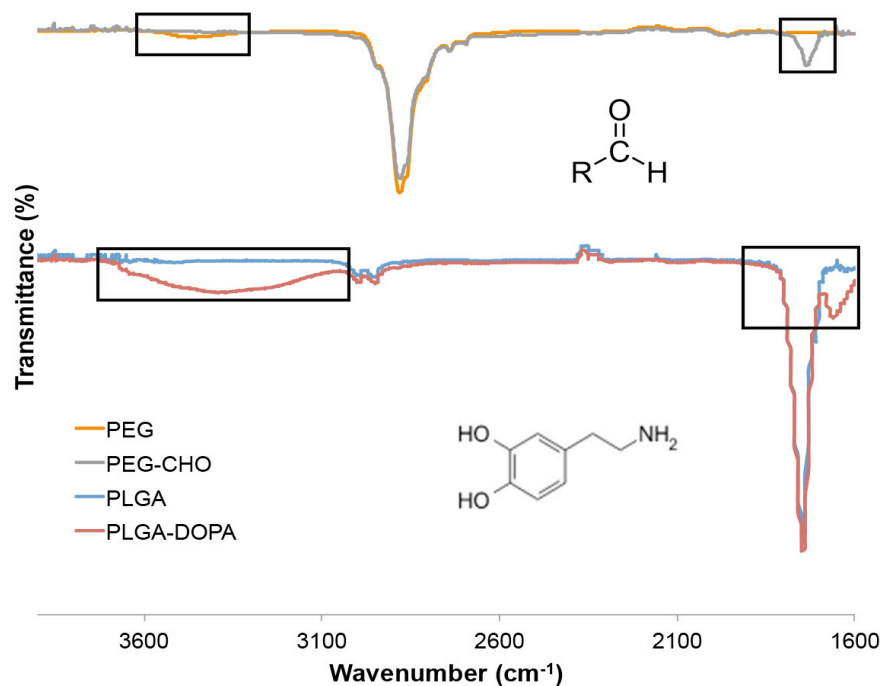


Figure 5.4. Fourier transform infrared spectroscopy (FTIR) of PEG, aldehyde (CHO) functionalized PEG, PLGA and dopamine (DOPA) functionalized PLGA. Aldehyde functionality imparts reactivity towards thiols and amines contained in tissue. Dopamine introduces catechol functionality that will react with primary amines and sulfur groups present in tissue through Michael addition and Schiff base reaction.

5.4 Polymers, Copolymers, and Block Copolymers

As we have demonstrated the ability to modulate mechanical properties and adhesion through blending, more polymers, copolymers, and block copolymers should be investigated to achieve application specific properties. It may also be beneficial to use different polymer architectures such as star or brush polymers in combination with chemical functionalization to improve functional group or ligand density. A microfiber mat generated from a PLGA and 8-arm PEG can be seen in Figure 5.5. When investigating new polymer/solvent combinations special consideration must be given to safety. Non-degradable polymers have the potential to cause harm if inhaled

and most volatile solvents are too toxic to be considered for direct application, but may be suitable for non *in situ* deposition.

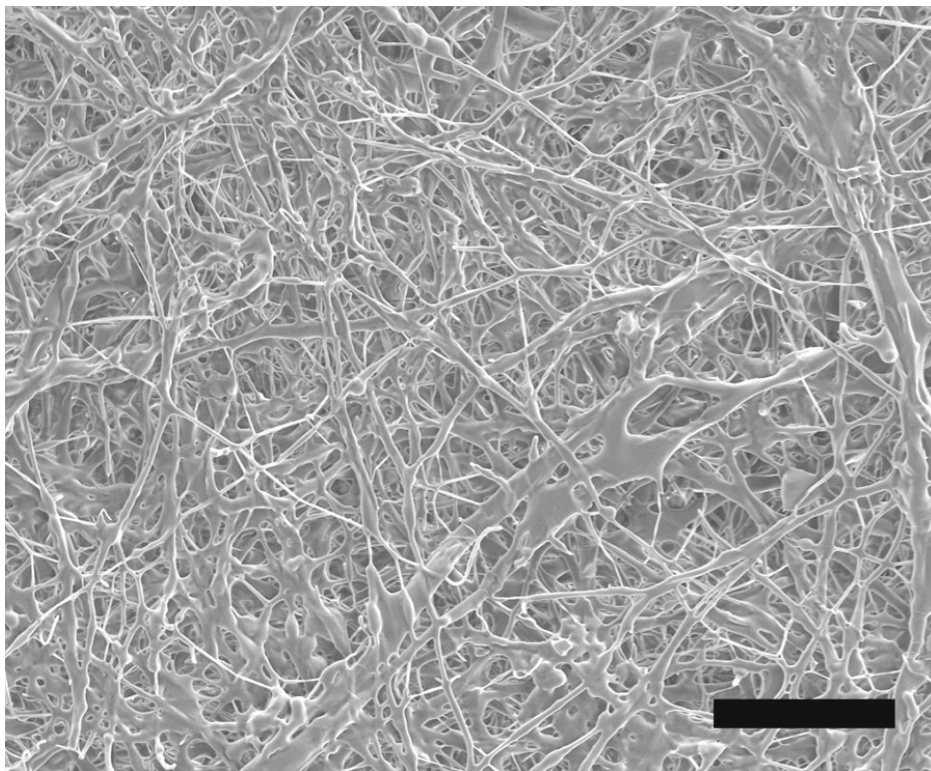


Figure 5.5. SEM of solution blow spun a PLGA and 8-arm PEG blend. Scale bar represents 100 μm .

5.5 Solution Blow Spinning Apparatus Investigation

Due to the relatively limited investigation of solution blow spinning as a technique, control over fiber diameter and deposition rate have not yet been well documented. Analogies have been drawn to electrospinning in which polymer molecular weight, concentration, and correspondingly solution viscosity, have been shown to drastically impact distinct morphology regimes after spraying (corpuscular, beads on a string, fibers).¹⁹¹ Parameters like nozzle size, gas flow rate, gas type, polymer solution flow rate, solution viscosity, and temperature have not been varied in a systematic way with consistent results. Controlling morphological parameters like fiber length,

orientation, and diameter, would further strengthen the capabilities of this technique. This is specifically true in fields involving cellular interaction like tissue engineering, where cellular response is greatly impacted by both fiber diameter and fiber orientation.²⁵²⁻²⁵⁴ Determining regimes of operation for maintaining specific morphologies may also be of significant importance when considering speed and scalability of any solution blow spinning for either single use *in situ* applications or at larger scales such as manufacturing tissue engineering scaffolds, or for non-biologically related applications such as textile²⁵⁵ or electronics development.²³⁵ Co-spinning multiple fiber types or core-shell fibers through the use of multiple nozzles in parallel or concentric nozzles could open other interesting avenues for investigation. These approaches could be used to develop hybrid material systems from a variety of standpoints, allowing for modulation of properties such as hydrophobicity, hydrophilicity, drug delivery, drug solubility, and mechanical properties. Fabricating a modular blow spinning apparatus that would allow for these design parameters to be easily varied could be of great scientific value towards these aims.

Chapter 6: Contributions

My research has resulted in 7 publications that are in press or accepted and 2 publications that are currently in the preparation or submission processes. A subset of this work has resulted in 4 full patent applications that have seen significant commercial interest and will be potentially be licensed in the near future. This same work has been presented over 15 times at a variety of conferences, including American Chemical Society, Biomedical Engineering Society, Materials Research Society, and Society for Biomaterials annual meetings. I have been awarded the Ruth L. Kirschstein National Research Service Award (NIH F31) and the Warren Citrin Graduate Fellowship in support of my research. My work on solution blow spinning has helped popularize a powerful technique for biomaterials fabrication. We were the first group to truly investigate using this approach to deposit non-woven polymer fiber mats *in situ*. This work's potential to impact the field is reflected in media and academic interest. It has been highlighted in *Chemical and Engineering News*, has been carried by international media outlets, and was on the most read list in *ACS Macro Letters* for over a year. More importantly, the work described in Chapter 5 has great potential to significantly improve a wide variety of surgical procedures.

Chapter 7: References

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