Seaweed to Sealant: Multifunctional Polysaccharides for Regenerative Medicine and Drug Delivery Applications

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SEAWEED TO SEALANT : MULTIFUNCTIONAL POLYSACCHARIDES FOR REGENERATIVE MEDICINE AND DRUG DELIVERY APPLICATIONS

A Dissertation Presented

by

Spencer Lincoln Fenn

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ABSTRACT

Pneumothorax, or a collapsed lung, is a serious medical condition resulting when air or fluid escapes the lung into the chest cavity and prevents the lung from inflating. Few viable means of sealing the damaged and leaking tissues are currently available, leading to longer hospital stays, multiple interventions, and increasing costs of care. The motivation of this dissertation is to engineer a novel polysaccharide-based therapeutic surgical sealant, which can be utilized to seal trauma-induced damage to the outer lining of the lung, i.e. pleura, preventing or reversing lung collapse to restore normal breathing function.

The use of polysaccharides, such as alginate and hyaluronan, has become increasingly prevalent in biomedical and tissue engineering applications due to the ability to add functionality through chemical modification, allowing for tunable mechanical and physical properties. These hydrophilic polymer chains can be crosslinked to form hydrogels, which can retain large volumes of water and can mimic the properties of tissues found within the body. In this work, polysaccharide hydrogel sealants were engineered with well-regulated gelation and mechanical properties, and further modified to achieve adhesion to biological tissues. This was accomplished by mimicking the mechanical and physical properties of the complex tissues, and crosslinking the hydrogels \textit{in situ} using a visible light-initiated system.

Methacrylated alginate and oxidized alginate were successfully synthesized and utilized to fabricate adhesive sealant patches, which can adhere and seal damaged tissues \textit{in vivo}. Methacrylation was implemented to allow covalent photo-crosslinking between adjacent polymer chains in solution. Here, a novel anhydrous chemistry was developed to allow for precise control over the degree of methacrylation and thus tune the mechanical properties of the resulting hydrogels by modulating the number of crosslinkable side-groups attached to the polysaccharide chain. To increase the adhesive properties of the resulting hydrogels, oxidation of the polysaccharide chain was subsequently implemented to form functional aldehyde groups capable of protein interactions through the formation of imine bonds on biological tissue surfaces. To test the performance of this multifunctional material, burst pressure testing was executed, revealing the relationship between the two distinct chemical modifications performed and the mechanical and adhesive properties of the resulting sealant.

In addition, methacrylated alginate was utilized to synthesize therapeutic, drug-encapsulating hydrogel nanoparticles, which when incorporated within the polysaccharide-based surgical sealant allow for local drug release. The ability to control drug release at the site of application further broadens the potential uses of this surgical sealant patch and will be discussed further within this dissertation.
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Figure 17: Quantitative cumulative release of doxorubicin (DOX) from Alg-MA sub-microspheres for 11 days (average ± standard deviation, n = 6 hydrogel samples per group). Various formulations of sub-microspheres were assessed: green photo-crosslinked (Green), green + Ca2+ dual-crosslinked (Green+C), UV photo-crosslinked (UV), UV + Ca2+ dual-crosslinked (UV+C). Sample aliquots were collected and the DOX concentration was determined using a standard curve at an absorption wavelength of 485 nm. (A) Cumulative DOX release profile during the first 8 hours. (B) Cumulative DOX release profile during 11 days.

Figure 18: Flow cytometry analysis of Alg-MA sub-microspheres after 12 hours of co-culture with human lung epithelial carcinoma (A549) cells. (A) Non-treated cell control, (B) cells cultured with non-labeled blank sub-microspheres, (C) cells cultured with green photo-crosslinked sub-microspheres, (D) cells cultured with green photo-crosslinked and calcium crosslinked sub-microspheres, (E) cells cultured with UV photo-crosslinked sub-microspheres, and (F) cells...
cultured with UV photo-crosslinked and calcium crosslinked sub-microspheres. (G) Flow cytometry histograms were presented to show the different fluorescence intensity between control cells and different Alg-MA sub-microsphere groups. Figure 19: Human lung epithelial carcinoma (A549) cells were cultured in the presence of hydrogel sub-microspheres for 24 hours in standard growth culture medium at 37°C and 5% CO₂. A549 mitochondrial activity was determined using an absorbance-based quantitative assay; absorbance data for the groups treated with sub-microspheres were normalized to the non-treated cell control (average ± standard deviation, n = 6 hydrogel samples per group). The cytotoxicity of Alg-MA sub-microspheres was analyzed on (A) blank (non-loaded) sub-microspheres. The bioactivity of doxorubicin (DOX) was verified using (B) DOX-loaded sub-microspheres. Various groups (white diamonds = green photo-crosslinked, white circles = UV photo-crosslinked, black diamonds = green + Ca²⁺ dual crosslinked, black circles = UV + Ca²⁺ dual crosslinked) and sub-microsphere concentrations (10, 50, 100 µg/mL) were characterized. Figure 20: The efficacy of doxorubicin (DOX)-loaded Alg-MA sub-microspheres as chemotherapeutic delivery vehicles was assessed using a MTT-based assay, to quantify cell proliferation over a 5-day period. A549 activity was recorded as mitochondrial activity and normalized to non-modified cell controls. Various formulations and concentrations (10-100 µg/mL) of sub-microspheres were assessed: green photo-crosslinked (Green), green + Ca²⁺ dual-crosslinked (Green+C), UV photo-crosslinked (UV), UV + Ca²⁺ dual crosslinked (UV+C). DOX was added exogenously (Free DOX) to the cell culture medium at various concentrations to test the effect of intracellular versus extracellular DOX delivery. (A) Effect of Alg-MA sub-microsphere concentration for each crosslinking type on A549 mitochondrial activity; (B) Effect of ‘free dox’ concentration on A549 mitochondrial activity on days 1, 3, and 5; (C) Effect of DOX concentration encapsulated within Alg-MA sub-microspheres on A549 mitochondrial activity on days 1, 3, and 5. Figure 21: Fabrication of tissue sealant patches is performed using an injection molding technique in combination with lyophilization to form dry sheets of sealant material which can be cut to size using a biopsy punch. Figure 22: Verification of the peak absorbance of doxorubicin hydrochloride was performed via a spectral sweep at 1 mg/mL in PBS (pH 7.4). The peak absorbance is shown at 485 nm wavelength which will be used for subsequent drug-release assays. Figure 23: A) Molecular repeat units for sodium alginate, methacrylated alginate, and methacrylated alginate dialdehyde are presented. B) The ¹H-NMR spectra of 25% and 50% oxidized AMA-DA, AMA and unmodified alginate are shown as labelled. Peaks of interest identified with arrows. The peaks at 5.78 and 6.24 ppm indicate that the hydrogens on the methylene of the methacrylate groups were present on the alginate backbone after modification. The degree of methacrylation for AMA was calculated to be 58%. The peaks at 5.17, 5.48
and 5.68 ppm are indicative of oxidation and the opening of the uronate residues to form aldehyde groups. The experimental degree of oxidation for the 25 DOO and 50 DOO AMA-DA were calculated to be 19% and 42% respectively. Degrees of methacrylation were found to decrease after oxidation/aldehyde-modification to 38% and 36% respectively.

Figure 24: Viscosity of 6% w/v polymer solutions was assessed over shear rates of 1-100 (1/s) at 37°C and shown in log₁₀ scale. Notice the viscosity decreases significantly for the oxidized/aldehyde-modified solutions, but is partially recovered when blended with AMA at a 1:1 ratio.

Figure 25: Gelation kinetics were assessed by oscillatory time sweeps at 10% radial strain and 1 Hz frequency while pre-cursor solutions were exposed to visible green light (525 nm) and maintained at 37°C. Storage moduli (G') of 6% w/v polymer solutions is shown over a 5 minute (300 s) period.

Figure 26: A&B) Dry sheet of non-crosslinked sealant material after injection molding and lyophilization. This material can be cut to required size, and contains all required initiators for visible green light photo-crosslinking. C) Hydrated and crosslinked adhesive hydrogel on fingertip.

Figure 27: (A) Viability, (B) cytotoxicity, (C) apoptosis and (D) mitochondrial activity as demonstrated human mesothelial cells after 24 h exposure to sealant patch materials as compared to non-treated control. Increases in overall cell viability were shown in AMA and 25% oxidized AMA-DA, simultaneously showing decreased cytotoxicity when compared to control. No significant differences in apoptosis and mitochondrial activity was observed.

Figure 28: Burst pressure at failure is shown for all tested formulations in inH₂O. Physiological lung pressures typically remain below 12 inH₂O, which is denoted with a red dotted-line. Statistical significance is shown between specific groups using an asterisk.

Figure 29: Graphic representations of both mechanisms of sealant failure. (A) Material failure occurs when a rupture is formed through the sealant material, yet the patch remains adhered to the surrounding substrate. (B) Adhesive failure occurs when the patch delaminates, either partially or completely (as shown above), from the substrate beneath.

Figure 30: The effects of degradation were assessed using 6% w/v sealant patches over a 14 day period at 37°C. (A) Mass decreased by more than 50% over the two-week period. (B) Similarly, swell ratios decreased dramatically over the 14 day period due to the reduction in polysaccharide molecules retaining water. (C) Images of gel patches on day 1 and 14 are presented. After 14 days of degradation, visible pitting/surface-erosion can be observed on the surface of the hydrogel.

Figure 31: DOX-encapsulating nanoparticle size distribution was assessed using dynamic light scattering in PBS (pH 7.4, 37°C), and is presented in the above histogram by number. The peak size by number is located at 150 nm diameter, with a standard deviation of 19 nm.
Figure 32: Drug release profiles (micrograms DOX per milligram particles) of DOX-loaded patches and nanoparticles over 48 hours at 37°C.

Figure 33: A549 cell viability after 24 h exposure to non-loaded and DOX-loaded sealant patches and nanoparticles. No significant differences in viability were detected between control and non-loaded sealant patches. Viability is however decreased significantly for both DOX-loaded patch formulations tested. DOX-loaded nanoparticles show a slight reduction in viability but is not statistically significant at p=0.05.
CHAPTER 1: INTRODUCTION

1.1. Motivation and Rationale

Hydrogel materials have long been considered ideal for medical and biological applications due to their high water content, and similarity to soft tissues within the body.\(^1\) For this reason, their use in drug delivery, tissue engineering, and regenerative medical applications has increased dramatically.\(^2-18\) Within this category fall polysaccharide-based hydrogel biomaterials, which consist of a network of long polymer chains hydrated in an aqueous solution; these chains are crosslinked to one another forming a solid or semi-solid gel. Polysaccharides of various types can be found throughout biological systems, and thus can often be exceedingly biocompatible when compared with more traditional synthetic polymer materials, often referred to as plastics.\(^3, 19\) This increase biocompatibility is, in part, due to the biochemical similarity between these molecules and those found in the extracellular matrix within many tissues and thus make these materials ideal for a vast array of medical applications.\(^19\) The basis and primary focus of this dissertation is to investigate the chemical functionalization of two polysaccharide molecules, hyaluronan and alginate, to promote specific mechanical and physical properties necessary for applications in injectable tissue repair, controlled therapeutic drug delivery and the adhesive closure of wounds in tissue.

1.1.1. Hydrogel Biomaterials with Tunable Mechanical Properties

The dawning era of regenerative medicine has introduced a paradigm shift in the treatment of diseased, degenerated or damaged biological tissues.\(^17, 20-25\) Rather than
engineering synthetic replacements for tissues, such as used in joint replacement therapies, there is increasing interest in tissue engineering using biomaterial scaffolds that encourage and support the innate healing processes of tissue.\textsuperscript{17, 26} One issue often found with biomaterial replacements is the mismatch of mechanical properties to the tissues they are designed to replace. For example, a metallic hip replacement far exceeds the mechanical properties exhibited by the natural bone making up the hip joint.\textsuperscript{27-28} Negative side effects due to this property mismatch are prevalent, as the excessive mechanical properties cause alterations in the dissipation of mechanical stresses.\textsuperscript{27-30} This, in-turn, results in stress concentrations on the metal/bone interfaces, and can result in loosening or further tissue damage over time.\textsuperscript{31} Many replacement biomaterials have limited lifespans, and may indeed require repeated replacement/revision procedures throughout the life of a patient.\textsuperscript{29, 31-36} By engineering biomaterial scaffolds with properties more closely aligned with innate tissue properties, it is thought that cellular and immune responses will be better controlled, and allow for improved healing of damaged tissues.\textsuperscript{37-44} It is also hoped that regenerating damaged tissues, rather than replacing them, will prevent the need for revision surgeries in the future.\textsuperscript{45} For example, rather than replacing an entire joint, hydrogel scaffolds could be injected into osteochondral defects within the joint, temporarily replacing the cartilage surface while simultaneously fostering tissue regeneration and healing of the articular surface.\textsuperscript{21-22, 46-48} This concept and other applications which could benefit from injectable hydrogel biomaterials with tunable properties will be discussed at length in Chapter 3 of this work.
Biocompatibility can also be a challenge. When implanted in the body, materials can be recognized as foreign objects by the human immune system leading to a cascade of immune responses, increased/premature degradation of the material and further negative side effects resulting from released degradation by-products.\textsuperscript{35} Tissue engineering using biocompatible polysaccharide-based hydrogel scaffolds with tunable mechanical and physical properties has the potential to eliminate many of the concerns facing biomaterial tissue replacement, by matching innate tissue properties and allowing for cell infiltration and extracellular matrix deposition, with the hydrogel material slowly degrading as it is replaced by the individual’s own natural tissue.\textsuperscript{49} A main priority of the work undertaken in this dissertation will be to develop several polysaccharide modification chemistries which can be utilized to achieve tunable mechanical properties, such as stiffness and elasticity, and ultimately provide a flexible, and biocompatible biomaterial hydrogel system for multiple tissue engineering and medical applications.\textsuperscript{3, 48, 50}

1.1.2. Advancing Surgical Closure Techniques

There is significant clinical need for improved surgical closure techniques in soft tissues. Current methods vary based on tissue type and function, with tissues under dynamic stresses such as the lung being more difficult to repair after surgery or injury due to the constant inflation and stretching of the surrounding tissues.\textsuperscript{51-54} The lung also poses additional challenges as any residual leakage of air or fluid from the surgical site or perforating wound can be trapped within the chest cavity, preventing the lung from
inflating. This life-threatening phenomenon is known as pneumothorax, or lung collapse, and requires immediate medical intervention to drain the air or fluid from the chest with a chest tube, allowing the lung to re-inflate and the patient to breath.53, 55-61 Unfortunately, this process does not resolve the underlying tissue damage and often leaves patients waiting in the intensive care unit for the tissue to heal on its own. Many commonly used wound closure techniques such as staples or sutures often fail to completely seal the defect or require secondary surgical removal.51-53, 55-56, 58, 61-64 Tissue sealants offer promise for these applications as they have the potential to seal wounds completely and can also be engineered to degrade naturally over time, resolving many of the issues of traditional closure techniques.65-82 For these reasons, this dissertation will feature a thorough investigation into the design, synthesis, and performance of a novel polysaccharide based tissue sealant which was designed to be applied to damaged lung or soft tissues as a liquid adhesive or a bandage-like patch.

1.1.3. Particle-Mediated Drug Delivery Mechanisms

Current routes of therapeutic drug administration are primarily limited to broad systemic distribution of the medication typically through enteral/enteric administration (oral, rectal), or intravenous injection. There are many applications where site-specific or cell-specific drug delivery would be more beneficial or prevent systemic side effects, such as the case with chemotherapeutics in the treatment of certain cancers.83 Chemotherapeutics, when delivered systemically, can often induce significant negative side effects related to non-specific drug action, which induces apoptosis in rapidly
Although cancer cells are rapidly dividing cells, there are healthy cells in the body that divide similarly, such as cells of hair follicles, skin, and the lining of the digestive system which exhibit increased cell death during chemotherapy and thus cause side effects. There is a dire need for cancer drug delivery systems which allow for increased efficacy in cancer cell clearance and yet decreased systemic toxicity. Drug encapsulating particles (microparticles, nanoparticles, micelles) are of increasing interest to biomaterials scientists in hopes that through the inclusion of specific targeting features and chemical modifications, we can improve the delivery mechanism and the efficiency of drug delivery to the target tissues and cells. In this dissertation, an investigation into the use of doxorubicin encapsulating alginate-based hydrogel particles will be performed to assess the benefits of intracellular drug/payload delivery in the treatment of human lung cancer.

1.1.4. Sustainable Biomaterials for Alternative Medicine

In addition to the primary motivations of this work, a supplementary theme of this work is the use of sustainable and naturally-derived materials. Medical waste of consumable materials and medical devices should be considered of major environmental concern. Many materials and biomaterials currently utilized for medical applications are synthetically-derived, often plastics, who’s synthesis, usage and disposal can impose negative environmental effects. The production of many of these products can involve by-products of the fossil fuel industry, or release harmful chemicals into our air, ground, and water supplies. These often non-degradable materials will likely remain in our landfills for hundreds of years. In the work described in this dissertation, two
naturally-derived polysaccharides, hyaluronan and alginate, were utilized not only for their superior biocompatibility but also in hopes of reducing environmental side effects of production and disposal. Hyaluronan is a molecule found ubiquitously in mammals and can be bacterially or enzymatically synthesized.\textsuperscript{99-103} Alginate can be extracted from brown algae, which lines many ocean floors and can be sustainably farmed, harvested and purified and thus was chosen as the primary material of interest in this dissertation.\textsuperscript{11,104}

\section*{1.2. Specific Aims of Work}

\subsection*{1.2.1. Development of a Novel Polysaccharide Methacrylation Chemistry}

The mechanical properties of biomaterials used in tissue engineering and regenerative medical applications are known to influence cell behavior, differentiation and tissue regeneration. Thus, one primary aim of this research is to develop a reliable means of modulating the mechanical properties of polysaccharide-based hydrogels for specific applications. It is hypothesized that through the development of a novel anhydrous polysaccharide methacrylation chemistry, it will be possible to control the degree of crosslinking occurring between methacrylate groups on adjacent polymer chains and ultimately tune the mechanics of the resulting hydrogel.

\subsection*{1.2.2. Design and Synthesis of an Alginate-Based Tissue Sealant}

Surgical sealants have the potential to offer improved performance over traditional surgical closure techniques and can be engineered to degrade naturally as
tissue regeneration and healing occur. Utilizing methacrylated and oxidized alginates, an investigation into the use of a novel polysaccharide-based aldehyde-mediated adhesive will be performed to assess its potential use as a pulmonary tissue sealant. It is hypothesized that by modulating the degree of alginate oxidation, improved adhesion to tissue surfaces will be possible as assessed through burst pressure testing. Furthermore, methacrylated and oxidized alginates will be used to fabricate ready-to-use photo-crosslinkable tissue adhesive patches, which can be applied to tissues like a bandage to seal leaks or perforations.

1.2.3. Synthesis of Drug Eluting Alginate-Based Sub-Microparticles

The ability to control the delivery and release of therapeutic drugs can improve treatment efficacy, and reduce side effects imposed by systemic drug administration especially in the case of chemotherapies.\textsuperscript{95, 105-107} Using photo-polymerization techniques it is possible to encapsulate chemotherapeutic drug within hydrogel nanoparticles which can be readily internalized by cancer cells. For this portion of my work, I hypothesize that doxorubicin hydrochloride, a common chemotherapeutic drug, can be encapsulated within methacrylated alginate nanoparticles and can subsequently be utilized for controlled intracellular drug delivery and the treatment of lung cancer.

1.2.4. Fabrication of a Therapeutic Tissue Sealant Patch

Medications such as antibiotics or chemotherapeutics are often systemically administered after surgeries to prevent infection or eliminate residual cancer cells from
resected tumors. The ability to seal a surgical site or wound while simultaneously providing controlled local therapeutic drug release at the application site is hypothesized to simplify and improve the standard of surgical care while providing benefits to both the surgeon and the patient with ease of use and improved health outcomes. In this work, doxorubicin encapsulating nanoparticles are incorporated within alginate-based tissue sealant patches and utilized to successfully control the local release of the drug to human lung cancer cells.
1.3. References


CHAPTER 2: SIGNIFICANCE AND BACKGROUND

2.1. Introduction

Hydrogels are natural or synthetic-based crosslinked polymer networks that swell but do not dissolve in aqueous media. Naturally-derived hydrogel biomaterials are advantageous compared with their synthetic counterparts due to heightened compatibility, degradability, sustainable resources, and intrinsic bioactive qualities. Alginate and alginate-based hydrogels are investigated for biomedical applications due to their inherent non-toxicity, biocompatibility, and ready availability from sustainable sources. Derived from brown algae, alginate is desirable not only for those attributes already listed, but also for its relatively low cost and the various applications it can be used for drug delivery and tissue engineering. Alginate will degrade in the body, but only hydrolytically. The use of polysaccharides, such as hyaluronan (HA), in biomaterials imparts biodegradability through enzymatic activity. HA is a linear, anionic high molecular weight polysaccharide found in soft tissue and synovial fluid that swells in the presence of water and provides lubrication to articulating surfaces and resistance to compressive loads in vivo. A wide variety of cell types express the archetypal HA receptor-CD44. These cellular interactions promote wound healing and tissue regeneration. Blends of alginate and other polysaccharides such as HA can form interpenetrating network hydrogels, which exhibit long-range elasticity and improved dynamic strength. The use of HA can also enhance biodegradation in vivo.
Chemical modification of alginate and other similar polysaccharides, such as hyaluronan, have been studied extensively for their ability to improve upon the physical and mechanical properties of crosslinked hydrogels for various applications.\textsuperscript{4-6} Methacrylation of alginate imparts a functional group capable of light-activated covalent crosslinking by free radical polymerization in the presence of a photoinitiator. This review will discuss several relevant applications of methacrylated polysaccharides, and will suggest ways in which this functionalization can be improved for usage in injectable tissue repair and tissue engineering.

One consideration which will also be discussed is how alginate is inherently non-adhesive; however, oxidation of the backbone structure\textsuperscript{7-8} will elicit functional aldehyde groups capable of forming crosslinks with extracellular matrix proteins, such as those found on many tissue surfaces.\textsuperscript{9-10} Aldehyde-mediated protein adhesion has been investigated for biomaterial and tissue engineering applications.\textsuperscript{3, 11-24}

In the following sections, I hope to provide background on the use of methacrylated and aldehyde-modified materials for use in regenerative medicine, providing further justification for the work performed in this dissertation. Subsequently, a discussion of additional adhesive tissue sealant materials for biomedical, and pulmonary use will be included along with relevant background information on lung biology and relevant pulmonary disease. Controlled drug delivery will also be investigated through
assessment of the varied biomaterial systems currently in development for controlled
drug administration, including particle-mediated drug delivery.

2.2. Methacrylated Polysaccharides for Tissue Engineering and Regenerative

Medicine

Methacrylation of polysaccharides provides the ability to covalently crosslink
adjacent polymer chains through free radical polymerization, forming a solid hydrogel
material that can be used in a vast array of biomedical applications.\textsuperscript{25} The process
involves the chemical substitution of free hydroxyl groups with crosslinkable
methacrylate side-groups.\textsuperscript{26} Although methacrylation has been used for decades for non-
medical applications, the dawning era of regenerative medicine and the growing interest
in engineering biological tissues using biomaterial scaffolds has increased the use of this
modification in the literature dramatically.\textsuperscript{1-2, 6-7, 9-10, 20, 25-103} The degree of methacrylation
has been shown to effect the mechanical properties of the resulting hydrogel material,
suggesting the ability to tailor the material to the designated tissue engineering
application.\textsuperscript{39, 104-105} Several chemistries, with varying levels of control over the degree of
methacrylation, have been developed to methacrylate polysaccharide molecules and yield
hydrogel materials with a broad range of mechanical properties.\textsuperscript{27, 39} These methods and
their associated applications will be discussed in the following sections.
2.2.1. Aqueous methacrylation of polysaccharides

One of the more prevalent methods used to methacrylate polysaccharides is via an aqueous, or water-based, reaction.\textsuperscript{1, 27, 29, 97} Many of these chemistries utilize a simple reaction based on a large excess of reactant, such as methacrylic anhydride\textsuperscript{27} or glycidyl methacrylate\textsuperscript{29} blended into a 1-2 wt\% aqueous polysaccharide solution and allowed to react overnight.\textsuperscript{34, 105-109} Although this reaction is reasonably simple to implement, it is not without pitfalls. One concern is the formation of methacrylic acid when the large excess of methacrylic anhydride blends with aqueous solutions.\textsuperscript{26, 38, 104-105} If the acidity within the reaction vessel increases uncontrolled, polymer degradation will occur, and the reaction may cease to continue due to alterations in polysaccharide solubility (i.e. precipitation). To reduce the risk of deleterious effects, the pH within the reaction vessel must be monitored and frequently adjusted to maintain a slightly basic environment, typically using sodium hydroxide.\textsuperscript{27}

Yet another downside to using an aqueous methacrylation chemistry is the lack of control afforded over the degree of modification/substitution. In theory, when utilizing such a large molar excess of reactants there is no limit to the number of free hydroxyl groups that could be modified. High degrees of methacrylation, however, are not commonly observed using these reactions due to much of the reactant being spent in the aforementioned acid formation.\textsuperscript{26} Additionally, there are limited means of controlling or confirming the level of modification during the reaction process other than guesswork and prior experience. This is due to various factors, such as alterations in pH and
temperature which will affect the reaction process, and thus batch to batch variation is prevalent. Some general control over the degree of methacrylation can be had by limiting the reaction time, but better methods of controlling these reactions are required by tissue engineers seeking specific mechanical properties in their end-product.

2.2.2. Anhydrous methacrylation of polysaccharides

Anhydrous methacrylation chemistries, performed in organic solvents, are sought to eliminate many of the concerns experienced with aqueous based chemistries. It is thought that due to the lack of water in the reaction vessel, acid formation will not occur, reducing the quantities of reactants which are required to specific molar ratios. These molar ratios can be calculated and stoichiometrically modulated to synthesize methacrylated polysaccharides with predetermined levels of modifications. In one study, researchers performed both aqueous and anhydrous methacrylation chemistries on hyaluronan to elucidate the different levels of control over the degree of hydroxyl substitution provided by each reaction. In this study, anhydrous reactions are shown to allow for improved control over the degree of methacrylation, and thus modulate the mechanical properties of the resulting crosslinked hydrogels. This same degree of control was not possible to duplicate with methacrylated hyaluronan synthesized via the commonly used aqueous methacrylation chemistry. Others have also successfully implemented anhydrous methacrylation chemistries that allow for superior control over the level of polysaccharide functionalization using hyaluronan, alginate and sucrose.
2.3. Aldehyde-Modified Biomaterials

The ability of aldehyde groups to crosslink with proteins is well established in the literature, and is of increasing interest to those developing materials for bioadhesive applications.\textsuperscript{3, 11-24} Additionally, these functional groups are often utilized as crosslinkable side-groups within biomaterial scaffolds for tissue engineering purposes. It has also been shown that by opening the uronate residues in alginate, forming aldehyde groups, the degradation rates of the resulting hydrogel materials increase by altering the molecules susceptibility to hydrolysis.\textsuperscript{10} Increased degradation rates can be ideal for biomedical applications where the implanted materials are meant to be temporary, or in tissue engineering applications where the material will be replaced by natural tissue over time.\textsuperscript{9-10, 49} In the following sections, examples of both applications of aldehyde modification will be explored.

2.3.1. Aldehyde-Mediated Bioadhesive Materials

Some of the largest contributions to the field of aldehyde-modified polysaccharide research comes from the laboratory of Dr. Eben Alsberg of Case Western Reserve University. Their work pioneered the ability to form functional aldehyde groups on the alginate molecule through the implementation of an oxidation reaction using sodium periodate.\textsuperscript{9-10, 49, 70, 110} It was also shown that degradation rates can be increased by increasing levels of oxidation, resulting in tunable degradation rates.\textsuperscript{10, 110} Additionally, the use of aldehyde-modified alginate/poly(ethylene glycol) hydrogels for
use in bioadhesive applications was explored and found to be superior to the more commonly used fibrin-based sealants when tested using a porcine skin model. Others have used aldehyde modified alginates as hemostatic clotting agents\textsuperscript{24} and sealants,\textsuperscript{18, 97, 111-113} often blending with gelatin\textsuperscript{18-19}, a protein molecule.

2.3.2. Aldehyde-Mediated Crosslinking for Tissue Engineering

Another use of aldehyde functionalization is to provide a crosslinkable site between polymer or polysaccharide molecules.\textsuperscript{12-13, 17, 20} Here, through the incorporation of protein molecules, such as gelatin or collagen, within the aldehyde-modified polymer solution, a gelation reaction is initiated forming a hydrogel material which can be used for tissue engineering and drug delivery applications.\textsuperscript{12-13, 17, 92}

2.4. Controlled Drug Release

Tissue regeneration is a promising field which explores the use of bioactive materials and cell signaling molecules to aid the body in the wound healing and regenerative processes. Lung tissue engineering and regeneration focus on the repair of the lung and pleural tissues after injury or surgery. Therapeutic agents are being developed and investigated as potential strategies for tissue regeneration in disease models such as chronic obstructive pulmonary disease.\textsuperscript{114} Modern drug delivery systems are designed to maintain the structure and bioactivity of biomolecules and to release therapeutics in a controlled and predictable manner. Micro-encapsulation is one of the core technologies used in polymer drug delivery systems.\textsuperscript{115} Microspheres for controlled
drug delivery applications are designed to provide uniform dimensions, shield the drug from the extracellular environment such as enzymatic attack, and be biocompatible and/or bio-resorbable.\textsuperscript{116} Alginate microspheres have attracted much attention for the development of controlled- and sustained-release drug delivery systems for growth factors,\textsuperscript{117} proteins,\textsuperscript{118} cytokines,\textsuperscript{58} and cells.\textsuperscript{5, 119} The fabrication of alginate microspheres is favorable for drug delivery due to the relatively mild yet rapid gelation process that omits the use of harsh chemicals to ensure stability of encapsulated biomolecules.\textsuperscript{120-123}

2.5. Lung Pathologies and Damage

The lung is a complex and heterogeneous tissue comprised of airways, bronchi, bronchioles, alveoli and a vast network of blood vessels. Injury to the connective tissue that lines the lung, the pleura, or to the lung itself can occur from many causes including trauma or surgery, as well as lung diseases or cancers.\textsuperscript{124-125} Bronchopleural fistulas, malignant pleural effusions, and traumatic or ventilator-induced pleural injuries are a continuing source of morbidity, mortality, and increased health care expenditures in the clinic.\textsuperscript{124-127} All of these conditions can result in lung collapse due to air or fluid filling the chest cavity, a serious and life threatening condition that requires immediate intervention.\textsuperscript{128-136} There are currently only limited methods of patching significant injuries to stop the air or fluid leak quickly and subsequently allow for healing processes to repair the underlying tissue.\textsuperscript{129-130, 132-133, 135-142} In the following sections, the current standards of care for those suffering from these ailments will be discussed in an effort to convey the urgent need for improved biomaterial sealant technologies.
2.5.1. Lung Collapse

Pneumothorax, or lung collapse, occurs when a perforation is formed through the pleura and into the underlying lung tissue causing air or fluid to leak into the chest cavity.\textsuperscript{129-132, 135-137} If this air or fluid is not released, the lung will be prevented from inflating and thus collapse. These injuries are often caused by trauma or surgery but can often occur spontaneously in individuals with underlying lung diseases.\textsuperscript{131, 138, 143} As one can imagine, a collapsed lung is immediately life threatening, as it prevents the individual from breathing, and requires immediate medical intervention. The insertion of a chest tube, through the chest wall, allows for draining of fluids and air from the chest cavity, but fails to address the underlying trauma which is causing the leak.\textsuperscript{134} Unfortunately, there are few means of resolving perforations in lung tissue, so many individuals are required to remain in the intensive care unit, with a chest tube and vacuum device in place, until the wound heals naturally. This can translate to long and repeated hospital stays, as well as substantial healthcare expenditures.\textsuperscript{124} A means of sealing the leaks in the lung tissue could reduce hospital stays, by eliminating or reducing the need for a chest tube and vacuum system.\textsuperscript{11, 97, 139, 144-146}
2.5.2. Pleurodesis

One technique used in the treatment of alveolar or lung tissue leaks is called pleurodesis.\textsuperscript{138, 140-141, 147-148} This procedure involves the chemical or mechanical irritation of tissues surrounding the wound or defect to induce inflammation and swelling, in an effort to seal the perforation. Chemical pleurodesis involves the application of an irritant material, such as talc\textsuperscript{139, 141-142, 149}, parenteral tetracycline\textsuperscript{150}, doxycycline, and bleomycin.\textsuperscript{149} Although these procedures show some efficacy, several of these antibiotic substances can induce allergic reaction or fatal alveolar injury.\textsuperscript{149} Unfortunately some of these treatments are painful due to the irritation\textsuperscript{142}, causing difficulty in breathing, and requiring additional applications to maintain the tissue in an inflamed state.\textsuperscript{149}

Mechanical pleurodesis induces inflammation using an abrasive material such as a nylon scouring pad (e.g. Marlex mesh scratch pad).\textsuperscript{143} These methods have been criticized for being too invasive and sometimes do more harm than good due to the side effects they can cause.\textsuperscript{140} Yet, until better alternatives are developed, pleurodesis will continue to be offered as the standard of care for those suffering from non-resolving pneumothorax and pleural effusions. Thus, our work strives to address the issues of current treatments by providing a natural and biocompatible sealant material which could be used as an alternative to pleurodesis.
2.6. Adhesive Biomaterials for Pulmonary Applications

Starting in the late 1990’s, fibrin began to be studied as a natural-based polymer sealant. Fibrin sealant is a hemostatic consisting of blood coagulation factors fibrinogen, factor XIII, thrombin, aprotinin and calcium chloride. This mixture gives rise to the formation of a stable, crosslinked fibrin clot. A reduction in the incidence of pulmonary or bronchopleural air leakage of 41% has been associated with the use of fibrin sealant. A 50% reduction of alveolar air leaks after pulmonary resection was achieved with the use of an aerosolized fibrin glue (HemaMyst® System) compared to standard stapling procedures. To improve upon the fibrin sealant, TachoComb® was subsequently developed, which combines collagen sheets with a fibrin coating. The limitation with these methods is that fibrin is a blood derivative and has the possibility of transmitting blood-borne diseases. One report describes the clinical use of non-crosslinked alginate to close pleural defects in vivo. Non-crosslinked alginate can provoke inflammation, an effect not observed with the use of crosslinked alginates.

While the use of hydrogels as surgical sealants and wound treatments began developing in the 1960’s, the use of hydrogels to treat pulmonary and alveolar air leaks initiated in the late 1980’s. Poly(ethylene glycol) (PEG) is the primary polymer used in repairing pulmonary and alveolar air leaks.

One of the first PEG-based pulmonary seals, FocalSeal®, utilizes a two-solution method with a photoinitiator, Eosin-Y, to form a sealant. Upon exposure of blue-green light, the sealant polymerizes to form a crosslinked, flexible hydrogel network. Commercially available Progel® is a two solution sealant consisting of
dilute PEG-hydrogen chloride and PEG-sodium phosphate/sodium carbonate solutions, which come in a preassembled, dual syringe applicator. Progel® is the only FDA approved pleural sealant, however, it is bioinert and does not aid in therapeutic tissue regeneration. Similar to Progel®, PEG and trilysine solutions are mixed upon application and crosslink within seconds to form a flexible hydrogel. This technology was introduced as a surgical sealant in Europe as PleuraSeal®, but never obtained FDA approval and later underwent a voluntary recall. These completely synthetic systems do not provide a mechanism for therapeutic drug delivery and thus motivate our work to develop an alginate based tissue sealant capable local/site-specific drug administration at the application site.

Many of the sealants discussed above contain toxic materials and their application consists of multiple steps, making it an involved process for the end user. In addition, the above studies lacked the incorporation of therapeutics into the sealant material to address underlying diseases, infections, and cancers. The investigative use of natural-based hydrogels as therapeutic agents to treat lung leaks and encourage tissue regeneration while simultaneously delivering therapeutic molecules has yet to be performed and is the focus of the following work.
2.7. References


CHAPTER 3: VISIBLE LIGHT CROSSLINKING OF METHACRYLATED HYALURONAN HYDROGELS FOR INJECTABLE TISSUE REPAIR

Spencer L. Fenn, Rachael A. Oldinski

Contributions

The experimental research described in this chapter was performed solely by myself, the first and primary author, including the majority all data analysis, figure preparation, and manuscript drafting. The manuscript was co-authored in partnership with my supervisor, Rachael Oldinski, who is listed as senior author.

3.1. Abstract

Tissue engineering hydrogels are primarily cured in situ using ultraviolet (UV) radiation which limits the use of hydrogels as drug or cell carriers. Visible green light activated crosslinking systems are presented as a safe alternative to UV photocrosslinked hydrogels, without compromising material properties such as viscosity and stiffness. The objective of this study was to fabricate and characterize photocrosslinked hydrogels with well-regulated gelation kinetics and mechanical properties for the repair or replacement of soft tissue. An anhydrous methacrylation of hyaluronan (HA) was performed to control the degree of modification (DOM) of HA, verified by $^1$H-NMR spectroscopy. UV activated crosslinking was compared to visible green light activated crosslinking. While the different photocrosslinking techniques resulted in varied crosslinking times,
comparable mechanical properties of UV and green light activated crosslinked hydrogels were achieved using each photocrosslinking method by adjusting time of light exposure. Methacrylated HA (HA-MA) hydrogels of varying molecular weight, DOM and concentration exhibited compressive moduli ranging from 1 kPa to 116 kPa, for UV crosslinking, and 3 kPa to 146 kPa, for green light crosslinking. HA-MA molecular weight and concentration were found to significantly influence moduli values. HA-MA hydrogels did not exhibit any significant cytotoxic affects towards human mesenchymal stem cells. Green light activated crosslinking systems are presented as a viable method to form natural-based hydrogels in situ.

3.2. Introduction

The possibility of transplanting stem cells in vivo to treat diseases and injuries, in a regenerative way, is becoming a reality. While challenges exist in the successful translation of stem cell-based engineered tissues, such as articular cartilage,\textsuperscript{1-3} stem cell transplantation to aid in tissue regeneration may be a viable option. The necessary requirements of a tissue engineering construct for a long-term successful regenerative approach include physiologically relevant bulk material properties for congruency in mechanical loading and energy dissipation, and the ability to biologically guide tissue regeneration.\textsuperscript{4} However, the translation of engineering techniques in situ relies on the improvement of hydrogel implantation. One minimally invasive approach is an injectable system, in which the hydrogel sets or cures in situ to provide mechanical stability and/or serve as a cell and/or drug carrier.\textsuperscript{5} Hydrogels are used as minimally invasive injectable
scaffolds that fill focal articular cartilage lesions. Injectable hydrogels offer an alternative to traditional surgical procedures by developing 3-D scaffolds that promote regeneration of cartilage.

Natural, biocompatible materials, such as extracellular matrix (ECM) proteins and polysaccharides, have been used to explore the effect of substrate stiffness on mesenchymal stem cell (MSC) differentiation and tissue growth. Hyaluronan (HA) is a linear, anionic, high molecular weight polysaccharide found in soft tissue and synovial fluid that swells in the presence of water and provides lubrication to articulating surfaces and resistance to compressive loads in vivo. A wide variety of cell types express the archetypal HA receptor CD-44; CD-44-HA interactions are essential for maintaining normal cartilage homeostasis. These cellular interactions are advantageous for promoting wound healing and tissue regeneration. Integrin-mediated cell-material interactions and substrate stiffness of hydrogels impact MSC differentiation. The focus of the current study was to mechanically analyze methacrylated HA (HA-MA) hydrogels undergoing either ultraviolet (UV) or visible green light crosslinking for the purpose of achieving a wide range of elastic moduli values for various tissue engineering applications.

Through careful selection of both the DOM and molecular weight of HA, a range of mechanical properties can be achieved and optimized for the regeneration of desired tissues. Methacrylation of polysaccharides, including HA, have been reported in
the literature. However, most studies have not examined non-aqueous methacrylation reactions, which allow for more precise control over stoichiometric ratios and the DOM, nor do previous studies report on variance of photocrosslinking methods via different light sources and photoinitiator systems. Accurate control of the DOM allows for tunable crosslink densities and mechanical properties, with future direction focused on injectable tissue repair. This work aimed to determine how the source of light-activated covalent crosslinking of HA-based hydrogels affects rheological and mechanical properties for the development of an injectable hydrogel for tissue engineering applications. The goal of this study was to design and fabricate green light activated crosslinking systems are presented as a safe alternative to UV photocrosslinked hydrogels, without compromising material properties such as viscosity and stiffness.

3.3. Materials & Methods

3.3.1. Synthesis of Methacrylated Hyaluronan (HA-MA)

Sodium HA (Lifecore Biomedical) lyophilized powders of two different molecular weights (Mw = 100 and 700 kDa) were rendered soluble in anhydrous dimethyl sulfoxide (DMSO, 99% anhydrous, Sigma Aldrich) through an ion exchange with hexadecyltrimethylammonium bromide salt (CTAB, Sigma Aldrich). Ion exchange resin (Dowex 50WX8-400) was loaded with CTA+ ions by submersion in a 1-2% (w/v) CTAB ethanol:water solution (1:1 ratio), then mixed with 1% (w/v) HA in deionized water for 24 hours at 40°C. The polymer solution was filtered to remove ion exchange resin, frozen and lyophilized (See Appendix II for detailed protocol). A 1% (w/v) HA-
CTA/DMSO solution was reacted with methacrylic anhydride (MA, Sigma Aldrich) in the presence of a catalyst, 4-(dimethylamino)pyridine (DMAP, Sigma Aldrich), for 24 hours at room temperature. The amount of MA was adjusted to achieve varying DOMs based on molar ratios of the hydroxyl groups (modification sites) per HA repeat unit to MA. The amounts of MA utilized in this study were 1x, 1.5x, and 2x the molar quantity of hydroxyl groups (See Appendix IV for calculations performed). The resulting solution was hydrolyzed through extensive dialysis and periodic adjustment to pH 8 with 5N sodium hydroxide. The DOM, or ratio of methacrylate groups per repeat unit of HA-MA, was determined using $^1$H-NMR spectroscopy (Bruker AVANCE III 500 MHz high-field NMR spectrometer). A 1% (w/v) polymer in deuterium oxide solution was analyzed at room temperature, spinning at 20 Hz for 16 scans. The DOM was calculated by taking the ratio of the relative integrations of the methacrylate peaks (6.1, 5.6, and 1.8 ppm), and HA methyl protons (1.9 ppm).$^{23,26}$

3.3.2. Green Light Crosslinking

HA-MA solutions of different concentrations (2, 3 or 4% (w/v)) and having various molecular weights and DOMs were prepared for visible green light crosslinking. To analyze different combinations of crosslinking reactants, aqueous solutions of the following concentrations were prepared (See Appendix VII for detailed protocol): 1 mM Eosin Y (EY, photosensitizer), 125 mM triethanolamine (TEOA, initiator), 20 mM 1-vinyl-2-pyrrolidinone (VP, catalyst).$^{23,27-28}$ To determine the most effective photoinitiator system for green light activation, an absorption assay was conducted on solutions
containing EY, EY supplemented with TEOA, EY supplemented with VP, and EY supplemented with TEOA and VP. The solution with the highest absorbance at 530 nm was chosen as the photoinitiator system for subsequent tests.

### 3.3.3. Photo-Rheometry

Viscosity and shear stress, as functions of shear rate, were evaluated on the reaction solutions for the preparation of photocrosslinked HA-MA hydrogel. All measurements were carried out on a rheometer (AR2000, TA Instruments) equipped with a Peltier plate maintained at 25°C and a 40 mm diameter 1°59'47" steel cone geometry. 100 kDa and 700 kDa HA-MA with varying DOMs were mixed with photoinitiator solutions for either UV or green light-activated chemistries to form 3% (w/v) concentrated hydrogel pre-cursor solutions. To form solutions for UV-activated photocrosslinking, a 0.05% (w/v) Irgacure D-2959 (Ciba) solution in phosphate buffered saline (PBS) was prepared (See Appendix VI for detailed protocol). To form solutions for green light crosslinking, a 1 mM EY, 125 mM TEOA, 20 mM VP solution was prepared (See Appendix VII for detailed protocol). Aliquots of 500 µL were placed within a 27 µm gap between the Peltier plate and cone geometry. Viscosity (Pa*s) and shear stress (Pa) of the polymer solutions were determined at varied shear rates, 1-50 (1/s), at 1% radial strain over 2 minutes (n = 3) and analyzed using analytical software (TA Data Analysis).
The gelation of HA-MA hydrogels was examined immediately following shear sweep experiments. Oscillatory time sweeps were conducted at 10% radial strain and 1 Hz during exposure to UV (320 - 390 nm, Uvitron Intelliray 400) or green light (530 nm, custom 20 LED array, 14 kmCd, Victory Rush Electronics) (n = 3). Data collection was initiated upon the start of light exposure. Shear loss (\(G'\)) and storage (\(G''\)) moduli were recorded; tan delta (ratio of \(G''\) to \(G'\)) was analyzed using analytical software (TA Data Analysis). HA-MA gelation initiation was identified at the inflection point in the tan delta curve and the terminal gelation time was approximated to occur as \(G'\) plateaus.

3.3.4. Unconfined Compression Testing

Uniaxial unconfined compressive testing was performed on 2, 3 and 4% (w/v) HA-MA hydrogels crosslinked using either UV or green light to elucidate any differences in mechanical properties between the two crosslinking systems (n = 4). The effects of molecular weight and DOM were also investigated. Hydrogel pre-cursor solutions were placed in a custom mold consisting of glass microscope slides and 1.6 mm thick Teflon® spacers. Molds were exposed to either UV light for 10 minutes or green light for 20 minutes at room temperature; photocrosslinking times were chosen to ensure that the hydrogels were terminally crosslinked using each method. Crosslinked samples were equilibrated in PBS, pH 7.4, for 24 hours at room temperature (n = 5) prior to testing. Specimens 6 mm in diameter and 1.6 mm in height were stamped using a biopsy punch. Compression testing was performed on a rheometer, equipped with a normal force transducer using an 8 mm parallel plate geometry and an opposing Peltier plate at 25°C.
A 10% uniaxial compressive strain was applied at a rate of 10 µm/s. Force (N) and changes in gap height (µm) were obtained using analytical software (TA Universal Analysis) and were subsequently used to calculate elastic strain (ε, %) and stress (σ, kPa). The elastic modulus (E) was calculated as the slope of a linear fit between 4 and 10% compressive strain within the linear-elastic region.

3.3.5. Cytotoxicity Assay

The cytotoxicity of photocrosslinked hydrogels to primary bone-marrow derived human MSCs was assessed spectrophotometrically as a function of mitochondrial activity in living cells using an MTT based assay. Primary bone-marrow derived human MSCs (passage 7) were seeded in treated 48-well tissue culture polystyrene plates at a density of 20,000 cells/well in 100 µL/well of standard MSC growth medium (alpha minimum essential medium, 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin) and allowed to adhere for 24 hours. MSCs were incubated in the presence of various photocrosslinked HA-MA hydrogels (n = 4) at 37°C and 5% CO₂. Hydrogels were fabricated using 100 kDa or 700 kDa HA-MA, 2 or 4% (w/v) polymer concentrations, and high DOMs. Hydrogels were terminally photocrosslinked prior to cell culture during exposure to either UV for 10 minutes or green light for 20 minutes. Hydrogel specimens were 6 mm wide and 2 mm tall. After 24 hours of incubation, medium was removed and cells were rinsed two times in sterile PBS. Mitochondrial activity was analyzed using an MTT-based In Vitro Toxicology Assay Kit (Sigma) following the manufacturer’s protocol with a plate reader (H1 Synergy, BioTek). Briefly,
mitochondrial dehydrogenases in viable cells cleave the tetrazolium ring in MTT, yielding formazan crystals which can be dissolved and measured spectrophotometrically. Absorbance values were recorded at 570 nm with background absorbance at 690 nm deducted. Average absorbance values for the experimental samples were compared to positive control values recorded for wells containing media and cells alone (See Appendix VIII for detailed protocol).

3.3.6. Statistical Analysis

All experiments were performed in triplicate with results reported as mean ± standard deviation. Statistical analysis was performed with a GLM procedure using Statistical Analysis System software. A fixed effect tri-factorial (concentration, molecular weight, and crosslinking method) model was generated to study the contribution of each factor to HA-MA hydrogels with a range of compressive elastic moduli. The multiple comparisons were performed based on a two-way ANOVA of individual and interaction effects of the three factors. For analyzing cytotoxicity, similar GLM procedures were preformed to obtain one-way ANOVA results. A p < 0.05 was considered significantly different.

3.4. Results

3.4.1. HA-MA Synthesis and Characterization

An anhydrous methacrylation of HA resulted in a functionalized polymer with a controllable DOM. Relative integrations of methacrylate groups, as identified in the
chemical structure and $^1$H-NMR spectra (Figure 1), resulted in varied DOM for both 100 kDa and 700 kDa HA. The various DOMs achieved were 71% (low), 81% (medium), and 90% (high) for 100 kDa HA-MA, and 43% (low), 54% (medium), and 68% (high) for 700 kDa HA-MA. Higher DOMs were achieved for the lower molecular weight HA. The CTA$^+$ groups were removed and replaced with Na$^+$ as confirmed through HA-MA dissolution in aqueous based solutions (i.e., DI water and PBS) and $^1$H-NMR spectra.

![Chemical structure schematic of methacrylated hyaluronan (HA-MA) repeat unit (top) and $^1$H-NMR spectra of various HA-MA polymers. Varying degrees of modification (DOM) were achieved through controlled stoichiometric ratios of HA and methacrylic anhydride. The DOM was calculated by taking the ratio of relative integrations of methacrylate peaks (6.1, 5.6, or 1.8 ppm), and HA methyl protons (1.9 ppm). Methacrylate protons at a, b, c and HA’s methyl proton at d are identified in the chemical structure and associated $^1$H-NMR spectra peaks.](image)

Figure 1: Chemical structure schematic of methacrylated hyaluronan (HA-MA) repeat unit (top) and $^1$H-NMR spectra of various HA-MA polymers. Varying degrees of modification (DOM) were achieved through controlled stoichiometric ratios of HA and methacrylic anhydride. The DOM was calculated by taking the ratio of relative integrations of methacrylate peaks (6.1, 5.6, or 1.8 ppm), and HA methyl protons (1.9 ppm). Methacrylate protons at a, b, c and HA’s methyl proton at d are identified in the chemical structure and associated $^1$H-NMR spectra peaks.
3.4.2. Green Light Crosslinking

The photoinitiator system for green light crosslinking and the effects of including an initiator and a catalyst were investigated for optimum absorbance upon light exposure. An absorption assay, performed on a microplate reader, was utilized to quantify light absorbance of various photoinitiator solutions (Figure 2). The photoinitiator solution comprising 1 mM EY, 125 mM TEOA, 20 mM VP exhibited the highest absorbance at 530 nm and was thus used for green light crosslinked HA-MA hydrogels.

![Figure 2: Visible light absorption of green light-activated crosslinking reactants was quantitatively determined using an aqueous-based absorbance assay. Reactants were placed in DI water at the following concentrations: 1 mM Eosin Y (EY, photosensitizer), 125 mM triethanolamine (TEOA, initiator), 20 mM 1-vinyl-2-pyrrolidinone (VP, catalyst). Using a microplate reader, absorbance at 530 nm was recorded for solutions containing EY (dashed and dotted line), EY supplemented with TEOA (dashed line), EY supplemented with VP (dotted line), and EY supplemented with TEOA and VP (solid line).]
3.4.3. Shear Strain Sweep Measurements

Viscosity and shear rate values for various HA-MA hydrogel pre-cursor solutions varied; the effects of shear rate on viscosity and shear stress values of 3% HA and HA-MA solutions are shown in Figure 3. Viscosity values for 100 kDa HA-based solutions were lower compared the 700 kDa HA-based solutions. Solution viscosities decreased exponentially with increasing shear for all solutions tested (Figure 3A,B). The HA-MA solutions exhibited lower viscosity values compared to the HA controls. At a shear rate of 10 (1/s) and 1% displacements, all three of the 100 kDa HA-MA solutions (DOM = low, medium, high) exhibited an 11-fold decrease in viscosity values compared to the non-modified HA control (Figure 3A), while the 700 kDa HA-MA solutions exhibited 3, 9, and 16-fold decreases in viscosity values for low, medium, and high DOM compared to the HA control (Figure 3B). For the experimental groups, viscosity decreased with increasing DOM.

Analogous to the viscosity results, the 700 kDa HA-based solutions exhibited higher shear stress values compared to the 100 kDa HA-based solutions (Figure 3C,D). The 100 kDa HA-based solutions displayed a linear increase in shear stress with increasing shear rate (Figure 3C); however, shear stress increased exponentially with increasing shear rate for all 700 kDa HA-based solutions tested (Figure 3D).
3.4.4. Gelation Behavior of HA-MA

HA-MA solutions of different concentrations, in the presence of photoinitiators, were successfully crosslinked and formed into hydrogels upon exposure to either UV or green light. The gelation times of HA-MA solutions activated by either UV or green light were approximated from oscillatory time sweeps. Tan delta was plotted versus time (Figure 4A,C) and the onset of gelation was determined to be the point of inflection where the slope of the tan delta curve became negative. Due to this clear inflection, the tan delta is a
clearer indication of the initiation of gelation, whereas the $G'$ is more indicative of terminal crosslinking as it plateaus (Figure 4B,D).

Due to air exposure and warming to 37°C, dehydration was observed in polymer solutions after 800 seconds causing high variability and unreliable data (data not shown) and thus approximations for terminal gelation times for both crosslinking systems were made as follows. For the UV crosslinked hydrogels, $G'$ appears to plateau at approximately 600 seconds. For green light crosslinked gels, tan delta exhibits delayed gelation initiation as compared to UV and $G'$ fails to plateau by 800 seconds indicating that gelation had not terminated by this point. The extended gelation time for green light crosslinked gels can be attributed to the lower intensity of the LEDs used as compared to the high intensity lamp used to produce UV light. Terminal crosslinking densities were approximated to be achieved after 10 and 20 minutes for UV and green light crosslinking, respectively. Thus, these exposure times were used to form hydrogels for mechanical testing.
Figure 4: The effect of photocrosslinking light source on gelation kinetics and hydrogel formation was analyzed using 3% (w/v) solutions of HA-MA with high degrees of modification (DOMs). Oscillatory time sweep experiments were conducted at 10% radial strain and 1 Hz. Tan delta values for (A) 100 kDa and (C) 700 kDa are plotted alongside corresponding storage (G') and loss (G'') shear moduli values for (B) 100 kDa and (D) 700 kDa. Solutions were exposed to either UV (320–390 nm, Uvitron Intelliray 400) or green light (530 nm, custom 20 LED array, 14 kmCd, Victory Rush Electronics) to activate photocrosslinking (n = 3).

3.4.5. Unconfined Compression Testing

Uniaxial unconfined compression tests were conducted on UV and green light crosslinked HA-MA hydrogels. The UV crosslinked hydrogels exhibited elastic moduli values ranging from 1 kPa to 116 kPa (Figure 5A), while the green light crosslinked hydrogels exhibited elastic moduli values ranging from 3 kPa to 146 kPa (Figure 5B). The high molecular weight hydrogels (700 kDa HA) exhibited higher elastic moduli values compared to the low molecular weight hydrogels (100 kDa HA) for both photocrosslinking systems. The elastic moduli of photocrosslinked HA-MA hydrogels
demonstrated significant correlations with molecular weight, DOM, and polymer concentration after performing a factorial analysis of the compressive data (p ≤ 0.003).

![Graph showing compressive modulus for different conditions](image)

**Figure 5:** Uni-axial unconfined compression experiments were conducted on photocrosslinked HA-MA hydrogels. The effect of polymer concentration (w/v), molecular weight, degree of modification (DOM), and light source were evaluated. (A) UV (10 minute terminal crosslinking time) and (B) green light (20 minute terminal crosslinking time) crosslinked HA-MA hydrogels consisted of low, medium, and or DOM and HA-MA concentrations of 2, 3 or 4% (w/v). Results are shown as average ± standard deviation, n = 5.

### 3.4.6. Cytotoxicity Assay

Primary human MSCs were cultured with photocrosslinked HA-MA hydrogels for 24 hours in standard MSC culture medium. Hydrogels varied by crosslinking method, molecular weight, and HA-MA concentration. Measurements were made using a MTT assay to quantify mitochondrial activity and HA-MA experimental hydrogel groups were compared to a positive control consisting of medium and cells alone (see Figure 6). Elevated absorbance corresponds to increased mitochondrial activity and viability; the
HA-MA hydrogel scaffolds were found not to be cytotoxic as compared to a positive control. Increased cell mitochondrial activity was found in all four of the tested experimental groups.

Figure 6: The cytotoxicity of photocrosslinked HA-MA hydrogels was evaluated after 24 hours of culture with primary human MSCs in standard MSC culture medium. Using a MTT assay to quantify mitochondrial activity, average absorbance values for each hydrogel experimental group were recorded and compared to control wells containing medium and MSCs alone. HA-MA with a high DOM was used. Hydrogels varied by crosslinking method (UV or green light), molecular weight (100 kDa or 700 kDa), and HA-MA concentration (2 or 4%, w/v). Elevated absorbance values indicate increased mitochondrial activity in all experimental groups.
3.5. Discussion

The implementation of injectable materials curable in situ relies on hydrogel systems that are tunable and easily manipulated. Photocrosslinking systems require light exposure of a polymer solution to induce covalent crosslinking between neighboring chains. Depending on the light source and the polymer/solution conditions, the hydrogel network can vary dramatically resulting in different mechanical properties. In addition to controlling the mechanical properties of the hydrogel, ease of use is an essential requirement of the end user, with low viscosities and short gelation times preferred. Furthermore, careful selection of the light source may eliminate unwanted side effects, such as cell death, and drug and material degradation. Injectable material systems can be accompanied by a laparoscopic lens and LEDs to photocrosslink materials in situ.\(^5\)

The methacrylation chemistry utilized in this study allowed for the controlled modification of HA and thus a broad range of DOMs was achieved. The 100 kDa HA was found to react more efficiently and increased DOMs were achieved compared to the 700 kDa HA-MA hydrogels under the same reaction and crosslinking conditions. This increase in reaction efficiency may be attributed to decreased solution viscosities in the reaction vessel, as a result of fewer intramolecular and intermolecular interaction between shorter polymer chains. Furthermore, shear strain sweeps revealed that a higher DOM resulted in decreased viscosity (Figure 3A). This is attributed to degradation of the polymer chains during the ion exchange process and the use of sodium hydroxide. Decreased viscosity may also be attributed to reduced physical interactions between
adjacent HA-MA chains as a result of the conversion of hydroxyl groups to methacrylate groups. In addition, the higher molecular weight HA-based solutions, including the HA-MA experimental groups, exhibited higher viscosities compared to the lower molecular weight polymer. The trends that were seen in the viscosity data were also revealed in the shear stress data, which was expected since the two values are mathematically related. Indeed the viscosity and shear stress values complement the mechanical data collected during compression testing.

Photo-rheometry experiments revealed that the reaction kinetics for the two crosslinking methods are different. The initiation of gelation, or the gel point, was identified as the inflection point of the tan delta curve, and this value varied for each of the polymer formulations tested (Figure 4). As shown in Figure 4, the initiation of gelation for UV and green light crosslinking were different and so were the times reached as tan delta approached zero, which indicates the rate of increase of $G'$, the storage modulus, compared to $G''$, the loss modulus. For both molecular weights investigated, the green light crosslinked hydrogels took approximately twice as long as the UV crosslinked hydrogels for the tan delta to approach zero, indicating that green light required twice as much time to crosslink and for the storage modulus to equilibrate (i.e., to cease increasing). Indeed, the hydrogels are considered to be terminally crosslinked when the storage modulus, $G'$, no longer increases with light exposure time. The UV crosslinked hydrogels demonstrated terminal crosslinking at approximately 10 minutes, however the green light crosslinked hydrogels exhibited increasing $G'$ values after 10 minutes.
Limitations of the material and the testing equipment resulted in fluctuating data (noise) after 13 minutes. Thus, combing the photo-rheometry, it was decided to use 10 minutes for UV crosslinked hydrogels and 20 minutes for the green light crosslinked hydrogels. Gelation times are dependent on light intensity; therefore, these times may be adjusted depending on light source and distance. Indeed, future work will also focus on optimizing the reaction kinetics of the visible light crosslinking system through chemical modifications.

The unconfined compressive elastic moduli results of the HA-MA hydrogels demonstrated that the method of photocrosslinking did not significantly impact mechanical properties, as long as the terminal degree of crosslinking was reached, which was approximated from photo-rheometry data. As shown in the literature, the molecular weight and solution concentration had more of an influence on the compressive stiffness; increasing elastic moduli were achieved with higher concentrations and higher molecular weights of HA.\textsuperscript{11}

Indeed, the results show that the two photocrosslinking methods result in hydrogels with comparable mechanical properties; thus, visible green light it is a safe alternative to UV without comprising material performance. It is hypothesized that the green light crosslinking system may be better suited for curing \textit{in situ}. All of the hydrogel formulations tested for toxicity to primary human MSCs displayed enhanced mitochondrial activity, further demonstrating terminal crosslinking and the lack of
toxicity due to residual photoinitiators and or methacrylation byproducts. While the crosslinking time for the HA-MA hydrogels exposed to green light is rather long at 20 minutes, the elastic moduli achieved are relatively high for low concentration systems.\textsuperscript{36} The use of high molecular weight HA decreases crosslinking efficiency, but results in a stiffer and tougher network hydrogel.

Hydrogel crosslink density has also been shown to influence hypertrophic differentiation of MSCs.\textsuperscript{37} It is desirable to achieve moderate stiffness values while maintaining moderate to low crosslink densities. With the use of a relatively low viscosity HA-MA solution, the material can be injected and cured \textit{in situ} to form a hydrogel using a non-invasive laparoscopic procedure. The use of visible light will enable a safer approach for the use of injectable hyaluronan hydrogel systems.

### 3.6. Conclusions

In this study, an anhydrous HA methacrylation was performed to control the DOM of HA, thus controlling the mechanical properties of photocrosslinked HA-MA hydrogels. UV and green light activated crosslinking systems resulted in varied gelation reaction kinetics; however, terminally crosslinked hydrogels exhibited comparable mechanical properties after exposure to UV or green light. The terminal crosslinking densities were achieved after 10 and 20 minutes for UV and green light crosslinking, respectively. Thus, these exposure times were used to form hydrogels for mechanical testing. The hydrogel elastic moduli demonstrated significant correlations with molecular
weight, DOM, and polymer concentration. In addition to presenting green light as a viable crosslinking methodology for natural-based materials, the compressive elastic moduli of visible light crosslinked HA-MA hydrogels reported here expand upon values in the literature, providing a more mimetic moduli range for biomedical applications.

3.7. Acknowledgements

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3.8. References


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CHAPTER 4: MECHANICAL PROPERTIES AND FAILURE ANALYSIS OF VISIBLE LIGHT CROSSLINKED ALGINATE-BASED TISSUE SEALANTS

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*Indicates Dual First-Authorship

Contributions

The following manuscript was drafted jointly, between two co-first-authors, myself and Patrick N. Charron M.S.. All experimental work disclosed in this chapter was shared between the two first authors. I was personally responsible for the methacrylation and photo-polymerization chemistries & analysis, protocol development, figure preparation, and manuscript revisions. Patrick Charron was responsible for burst pressure testing and rheometry. Third author, Alex Poniz, was responsible for burst pressure device fabrication. Senior author Rachael Oldinski was involved in all stages of manuscript development and editing.

4.1. Abstract

Engineered surgical sealants and tissue adhesives are becoming more prevalent in surgical suites as current medical and robotic technologies improve, simplifying the procedures of complex internal surgeries. These tissue sealants serve as an alternative method for rejoining and sealing tissues over sutures and staples. This study investigated the potential for chemically-modified alginate hydrogels to serve as effective surgical tissue sealants. Burst pressure properties and adhesion characteristics were used to examine the efficacy of an alginate-based tissue sealant. Burst pressure experiments were conducted on a custom-fabricated burst pressure device using constant air flow; pressure-
time plots quantified burst pressure and total volume at failure. Experimental observation and digital videography verified cohesive and adhesive failure of experimental and control sealants. In summary, visible light crosslinked alginate tissue sealants formed effective seals and maintained pressures up to 50 mm Hg.

4.2. Introduction

For several decades, breakthroughs in medical technologies have improved patient care while also reducing the invasiveness and recovery time for many procedures. In particular, advances in laparoscopic surgery have allowed for internal procedures to be performed through small incisions, decreasing the need for many open-body operations.\textsuperscript{1-3} In these minimally-invasive surgeries, common approaches to rejoin or seal tissues, such as sutures or staples, can be difficult to implement, may cause further damage to tissue, and often fail to completely seal tissue resulting in the leakage of bodily fluids or air.\textsuperscript{1,4} An alternative method for providing a simple, immediate seal for wound closure is the use of new engineered tissue sealants, which are applied as a liquid and activated on demand to adhere, cure, and bond \textit{in situ}.\textsuperscript{1,5}

Tissue sealants are commonly formed using natural or synthetic polymers, or both. Synthetic sealants, capable of higher mechanical strengths and tissue-bonding properties, are potentially cytotoxic, may cause chronic inflammation, and exhibit low adherence to wet tissues.\textsuperscript{4} Natural based sealants are advantageous compared to their synthetic counterparts due to heightened biocompatibility, degradability, sustainable
derivation, and intrinsic bioactive qualities.\textsuperscript{6-11} The abundant resource, propensity for chemical modification, and enhanced biocompatibility, degradability, and non-toxicity make alginate and alginate-based hydrogels prime candidates for tissue sealant applications.\textsuperscript{7, 12-13} Alginate, derived from brown algae, is desirable not only for those attributes already listed, but also for its relatively low cost and historical use in drug delivery and tissue engineering.\textsuperscript{7, 13} While alginate hydrogels can form films \textit{in situ} via calcium crosslinking, these materials are relatively weak (i.e., low elasticity, toughness), and exhibit relatively quick degradation times.

Chemical modification of alginate and similar polysaccharides allows for designed physical and mechanical properties of engineered alginate hydrogels through controlled covalent crosslinking, thus addressing limitations associated with ionically crosslinked alginate hydrogels.\textsuperscript{7, 11, 14-15} The methacrylation of alginate imparts an acrylate functional group capable of covalent crosslinking with itself by free radical polymerization in the presence of a photoinitiator.\textsuperscript{7, 15-16} While tradition photo-crosslinking mechanisms often employ ultraviolet (UV) light activation, UV exposure is cytotoxic and degrades biological molecules (e.g., drugs).\textsuperscript{17-22} As a safer alternative, methacrylated alginate (Alg-MA) also covalently crosslinks upon exposure to visible light in the presence of the photosensitizer, Eosin Y.\textsuperscript{12, 16, 23-24} Furthermore, while alginate is inherently non-adhesive, oxidation of the backbone structure \textsuperscript{25-26} will elicit functional aldehyde groups capable of forming covalent bonds with amine groups present.
on tissue (i.e., imine bond formation) like those found in extracellular matrix (ECM) proteins.\textsuperscript{27-29}

Burst pressure testing is a useful means of analysis for tissue sealants, as it allows for quantification of the sealant mechanical strength under characteristic loading conditions. Burst pressures were collected following a modified standard (ASTM F2392), which quantifies not only maximum burst pressures, but also the mode of failure (cohesive, i.e., material failure, versus adhesive failure) for the various hydrogel compositions.\textsuperscript{30} In addition, a video recording system may be employed to further characterize the mode of sealant failure. Burst pressure and failure data analysis provides a direct comparison of tissue sealant mechanical behavior and also enables indirect insight into the effects of material integrity and tissue adhesion; burst pressure experiments and rheological characterization of novel hydrogels provides insight into material structure-function relationships, in order to optimize material properties for biomedical applications.

This paper presents the fabrication and characterization of Alg-MA-Ox tissue sealants, specifically for use in sealing damaged lung tissue. Our investigation seeks to quantitatively assess the effect of modulating the degree of methacrylation and oxidation on adhesion and burst pressure properties. The novelty of our approach lies in the implementation of both methacrylation and oxidation chemistries to vary mechanics and adhesion, as well as our use of visible light crosslinking to reduce the cytotoxic effects
observed with UV-cured sealants. We hypothesized that an Alg-MA-Ox hydrogel will form an effective seal over an air leak upon exposure to visible light. Furthermore, we hypothesized that the Alg-MA-Ox hydrogels would adhere to an ECM substrate and exhibit high burst pressures in a physiologically-relevant environment. To test these hypotheses, a custom burst pressure device was designed, fabricated, and implemented for the quantitative and repeatable mechanical analysis of visible-light crosslinked alginate-based hydrogel tissue sealants.

4.3. Materials and Methods

4.3.1. Synthesis of methacrylated alginate (Alg-MA)

Alg-MA was synthesized using an aqueous-based reaction. One percent (w/v) sodium alginate (Manugel, Mw ≈ 200 kDa, FMC Biopolymer) solution, in deionized (DI) water, was reacted with a 20-molar excess of methacrylic anhydride (Sigma Aldrich) for 12 hours.\textsuperscript{12, 16, 31-33} The reaction product was treated with sodium hydroxide and dialyzed in DI water for 3 days (See Appendix I for detailed protocol). The degree of methacrylation (DOM), or ratio of methacrylate groups per repeat unit of alginate, was determined using \textsuperscript{1}H-NMR spectroscopy (Bruker AVANCE III 500 MHz high-field NMR spectrometer). Briefly, Alg-MA was dissolved in deuterium oxide (D\textsubscript{2}O) at 1% (w/v) solution and was analyzed at room temperature, spinning at 20 Hz for 16 scans. The DOM was determined by calculating ratios of methylene (6.1, 5.6 ppm) and alginate methyl (1.9 ppm) peak integrations.\textsuperscript{16} Alg-MA material with a single DOM was used for subsequent rheological and mechanical analysis, and modification via oxidation.
4.3.2. Synthesis of oxidized methacrylated alginate (Alg-MA-Ox)

Alg-MA was reacted with sodium periodate (Sigma) to open the uronate residues, which form the alginate backbone, creating an aldehyde group.8,27 One percent (w/v) alginate solutions were prepared by dissolving sodium alginate or Alg-MA in DI water. Different volumes of a 3% (w/v) sodium periodate solution were added to the alginate solutions and reacted in the dark for 24 hours at room temperature to achieve distinctly different degrees of oxidation (DOO). The Alg-MA and sodium periodate solutions were mixed together and stirred for 24 hours in a dark environment at room temperature. The product, Alg-MA-Ox, was purified by dialysis in DI water (MWCO 6 kDa, Thermo-Fisher) for three days and lyophilized (See Appendix IX for detailed protocol). Theoretical DOO values were 10, 30 and 50% modification; experimental values were determined by comparing the ratio of alginate methyl protons (5.5 and 5.0 ppm), to the newly formed methyl protons (5.0 ppm).27 In addition, the DOM was reassessed after the oxidation reaction (see methods above).

4.3.3. Polymer characterization and visible light crosslinking

Viscosity and shear stress, as functions of shear rate, were evaluated on Alg-MA and Alg-MA-Ox hydrogel sealant precursor solutions (i.e., prior to photo-crosslinking). All measurements were carried out on a rheometer (AR2000, TA Instruments) equipped with a Peltier plate maintained at 25°C, using a 40 mm diameter 1°59′47″ hard anodized aluminum cone geometry. Alginate and chemically-modified alginate powders were
added to DI water to form 3% (w/v) alginate solutions of the following compositions: alginate controls, Alg-MA (DOM = 77), Alg-MA-Ox (DOO = 10, 30, 50), and 50:50 polymer blends of Alg-MA and Alg-MA-Ox materials. The control and modified alginate solutions were blended with photo-activators to enable photo-crosslinking (See Appendix VII for detailed protocol): 1 mM Eosin Y (photo-sensitizer), 125 mM triethanolamine (photo-initiator), 20 mM 1-vinyl-2-pyrrolidinone (catalyst).\textsuperscript{16, 23-24} Aliquots of 580 µL were placed within a 27 µm gap between the Peltier plate and cone geometry. Shear sweeps were performed to determine the viscosity (Pa.s) and shear stress (Pa) of the alginate-based solutions at 1% radial strain, 1-100 (1/s), over one min (n = 4). Data was analyzed using analytical software (TA Data Analysis); data sets for various samples within a single group are merged and sorted by shear rate, and plotted on the same graph.

Oscillatory time sweeps at 10% radial strain and 1 Hz were conducted on alginate-based hydrogel precursor solution during exposure to green light (525 nm, custom 9.84 cm diameter LED ring, NFLS-G30X3-WHT, SuperBrightLEDs) over a period of 10 minutes (600 seconds).\textsuperscript{15} Data collection started upon the start of light exposure (i.e., photo-crosslinking activation). Shear storage ($G'$) and loss ($G''$) moduli were calculated, and tan delta (ratio of $G''$ to $G'$) was analyzed using analytical software (TA Data Analysis).
4.3.4. Burst pressure testing and failure analysis

Burst pressure values were determined using ASTM F2392 as a guide. Burst pressure was recorded using a custom-designed pressure-chamber testing device according to the ASTM standard (Figure 7).

![Figure 7: Schematic illustration of experiment setup for mechanical burst pressure testing of alginate-based sealants.](image)

The main chamber was machined from polyether ether ketone (PEEK) to provide low cost fabrication and fluid-related degradation resistance; it was designed to be two sections fastened by two bolts with a fluoroelastomer o-ring to provide an airtight seal, for easy specimen loading between the sections. A syringe pump (Harvard Apparatus PHD 2000 Infusion) controlled the fluid injection rate into the chamber; for this experiment we used air pressure. A pressure transducer (Omega PX-409-030AUSBH) was connected to the pressure chamber through a NPT port, allowing for accurate, real-time data acquisition via a connected computer (Figure 7).
Collagen substrates (collagen casing, The Sausage Maker Inc.) were hydrated in DI water and tested on the pressure device before creating a defect (i.e., puncture) to ensure that the substrate was intact and void of defects, and formed a leak-free seal. The substrates were punctured with a 3 mm diameter biopsy punch and re-tested to ensure a through-thickness defect was formed resulting in a drop in pressure (or the inability to hold pressure). Punctured collagen substrates were clamped between a glass slide and a Teflon mold, with a 1.5 cm diameter hole, centered over the collagen puncture. Hydrogel precursor solution (0.5 mL) was deposited into the mold, over the collagen puncture, and photo-crosslinked for 10 minutes under visible green light (523 nm). The collagen substrate with the hydrogel sealant was removed from the mold and loaded onto the burst pressure device. Air volume, via the syringe pump, was increased at a constant air flow rate into the pressure chamber, until the seal failed; failure was defined as a loss in pressure due to a leak in seal (i.e., experimental sample). All burst pressure tests were conducted at room temperature with compressed air at an infusion rate of 75 mL/hr.

The hydrogel sealant failure mechanism, either hydrogel delamination from the collagen substrate or hydrogel material failure (i.e., failure of the material under loading), was assessed via visual inspection of the hydrogel materials and a digital microscope (Dino-Lite Pro AM413TA, Dino-Lite Digital Microscopes) during and after testing. Video was collected directly above the burst pressure device for the duration of the test. The digital microscope was positioned 10-15 cm above the hydrogel sealant, and recording was initiated just prior to the activation of the syringe pump and halted several
seconds after sealant failure (Supplemental Video 1 has been edited to show failure activity). Delamination, i.e., adhesive failure, was classified as bond failure between the sealant and substrate, whereas material failure was classified as bond failure within the material itself.

4.4. Results and Discussion

4.4.1. Rheology

The chemical modification of alginate, and other polysaccharides, may induce chain scission and a reduction in molecular weight of the alginate backbone; polymer degradation depends on the nature of the chemical reaction.\textsuperscript{8, 22, 27-28} Also, sequential chemical reactions will affect the degree of modifications of various functional groups. Therefore, DOO and DOM were calculated for each modified alginate formulation. All of the Alg-MA starting materials had a DOM = 77; however, as evidenced by the DOM calculations performed on the Alg-MA-Ox materials, the oxidation reaction slightly reduced the DOM, as shown in Table 1.

Table 1: Summary of the chemical characterization, burst pressure values, and mode of failure for Alg-MA, Alg-MA-Ox, and homogenous 50:50 polymer blends of Alg-MA and Alg-MA-Ox hydrogel sealants.

<table>
<thead>
<tr>
<th>Group</th>
<th>DOM (%)</th>
<th>DOO (%)</th>
<th>$G'$ (Pa) at $t = 600$ s</th>
<th>Burst Pressure (mmHg)</th>
<th>Principal Mode of Failure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alg-MA</td>
<td>77</td>
<td>0</td>
<td>~1560</td>
<td>49.41 ± 22.46</td>
<td>D</td>
</tr>
<tr>
<td>10 Alg-MA-Ox</td>
<td>70</td>
<td>10</td>
<td>~113</td>
<td>26.11 ± 13.13</td>
<td>M</td>
</tr>
<tr>
<td>30 Alg-MA-Ox</td>
<td>64</td>
<td>25</td>
<td>~0.01</td>
<td>7.09 ± 9.85</td>
<td>M</td>
</tr>
<tr>
<td>10 Alg-MA-Ox Blend</td>
<td>74</td>
<td>5</td>
<td>~440</td>
<td>48.04 ± 10.64</td>
<td>M</td>
</tr>
<tr>
<td>30 Alg-MA-Ox Blend</td>
<td>71</td>
<td>13</td>
<td>~235</td>
<td>35.40 ± 10.66</td>
<td>M</td>
</tr>
<tr>
<td>50 Alg-MA-Ox Blend</td>
<td>54</td>
<td>20</td>
<td>~150</td>
<td>27.66 ± 12.36</td>
<td>M</td>
</tr>
</tbody>
</table>
Physical changes, such as a reduction in molecular weight, result in variable mechanical responses. Thus, we quantified viscosity and shear stress after each chemical reaction; the effects of shear rate on the viscosity and shear stress of 3% (w/v) alginate solutions are shown in Figure 8.

Figure 8: (A) Viscosity (Pa.s) and (B) shear stress (Pa) values were collected for alginate-based tissue sealant precursor solutions, including: Alg-MA, oxidized Alg-MA, and homogenous 50:50 blends of Alg-MA and Alg-MA-Ox. Representative plots for each control and experimental group are shown as average values (n = 4).

Solution viscosities decreased exponentially with increasing shear rates for all solutions tested. Compared to non-modified alginate solutions (i.e., controls), the methacrylated alginate (Alg-MA) solution maintained high viscosities and shear stresses (Figure 8). In contrast, the oxidized and methacrylated alginate (Alg-MA-Ox) solutions demonstrated much lower viscosities and shear stresses when compared to alginate and Alg-MA solutions. Methacrylation chemistry is deleterious to the alginate backbone, although this is more pronounced in Alg-MA-Ox materials.\textsuperscript{8, 27-28} During the oxidation reaction, alginate molecular weight was reduced, and resulted in a > 100-fold reduction in viscosity. During the oxidation reaction, Alg-MA was exposed to high concentrations of
sodium periodate to open the uronate residues on the acidic repeat units, rendering the polymer reactive with ECM proteins (i.e., imine formation) through the introduction of aldehyde functional groups.\textsuperscript{22, 34} Additionally, the oxidation of alginate increases susceptibility to hydrolytic degradation, improving biodegradability of the sealant.\textsuperscript{8} Thus, tissue adhesion and \textit{in vivo} degradation time can be controlled through the optimization of the addition and creation of acrylate and aldehyde functionalities to alginate. Adhesion may be further assisted through the formation of weak hydrogen bonds between the biomacromolecules comprising the sealant and the proteins present on the tissue surface.\textsuperscript{35}

To effectively seal a leak or damaged tissue, a liquid tissue sealant must display a relatively high viscosity to maintain a film over a tissue defect during the gelation, or curing, process. While the Alg-MA-Ox material is required to maintain adhesion to underlying tissue, the requirements of the amount and/or degree of oxidation have not been studied. Thus, Alg-MA was blended with Alg-MA-Ox materials to enhance the viscosity of the solutions and further improve material properties. Alg-MA and Alg-MA-Ox blends (1:1) were created from the same formulation of Alg-MA, and different Alg-MA-Ox polymers with varying degrees of oxidation (10, 30 and 50\%). Upon blending Alg-MA with Alg-MA-Ox, viscosity and shear stress values were partially recovered compared to Alg-MA-Ox solutions. The viscosity and shear stress values for all of the polymer blend solutions were similar, suggesting that the properties of the Alg-MA polymer were dominant and served as an equalizing agent (see Figure 8).
4.4.2. Visible light crosslinking and gelation time

Alg-MA and Alg-MA-Ox solutions, in the presence of photo-activators, were crosslinked and formed into hydrogels upon exposure to green light. The gelation times of the hydrogel solutions were determined from oscillatory time sweep plots (Figure 9).

Figure 9: Gelation of alginate-based sealants were assessed using oscillatory time sweeps at 10% radial strain and 1 Hz during exposure to green light (525 nm) over a period of 10 minutes. (A, B) Alg-MA and oxidized Alg-MA, and (C, D) homogenous 50:50 blends of Alg-MA and Alg-MA-Ox. Delta values decreased as crosslinking occurred via visible light exposure to form hydrogels (A, C). Storage moduli values, $G'$, and loss moduli values, $G''$, were collected during gelation (B, D). Representative plots for each control and experimental group are shown as average values (n = 4).
The initiation of gelation, i.e., gelation time, was determined to be at the inflection point of the delta versus time curve. The Alg-MA solution and Alg-MA-Ox polymer blend solutions indicate a clear inflection in the delta curve, indicative of successful photo-initiation and subsequent crosslinking. The Alg-MA-Ox solutions with DOO ≥ 30 failed to form a gel (i.e., photo-crosslink) rheometry. The storage moduli curves versus time are a more reliable indicator for terminal crosslinking as it plateaus and indicates no further crosslinking (Figure 9B, D). Upon inspection of the G’ curves, a majority of the crosslinking was completed by 600 seconds, and increased by several orders of magnitude. Thus, an exposure time of 10 minutes (600 seconds) was used to form hydrogels for burst testing.

4.4.3. Burst pressure properties

To perform effectively, a tissue sealant must adhere to underlying tissue and exhibit material strength exceeding physiological loading conditions. Burst pressure measurements and the subsequent failure analysis quantified the ability for alginate-based hydrogels to perform as tissue sealants under increasing pressure. Two modes of failure, delamination of the material from the substrate, and material rupture due to a loss in strength, were determined via video during experimentation (Figure 10A). Representative pressure versus time curves from burst pressure testing are shown in Figure 10B.

While the Alg-MA hydrogel saw the highest individual burst pressure, it was also the only material to fail by delamination. Burst volume measurements were
calculated based on the amount of air pumped into the device before failure. The burst pressure volumes relate to the qualitative flexibility of the tissue sealant (Figure 10C). Comparing the 10 Alg-MA-Ox and 10 Alg-MA-Ox blend sample groups, the flexibility of the blend material resulted in a higher burst pressure and burst volume.

As the DOM decreases, the number of methacrylate functional groups available for covalent crosslinking decreases. With less methacrylate groups available, the resulting polymerized hydrogel is more loosely bound together than its highly methacrylated counterpart. Polymer networks that are more loosely bound behave more as a viscous
material rather than an elastic material, and exhibit lower storage moduli, as shown in Error! Reference source not found. (see above). Because our polymer hydrogels rely more on elastic than viscous behavior, post-crosslinking, for its application as a sealant, it is expected that storage modulus would correlate with burst pressure. The relation between DOM, storage modulus, and burst pressure are highlighted in Error! Reference source not found..

Delamination, or adhesive failure, occurred when the hydrogel patch separated from the substrate with no visible sign of material damage (Figure 10A). Material failure was characterized as the failure of the sealant material immediately above the puncture, and showing visible damage to the material (Figure 10A). The Alg-MA hydrogel sealants failed exclusively through delamination, while the Alg-MA-Ox materials exhibited material failure, with the exception of a single 10 DOO Alg-MA-Ox:Alg-MA blend experimental group. Alginate is inherently non-adhesive, and there are few forces keeping the Alg-MA hydrogel sealant adhered to the collagen substrate. However, the Alg-MA-Ox hydrogels and blends interact with the collagen substrate through functional aldehyde groups, preventing delamination before material failure. The only case of delamination outside the Alg-MA group occurred in the blend with the lowest theoretical DOO, resulting in fewer aldehyde groups to interact with the substrate, possibly explaining the delamination. In summary, aldehyde functionality is required to ensure adhesion and the formation of a seal. However, the optimum DOO required for
maintaining tissue adhesion while exhibiting high burst pressures has yet to be determined.

4.5. Conclusions

Alginate, a biomacromolecule derived from brown algae, was investigated as a hydrogel tissue sealant. Chemical modifications were tuned to optimize the mechanical and adhesive properties, which are essential in developing a tissue sealant. Specifically, methacrylation resulted in acrylate groups that were photo-responsive in the presence of photo-activators and visible green light. Alginate oxidation created aldehyde groups for sealant-substrate interactions and increased adhesion to tissue matrix proteins. A custom-designed burst pressure device was fabricated and implemented in the mechanical analysis of alginate-based hydrogel sealants. While Alg-MA hydrogels were able to withstand high pressures without mechanically failing, these materials delaminated from the substrate and failed adhesively. Conversely, Alg-MA-Ox formed hydrogels that adhered to the substrate; however, these materials failed at lower burst pressures. Alg-MA and Alg-MA-Ox polymer blends benefited from the high mechanical strength of the Alg-MA and the adhesiveness of the Alg-MA-Ox. Most notably, higher DOO Alg-MA-Ox did not result in improved adhesion, which may also be the result of polymer degradation during the oxidation reaction. Indeed, minimal oxidative degradation (1-5% DOO) is required to induce hydrogel sealant adhesion while maintaining hydrogel network integrity.
4.6. Acknowledgements

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4.7. References


CHAPTER 5 : DUAL-CROSSLINKED METHACRYLATED ALGINATE SUB-MICROSPHERES FOR INTRACELLULAR CHEMOTHERAPEUTIC DELIVERY.

Spencer L. Fenn, Tianxin Miao, Ryan M. Scherrer, Rachael A. Oldinski

Contributions

As the first author of the following chapter, I was responsible for all experimental work and the majority of all data analysis, manuscript preparation and editing. Tianxin Miao, Ph.D., assisted in experimental design, data analysis, manuscript and figure development. Co-author, Ryan Scherrer, assisted in experimental design and the drafting of the introduction. Senior author, Rachael Oldinski Ph.D., oversaw all experimental work and subsequent manuscript preparation.

5.1. Abstract

Intracellular delivery vehicles comprised of methacrylated alginate (Alg-MA) were developed for the internalization and release of doxorubicin hydrochloride (DOX). Alg-MA was synthesized via an anhydrous reaction, and a mixture of Alg-MA and DOX was formed into sub-microspheres using a water/oil emulsion. Covalently crosslinked sub-microspheres were formed via exposure to green light, in order to investigate effects of crosslinking on drug release and cell internalization, compared to traditional techniques such as ultra violet (UV) light. Crosslinking was performed using light exposure alone, or in combination with ionic crosslinking using calcium chloride (CaCl₂). Alg-MA sub-microsphere diameters were between 88 – 617 nm, and zeta-potentials were
between -20 and -37 mV. Using human lung epithelial carcinoma cells (A549s) as a model, cellular internalization was confirmed using flow cytometry; different sub-microsphere formulations varied the efficiency of internalization, with UV-crosslinked sub-microspheres achieving the highest internalization percentages. While blank (non-loaded) Alg-MA sub-microspheres were non-cytotoxic to A549s, DOX-loaded sub-microspheres significantly reduced mitochondrial activity after five days of culture. Photo-crosslinked Alg-MA sub-microspheres may be a potential chemotherapeutic delivery system for cancer treatment.

### 5.2. Introduction

Lung cancer is one of the most widespread type of carcinoma, resulting in the largest number of cancer-related deaths around the world.\(^1\) Greater than 85% of lung cancer cases are currently classified as non-small-cell lung cancer (NSCLC), including adenocarcinoma, squamous-cell carcinoma and large-cell carcinoma. Despite the recent advances in early detection and cancer treatment, NSCLC is often diagnosed at an advanced stage and has a poor prognosis.\(^1\) Chemotherapy is one of the current recommended treatments to prevent or reduce tumor-induced symptoms, prolong patient survival, and maintain patient quality of life.\(^4\) Chemotherapy treatments can last as long as 6 months at high parenteral dosages, and are frequently associated with systemic toxicity.\(^5\)-\(^6\)
Doxorubicin hydrochloride (DOX) is one of the most widely used chemotherapeutic drugs, and is known as an anthracycline antibiotic. The main anti-cancer mechanisms that have been suggested for DOX fall into the following categories: 1) DOX intercalation into DNA, shutting down protein synthesis and DNA replication; 2) DOX-induced production of reactive oxygen species (ROS), inducing DNA damage and/or lipid membrane peroxidation; 3) DNA crosslinking, binding and alkylation; 4) DOX interference with DNA unwinding, strand separation and helicase activity; 5) damage to the bilayer structure of cell membranes; 6) DNA inhibition of topoisomerase II, initiating DNA damage pathways. All the above activities require that DOX be presented inside the cytoplasm, which requires intracellular delivery of DOX to cancer cells. DOX treatment induces several side effects including nausea, vomiting, and fever in patients. A significant incidence of cardiovascular side effects – hypotension, tachycardia, arrhythmias, and ultimately congestive heart failure – are also reported. Therefore, there is a need for drug delivery systems which efficiently encapsulate and deliver chemotherapeutics while reducing adverse events. As a small molecule, concerns of low encapsulation efficiency, drug leakage, and aggregation limit the therapeutic efficacy of DOX, and complications associated with sterilization have not been resolved.

Modern drug delivery systems are designed to maintain the structure and bioactivity of biomolecules and to release therapeutics in a controlled and predictable manner. Micro-encapsulation is one of the core technologies used in polymer drug delivery systems. However, the relatively large micron-size (> 10 µm) of the drug
delivery particles limits cellular internalization. Therefore, the association of DOX to sub-micron carriers has drawn greater interest,\textsuperscript{14} including liposomes,\textsuperscript{15} nanospheres and sub-microspheres,\textsuperscript{16} and micelles.\textsuperscript{17}

Alginate is an unbranched polysaccharide consisting of $1\rightarrow 4$ linked $\beta$-D-mannuronic acid (M) and its C-5 epimer $\alpha$-L-guluronic acid (G). Alginate is extracted from brown seaweed, and has been investigated for biomedical and pharmaceutical applications due to its relatively low cost, low toxicity, biocompatibility, and biodegradability.\textsuperscript{18-21} Alginate particles have increasingly been shown to offer controllable drug encapsulation efficiencies and release profiles, while maintaining the bioactivity of various drugs, including proteins,\textsuperscript{22} cytokines,\textsuperscript{23} and small molecules.\textsuperscript{24} Through the formation of a water/oil emulsion and subsequent exposure to calcium ions, alginate particles within the micrometer – nanometer size scale can be generated, and are often referred to as ionically crosslinked alginate particles.\textsuperscript{20, 22, 25}

The fabrication of alginate microspheres and sub-microspheres is favorable for drug delivery due to the relatively mild ionic gelation process.\textsuperscript{22, 26} However, limitations associated with the relatively weak ionic bonds include low drug encapsulation efficiency and rapid drug-release rates ($< 24$ h).\textsuperscript{27} To overcome these limitations, methacrylated alginate (Alg-MA) was synthesized\textsuperscript{28} and sub-microspheres were generated utilizing a water/oil emulsion\textsuperscript{22} and subsequent crosslinking. Alg-MA sub-microspheres were covalently crosslinked using photoinitiators and visible (i.e., green) or UV light
irradiation. Dual-crosslinked sub-microspheres were generated with the subsequent addition of calcium chloride. To evaluate the efficiency of internalization and the bioactivity of DOX-loaded Alg-MA sub-microspheres, human lung epithelial carcinoma cells (A549s) were utilized as a model system. We hypothesized the dual-crosslinking would result in a tighter hydrogel network for more efficient intracellular DOX delivery (Figure 11). DOX encapsulation efficiency and in vitro release were quantified using an absorbance assay. While blank (non-loaded) Alg-MA sub-microspheres were non-cytotoxic to A549s, DOX-loaded sub-microspheres significantly reduced mitochondrial activity after five days of in vitro culture.

Figure 11: Schematic of the hydrogel network structure comprising photo-crosslinked and dual-crosslinked Alg-MA sub-microspheres. (I) Photo-crosslinked sub-microspheres exhibit a porous hydrogel network with intermolecular covalent crosslinks, encapsulating DOX. (II) Upon the addition of ionic crosslinking, the hydrogel network tightens, resulting in reduced drug loss and slower diffusion-based drug release; this is the desired product. (III) However, the introduction of aqueous-based calcium chloride (CaCl₂) solution may result in drug loss during the ionic crosslinking step. (IV) The non-ideal dual-crosslinked product may exhibit lower drug loading capacity due to the additional steps in the fabrication process.
5.3. Materials & Methods

5.3.1. Materials and reagents

Sodium alginate (M\textsubscript{w} = 65–75 kg/mol, 60-70% guluronic acid residues) was generously donated by FMC BioPolymer. Irgacure D2959 was generously donated by Ciba Inc. Biology-grade mineral oil, Span 80, Tween 80, ethylenediaminetetraacetic acid (EDTA), deuterium oxide (D\textsubscript{2}O), dimethyl sulfoxide (DMSO, 99% anhydrous), dodecyltrimethylammonium bromide salt (DTAB), methacrylic anhydride (MA), 4-(dimethylamino)pyridine (DMAP), DOX, N-ethyl-N'(3-dimethylaminopropyl)carbodiimide hydrochloric acid (EDC), N-hydroxysuccinimide (NHS), and an in vitro toxicology assay kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based) were purchased from Sigma-Aldrich. One molar hydrochloric acid (HCl) and 1 M sodium hydroxide (NaOH) were purchased from BDH ARISTAR® PLUS. Dichloromethane (DCM, 99.9%), sodium citrate, isopropanol, calcium chloride (CaCl\textsubscript{2}), sodium chloride (NaCl), sodium citrate, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) mammalian cell culture medium, Alexa Fluor® 647 cadaverine and 20X phosphate buffered saline (PBS) were purchased from Fisher Scientific. Fetal bovine serum (FBS) was purchased from Atlanta Biologics. Penicillin, streptomycin, and 0.25% trypsin EDTA were purchased from Corning Cellgro. A549 (CCL-185™) human lung epithelial carcinoma cells were purchased from ATCC®.
5.3.2. Synthesis and characterization of methacrylated alginate (Alg-MA)

Alg-MA was synthesized utilizing an anhydrous reaction to control the degree of methacrylation (DOM). Sodium alginate was rendered soluble in anhydrous DMSO through an ion exchange with DTAB. Aqueous solutions of sodium alginate (1%, w/v) and DTAB (2%, w/v) were prepared and slowly mixed while stirring at 1000 rotations per minute (See Appendix III for detailed protocol). The precipitate was washed in DI water and lyophilized. A 1% (w/v) alginate-DTA/DMSO solution was reacted with MA in the presence of a catalyst, DMAP, for 24 hours at room temperature (See Appendix IV for detailed protocol). The solution was hydrolyzed through extensive dialysis in 0.2 M sodium phosphate dibasic salt solution followed by further dialysis in DI water. Alginate methacrylation was confirmed using ¹H-NMR spectroscopy, (Bruker AVANCE III 500 MHz high-field NMR spectrometer) by the presence of methacrylate (6.25, 5.75 ppm) and alginate methyl resonances (2.0 ppm). A 1% (w/v) polymer solution in D₂O was analyzed at room temperature, spinning at 20 Hz for 16 scans. The DOM was quantified by peak integration and calculation of the ratio between of the methyl protons at 2.0 ppm and the newly formed methylene protons of methacrylate at 5.75 ppm and 6.25 ppm.

5.3.3. Dual-crosslinked Alg-MA sub-microsphere design and fabrication

Aqueous Alg-MA solutions were mixed with photoinitiators for UV (0.05%, w/v, Irgacure D2959) or visible green light activation [1 mM eosin Y (photosensitizer), 125 mM triethanolamine (initiator) and 20 mM 1-vinylpyrrolidone (catalyst)],
respectively. Two percent (w/v) Alg-MA solutions were mixed with 0.1% (w/v) DOX and formed into sub-microspheres using a water/oil emulsion and subsequent crosslinking (Figure 12).

Figure 12: (A) Chemical structure of methacrylated alginate (Alg-MA). Alg-MA was covalently crosslinked in the presence of photoinitiators under light activation, to form photo-crosslinked Alg-MA hydrogel networks. Alg-MA hydrogels were ionically crosslinked in the presence of calcium chloride (CaCl2) to form dual-crosslinked Alg-MA hydrogel networks. (B) Schematic representation of microsphere fabrication techniques. Microspheres with or without DOX were prepared by premixing Alg-MA solutions and creating a water/oil emulsion at room temperature. Alg-MA sub-microspheres were photo-crosslinked upon exposure to visible or UV light, respectively, and further dual-crosslinked in the presence of 1 M CaCl2.
Alg-MA sub-microspheres without DOX were fabricated as blank (i.e., non-loaded) controls. One milliliter of polymer/DOX solution was slowly added to 6.72 mL of biological-grade mineral oil containing 5% (v/v) Span 80, while mixing at 1200 rpm for 5 minutes at room temperature. Subsequently, 400 µL of 30% (v/v) Tween 80 (in biological-grade mineral oil) was added and mixed for an additional 5 minutes. Crosslinking was performed four different ways: 1) green light exposure for 10 minutes (Green, using 525 nm wavelength, NFLS-G30 3-WHT, SuperBrightLEDs); 2) UV light exposure for 10 minutes (UV, using 320–390 nm wavelength, Uvitron Intelliray 400); 3) green light plus 5 mL of 0.5 M CaCl₂, mixing for 15 minutes (Green+C); and 4) UV light plus 5 mL of 0.5 M CaCl₂, mixing for 15 minutes (UV+C). After crosslinking, 3 mL of isopropanol was added to the emulsion and mixed for 5 minutes, then centrifuged at 400 rpm for 5 minutes to precipitate sub-microspheres. Alg-MA sub-microspheres were washed sequentially with isopropanol (x2) and DI water (x2), respectively, and centrifuged after each wash.

The diameters and zeta-potentials (i.e., surface charge) for hydrated, blank and DOX-loaded Alg-MA sub-microspheres were quantified using dynamic light scattering (DLS, Zetasizer Nano ZSP, Malvern). Sub-microspheres were suspended in PBS, pH = 7.4, at room temperature. Hydrodynamic diameters were determined based on number averages, and the size distribution was plotted for each sub-microsphere group. After lyophilization, Alg-MA sub-microspheres were characterized by scanning electronic microscopy (SEM, JEOL 600); samples were sputter coated with 45 nm of Au-Pb prior to
imaging. SEM micrographs of various magnifications were used to visualize or attempt to visualize Alg-MA sub-microspheres.

**5.3.4. Drug loading and mechanisms of release**

Covalently and/or dual-crosslinked Alg-MA sub-microspheres were evaluated for use as chemotherapeutic delivery vehicles. DOX was utilized as a model drug for its intrinsic UV absorbance and ease of quantification, for drug encapsulation, drug release and effectiveness assays. To determine whether or not UV or green light exposure changed the chemical structure or bioactivity of DOX, aqueous DOX solutions were exposed to UV and green light for 10 minutes, and then characterized by $^1$H-NMR, using non-modified DOX as a control. DOX encapsulation efficiency, i.e., drug retention during sub-microsphere fabrication, was calculated as a percentage of the initial loading concentration. Covalent crosslinking of the sub-microspheres prevents dissolution; therefore, an extended diffusion process was utilized to quantify encapsulated drug. Briefly, 1 mg of sub-microspheres was suspended in 1 mL of PBS, incubated at 37°C and agitated for three weeks. The solution was centrifuged, and the supernatant was analyzed on a microplate reader (Synergy HT microplate reader, BioTek) at 485 nm absorbance, and compared to standard curve ($EE = \frac{\text{Actual Drug Encapsulated}}{\text{Theoretical Drug Loaded}}$). Detection of drug lost during washing procedures was not possible due to presence of multiple phases of emulsion additives and the absorbance detection limit of DOX on the equipment utilized.
To characterize in vitro release profiles, released DOX concentration was quantified using the intrinsic absorbance at 485 nm in a 48-well tissue culture polystyrene (TCPS) plate at 37°C (Synergy HT microplate reader, BioTek). One milligram of lyophilized DOX-loaded Alg-MA sub-microspheres was dissolved in 500 µL of PBS, pH 7.4 (n = 3). At 1, 2, 4, 8, 12, and 24 hours, and daily up to 11 days, 100 µL of PBS was removed for analysis, and replaced with 100 µL of fresh PBS to maintain the total volume. DOX concentration was determined using an absorbance assay and generating a standard curve. Cumulative DOX (µg) released over time was calculated by adding the mass of DOX released at each time point per mass of sub-microspheres.

5.3.5. Cellular uptake of Alg-MA sub-microspheres

Four different formulations of blank (i.e., non-loaded) Alg-MA sub-microspheres were reacted with Alexa Fluor® 647 cadaverine dye to form fluorescent sub-microspheres (Alexa 647-Alg-MA); the surface reaction chemistry was performed according to the manufacturers protocol through carbodiimide chemistry at room temperature catalyzed by NHS/EDC. Alg-MA sub-microspheres without DOX were used in order to avoid cell death during internalization and analysis. A549s were seeded in 48-well TCPS plates at 25,000 cells/well in 500 µL/well of standard growth culture medium (DMEM/F-12, 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin), and allowed to adhere for 24 hours at 37°C and 5% CO₂. Cells were then incubated with blank Alexa 647-Alg-MA sub-microspheres (n = 6 per group) at 100 µg/mL, 37°C and 5% CO₂. After 12 hours, culture medium containing Alexa 647-Alg-MA sub-microspheres was
removed, and adherent cells were thoroughly rinsed with PBS three times to remove non-internalized and cell-surface-bound sub-microspheres. Cells were trypsinized and re-suspended in PBS at 1x10^6 cells/mL, and analyzed by flow cytometry (BD LSRII Flow Cytometer) to quantify the percentage of A549s that internalized the sub-microspheres. A549s cultured with no sub-microspheres, and cells cultured with non-fluorescent sub-microspheres, were prepared and analyzed as controls.\textsuperscript{22}

### 5.3.6. Cytotoxicity of blank and drug-loaded Alg-MA sub-microspheres

The cytotoxicity of blank (i.e., non-loaded) and DOX-loaded sub-microspheres was evaluated using a toxicology, MTT-based assay. A549s were seeded in 48-well TCPS plates at 25,000 cells/well in 500 µL/well of standard growth culture medium, and allowed to adhere for 24 hours at 37°C and 5% CO\textsubscript{2}. Cells were then incubated in the presence of blank Alg-MA sub-microspheres or DOX-loaded Alg-MA sub-microspheres (n = 6 per group, per fabrication type) at sub-microsphere concentrations of 10, 50, 100 µg/mL. After 24 hours, medium containing sub-microspheres (blank groups and DOX-loaded groups) was removed, cells were rinsed two times in sterile PBS, and then analyzed using a MTT-based assay according to the manufacturer’s protocol. The optical density was measured at 570 nm; background absorbance at 690 nm was subtracted from the measured absorbance at 570 nm (Synergy HT microplate reader, BioTek). Absorbance values for the experimental samples were normalized to controls and reported as normalized mitochondrial activity.\textsuperscript{22}
5.3.7. Effect of intracellular vs. extracellular drug delivery on cell proliferation

The bioactivity of the DOX-loaded sub-microspheres was evaluated using a similar method discussed in section 2.6.1. A549s were seeded in 48-well TCPS plates at 10,000 cells/well in 500 µL/well of standard growth culture medium, and allowed to adhere for 24 hours. Cells were incubated in the presence of DOX-loaded Alg-MA sub-microspheres (n = 6 per fabrication type) at sub-microsphere concentrations of 10, 50, and 100 µg/mL. A549s and Alg-MA sub-microspheres were co-cultured for 5 days with media exchanges. Free DOX (i.e., DOX contained within the cell culture medium) was added to A549s at different concentrations (5, 4, 3, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015 and 0 µg/mL) to compare the effect of intracellular versus extracellular DOX delivery. After 1, 3 and 5 days of culture, a MTT-based assay was performed according to the manufacturer’s protocol, to quantify the effects of DOX-loaded Alg-MA sub-microspheres and free DOX on in vitro cancer cell proliferation. Absorbance values for the experimental samples were normalized to controls and reported as normalized mitochondrial activity.22

5.3.8. Data analysis

The quantitative results for all experiments are reported as mean ± standard deviation. Statistical analysis was performed on Alg-MA sub-microsphere co-cultured cell assays, using one-way ANOVA with DUNNETs method (α = 0.05) via the SAS statistics program in the GLM procedure, as the post-test to compare all of the groups. A p < 0.05 was considered significantly different.
5.4. Results & Discussion

5.4.1. Synthesis and characterization of Alg-MA

The chemical modification of alginate rendered it hydrophobic and soluble in organic solvents. An anhydrous methacrylation of alginate resulted in a functionalized biomacromolecule with a controllable DOM.\textsuperscript{28} $^1$H-NMR spectra for Alg-MA and non-modified alginate are shown in Figure 13. The DOM for the Alg-MA used as the base material in the sub-microspheres was approximately 64\%.\textsuperscript{31-33} Peaks between 3.0 and 3.5 ppm indicate methyl groups at the end of alginate chains resulting from degradation during the methacrylation chemistry.

![Figure 13: The $^1$H-NMR spectra of Alg-MA and alginate are shown. The peaks at 5.75 and 6.25 ppm indicate that the hydrogens on the methylene of the methacrylate groups were present on the alginate backbone after modification. The degree of methacrylation was calculated to be 64\%.](image)
5.4.2. Fabrication and characterization of dual-crosslinked Alg-MA sub-microspheres

The formation of Alg-MA sub-microspheres was indicative of a crosslinked hydrogel network, obtained through either covalent crosslinking\(^{34}\) (e.g., via photocrosslinking) alone, or in combination with ionic crosslinking (e.g., by the addition of CaCl\(_2\)), as illustrated in Figure 12.\(^{27, 34-35}\) The DOM for the Alg-MA base material was 64%, and this moderate-DOM sustained both covalent and ionic crosslinking. Photocrosslinking occurred upon UV or green light activation between adjacent acrylate groups, while the subsequent presence of CaCl\(_2\) induced ionic crosslinking between adjacent carboxyl groups. While methacrylation took place at available hydroxyl groups, ionic crosslinks formed between adjacent carboxyl side-groups on neighboring alginate chains, thus allowing Alg-MA to sustain dual-crosslinking.

DOX-loaded sub-microsphere hydrodynamic diameters were quantified using DLS analysis (Table 2). The largest populations of DOX-loaded sub-microspheres were sized between 243 – 391 nm: UV = 243 nm, Green = 391 nm, UV+C = 346 nm, Green+C = 358 nm. The variability of the sub-microsphere diameters, plotted as size distributions in Figure 14A+B, is an almost unavoidable result of the emulsion process, and is indeed a limitation of the fabrication method; however, the linear size distribution plots indicate the following: Alg-MA sub-microspheres exhibited size populations within the same size scale, thus demonstrating consistency in fabrication method. SEM images (Figure 15) also indicated that Alg-MA sub-microspheres were spherical in shape, however
heterogeneous in size. The zeta-potentials ranged between -20 mV and -37 mV, and none of the groups demonstrated any significant outlying data.

Table 2: Dynamic light scattering (DLS) quantitative analysis of hydrodynamic diameters and zeta-potentials of blank (i.e., non-loaded) and DOX-loaded photo-crosslinked and dual-crosslinked Alg-MA sub-microspheres. DOX encapsulation efficiencies were determined using an absorbance assay after sub-microsphere fabrication.

<table>
<thead>
<tr>
<th>Sub-Microsphere Group</th>
<th>Hydrodynamic Diameter by Number (nm)</th>
<th>Zeta-Potential (mV)</th>
<th>Encapsulation Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blank DOX Loaded</td>
<td>Blank DOX Loaded</td>
<td>DOX Loaded</td>
</tr>
<tr>
<td>Green Light</td>
<td>334 391</td>
<td>-37 -27</td>
<td>28</td>
</tr>
<tr>
<td>UV Light</td>
<td>331 243</td>
<td>-21 -21</td>
<td>84</td>
</tr>
<tr>
<td>Green Light + Calcium</td>
<td>88 358</td>
<td>-29 -33</td>
<td>26</td>
</tr>
<tr>
<td>UV Light + Calcium</td>
<td>197 346</td>
<td>-27 -25</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 14: Dynamic light scattering size distribution plots for photo-crosslinked and dual-crosslinked Alg-MA sub-microspheres both non-loaded/blank (A) and DOX-Loaded (B): green photo-crosslinked (Green), green + Ca2+ dual-crosslinked (Green+C), UV photo-crosslinked (UV), UV + Ca2+ dual-crosslinked (UV+C). Hydrodynamic diameters were based on number-average calculations.
Figure 15: Scanning electron microscopy (SEM) images representing dehydrated, Alg-MA sub-microparticles for qualitative assessment of morphology.

5.4.3. Swelling and diffusion-based drug release

The efficacy of Alg-MA sub-microspheres as chemotherapeutic delivery vehicles was investigated. DOX was utilized as a model drug for its intrinsic UV absorbance and ease of quantification for subsequent drug encapsulation, drug release and effectiveness assays. Alg-MA sub-microspheres were designed to encapsulate DOX without interfering with the detectability or bioactivity of DOX. Both photo-crosslinking alone or dual-crosslinking were successful in fabricating DOX-loaded sub-microspheres. The low level of UV or green light exposure required for sub-microsphere fabrication did
not change the chemical structure of DOX, verified by \(^1\)H-NMR spectroscopy (Figure 16), and the toxic effects of DOX were still active.\(^{36}\) The mild-gelation techniques used to form Alg-MA sub-microspheres may retain the functionality and bioactivity of other therapeutics. Indeed, it was hypothesized that secondary, ionic crosslinking may show no beneficial effect on DOX encapsulation efficiency, however, the effect of ionic crosslinking may result in sustained drug release due to a tighter hydrogel network structure.

Figure 16: The \(^1\)H-NMR spectra of DOX solutions before and after light exposure are shown in Supplemental Figure S3. No significant differences are present in the spectra for UV and green light exposed DOX solutions and the spectrum for the non-modified DOX solution, showing molecular structure and bioactivity are preserved.
The cumulative mass of DOX released over time was calculated from four different types of Alg-MA sub-microspheres, and release profiles are shown in Figure 17. The DOX release profiles followed two different trends – a linear release profile was seen during the first 8 hours of release, consistent with hydrogel-swelling induced drug release (see Figure 17A). Cumulative DOX release profiles through 11 days are shown in Figure 17B. The release profiles for UV, Green, and Green+C groups followed a logarithmic trend (trend line $R^2 \geq 0.92$), while the UV+C group followed a linear release profile up to 11 days (trend line $R^2 = 0.98$); however, these trends were not analyzed further. The amount of DOX released did show a similar trend with encapsulation efficiencies: dual-crosslinked sub-microspheres encapsulated less drug and released less drug over an 11 day period.\(^{37-38}\) The introduction of aqueous-based CaCl\(_2\) solution to the emulsion resulted in drug loss due to DOX solubility in aqueous solutions.\(^{39}\)

![Figure 17: Quantitative cumulative release of doxorubicin (DOX) from Alg-MA sub-microspheres for 11 days (average ± standard deviation, $n = 6$ hydrogel samples per group). Various formulations of sub-microspheres were assessed: green photo-crosslinked (Green), green + Ca\(_2\)+ dual-crosslinked (Green+C), UV photo-crosslinked (UV), UV + Ca\(_2\)+ dual-crosslinked (UV+C). Sample aliquots were collected and the DOX concentration was determined using a standard curve at an absorption wavelength of 485 nm. (A) Cumulative DOX release profile during the first 8 hours. (B) Cumulative DOX release profile during 11 days.](image)
The varying release kinetics with time suggest that the sub-microsphere structure may be optimized further for controlled release applications, by varying the degree of crosslinking to extend or delay the drug release rate. It is hypothesized that increasing the drug-loading concentration, increased efficacy over longer time periods could be achieved. Decreasing the variability in the diameter of the sub-microspheres and varying the Alg-MA DOM may also result in varied release rates due to changes in the network microstructure. However, sub-microsphere size homogeneity and drug release profile optimization were outside the scope of this study and may be addressed through further investigations.

5.4.4. Cellular uptake of Alg-MA sub-microspheres

Uptake of Alg-MA sub-microspheres into A549s was quantitatively determined via flow cytometry to detect the fluorescent signal of Alexa-647-labeled sub-microspheres. Non-treated A549s and cells cultured with non-fluorescently labeled sub-microspheres were used as controls. Utilizing gate settings based on the fluorescent intensity level of the probe, negative and positive populations were established, and it was found that all four types of Alg-MA sub-microspheres were readily internalized by A549s. The positive population was > 80% in all four treatment groups (Figure 18A-F). UV crosslinked Alg-MA sub-microspheres (single and dual-crosslinked) exhibited higher internalization rates compared to green light crosslinked groups (Figure 18G), which may
be related to sub-microsphere diameter; however, statistics were not performed on the internalization data.

Figure 18: Flow cytometry analysis of Alg-MA sub-microspheres after 12 hours of co-culture with human lung epithelial carcinoma (A549) cells. (A) Non-treated cell control, (B) cells cultured with non-labeled blank sub-microspheres, (C) cells cultured with green photo-crosslinked sub-microspheres, (D) cells cultured with green photo-crosslinked and calcium crosslinked sub-microspheres, (E) cells cultured with UV photo-crosslinked sub-microspheres, and (F) cells cultured with UV photo-crosslinked and calcium crosslinked sub-microspheres. (G) Flow cytometry histograms were presented to show the different fluorescence intensity between control cells and different Alg-MA sub-microsphere groups.

5.4.5. Cytotoxicity of blank and drug-loaded Alg-MA sub-microspheres

To verify the non-toxicity and retention of DOX bioactivity after sub-microsphere encapsulation, MTT assays were performed on blank Alg-MA sub-microspheres and DOX-loaded Alg-MA sub-microspheres after 24 hours of culture with A549s to quantify mitochondrial activity. A549 viability was assessed in the presence of Alg-MA sub-microspheres at increasing concentrations (10, 50, 100 µg/mL, Figure 19). Blank Alg-MA sub-microspheres (with no drug content) were minimally cytotoxic to
A549s (mitochondrial activity > 80%) at concentrations up to 100 µg/mL (Figure 19A). Increased mitochondrial activity may be attributed to low molecular weight soluble alginate (i.e., sugar) in the culture media. Additionally, we hypothesize the reduced cytotoxicity seen in the UV+C group may be due to enhanced clearance of residual UV photoinitiator upon secondary crosslinking with an aqueous calcium chloride solution. DOX-encapsulated sub-microspheres delivered bioactive drug, significantly reducing mitochondrial activity within 24 hours (Figure 19B).

Figure 19: Human lung epithelial carcinoma (A549) cells were cultured in the presence of hydrogel sub-microspheres for 24 hours in standard growth culture medium at 37°C and 5% CO₂. A549 mitochondrial activity was determined using an absorbance-based quantitative assay; absorbance data for the groups treated with sub-microspheres were normalized to the non-treated cell control (average ± standard deviation, n = 6 hydrogel samples per group). The cytotoxicity of Alg-MA sub-microspheres was analyzed on (A) blank (non-loaded) sub-microspheres. The bioactivity of doxorubicin (DOX) was verified using (B) DOX-loaded sub-microspheres. Various groups (white diamonds = green photo-crosslinked, white circles = UV photo-crosslinked, black diamonds = green + Ca²⁺ dual crosslinked, black circles = UV + Ca²⁺ dual crosslinked) and sub-microsphere concentrations (10, 50, 100 µg/mL) were characterized.
5.4.6. Effect of intracellular vs. extracellular drug delivery on cell proliferation

Four different types of DOX-loaded Alg-MA sub-microspheres were cultured with A549s at concentrations of 10, 50 and 100 µg/mL. In addition, different concentrations of free DOX was added to the culture media and served a control. A short-term cell proliferation study (utilizing an MTT assay) was performed for 5 days. On day 1, all of the Alg-MA sub-microsphere groups reduced A549 proliferation, regardless of the crosslinking type or concentration (Figure 20A). On days 3 and 5, the UV crosslinked Alg-MA sub-microspheres showed the greatest reduction in the A549 proliferation. For comparison, normalized mitochondrial activity was plotted versus free DOX, Figure 20B, or DOX-loaded sub-microspheres calculated, Figure 20C.

Compared to the free DOX control, Alg-MA sub-microsphere-mediated delivery shows a similar decreasing trend as drug concentration increases, though remains less effective. Also, it is likely that drug remains within the sub-microspheres and is not released intracellularly. Another consideration is that due to the extended drug release profile of DOX from sub-microspheres (Figure 17B), improved efficacy beyond 5 days may be achieved. Indeed, photo-crosslinked microspheres alone are advantageous for delivering a chemotherapeutic to cancer cells, at clinically-relevant dosages, and decreased lung cancer cell mitochondrial activity.
Figure 20: The efficacy of doxorubicin (DOX)-loaded Alg-MA sub-microspheres as chemotherapeutic delivery vehicles was assessed using a MTT-based assay, to quantify cell proliferation over a 5-day period. A549 activity was recorded as mitochondrial activity and normalized to non-modified cell controls. Various formulations and concentrations (10-100 µg/mL) of sub-microspheres were assessed: green photo-crosslinked (Green), green + Ca2+ dual-crosslinked (Green+C), UV photo-crosslinked (UV), UV + Ca2+ dual-crosslinked (UV+C). DOX was added exogenously (Free DOX) to the cell culture medium at various concentrations to test the effect of intracellular versus extracellular DOX delivery. (A) Effect of Alg-MA sub-microsphere concentration for each crosslinking type on A549 mitochondrial activity; (B) Effect of ‘free dox’ concentration on A549 mitochondrial activity on days 1, 3, and 5; (C) Effect of DOX concentration encapsulated within Alg-MA sub-microspheres on A549 mitochondrial activity on days 1, 3, and 5.
5.5. Conclusions

The study reported here in focused on the efficacy of utilizing crosslinked Alg-MA sub-microspheres to intracellularly deliver a chemotherapeutic. Photo-crosslinked and dual-crosslinked Alg-MA sub-microspheres successfully encapsulated DOX, were internalized by A549s, and delivered DOX to A549s, reducing mitochondrial activity compared to non-modified cell controls. The outcome of this study suggests that photo-crosslinking alone, and in particular green light activation, is an effective means of producing drug delivery vehicles, and perhaps additional crosslinking steps or procedures are not beneficial, perhaps even detrimental, to drug encapsulation efficiencies. Based on drug encapsulation predictions and calculations, effective clinical drug dosages were achieved, as compared to free DOX delivery, and were controllable. While the efficacy for using photo-crosslinking Alg-MA sub-microspheres was shown during a short time frame (5 days) in vitro, future in vivo work may show enhanced drug efficacy using microsphere-mediated delivery compared to exogenous intravenous chemotherapy over extended periods of time. Further discussion of this work can be found in Appendix XI.

5.6. Acknowledgements

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5.7. References


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CHAPTER 6: THERAPEUTIC ALGINATE-BASED ADHESIVE HYDROGELS FOR LUNG TISSUE REPAIR

Spencer L. Fenn, Patrick Charron, Rachael A. Oldinski

Contributions

The research discussed in this chapter was performed by the first author, Spencer L. Fenn. The first author was also responsible for manuscript drafting and figure development with input and revisions being provided by the second author, Patrick N. Charron, and the senior author Rachael A. Oldinski.

6.1 Abstract

Injury to the connective tissue that lines the lung, the pleura, or to the lung itself can occur from many causes including trauma or surgery, as well as lung diseases or cancers. Bronchopleural fistulas, malignant pleural effusions, and traumatic or ventilator-induced pleural injuries are a continuing source of morbidity, mortality, and increased health care expenditures in the clinic. There are currently only limited methods of patching significant injuries to stop the air or fluid leak quickly and subsequently allow for healing processes to repair the underlying tissue. We have devised an adherent hydrogel sealant patch system, based on the blending of methacrylated alginate (AMA) and AMA di-aldehyde (AMA-DA), which is capable of sealing damaged tissues and sustaining physiological pressures. Methacrylation of alginate hydroxyl groups rendered the polysaccharide capable of photo-crosslinking when mixed with an eosin Y based photoinitiator system and exposed to visible green light. To improve tissue adhesion,
oxidation of alginate was performed, opening uronate residues on the carbohydrate backbone to yield functional aldehyde groups capable of imine bond formation with proteins found in many tissues. These two chemical modifications, when utilized in combination, allow for the synthesis of an adhesive hydrogel sealant. The resulting bulk material was subsequently mixed at various ratios and concentrations, blended with photoinitiators and fabricated into film patches using a manual injection molding technique. To test efficacy of the alginate-based patch system, a custom burst pressure testing device was designed and fabricated according to ASTM Standard F2392-04R10, utilizing punctured collagen casings as a test substrate. High-speed videography was performed during each test to assess the mechanism of failure, either adhesive failure (delamination from the substrate), or material failure (burst through the patch). Cell viability and cytotoxicity were assessed using human mesothelial cells (MeT-5A, ATCC) after 24 hours culture in the presence of test materials. Additionally, drug-eluting adhesive patches were fabricated through incorporation of doxorubicin encapsulating nanoparticles, and used to successfully treat human lung cancer cells (A549, ATCC).
6.2. Introduction

Lung leaks, due to damage in the pleural lining or resected tissue, are a major medical problem thoracic surgeons are currently facing, and are associated with increased morbidity, mortality, and health care costs. Trauma or complications of pulmonary surgery can result in air or liquid leaking out of the lung and into the pleural space, i.e., chest cavity, decreasing lung volume and causing the lung to collapse (pneumothorax), ultimately requiring emergent placement of a chest tube, an invasive approach associated with complication and limitations, to re-inflate the lung.

Chronic pleural effusions related to underlying lung cancers or cancers metastatic to lung (malignant effusions) are especially difficult to treat, apart from surgical interventions or pleurodesis. Physical pleurodesis is a surgical technique involving suturing or pleural abrasion procedures through the use of thoracotomy.\(^1\) Chemical pleurodesis consists of applying a nocuous material, such as talc, on the lung wall to cultivate the inflammatory response.\(^2-3\)

Few effective means exist of patching the injury to stop the air or fluid leak and allow appropriate healing to occur. Approaches using glues or other sealants applied either directly to the lung surface or instilled into the airways have not been widely successful to date. In addition, prolonged tissue damage due to an underlying disease, infection or cancer has not been addressed by the current methods. Few-effective options are available and new therapeutic approaches are desperately needed.
Indeed, surgical sealants tools available to surgeons when closing or sealing surgical sites or wounds in tissue, yet due to the dynamic movement and stretch imposed on lung tissues, few have shown efficacy at physiological lung pressures. Hydrogels are natural or synthetic-based crosslinked polymer networks that swell but do not dissolve in aqueous media. While the use of hydrogels as surgical sealants and wound treatments began developing in the 1960’s, the use of hydrogels to treat pulmonary and alveolar air leaks initiated in the late 1980’s.\(^4\) Natural based biomaterials are advantageous compared to their synthetic counterparts due to heightened compatibility, degradability, sustainable resources, and intrinsic bioactive qualities. Alginate and alginate-based hydrogels are investigated for biomedical applications due to their inherent non-toxicity, biocompatibility, and are readily available.\(^5\) Derived from brown algae, alginate is desirable not only for those attributes already listed, but also for its relatively low cost and the various applications it can be used for drug delivery and tissue engineering.\(^6\) Chemical modification of alginate has been studied extensively for improving upon the physical and mechanical properties of ionically crosslinked alginate gels.\(^7-9\) Methacrylation of alginate imparts a functional group capable of light-activated covalent crosslinking by free radical polymerization in the presence of a photoinitiator. Alginate is inherently non-adhesive; however, oxidation of the backbone structure will elicit functional groups capable of forming crosslinks with extracellular matrix proteins, such as those found on the pleural surface.\(^10-13\)
In order to treat lung leaks, we ultimately aim to develop a sealant that not only provides a reliable seal, but that can also control the release of soluble drugs and growth factors to treat disease and/or stimulate tissue regeneration. We illustrate the ability to control therapeutic drug release through nanoencapsulation techniques and subsequent mixing with the polymer precursor solutions prior to tissue sealant patch fabrication.

6.3. Materials and Methods

6.3.1. Synthesis of methacrylated alginate (AMA)

Methacrylated alginate (AMA) was synthesized as previously described.\textsuperscript{5, 14} Briefly, a 2% (w/v) solution of Manugel\textsuperscript{®} GMB (MW ≈ 170-240 kDa, FMC Biopolymer) in deionized water was mixed with a 20-fold molar excess of methacrylic anhydride (Sigma-Aldrich). The solution was maintained at pH 8 using 5 N sodium hydroxide (Fisher) for 24 hours. Purification was performed via dialysis (MWCO=6000-8000 Da) against deionized water for 5 days, and lyophilized to yield a dry product. A 1% (w/v) AMA was prepared in deuterium oxide (Acros Organics) and subsequently analyzed using \textsuperscript{1}H-NMR spectroscopy (Bruker AVANCE III 500 MHz high-field NMR spectrometer), for 64 scans at 20 Hz. The degree of methacrylation (DOM) was established through integration of the peaks associated with methacrylate (6.24, 5.78 ppm) and alginate methyl resonances (1.96 ppm), and calculation of the ratios between.\textsuperscript{14-16}

6.3.2. Synthesis of methacrylated alginate dialdehyde (AMA-DA)
To retain the ability to photo-crosslink while simultaneously improving the adhesive properties to tissue/proteins, AMA was oxidized to yield AMA dialdehyde or (AMA-DA). A 1% AMA solution was prepared in deionized water and reacted with sodium periodate (Sigma) at 0.25% (w/v) or 0.5% (w/v) to yield AMA-DA solutions with theoretical degrees of oxidation (DOO) of 25% (AMA-25DA) and 50% (AMA-50DA) respectively.\textsuperscript{11-14, 17} The products were dialyzed against deionized water for 48 hours, and lyophilized to obtain dry AMA-DA. 1% (w/v) AMA-DA solutions were prepared in deuterium oxide (Acros Organics) and subsequently analyzed using \textsuperscript{1}H-NMR spectroscopy (Bruker AVANCE III 500 MHz high-field NMR spectrometer), for 64 scans at 20 Hz. The degree of oxidation (DOO) was calculated by taking integrals of the alginate methyl protons at 5.0 ppm and the newly formed methyl protons at 5.17, 5.48 and 5.68 ppm and subsequent comparison of ratios.\textsuperscript{12-13}

6.3.3. Visible light crosslinking and hydrogel formation

AMA and AMA-DA solutions were analyzed using a rheometer (AR2000, TA Instruments) to if functionalization modulated the physical and mechanical properties of the molecules in solution or crosslinked as hydrogels. The viscosity and gelation kinetics of Alg, AMA, and AMA-DA precursor solutions and resulting hydrogels were determined. All tests were performed at 37°C using a 20-mm diameter 1°59’6” steel cone geometry with a truncation gap of 57 µm; 6% (w/v) polymer solutions were prepared in PBS with photo-initiators added at the following final concentrations: 1 mM Eosin Y (photo-sensitizer, Acros Organics), 125 mM triethanolamine (initiator, Sigma), 20 mM 1-vinyl-2-pyrrolidinone (catalyst, Sigma).\textsuperscript{18-20} Viscosity was measured at
shear rates ranging from 1-100 (1/s), over a 60 second time period, using non-crosslinked precursor solutions. Gelation kinetics of the precursor solutions were assessed using an oscillatory time sweep at 10% radial strain at 1 Hz during exposure to visible green light (525 nm, custom 9.84 cm diameter light emitting diode (LED) array, NFLS-G30X3-WHT, SuperBrightLEDs) for a total of 5 minutes (300 seconds). Shear storage ($G'$) and loss ($G''$) moduli, as well as tan delta (ratio of $G''$ to $G'$) were calculated using analytical software (TA Data Analysis).

6.3.4. Hydrogel Patch Fabrication

Lung sealant patches were fabricated using an injection molding technique. Polymer solutions (3% and 6% w/v) in deionized water were prepared using AMA, AMA-25DA, AMA-50DA and subsequently blended at 1:1 or 1:2 ratio. Polymer solutions were then supplemented with photo-initiators as described in Section 2.3 and were protected from exposure to light.

Precursor solutions were injected between two sheets of polytetrafluoroethylene (Teflon™) with a 1 mm spacer, using a syringe and 20-gauge needle, taking care to avoid the formation of air-bubbles. The mold was then rapidly frozen using liquid nitrogen (vapor phase) and freeze-dried using a lyophilizer (Freezone, Labconco). Once dry, the mold was disassembled and the newly formed dry sheet of material was cut into individual patches using circular biopsy punches. It is important to note that at this stage, the patches have not yet undergone photo-crosslinking yet contain photo-initiators required to induce photo-crosslinking once exposed to visible green light.
Figure 21: Fabrication of tissue sealant patches is performed using an injection molding technique in combination with lyophilization to form dry sheets of sealant material which can be cut to size using a biopsy punch.

6.3.5. Assessment of material cytotoxicity

To assess the cytotoxicity of AMA and AMA-DA patches, McT-5A human mesothelial cells (ATCC® CRL9444™) were cultured in the presence of test materials over a 24-hour period (n=3). Mesothelial cells were maintained at 37°C and 5% CO₂ in Medium 199 (Sigma) supplemented with the following components: 10% fetal bovine serum (FBS, Hyclone), 3.3 nM epidermal growth factor (EGF, Sigma), 400 nM hydrocortisone, 870 nM bovine insulin (Sigma), 20 mM HEPES (Sigma), 3.87 µg/L selenious acid (Aldrich), Trace Elements B Liquid used at 1,000 dilution (Corning). Cell viability, cytotoxicity and apoptosis were assessed using an ApoTox-Glo™ Triplex Assay (Promega Corporation) according to the manufacturers protocol, and metabolic
activity was assessed using an thiazolyl blue tetrazolium bromide (MTT, Sigma) based assay. Cells were treated with 10 mg/mL AMA, AMA-25DA and AMA-50DA.

After 24-hours culture with test materials, the ApoTox-Glo™ viability/cytotoxicity reagent was prepared and added to each well per the manufacturers protocol allowed to culture at 37°C for an additional 30 min. At this time, the plate was assessed using a plate reader (H1 Synergy, BioTek) measuring fluorescence intensity at the following excitation and emission wavelengths listed in nanometers: 400ₜₜ/505ₜₜ (viability) and 485ₜₜ/520ₜₜ (cytotoxicity). Successively the apoptosis reagent was prepared, added to each well, and allowed to culture for an additional 30 minutes at room temperature. Luminescence was then read using a plate reader.

After 24-hours of culture with test materials, a previously prepared MTT-solution in PBS was added to each test well, up to a final concentration of 0.5 mg/mL, mixed briefly on an orbital shaker, and incubated at 37°C for 3-hours. Next, the cell culture media and excess MTT-solution were aspirated from each well and replaced with dimethyl sulfoxide (DMSO, Sigma) to solubilize the formazan crystals using gentle orbital shaking. Absorbance was assessed at 540 nm wavelength, the peak absorbance of formazan in DMSO, using a plate reader. Background absorbance was assessed at 690 nm wavelength and subtracted from the absorbance observed at 540 nm to obtain a corrected absorbance reading of cell mitochondrial activity.
6.3.6. Burst pressure and sealant failure analysis

To assess the performance of sealant patch formulations, ASTM F2392-04 was modified to build and utilize a custom-built burst pressure testing device, as previously described and depicted. Briefly, a polyether ether ketone device was designed to securely fasten a membrane/substrate across an open chamber, using a fluoroelastomer o-ring and clamp, which can be pressurized using an air-filled syringe and syringe pump (PHD 2000 Infuser, Harvard Apparatus). Pressures within the chamber were recorded digitally using a USB-connected pressure transducer (Omega PX-409-030AUSBH) attached to the chamber through a side NPT-port. The entire apparatus was housed in an incubator held at 37°C. High-speed video (120 frames per second) is recorded during testing using a camera (Hero4, GoPro) with attached macro lens which has been mounted within the incubator. This video is utilized to assess the mechanism of failure, either adhesive failure (delamination) or material failure (burst).

Collagen-rich substrates (Collagen Casings, The Sausage Maker Inc.) were used as an in vitro test membrane per the standard. Each substrate was hydrated in PBS at 37°C for at least 30 minutes prior to testing. To verify each substrate was free from defects and leaks, the substrate was first clamped down in the device and pressurized to 12 in-H$_2$O at an infusion rate of 75 mL/hr, and held briefly ensuring no change in pressure (i.e., no leaking occurred). Once substrate integrity was confirmed, the membrane was removed from the device and a small defect was created in the membrane using a 1.5 mm diameter biopsy punch. Over these defects 8 mm diameter sealant patches
were applied, and hydrated using 20 µL of PBS at 37°C. The sealant was then photo-crosslinked for 5 minutes using a custom LED array (525 nm, NFLS-G30X3, SuperBrightLEDs). Once cured, the now-sealed membrane was returned to the burst-pressure testing apparatus and pressurized at 75 mL/hr until failure of the sealant was observed.

**6.3.7. In vitro degradation study**

To assess the effects of degradation on hydrogel mass and swell ratios, crosslinked hydrogels fabricated from 6% AMA:AMA-25DA at 1:1 ratio were first weighed (dry), and subsequently placed in cell culture media in a shaker-incubator at 37°C for 14 days. On days 1, 3, 7, and 14, samples were removed and weighed (wet), freeze-dried, then weighed (dry) to obtain both swell ratios and changes in mass.

**6.3.8. Fabrication of doxorubicin-loaded nanoparticles**

A 2% (w/v) AMA solution was prepared in deionized water, and subsequently supplemented with 0.1% (w/v) doxorubicin hydrochloride (DOX, Sigma). Additionally, photo-initiators were added to the mixture, as listed in Section 2.3. Drug-encapsulated hydrogel nanoparticles were formed using a water-in-oil emulsion technique and covalently photo-crosslinked using visible green light. At room temperature, one mL of DOX-loaded polymeric precursor solution was added dropwise to 6.75 mL of bioreagent-grade mineral oil containing 5% (v/v) Span 80, consistently mixing at 1200 rpm with an egg-shaped stir bar for 5 minutes. Next, 400 µL of 30% (v/v) Tween 80 (in bioreagent-grade mineral oil) was added and mixed for a further 5 minutes. At this point,
the emulsion droplets were photo-crosslinked for 5 minutes using visible green light produced by a custom LED-array (525 nm wavelength, NFLS-G30 3-WHT, SuperBrightLEDs). Particles were washed in isopropanol (2x) and de-ionised water (2x), using high-speed centrifugation between each washing step, and subsequently freeze-dried for storage. Particle size in PBS (pH 7.4) was assessed using dynamic light scattering (DLS, Zetasizer Nano, Malvern) at 37°C.

6.3.9. Fabrication of drug eluting sealant patch

Polymeric precursor solutions were prepared as described in Section 2.4 for the two highest performing formulations, 6% AMA:AMA-25DA and 6% AMA:AMA-50DA blended at a 1:1 weight ratio, and were subsequently blended with 800 µg/mL DOX-encapsulated nanoparticles. The solutions were quickly injected into molds (see Section 6.3.4.), frozen, and lyophilized to form DOX-eluting sealant patches.

6.3.10 Chemotherapeutic drug release

To assess drug-release kinetics of the DOX-eluting patches, 10 mg patches each containing approximately 160 µg DOX-nanoparticles, were prepared from the molded sheet using a biopsy punch, and placed in a 48-well plate. The patches were hydrated with 50 µL PBS (pH 7.4), and crosslinked for 5 minutes. Subsequently, 500 µL PBS was added to each well, and the entire plate was placed in an incubator shaker at 37°C at 30 rotations per minute (rpm). At 1, 2, 4, 6, 12, 24, 48 hours, 100 µL of supernatant were collected from each well and immediately replaced with 100 µL of fresh PBS. To
quantify cumulative drug released at each time point, the absorbance of collected aliquots was assessed at 485 nm wavelength using a plate reader and compared to a DOX standard curve.\textsuperscript{15,23} Peak absorbance of DOX in PBS (pH 7.4) was previously confirmed to be 485 nm when assessed using an absorbance spectral sweep from 300-700 nm at 1 mg/mL concentration (Figure 22).

![Figure 22: Verification of the peak absorbance of doxorubicin hydrochloride was performed via a spectral sweep at 1 mg/mL in PBS (pH 7.4). The peak absorbance is shown at 485 nm wavelength which will be used for subsequent drug-release assays.](image)

6.3.11. Bioactivity of chemotherapeutic patch

The bioactivity of the DOX-eluting patches was assessed using human lung epithelial carcinoma cells (A549, ATCC). Cells were seeded in the lower portion of a 48-well Transwell\textsuperscript{®} plate (0.4 µm pore diameter) at 25,000 cells per well, and allowed to adhere to substrate overnight at 37°C in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12, Gibco) mammalian cell culture medium supplemented with
10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Prior to starting the assay, 10 mg patches, containing 160 µg of DOX-encapsulating nanoparticles each, were hydrated and photo-crosslinked on the permeable membrane insert of the Transwell® plate (n=3 per group), and subsequently added to the seeded wells. After 24 hours of culture at 37°C, viability was assessed as previously described in Section 2.5.

6.3.12. Statistical analysis

All numerical results are presented as a mean ± standard deviation unless otherwise stated. A one-way ANOVA with Dunnett’s method for multiple comparisons was utilized for viability, cytotoxicity, apoptosis and MTT assays. A one-way ANOVA with Tukey’s method for multiple comparisons was used for burst pressure data analysis. A p-value of 0.05 or less was considered statistically significant for all statistics performed.

6.4. Results

6.4.1. Synthesis of AMA and AMA-DA

Alginate methacrylation was performed to enable covalent photo-crosslinking between adjacent polysaccharide chains in aqueous solutions, forming a hydrogel.5, 9, 12, 14-15, 24-31 The aqueous methacrylation chemistry yielded a functionalized biomacromolecule capable of covalent crosslinking, as confirmed by 1H-NMR (see Figure 23). The degree of methacrylation (DOM) was calculated to be 58% for AMA, and 36% and 38% for AMA-25DA and AMA-50DA respectively. The degree of
oxidation (DOO, i.e. aldehyde-modification) was calculated to be 19% for AMA-25DA and 42% for AMA-50DA.

![Diagram](image)

**Figure 23:** A) Molecular repeat units for sodium alginate, methacrylated alginate, and methacrylated alginate dialdehyde are presented. B) The $^1$H-NMR spectra of 25% and 50% oxidized AMA-DA, AMA and unmodified alginate are shown as labelled. Peaks of interest identified with arrows. The peaks at 5.78 and 6.24 ppm indicate that the hydrogens on the methylene of the methacrylate groups were present on the alginate backbone after modification. The degree of methacrylation for AMA was calculated to be 58%. The peaks at 5.17, 5.48 and 5.68 ppm are indicative of oxidation and the opening of the uronate residues to form aldehyde groups. The experimental degree of oxidation for the 25 DOO and 50 DOO AMA-DA were calculated to be 19% and 42% respectively Degrees of methacrylation were found to decrease after oxidation/aldehyde-modification to 38% and 36% respectively.

### 6.4.2. Rheology

Viscosities for 6% w/v polymer solutions are presented in Figure 24, over a range of increasing shear rates. AMA solutions (blue circles) demonstrated the highest viscosity over all shear rates tested. Significant decreases in viscosity were observed after oxidation of AMA, to yield functional aldehyde groups, as shown by AMA-25DA (red square) and AMA-50DA (green triangle), with viscosity decreasing more with higher levels of oxidation. The decrease in viscosity was partially recovered when blended with
non-oxidized AMA, as shown by both 1:1 blends presented (orange diamond, violet triangle).

Figure 24: Viscosity of 6% w/v polymer solutions was assessed over shear rates of 1-100 (1/s) at 37°C and shown in log10 scale. Notice the viscosity decreases significantly for the oxidized/aldehyde-modified solutions, but is partially recovered when blended with AMA at a 1:1 ratio.

6.4.3. Visible light crosslinking and gelation kinetics

Oscillating a shear force during hydrogel crosslinking reveals the sealants gelation kinetics. Storage modulus, or G’, is a measurement of the elastic response of the material, and will increase as crosslinking between adjacent polysaccharide chains and their methacrylate side-groups occurs, resulting in a plateau when crosslinking has reached its terminal maximum. In Figure 25, G’ values are presented and show similar plateauing behavior after 100 seconds for all groups tested. Non-oxidized AMA exhibited the highest storage moduli after 5 minutes of crosslinking, followed by the AMA-DA blends, and lastly the pure AMA-DA exhibited the lowest moduli.
Figure 25: Gelation kinetics were assessed by oscillatory time sweeps at 10% radial strain and 1 Hz frequency while pre-cursor solutions were exposed to visible green light (525 nm) and maintained at 37°C. Storage moduli ($G'$) of 6% w/v polymer solutions is shown over a 5 minute (300 s) period.

6.4.4. Hydrogel Patch Fabrication

Thin polymer films were successfully formed using an injection molding technique, followed by subsequent lyophilization (Figure 26A&B). These films contain all photoinitiators required for crosslinking to form a hydrogel, when hydrated and exposed to visible green light (Figure 26C). Due to the blending of AMA and AMA-DA in the fabrication process (as denoted in Table 3), the DOM and DOO varied between each formulation. The DOM and DOO for each patch formulation was calculated and is listed in Table 3.
Figure 26: A&B) Dry sheet of non-crosslinked sealant material after injection molding and lyophilization. This material can be cut to required size, and contains all required initiators for visible green light photo-crosslinking. C) Hydrated and crosslinked adhesive hydrogel on fingertip.

Table 3: Summary of patch formulations tested, including polymer concentrations, blend ratios, degrees of methacrylation (DOM) and oxidation/aldehyde-modification (DOO), average burst pressures achieved prior to failure, and the mode of sealant failure (delamination/adhesive-failure is denoted by D, and material failure/rupture is denoted by M)

<table>
<thead>
<tr>
<th>Conc. (% w/v)</th>
<th>Formulation</th>
<th>Blend Ratio</th>
<th>DOM (%)</th>
<th>DOO (%)</th>
<th>Average Burst Pressure (inH₂O)</th>
<th>Predominant Mode of Failure</th>
</tr>
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<tr>
<td>3</td>
<td>AMA</td>
<td>n/a</td>
<td>58</td>
<td>0</td>
<td>41.0 ± 23.7</td>
<td>D</td>
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<tr>
<td></td>
<td>AMA:AMA-25DA</td>
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<td>48</td>
<td>10</td>
<td>7.7 ± 8.4</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>AMA:AMA-50DA</td>
<td>1:1</td>
<td>47</td>
<td>21</td>
<td>5.3 ± 6.6</td>
<td>M</td>
</tr>
<tr>
<td>4.5</td>
<td>AMA</td>
<td>n/a</td>
<td>58</td>
<td>0</td>
<td>51.8 ± 34.9</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>AMA:AMA-25DA</td>
<td>1:1</td>
<td>48</td>
<td>10</td>
<td>25.3 ± 5.8</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>AMA:AMA-50DA</td>
<td>1:1</td>
<td>47</td>
<td>21</td>
<td>5.9 ± 7.48</td>
<td>M</td>
</tr>
<tr>
<td></td>
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<td>1:2</td>
<td>45</td>
<td>13</td>
<td>20.6 ± 13.8</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>AMA:AMA-50DA</td>
<td>1:2</td>
<td>43</td>
<td>28</td>
<td>12.0 ± 3.9</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>AMA:AMA-25DA</td>
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<td>51</td>
<td>6</td>
<td>65.3 ± 50.3</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>AMA:AMA-50DA</td>
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<td>77.3 ± 53.3</td>
<td>D</td>
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<td>0</td>
<td>142.0 ± 34.5</td>
<td>D</td>
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<tr>
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<td>10</td>
<td>200.8 ± 11.9</td>
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<tr>
<td></td>
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<td>47</td>
<td>21</td>
<td>119.2 ± 21.6</td>
<td>M</td>
</tr>
</tbody>
</table>

6.4.5. Cell viability, apoptosis, and material cytotoxicity

After 24 hours exposure to the hydrogel patch material (not containing DOX), no statistically significant decreases in mesothelial cell viability were observed (Figure
27A), though significant increases in cell viability were witnessed by the cells exposed to AMA and AMA-50DA. Similarly, though no significant increases in cytotoxicity were observed in the cells exposed to sealant materials, significant decreases in cytotoxicity are shown in both AMA and AMA-50DA groups (Figure 27B) which aligns with viability test results. Cell metabolism as indicated by mitochondrial activity is often used as an indicator of cytotoxicity and proliferation using MTT-based assays. MTT is metabolized by the cell mitochondria resulting in the formation of formazan crystals which can subsequently be dissolved and quantified via absorbance detection on a plate reader. Increased formazan concentrations result in heightened absorbance, signifying higher mitochondrial activity, and decreased cytotoxicity. No significant differences in cell apoptosis or mitochondrial activity were found with any test material (Figure 27C & D).
Figure 27: (A) Viability, (B) cytotoxicity, (C) apoptosis and (D) mitochondrial activity as demonstrated human mesothelial cells after 24 h exposure to sealant patch materials as compared to non-treated control. Increases in overall cell viability were shown in AMA and 25% oxidized AMA-DA, simultaneously showing decreased cytotoxicity when compared to control. No significant differences in apoptosis and mitochondrial activity was observed.

6.4.6. Burst pressure properties

Burst pressure testing of alginate-based sealant patches yielded pressures at failure ranging from 5-200 in-H$_2$O as shown in Figure 28. A red dotted-line is shown at 12 inH$_2$O, representing the normal maximum physiological lung pressure, and acts as a threshold which must be achieved for the sealant to be considered for the desired application. Although several 3% and 4.5% w/v formulations achieve average burst pressures above this threshold value, the variation (as shown by standard deviation error
bars) disqualifies many of these formulations as they do not provide any margin of error. The only groups which consistently yielded burst pressures above the threshold with acceptable levels of variation were the 6% w/v formulations, of which the AMA:AMA-25DA 1:1 blend performed significantly better than the other tested 6% w/v formulations with an average burst pressure above 200 in-H₂O.

![Figure 28: Burst pressure at failure is shown for all tested formulations in inH₂O. Physiological lung pressures typically remain below 12 inH₂O, which is denoted with a red dotted-line. Statistical significance is shown between specific groups using an asterisk.](image)

The mechanism of sealant failure was assessed using high-speed videography. Material failure occurs when a rupture is formed through the sealant material, with the sealant maintaining adhesion to the substrate, as indicated in Figure 29A by a small perforation. Adhesive failure, or delamination, occurs when the sealant peels off of the substrate, either partially or completely (as shown in Figure 29B). The predominant mode of failure for each patch formulation is listed in Table 3. Sealants blended with AMA-DA...
were more prone to material failure (rupture), while pure AMA-based sealants failed exclusively through adhesive failure (delamination) due to lack of crosslinking to the substrate. Blends of AMA and AMA-DA at a 1:2 ratio failed predominantly via material failure, though reverted to adhesive failure when blended at an inverse ratio of 2:1.

Figure 29: Graphic representations of both mechanisms of sealant failure. (A) Material failure occurs when a rupture is formed through the sealant material, yet the patch remains adhered to the surrounding substrate. (B) Adhesive failure occurs when the patch delaminates, either partially or completely (as shown above), from the substrate beneath.

6.4.7. Degradation of alginate-based sealant patches

Over a 14-day period, sealant patches made from 6% w/v AMA:AMA-25DA blended at a 1:1 ratio, lost over 50% of their initial dry mass (Figure 30A). Swell ratios also decreased significantly during the 14-day period (Figure 30B), from 2667% to
1902%, which relates to the decrease in water-retaining polysaccharide content. Visual changes in appearance can also be observed, with pitting and surface erosion observed on the hydrogel patch surface (Figure 30C).

![Graphs showing mass and swell ratio decrease over 14 days.](image)

**Figure 30:** The effects of degradation were assessed using 6% w/v sealant patches over a 14 day period at 37°C. (A) Mass decreased by more than 50% over the two-week period. (B) Similarly, swell ratios decreased dramatically over the 14 day period due to the reduction in polysaccharide molecules retaining water. (C) Images of gel patches on day 1 and 14 are presented. After 14 days of degradation, visible pitting/surface-erosion can be observed on the surface of the hydrogel.

### 6.4.8. Doxorubicin encapsulating nanoparticle size evaluation

Doxorubicin encapsulating AMA nanoparticles were successfully synthesized using a water-in-oil emulsion technique, and their size distribution was assessed by
dynamic light scattering. Particles were found to be under 200 nm diameter in general, with the peak size being 150 nm in diameter (see Figure 31).

![Size Distribution by Number](image)

Figure 31: DOX-encapsulating nanoparticle size distribution was assessed using dynamic light scattering in PBS (pH 7.4, 37°C), and is presented in the above histogram by number. The peak size by number is located at 150 nm diameter, with a standard deviation of 19 nm.

6.4.9. Mechanisms of drug release

Drug release profiles for DOX-eluting chemotherapeutic patches and DOX-encapsulated AMA nanoparticles were assessed over a 48 hour time period and presented in Figure 32 in micrograms DOX per milligram particles. During the initial 12 hours, the drug release expressed by the DOX-encapsulated AMA nanoparticles appears to be more rapid, with DOX-eluting sealant patches exhibiting delayed release of the chemotherapeutic drug. After 24 hours, little further drug is released, with no significant difference in the total amount of drug released by any test group after 24 or 48 hours respectively.
Figure 32: Drug release profiles (micrograms DOX per milligram particles) of DOX-loaded patches and nanoparticles over 48 hours at 37°C.

6.4.11. Bioactivity of DOX-eluting chemotherapeutic patch

The bioactivity of DOX-eluting adhesive patches, as compared to DOX-encapsulating nanoparticles and non-loaded patches, was assessed using A549 cells, after 24 hours exposure to the test materials and reported as viability in Figure 33. As can be seen, there was no decrease in viability observed after 24 hours exposure to non-loaded patch materials, whereas a significant decrease in cell viability was demonstrated by both the DOX-eluting patch formulations tested. Although DOX-encapsulating nanoparticles showed a decrease in cell viability, this difference was not significant.
Figure 33: A549 cell viability after 24 h exposure to non-loaded and DOX-loaded sealant patches and nanoparticles. No significant differences in viability were detected between control and non-loaded sealant patches. Viability is however decreased significantly for both DOX-loaded patch formulations tested. DOX-loaded nanoparticles show a slight reduction in viability but is not statistically significant at p=0.05.

6.5. Discussion

In a prior study, our laboratory investigated the sealant performance of AMA and AMA-DA blends applied in liquid form, to assess how varying the level of oxidation influenced the adhesion and burst pressure. This subsequent study further expands our work in functionalized alginate-based sealants by introducing a novel patch application system, which is intended for use as a lung tissue bandage.

Pure AMA is inherently non-adhesive, and thus oxidation of the molecule was performed to take advantage of aldehyde-mediated adhesion to proteins commonly found on the tissue surface. Aldehyde modification of polymers has been shown to improve
adhesion to proteins found on or within biological tissues. Using an oxidation reaction, it is possible to open the uronate residues found on the alginate backbone to form two functional aldehyde groups. There is, however, a balance which must be achieved between the degree of oxidation/aldehyde-modification required to improve sealant adhesion, and the viscosity and mechanical performance of the material. As previously reported, and shown in Figure 24, the oxidation of alginate and AMA results in significant decreases in solution viscosity. This is due to a reduction in molecular weight or chain length of the polysaccharide molecules resulting from the deleterious oxidation reaction. A reduction in DOM can also be observed when increasing the level of oxidation, as quantified from the $^1$H-NMR spectra shown in Figure 23. For these reasons, the DOO was kept below 50% for the materials tested in this study. Additionally, the AMA-DA material was blended with non-oxidized AMA to partially recover the viscosity and mechanical performance of the sealant while still maintaining the important presence of aldehyde groups for adhesion. All the patch formulations/blends tested successfully photo-crosslink in under 5 minutes when hydrated and exposed to visible green light (525 nm) as confirmed by the plateauing storage moduli presented in Figure 25.

Using a custom-made burst pressure testing device, a wide-range of sealant performance was observed between concentrations and formulations with several achieving burst pressures consistently above the physiological threshold of 12 in-H$_2$O (Figure 28). At 3% and 4.5% w/v polymer concentration, variation between tests
remained excessively high, eliminating any margin of safety. Sealant patches fabricated from 6% w/v polymer solutions yielded improved results at reliably high burst pressures over 100 in-H2O on average. AMA, when used alone, was lacking any aldehydemediate adhesion and thus was prone to delamination or adhesive failure (Table 3). In our study, we found that formulations prone to delamination also yielded the highest levels of variation between tests. Sealant patches containing AMA and AMA-DA at 1:1 or 1:2 ratio fail almost exclusively via material failure, or rupture (Table 3), which is believed to be due to improved adhesion to the collagen substrate as well as weakened mechanical performance (Figure 24 & 5).

Alginate-based materials are commonly utilized, and increasingly so, in biological and medical applications and are widely accepted as highly biocompatible. Indeed, mesothelial cells demonstrated no deleterious effects when cultured in the presence of alginate sealant patch materials over 24 hours, as confirmed by viability, cytotoxicity, apoptosis and mitochondrial activity assays (Figure 27). Inversely, when DOX-encapsulating AMA nanoparticles are incorporated into the adhesive patch and cultured alongside A549 human lung cancer cells, a significant decrease in cell viability is observed revealing potential applications for such a drug-eluting tissue adhesive patch. As the nanoparticles are made from the same AMA material as the sealant, the particles are seamlessly crosslinked within the hydrogel sealant. Drug release assays reveal delayed DOX release from the drug-eluting patches as compared to nanoparticles alone (Figure 32). This is because the drug, which has a relatively low solubility in aqueous
solutions, must not only diffuse through the particle but subsequently through the surrounding sealant hydrogel before ultimately diffusing into the surrounding supernatant. This bi-phasic diffusion delays drug release from the drug-eluting patch as compared to the nanoparticles. Interestingly, though the DOX-encapsulating nanoparticles release the drug more rapidly than DOX-eluting patches, they appear less effective in killing cancer cells over a 24 hour period (Figure 33). Due to the small size of these particles, approximately 150 nm in diameter, and prior research performed using similar particles, it is thought that many of the particles within the culture are rapidly internalized by A549 cells. In prior studies, efficacy of DOX-encapsulating particles in clearing cancer cell populations improved over a 5 day period, and it is thought that once internalized the particle and drug may be temporarily trapped within an endosome and not immediately available to act on the cell nuclei and DNA. The pH within an endosome can also vary significantly from that of the cell culture media, also influencing drug release. Decreasing pH can induce protonation of the carboxylate side groups on the alginate backbone, causing hydrogen bonding to occur and an increase in viscosity, ultimately slowing diffusion processes further in internalized nanoparticles.

6.6. Conclusions

Dry sealant patches were successfully designed and fabricated including all necessary reactants for photocrosslinking using visible green light. Methacrylated alginates were found to require moderate aldehyde modification to improve adhesive properties of the hydrogel material. Blending of non-oxidized material with oxidized (aldehyde modified) alginates yields patches with improved burst pressure performance,
and decreased delamination as compared with pure AMA. Drug-eluting adhesive patches also show efficacy when loaded with DOX-encapsulating nanoparticles and cultured in the presence of A549 human lung cancer cells over a 24 hour period, suggesting diversified medical applications.

6.7. Acknowledgements

This work was funded in part by NIH Grants T32 HL076122 (SL Fenn training fellowship), R01 EB020964-01 (Oldinski), The University of Vermont SPARKVT Program, as well as the University of Vermont College of Engineering and Mathematical Sciences.
6.8. References


CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

7.1. Summary of Work and Concluding Remarks

The work performed and presented in this dissertation was primarily motivated by the clinical need for biocompatible and bioadhesive materials with tunable mechanical properties. To accomplish this, I began by developing a novel anhydrous methacrylation chemistry, which can be broadly applied to many polysaccharides including hyaluronan and sodium alginate. This work advances prior research utilizing methacrylated polysaccharides by demonstrating the ability to control the degree of crosslinking through calculated adjustment of reactants, thus modulating the mechanical properties of the resulting hydrogel, making these materials broadly applicable to many tissue engineering and regenerative medical purposes. Additionally, we show the ability to use visible green light for covalent photo-crosslinking of methacrylated polysaccharides through the use of an Eosin Y-based photoinitiator system, and yet still achieve mechanical properties equivalent to or exceeding those achieved using cytotoxic ultraviolet light initiated systems.

Subsequently, we utilize methacrylated alginate to synthesize a novel injectable tissue sealant capable of in situ tissue repair using visible green light initiated photo-crosslinking. In this work, we further functionalized the polysaccharide molecule through an oxidation reaction which opens the uronate residues found on the carbohydrate backbone to form two aldehyde groups per repeat unit. These aldehyde groups were shown to influence the adhesion of our hydrogel material to extracellular matrix proteins
found on tissue surfaces, such as collagen, through imine bonding. By modulating the degree of oxidation, we varied the number of aldehyde groups available for adhesion to proteins. We also found that the oxidation of polysaccharide molecules can reduce molecular chain length and further influence the mechanical properties of the adhesive material. This work builds on the literature by demonstrating the ability to blend oxidized-methacrylated alginates with methacrylated alginates to improve sealant performance as confirmed by burst pressure testing.

Methacrylated- and aldehyde-modified alginates were once again utilized in the design and fabrication of pulmonary sealant patches, introducing a novel mechanism for sealant application. The adhesive polysaccharide-based patches were formed using an injection molding technique, with subsequent lyophilization, to produce dry sheets of polymer material which can be applied to damaged tissues, hydrated and crosslinked to seal perforations and leaks. The performance and adhesion of these novel adhesive patches was assessed using a custom burst pressure testing device, and were shown to vastly exceed the performance of the aforementioned liquid sealant. The sealant materials were found not to be cytotoxic or induce apoptosis in human mesothelial cells, showing promise for the use of such a patch system on pulmonary tissues.

Another aim of this research was to provide a mechanism of controlled intracellular drug delivery in the treatment of disease and/or tissue engineering and regenerative medicine applications. Methacrylated alginate was blended with a model
drug, in this case doxorubicin hydrochloride, and subsequently formed into sub-micron and nano-sized particles through a water-in-oil emulsion technique. The chemotherapeutic drug was encapsulated within the individual particles, and trapped via covalent photo-crosslinking techniques. The resulting particles were shown to effectively deliver bioactive drug, intracellularly, inducing cell death in human lung cancer cells. It is hoped that by taking advantage of cell internalization pathways, improvements in the efficacy of anti-cancer therapies can be made while simultaneously reducing effective dosages and systemic side effects.

By combining the aforementioned doxorubicin encapsulating alginate nanoparticles within my novel adhesive alginate sealant patch, I have additionally shown the ability to effectively deliver chemotherapeutics to human lung cancer cells. These drug-loaded adhesive patches could be applied to many tissues internally, and remain in place, while allowing for local drug release. The impact of this work, although preliminary, could be broad due to the frequent need/want to administer therapeutic drugs in site-specific manner. The benefits to such a method of administration could translate to lower effective doses of drug, and a reduction in systemic side effects.

7.2. Future Directions

There are several directions I foresee this promising and exciting work proceeding in the future. With our in vitro burst pressure model showing sufficient performance, well exceeding physiological pressures, the most obvious next step to be
undertaken is the use of these adhesive hydrogels in vivo to seal lung defects. Indeed, this work has already been initiated in our laboratory in collaboration with researchers at the Larner College of Medicine at the University of Vermont. Having already performed several proof-of-concept tests using excised murine lungs in 2014, we have since begun preparation for in vivo testing of our novel adhesive sealant hydrogels. We plan to use the Scireq® FlexiVent™ system to monitor lung function and respiratory mechanics in healthy mouse lungs, then repeat measurements after perforating the lung with a small incision, and again after sealing the wound with our novel alginate sealant hydrogels. Relevant IACUC, surgical and experimental protocols have been developed, validated and approved for our future animal studies. We hope that these future experiments will prove to be successful, so that we may continue our ongoing technology commercialization efforts.

Additionally, having shown the ability to successfully deliver therapeutic molecules using the adhesive alginate hydrogels, we hope to begin an investigation into the delivery of growth factors to aid in the healing of damaged and diseased lung tissues. Recently we have shown that intracellular delivery of fibroblast growth factor-2 (FGF-2) can selectively induce cell death in A549 lung cancer cells, yet appears to have no effect on normal lung epithelial cells. It has also been shown that FGF-2 can aid in tissue and wound healing. Indeed, this very work was the subject of a recently issued NIH R01 awarded to our laboratory and will be a primary focus of the Oldinski laboratory over the upcoming years.


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Kesti, M.; Muller, M.; Becher, J.; Schnabelrauch, M.; D'Este, M.; Eglin, D.; Zenobi-Wong, M., A Versatile Bioink for Three-Dimensional Printing of Cellular Scaffolds


APPENDIX I: STANDARD OPERATING PROCEDURE: ALGINATE
METHACRYLATION WITH METHACRYLIC ANHYDRIE IN WATER
Author: Spencer L. Fenn, Rachael A. Oldinski, Patrick Charron

MATERIALS:
- Alginate (Alg) (FMC BioPolymer)
- Methacrylic anhydride (MA) (Sigma-Aldrich)
- 2N sodium hydroxide (NaOH) (Sigma-Aldrich)

METHODS:

a) Add 1 gram Alg to 100 mL DI water to form a 1% (w/v) solution. (Use 0.25g in 25 mL for quarter batch)
b) Add 8.46 mL MA (2.11 mL for quarter batch) to the Alg solution to form a 20-fold molar excess*. Adjust the pH of the solution to 8.5 with 2N NaOH. Stir the solution overnight (>12 h) at room temperature in a round bottom flask. Periodically adjust the pH with 2N NaOH as necessary.
c) Adjust the pH of the final solution to pH 7.0 and dialyze with DI water for 3 days, changing solution every 6-8 hours.
d) Freeze the solution in the -80 °C freezer in a lyophilizer flask and lyophilize.

*This method assumes 1 mol MA = 1 mol Alg (repeat unit)
Alg MW: 352.24 g/mol

REFERENCE:
APPENDIX II: STANDARD OPERATING PROCEDURE: AA-CTA SYNTHESIS
Author: Spencer L. Fenn

MATERIALS:
- Alginate (FMC BioPolymer)
- Hexadecyltrimethylammonium bromide (CTAB - Sigma Aldrich)
- Deionised water

METHODS:

1) Make 1% w/v polymer solution in deionized water and set aside for later use.

2) In a separate vessel, make an equal volume of 4% w/v CTAB in water solution, may need to heat slightly until solution goes clear.

3) Mix solutions.

4) After 24 hours centrifuge or filter off supernatant (or simply put solution in large beaker, cover and allow to sit in fume hood, polymer will settle and supernatant can be removed easier). Rinse several times with 70% ethanol in water.

5) Freeze at -80°C and lyophilize.

REFERENCE:

APPENDIX III: STANDARD OPERATING PROCEDURE: AA-DTA SYNTHESIS  
Author: Spencer L. Fenn

MATERIALS:
- Alginate (FMC BioPolymer)
- Dodecyltrimethylammonium bromide (DTAB)
- Deionised water

METHODS:

6) Make 1% w/v polymer solution in deionized water and set aside for later use.

7) In a separate vessel, make an equal volume of 2-3% w/v DTAB in water solution, may need to heat slightly until solution goes clear.

8) Gradually mix solutions together. Precipitate should immediately begin to form.

9) After 2-4 hours centrifuge or filter off supernatant (or simply put solution in large beaker, cover and allow to sit in fume hood, polymer will settle and supernatant can be removed easier). Rinse several times with DI water.

10) Freeze at -80°C and lyophilize.

REFERENCE:

APPENDIX IV: STANDARD OPERATING PROCEDURE: METHACRYLYATION OF ALGINATE WITH METHACRYLIC ANHYDRIDE IN DMSO
Author: Spencer L. Fenn, Patrick N. Charron

MATERIALS:
- AA-DTA (See AA-DTA synthesis SOP)
- Anhydrous dimethyl sulfoxide (DMSO) (*Sigma-Aldrich*)
- 4-Dimethylaminopyridine (DMAP: MW=122.17g/mol) (*Sigma Aldrich*)
- Methacrylic anhydride (MA: MW=154.16g/mol; density=1.035g/mL) (*Sigma-Aldrich*)
- HCl Solution
- Sodium Phosphate Dibasic (Fisher)

METHODS:

3) Add 25 mL DMSO to 0.25 g AA-DTA (See AA-DTA synthesis SOP) under nitrogen gas flow in a 250 mL round bottom flask to make a 1% AA-DMSO solution.
   e) Heat may need to be applied via mineral oil bath up to 100°C until in solution.
   f) Allow DMSO solution to cool to room temperature before adding DMAP and MA

2) Add DMAP using 1:2 molar ratio, DMAP:AA-DTA
   a) $0.25g \cdot \frac{122.17g/mol}{580.682g/mol} \cdot \frac{1}{2} = 0.0263g DMAP$

3) Add enough methacrylic anhydride to equal two times the molar requirements for 100% modification (0.464 mL) under nitrogen atmosphere and allow to stir overnight.
   a) $0.25g \cdot \frac{154.16g/mol}{580.682g/mol} \cdot 8 = 0.1327g \cdot \frac{1mL}{1.035g} = 0.128mL$
   b) The multiplication factor of 8 allows for 100% excess to insure 100% modification. Alter for lower degrees of modification

4) Add HCl to molar equivalent of DMAP used.

5) Dialyze against DI water for 3 days to remove DMSO and excess MA and DMAP, dialyze against sodium phosphate dibasic until clear, and then several days in DI water to remove excess sodium ions.

6) Freeze at -80°C and lyophilize.
7) See chemistry in schematic form on following page.
Appendix IV Continued: Anhydrous Methacrylation Chemistry Schematic
APPENDIX V: STANDARD OPERATING PROCEDURE: HYALURONAN METHACRYLYATION IN WATER WITH GLYCIDYL METHACRYLATE

Author: Spencer L. Fenn, Rachael A. Oldinski

MATERIALS:
- Hyaluronan (HA) (*Lifecore Biomedical*)
- Phosphate buffered saline (PBS, pH 7.4) (*Amresco*)
- Dimethylformamide (DMF) (*Sigma-Aldrich D119*)
- Glycidyl methacrylate (GMA) (*Sigma-Aldrich 779342*)
- Triethylamine (TEA) (*Sigma-Aldrich T0886*)
- Ethanol or Acetone

METHODS:

11) Add 0.25 g HA to 50 mL PBS solution and 16.75 mL DMF.
12) Add 3.325 g GMA (3.2 mL) and 1.675 g of TEA (2.3 mL) to the HA solution. Stir at room temperature for 10 days.
13) The reaction solution was precipitated with 180-200 mL ethanol or a 20-fold volume excess of acetone.
14) The reaction solution was filtered (or centrifuged), and dialyzed against DI water for 3 days, exchanging water every 6-8 hours.
15) The solution was then frozen at -80 °C and lyophilized. Final mass taken for yield calculations.

REFERENCE:
APPENDIX VI: STANDARD OPERATING PROCEDURE: UV PHOTO-CROSSLINKING OF METHACRYLATED BIO-POLYMERS
Author: Spencer L. Fenn

MATERIALS:
- Methacrylated Hyaluronan (HA-MA) or Alginate (AA-MA)
- Irgacure D2959 (Ciba), or other photoinitiator.
- De-ionized Water

METHODS:

NOTE: The majority of this protocol should be performed with minimal light exposure to prevent premature crosslinking, or deactivation of photoinitiator.

16) A 0.05% (w/v) solution of Irgacure D2959 in De-ionized water was prepared with minimal light exposure due to photosensitivity of the photoinitiator. As the photoinitiator is not readily soluble in water at room temperature, sonication may be used to facilitate this process (15 min).

17) A 2-4% (w/v) solution of HA-MA/AA-MA in the aforementioned solution was mixed until fully in solution.

18) Polymer solution was injected into required mold ensuring UV light was able to pass through solution fully, ie, using glass.

19) Using UV lamp, the mold and polymer solution were exposed to UV light for designated time points (i.e. 1, 5, 10, 15 min) which may alter degree of crosslinking.

REFERENCE:
APPENDIX VII: STANDARD OPERATING PROCEDURE: EOSIN Y CROSSLINKING HA-MA OR AA-MA

Author: Spencer L. Fenn

MATERIALS:
- Methacrylated Alginate or Hyaluronan (previously synthesized in lab)
- Eosin Y (Acros Organics MW: 691.85 g/mol)
- 1-vinyl-2-pyrrolidinone (Sigma, V3409, MW: 111.14 g/mol, ρ: 1.04 g/mL)
- Triethanolamine (Sigma, 90279, MW: 149.19 g/mol, ρ: 1.124 g/mL)

METHODS:

CAUTION: All steps prior to crosslinking should be performed in a darkened room with no visible green light sources. All solutions should be stored out of light, preferably in refrigeration.

1. Prepare stock crosslinking solutions (Nettles, Vail, Morgan, & Grinstaff, 2004):
   a. 2 mL methacrylated polymer solution in DI water (typically 1-4%).
   b. 0.5% eosin Y (photosensitizer, EY) in 1-vinyl-2-pyrrolidinone (catalyst, 1VP)
   c. 5M triethanolamine (initiator, TEOA) in DI water.

2. Add 5 µL of the EY/1VP solution to the 2 mL polymer solution and mix.

3. Add 50 µL of the TEOA solution to the polymer solution and mix thoroughly

4. Fill mold with polymer crosslinking solution.
   a. Molds should be fully translucent to allow for full crosslinking of gels.

5. Expose mold to visible green light of wavelength 510 nm for 2-6 minutes to crosslink hydrogel (Bahney, Lujan, & Hsu, 2011).

REFERENCE:


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APPENDIX VIII: STANDARD OPERATING PROCEDURE: IN VITRO CYTOTOXICITY ASSAY, MTT BASED
Author: Spencer L. Fenn (Modified from Sigma Protocol)

MATERIALS:
- 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT (Sigma: M5655)
- Dimethyl Sulfoxide (Bioreagent Grade)
- Cell Culture Media or Salt Solution WITHOUT Phenol Red (Phenol red has absorbance at 570 nm, contributing to increased background, and decreased test sensitivity)

METHODS:

Note: MTT is light sensitive, it is best to keep lights dimmed as much as possible. Do not leave solutions exposed to light for extended periods of time.

1) Prepare MTT Solution. Refrigerate/freeze until use.
   a. Add MTT to PBS or balanced salt solution (without phenol red and serum) at 5 mg/mL concentration. Mix until dissolved. Usually no more than 3 mL will be required.
   b. Excess solution can be stored frozen for up to 6 months.

2) Seed well plate with cells, incubate allowing cells to adhere and expand for at least 24 hours (or to desired confluency). Make sure to seed wells for positive control. Also leave several wells empty (with just media) for “blank” adjustment if desired.
   a. 48 well plates are best for MTT, avoid using anything with less surface area.
   b. Transwell plates can also be used when testing cytotoxicity of solid materials/scaffolds.
   c. If possible, use media without phenol red. If unavailable, media with phenol red can be utilized during initial cell expansion and culture with test material, but remove and replace with a balanced salt solution (PBS, etc) without phenol red when you add MTT solution. Expect decreased sensitivity in this case.

3) Add test solution/material and allow to culture in incubator for 24 hours.

4) Add previously prepared MTT solution at 10% of the media volume to each well, including controls/blanks.
   a. Incubate for 2-4 hours. Usually 2 hours is sufficient, you should see dark purple formazan crystals appear as the MTT is metabolized by cells. If your cell population is lower or their metabolic rate is slower, you may need to culture for up to 4 hours.

5) Carefully aspirate all media/MTT solution from each well.
a. Try not to remove any purple formazan crystals from the wells as this will increase your variation between wells.

b. If concerned about accidentally removing crystals during aspiration, you can leave a small (known) amount of MTT/Media/PBS in each well, but this will increase background and reduce sensitivity.

6) Add DMSO to each well, 150-200 uL typically if using a 48 well plate (consider if you are making duplicates or triplicates. You can add up to original media volume if needed). The DMSO will help dissolve the formazan crystals for detection on the plate reader.
   a. Using an incubator shaker (preferred, as doesn’t create bubbles) or gently pipetting up and down can aid in dissolving the crystals.
   b. The solutions should turn varying degrees of purple. The deeper the purple, the less cytotoxicity experienced. Your blank wells should remain clear.

7) Take aliquots (typically 50-100 uL, just be consistent throughout) from each test well and place in a 96 well plate (untreated).
   a. Usually duplicates/triplicates will be taken from each test well. In other words, if you started with an n=3, you should end up with 6 or 9 wells for plate reading.

8) Read absorbance at 540 nm and 690 nm (background) using plate reader.
   a. Subtract background at 690 nm from the absorbance at 540 nm to obtain raw absorbance of each well.
   b. Additionally, you can perform Blank Adjustment using your blank wells if desired (not always necessary, but can increase sensitivity)
   c. Data can be normalized to your positive control wells.

References:

APPENDIX IX: STANDARD OPERATING PROCEDURE: PARTIAL OXIDATION OF ALGINATE
Author: Patrick Charron, Spencer L. Fenn

Materials:
- Alginate (FMC BioPolymer)
- Sodium Periodate, NaIO₄ (Sigma Aldrich)
- Deionised water

Methods:
1) Prepare 1-2% w/v polymer solution in deionized water
2) In separate vessel, prepare 2-5% w/v NaIO₄ solution in deionized water for desired degree of oxidation
   a. Theoretical DOO equivalent to Alginate:NaIO₄ ratio
      e.g. 10 DOO → 1 g Alginate:0.1 g NaIO₄
3) Mix NaIO₄ solution into alginate solution and allow to stir for 24 hrs at room temperature in dark space
4) Dialyze against deionized water for 3 days
5) Freeze at -80°C and lyophilize

Note: Oxidation reaction can be stopped by addition of molar equivalent of ethylene glycol to NaIO₄

Reference:
APPENDIX X: STANDARD OPERATING PROCEDURE: PREPARATION OF CROSSLINKABLE AA-MA/HA-MA FILM PATCHES VIA SPIN COATING

Author: Spencer L. Fenn

MATERIALS:
- Spin Coater
- Dessicator/Lyophilizer
- Razor Blade
- Methacrylated Alginate or Hyaluronan
- Photoinitiators
  - Irgacure D2959
  - Eosin Y (photosensitizer, Acros Organics MW: 691.85 g/mol)
  - 1-vinyl-2-pyrrolidinone (catalyst, Sigma, V3409, MW: 111.14 g/mol, ρ: 1.04 g/mL)
  - Triethanolamine (initiator, Sigma, 90279, MW: 149.19 g/mol, ρ: 1.124 g/mL)

METHODS:

9) Prepare polymer solutions (higher concentration=denser/thicker films) with photoinitiators. Caution: Take care not to expose solutions to activating wavelengths, i.e. UV and Green Light (510nm). Please note that even fluorescent lighting will trigger gelation of eosin Y based solutions. It may be best to use mixing syringes to get fully homogenous solutions.
   a.) 0.05% Irgacure D2959 in water
   OR
   b.) 0.00125% (w/v) eosin Y (photosensitizer), 125mM triethanolamine (TEOA, initiator), and 19mM 1-vinyl-2-pyrrolidinone (1VP, catalyst).

10) Using spin coater, coat a glass slide with the polymer solution. Film thickness can be adjusted by altering speed and length of time. Suggested settings : 1500 rpm for 5-10 seconds.

11) Take coated slide and lay flat in vacuum oven/dessicator (in complete darkness, using vacuum if possible and low heat) until dry OR lay flat in -80C freezer and subsequently lyophilize, again ensuring to keep sample in complete darkness to prevent premature gelation. (Possible pitfall with lyophilization: when the polymer solution freezes, ice crystals form throughout and thus the final lyophilized film is highly porous. If this becomes an issue, rely on vacuum desiccation.)

12) Once dry, films can be removed from slide using razor blade.
13) To use films, simply cut to size, and stick to wet tissue. Allow film to hydrate by absorbing surrounding water, and then expose the patch to its activation light source, i.e. UV or green light for 10 min.

**REFERENCE:**
Spencer L. Fenn  
4th-year Ph.D. Candidate in Bioengineering, University of Vermont  

Title: Intracellular Delivery of Bioactive Chemotherapeutic Using Dual-Crosslinked Alginate Sub-Microparticles  
Presented to the Vermont Lung Center  
November 10th, 2015

In follow-up to the talk titled “Intracellular Delivery of Bioactive Chemotherapeutic Using Dual-Crosslinked Alginate Sub-Microparticles” presented to the Vermont Lung Center on November 10th, this report is intended to summarize the multidisciplinary aspects of this work as discussed during the subsequent Interdisciplinary Research Workshop (IRW). Present at the IRW were UVM faculty members, post-doctoral fellows and student trainees specializing in materials science, engineering, bioengineering, pulmonary medicine and cellular & molecular biology.

In this presentation, the synthesis of novel dual-crosslinked sub-microparticles was described as well as their application for use as intracellular chemotherapeutic delivery vehicles in lung cancer cells. This work bridges several fields including chemistry and materials science, engineering, pharmacology, cell biology, and pulmonary medicine. The primary hypotheses of the presented work were, 1) chemotherapeutic release profiles can be modulated or extended through secondary crosslinking of drug-encapsulated alginate sub-microparticles, and 2) intracellular delivery of bioactive chemotherapeutic to lung cancer cells will result in increased cell death and cytotoxicity at lower drug dosages as compared to extracellularly delivered chemotherapeutic dosed in cell growth media. The main topics of discussion for this talk included an introduction to the material used, sodium alginate, as well as the chemical
modification required to allow for covalent crosslinking; the fabrication of doxorubicin
encapsulated single and dual-crosslinked alginate sub-microparticles; characterization of
these particles by size, shape, surface charge and drug-release profiles; and lastly an
investigation into particle internalization or cell uptake, and the cytotoxicity/bioactivity
on human lung cancer cells (A549, epithelial carcinoma).

The first disciplines discussed during this presentation were that of materials
science and chemistry. Alginate is a long-chain anionic polysaccharide derived from
brown algae made up of guluronate and mannuronate sugar residues. The primary
motivations for utilizing this material are its inherent biocompatibility, chemically
modifiable hydroxyl and carboxyl side-groups (for material functionalization), as well is
its sustainable source. Alginate is also capable of reversible ionic cross-linking between
guluronate residues in adjacent polysaccharide chains in the presence of calcium ions.
Additionally, alginate can be covalently crosslinked by means of chemical
methacrylation of hydroxyl groups on the molecule repeat unit, forming methacrylate
alginate (Alg-MA). Methacrylation allows for free-radical initiated crosslinking between
polysaccharide chains, often using ultraviolet or visible light sensitive photoinitiators.
During this presentation, a brief overview of a novel anhydrous methacrylation chemistry
was described. The degree of methacrylation influences the degree of crosslinking with
more methacrylate groups equating to a tighter network structure. These two means of
crosslinking, both ionic and covalent, can be utilized alone or in combination to form
single or dual-linked hydrogels.
Following the discussion of chemistry, the engineering and synthesis of doxorubicin encapsulating, single and dual-crosslinked sub-microspheres and the subsequent assessment of physical properties were discussed. Aqueous solutions of Alg-MA and doxorubicin were blended, and added dropwise into oil/surfactant stirring rotationally at high speed forming a water-in-oil emulsion. As the solution stirs, non-crosslinked droplets of Alg-MA are broken into increasingly smaller and smaller droplets. Photocrosslinking is then performed by exposing the emulsion to visible or UV light for ten minutes, covalently crosslinking the droplets to form sub-micron size doxorubicin encapsulating particles. Some particles were then dual-crosslinked by the addition of an aqueous calcium chloride solution, forming a tighter network structure with additional ionic bonds between adjacent guluronate sugar residues. The particle size was quantified using dynamic light scattering, and shape by scanning electron microscopy. The particles were found to range from approximately 100-600 nanometers, a size range which is typically appropriate for cellular internalization. As cellular uptake is also influenced by particle surface charge, with more neutral particles providing enhanced internalization, the zeta-potential of the particles was also quantified with all particles found to be negative in charge ranging from -20 to -37 mV.

A discussion of drug release was then provided, showing that dual-crosslinked particles do indeed exhibit reduced and extended drug release as compared to single crosslinked particles, confirming the first hypothesis. The release profiles for the dual-crosslinked particles were found to release significantly less drug overall, which led us to believe that there may be reduced encapsulation efficiency in dual-crosslinking particles
due to the addition of aqueous calcium chloride solution to the emulsion which is capable of washing away drug. Several suggestions were provided by audience members to enhance the quantification and analysis of the data, such as integration of the release curves to determine encapsulation efficiency and show differences between each crosslinking type as well as developing models to elucidate the mechanisms dictating release from the hydrogel material. It was agreed that further investigation into the already recorded data could prove both interesting and valuable.

Internalization of the particles was then discussed in depth, with the audience asking many questions relating to verification of cellular uptake. At the time of the presentation, solely flow cytometry had been used to show increased fluorescent intensity of A549 cells cultured in the presence of fluorochrome labelled particles. Several audience members hypothesized that the particles could simply be aggregating just outside of the cell membrane, increasing the fluorescence reading. It was suggested that further analysis would be required to confirm cellular internalization, such as confocal microscopy using multiple stains to localize particles within the cell membrane, or using endosomal markers to show particles within endosomes. Since that time, we have investigated A549 particle internalization further and performed confocal microscopy at various time-points showing the gradual internalization process underwent by the particles. We now feel reasonably confident that the particles are indeed readily internalized by this cell line. Also discussed at the presentation and subsequent IRW was how surface charge relates to internalization. Upon investigation of the percentage of cells tested positive for particle uptake, it was found that the particles exhibiting the most
neutral charge (UV crosslinked) were far more readily internalized by the A549 cells, with over 90% testing positive as compared to only 60% testing positive for visible-light crosslinked particles. Initially it was though these differences could have been attributed to size of particles, but after consideration and discussion it was established that particle surface charge had a greater influence on cellular uptake than originally thought. Also suggested during the IRW was to investigate how particle uptake occurs and what happens to the particle upon internalization.

Lastly, the bioactivity/toxicity of the doxorubicin encapsulating particles over a 5 day period was described as compared to extracellularly delivered drug in media at various concentrations. The data presented showed decreased metabolic activity as particle/drug dosage increase as expected. When compared, day one results showed enhanced cell cytotoxicity using alginate particles despite theoretically delivering significantly reduced dosages of drug overall. It was suggested that the reduced dosage may still provide increased efficacy when delivered intracellularly, directly within the cell. Although not all of the particles performed as well over the longer time periods of 3 and 5 days, it is thought that these differences are more likely related to variances in initial drug encapsulation and reduced drug release as time progresses. It was also proposed that there may be fewer particles available for internalization at later time points. As advised by attendees previously, elucidation and model development of the drug release mechanisms may provide further insight into why efficacy decreases as time progresses and varies between the single and dual-crosslinked particles.
In conclusion, the discussion during my presentation and in the following
Interdisciplinary Research Workshop provided me with invaluable insight from experts
within and outside my immediate research field. Many questions raised were not things
that I, nor my supervisor, had initially considered and thus an outsider’s perspective was
extremely appreciated and well received. The encouragement to look deeper and further
analyze my data will also help to improve my overall understanding of my work and
enhance the conclusions drawn. I found the workshop particularly beneficial as it allowed
me to think more critically to develop and enhance my work prior to submission of a
manuscript and public presentation to a large society conference later this year.
APPENDIX XII: MET-5A HOMO SAPIENS MESOTHELIUM CELL CULTURE
Author: Adapted from ATCC Protocol by Spencer L. Fenn

MATERIALS:

Complete Growth Medium
- Medium 199 (Sigma Catalog No. M4530)
- 400 nM Hydrocortisone (Sigma Catalog No. H0888, MW: 362.46)
- 870 nM Bovine Insulin (Sigma Catalog No. I1882 or I6634, MW: 5733.49)
- 3.87 µg/L Selenious acid (Aldrich Catalog No. 211176)
- Trace elements B 1000X solution at 1000 dilution. (Mediatech/Corning Catalog No. 99-175)
- 20 mM HEPES (Life Technologies Catalog No. 15630)
- 10% Fetal Bovine Serum (ATCC Catalog No. 30-2020, not heat-inactivated)
- *3.3 nM Epidermal Growth Factor (EGF) (Corning Catalog No. 354001, MW: 6 kDa, 6000 g/mole) *ADD AFTER FILTERING

Note: All values listed above are final concentration. All components must be filter sterilized, except the EGF, which may be retained in filter membrane and thus can be added separately.

Additional Materials Required
- 0.25% (w/v) Trypsin-0.53 mM EDTA solution
- Dimethyl Sulfoxide (Sigma Cat. No. D2650)

METHODS:

Subculturing
Volumes are given for a T75 flask.
1) Remove and discard culture medium.
2) Rinse the cell layer with 1-2 mL Trypsin-EDTA solution to remove all traces of serum containing trypsin inhibitor.
3) Add 3.0 mL of Trypsin-EDTA solution to flask and incubate for 5-10 minutes at 37°C, or until cells release from flask and are freely floating. Do not tap or shake to release cells as this may cause aggregation to occur.
4) Add 6.0 to 8.0 mL of complete growth medium and mix by gently pipetting.
5) Transfer cell solution to centrifuge tube and spin, 125 x g for 5 to 10 minutes. Discarding supernatant, resuspend cells in fresh growth medium. Add appropriate aliquots of cell suspension to new culture vessels.
6) Incubate cultures at 37°C.

Subcultivation Ratio: A subcultivation ratio of 1:2 to 1:4 is recommended

Medium Renewal: Every 2 to 3 days
Note: Heavy floating debris is normal to observe, as are floating but viable cells. Please retain floating cells (using centrifugation) and return to flask for the first week after thawing cells, to allow for recovery from cryopreservation. The floating cells will attach when the culture has completely recovered from cryopreservation.

Cryopreservation

Freeze medium: Complete growth medium supplemented with 5% (v/v) DMSO

Storage temperature: liquid nitrogen vapor phase

REFERENCES:

2. https://www.atcc.org/products/all/CRL-9444.aspx#culturemethod
Research Plan

Injury to the connective tissue that lines the lung, the pleura, or to the lung itself can occur from many causes including trauma, as well as underlying lung diseases or lung cancers. Bronchopleural fistulas, malignant pleural effusions, and traumatic or ventilator-induced pleural injuries are a continuing source of morbidity, mortality, and increased health care expenditures. There are currently only limited means of patching significant injuries to stop the air or fluid leak and allow appropriate healing to occur. New approaches are desperately needed. I have devised a new technique based on methacrylated-alginate (Alg-MA) hydrogels delivered in an easy-to-apply patch formulation that can both seal the affected tissue, as well as deliver water-soluble drugs such as fibroblast growth factor 2 (FGF-2) to stimulate repair within the pleural lining.

The proposed pleural sealant patch, is fabricated through a process of photolithography using an oxidized form of methacrylated-alginate, and will be delivered in a ready to use dry form, for easy storage and application. Alginate, a natural polysaccharide derived from seaweed, is an ideal material for this application, as it is easily modified to add functionality such as crosslinking to form a hydrogel (through methacrylation) or increased adhesion to the tissue (through oxidation). The dried patch can simply be cut to desired size, applied to moist tissue, and exposed to visible light to crosslink and adhere the patch to the pleural surface. Promising preliminary results show that the Alg-MA patch is capable of withstanding pressures of 20 cm H\textsubscript{2}O after being applied to a punctured mouse lung. The proposed project focuses on the optimization and efficacy testing of the pleural sealant patch to both seal leaks and heal tissue, using both mechanical testing, as well as ex vivo, and in vivo studies.

Specific Aim 1: Determine how the physical properties of Alg-MA hydrogels impact their capacity to seal a pleural leak. Alginate will be chemically oxidized to encourage tissue adhesion. We will examine the effect of oxidation and degree of methacrylation on physical properties including burst pressure, adhesive strength, and cohesive strength. We will verify adhesion and seal formation immediately following injury using excised mouse lungs in an ex vivo lung pleural wound model and lung volume measurements.

Specific Aim 2: Determine how Alg-MA hydrogel properties affect their capacity for dynamic mechanical stability and controlled release of drugs. Drug release rates are controllable via the degree of crosslinking and encapsulation technique. We will develop hydrogel blends with methacrylated-hyaluronan to optimize degradation, which further controls release rate. To stimulate wound healing, FGF-2, known to stimulate lung tissue wound healing, will be encapsulated within microspheres to protect the drug. We expect the degradation rate and rate of drug release to depend on molecular weight, degree of modification, and polymer constituents. We will investigate how physical (mass loss, swell ratio) and mechanical properties (dynamic elasticity), and drug release rates change over time with different formulations.

Specific Aim 3: Assess the ability of drug-eluting Alg-MA hydrogels to repair damaged pleural tissue. As proof of concept, we will determine the ability of the optimized Alg-MA hydrogels with or without FGF-2-containing microspheres to repair pleural leaks in an in vivo rat model of lung collapse. Relevant endpoints include durability of repair, maintenance of lung volume, ex vivo burst pressure, and histological images.
Vermont Lung Center NIH T32 Multidisciplinary Training in Lung Biology  
Predoctoral Trainee: Spencer L. Fenn  
Mentor: Rachael A. Oldinski

Mr. Fenn is a fourth year predoctoral fellow in the cross-disciplinary bioengineering program working under the advisement of Dr. Rachael A. Oldinski and has been training on the Vermont Lung Center grant since January 2015.

**Project Summary:**

The primary objective of the Oldinski lab is to develop novel biocompatible materials for use in tissue engineering/regeneration and drug delivery applications. This multidisciplinary work focuses primarily on the engineering and chemical manipulation of polymeric materials to achieve varied physical and mechanical properties to suit diverse medical applications. The work of Mr. Fenn is dedicated to the use of polysaccharide-based hydrogels as a surgical sealant, 3-dimensional tissue scaffold, and also as intracellular drug delivery vehicles.

Alginate, a biocompatible polysaccharide sustainably derived from brown algae, and hyaluronan, a similar molecule found ubiquitously throughout the human body, serve as the base materials for Mr. Fenn’s work. Chemical modifications such as methacrylation are performed to allow for covalent and physical crosslinking between adjacent polysaccharide chains forming hydrogels which can be used to seal damaged tissue, act as a scaffold to encourage tissue growth, or encapsulate drug molecules for therapeutic release.
During the past year of training under the T32, Mr. Fenn has been highly involved in the development of a novel alginate-based tissue sealing bandage which is intended to seal punctured lung tissue, restoring lung function after collapse. The primary objective of this work is to improve upon the current standard of care for lung collapse which includes insertion of a chest tube, suturing and/or intentional chemical/mechanical irritation of the surrounding tissues to induce inflammation (pleurodesis). The alginate-based bandage has been shown to successfully adhere and seal excised and punctured mouse and rat lungs, restoring lung pressures to physiological norms. As a means of higher throughput analysis of modified bandages, a burst pressure device was developed and utilized to assess pressures required to induce failure of the bandages and the mechanisms of failure (delamination, or material failure). This data was then utilized to inform further chemical modifications of the alginate material to increase adhesion and elasticity, improving performance on lung tissues.

Mr. Fenn was also involved in the development of a novel shear-thinning, and thermo-responsive alginate-based hydrogel for injectable tissue engineering applications. This material has the ability to flow like a liquid at room temperatures and when shear forces are applied, allowing for easy application via a syringe, but quickly crosslinks to form a solid gel when exposed to temperatures above 34°C (e.g. body temperature). The implications of these properties are vast, with potential applications in tissue engineering and drug delivery.

Methacrylated alginate was also utilized my Mr. Fenn to develop dual-crosslinked sub-microparticles for intracellular chemotherapeutic delivery. Previous work
in the Oldinski lab has shown that many cell types readily internalize alginate sub-
microparticles via the endocytosis pathway, and subsequently they have shown
successful intracellular delivery of growth factors to induce stem cell differentiation. Mr.
Fenn utilized two crosslinking techniques to prolong the release profile of a
chemotherapeutic drug (doxorubicin) by tightening the polymer network structure
(reducing diffusive release), allowing sufficient time for human lung epithelial carcinoma
cells (A549) to internalize the particles (confirmed by flow cytometry and microscopy).
His work shows that lower doses of chemotherapeutic drug can be utilized with increased
efficacy when delivered intracellularly as compared to drug dosed in cell culture media.
Further work focused on the use of these particles to treat cancer resistant cell lines, as
well as inducing preferential internalization by cancer cells is currently being investigated
within the Oldinski Lab.

Publications:

Alginate Sub-Microspheres for Intracellular Chemotherapeutic Delivery." ACS
Applied Materials & Interfaces. (Accepted, 2016))

Fenn SL, Charron PN, Oldinski RA. “Mechanical Properties and Failure Analysis of
Visible Light Crosslinked Alginate-Based Tissue Sealants.” Journal of the Mechanical
Behavior of Biomedical Materials. (Accepted, 2015)

Miao T, Fenn SL, Charron PN, Oldinski RA. "Self-Healing and Thermo-Responsive
Dual-Crosslinked Alginate Hydrogels based on Supramolecular Inclusion
Complexes." Biomacromolecules. (Accepted, 2015)

Fenn SL, Oldinski RA. "Visible light crosslinking of methacrylated hyaluronan
hydrogels for injectable tissue repair." Journal of Biomedical Materials Research B
Applied Biomaterials. (Accepted, 2015).

Wagner, DE, Fenn, SL, et al. "Design and Synthesis of an Artificial Pulmonary Pleura
Accepted Abstract Submissions in 2015:


Other Activities:

Mr. Fenn is an active member of several academic societies including Materials Research Society (MRS), Society for Biomaterials (SFB), and Biomedical Engineering Society (BMES) and has also participated in the Summer Biomechanics Bioengineering and Biotransport Conference (SB3C) in 2015. Mr. Fenn is current President of the University of Vermont Chapter of the Materials Research Society, as well as Vice-Chairman of the Board of Directors for a local AIDS Service Organization (ASO). Additionally, Mr. Fenn is currently enrolled in the University of Vermont Graduate Teaching Program