

AWARD NUMBER: W81XWH-15-1-0107

TITLE: Development of a Novel Alginate-Based Pleural Sealant

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REPORT DATE: July 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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# REPORT DOCUMENTATION PAGE

*Form Approved*  
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<b>1. REPORT DATE</b> July 2016			<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED</b> 15 Jun 2015 - 14 Jun 2016	
<b>4. TITLE AND SUBTITLE</b>  Development of a Novel Alginate-Based Pleural Sealant					<b>5a. CONTRACT NUMBER</b>	
					<b>5b. GRANT NUMBER</b> W81XWH-15-1-0107	
					<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Daniel J. Weiss MD PhD  E-Mail:					<b>5d. PROJECT NUMBER</b>	
					<b>5e. TASK NUMBER</b>	
					<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of Vermont and State Agricultural College  Burlington, VT 05405					<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012					<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
					<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b>  Approved for Public Release; Distribution Unlimited						
<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT</b>  A variety of lung diseases such as emphysema, infections, and lung cancers as well as lung injury from trauma, including battlefield trauma, and complications of respirator life support of critically ill patients in intensive care units can result in lung collapse that can be immediately life-threatening or result in chronic leaking of air or fluid out of the lung. These remain challenging medical problems for which few good options are currently available and result in significant morbidity, mortality, hospital stays, health care costs, and other complications. New options are thus desperately needed. We are developing a novel approach to provide an easy-to-apply lung sealant which can repair lung leaks. This initially involved use of a chemically modified form of alginate, a naturally occurring seaweed derivative, increasingly being explored for a variety of biomedical applications. Particular attributes include easy availability, low cost, easy use, biodegradability, and lack of significant toxicity. In the studies to date, we have done extensive materials characterization not just of modified alginates but now a number of other biologic compounds that also have potential as pleural sealants. We have further extensively evaluated promising compounds using small (rodent) and large (pig) ex vivo lung models and have performed initial in vivo evaluations of several compounds in a non-survival surgery rat lung injury model. The studies to date have thus identified several promising compounds that will be further evaluated in the non-survival surgery and also a survival surgery rat lung injury model during the 6 month extension period of the grant. These will lead to a firm platform for further investigations in large animal survival surgery models and subsequent discussions with the FDA about new IND for a clinical investigation.						
<b>15. SUBJECT TERMS</b> Lung, lung health, lung disease, pneumothorax, pleura, pleural sealant, alginate						
<b>16. SECURITY CLASSIFICATION OF:</b> Unclassified			<b>17. LIMITATION OF ABSTRACT</b>  UU	<b>18. NUMBER OF PAGES</b>  43	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC	
<b>a. REPORT</b>  U	<b>b. ABSTRACT</b>  U	<b>c. THIS PAGE</b>  U			<b>19b. TELEPHONE NUMBER (include area code)</b>	

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**1. Introduction**

A variety of lung diseases such as emphysema, infections, and lung cancers as well as lung injury from trauma, including battlefield trauma, and complications of respirator life support of critically ill patients in intensive care units can result in lung collapse that can be immediately life-threatening or result in chronic leaking of air or fluid out of the lung. These remain challenging medical problems for which few good options are currently available and result in significant morbidity, mortality, hospital stays, health care costs, and other complications. New options are thus desperately needed. We are developing a novel approach to provide an easy-to-apply lung sealant which can repair lung leaks. This initially involved use of a chemically modified form of alginate, a naturally occurring seaweed derivative, increasingly being explored for a variety of biomedical applications. Particular attributes include easy availability, low cost, easy use, biodegradability, and lack of significant toxicity. In the studies to date, we have done extensive materials characterization not just of modified alginates but now a number of other biologic compounds that also have potential as pleural sealants. We have further extensively evaluated promising compounds using small (rodent) and large (pig) *ex vivo* lung models and have performed initial *in vivo* evaluations of several compounds in a non-survival surgery rat lung injury model. The studies to date have thus identified several promising compounds that will be further evaluated in the non-survival surgery and also a survival surgery rat lung injury model during the 6 month extension period of the grant. These will lead to a firm platform for further investigations in large animal survival surgery models and subsequent discussions with the FDA about new IND for a clinical investigation.

**2. Keywords**

Lung, lung health, lung disease, pneumothorax, pleura, pleural sealant, alginate

**3. Accomplishments**

a) **What were the major goals of the project?** Listed from the Statement of Work

<p><b>Specific Aim 1(specified in proposal)</b></p> <p><b>To optimize the modified alginate for use as a pleural sealant</b></p>
<p><b>Major Task 1: Develop chemically modified alginate (AA-MA) hydrogels and characterize material properties.</b></p>
<p>Subtask 1: Synthesize and chemically characterize AA-MA polymer formulations.</p>
<p>Subtask 2: Quantify the viscosity and shear mechanical properties of AA-MA solutions and hydrogels.</p>
<p>Milestone(s) Achieved: An elastic AA-MA hydrogel will be fabricated with controllable degrees of methacrylation and crosslinking.</p>
<p><b>Major Task 2: Assess the burst pressure strength and adhesiveness of AA-MA hydrogel sealants.</b></p>
<p>Subtask 1: Measure burst pressure and analyze cohesion and adhesion of AA-MA hydrogels on collagen substrates.</p>
<p>Subtask 2: Synthesize AA-MA hydrogels with the ability to covalently link to tissue proteins or create cell-material linkages.</p>
<p>Milestone(s) Achieved: AA-MA hydrogel sealant will exhibit burst pressures beyond the physiological range and will remain adhered to underlying substrate/tissue up to burst pressure.</p>

<b>Specific Aim 2(specified in proposal)</b>
<b>To assess the use of optimized modified alginates in an in vivo rat lung injury model</b>
<b>Major Task 1: Assess different modified alginate hydrogels and patches in an open-chest in vivo rat model.</b>
Subtask 1: Assess different alginate formulations in the non-survival rat surgery model: evaluation of lung mechanics.
Subtask 2: Assess different alginate formulations in the non-survival rat surgery model: histologic evaluation of lung tissues.
Milestone(s) Achieved: An elastic AA-MA hydrogel will be fabricated which completely seals a lung leak and is durable.

<b>Major Task 2: Assess optimal modified alginate gel/patch in a survival surgery model of lung injury in rats.</b>
Subtask 1: Assess survival and animal behavior over the 2 week post-surgical observation period.
Subtask 2: Assess serial chest-rays over the 2 week post-surgical observation period.
Subtask 3: Assess serial blood draws for toxicological evaluations over the 2 week post-surgical observation period.
Subtask 4: Assess lung histology at the end of the 2 week post-surgical observation period.
Milestone(s) Achieved: Demonstration of safety and efficacy of the optimal alginate formulation.

**What was accomplished under these goals?**

**1) Major activities**

We have made significant progress in both Major Tasks for Specific Aim 1 and for Major Task 1 in Specific Aim 2 and have approached milestones for all. This is described in detail in the below relevant sections. Due to ongoing modifications and optimization of the pleural sealant materials being studied, Major Task 2 in Specific Aim 2 has not yet been initiated.

**2) Specific objectives**

The major objective of the proposal is to develop a pleural sealant that will have optimized mechanical and biological properties, coupled with low cost, ease-of use, appropriate storage, and other logistical considerations. Based on promising preliminary data at the time of submission, the proposal was initially focused on methacrylated alginates (AA-MA). Continued study of the AA-MA formulations has defined strengths but also limitations on their use and has stimulated expansion of study into a range of additional biologic materials and other chemical modifications that have resulted in a series of compounds that appear to be more potent as sealants using the *ex vivo* lung models as well as in the pre-clinical (rat) non-survival surgery model. This has provided a firm basis for continuing studies.

**3) Significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative)**

**Specific Aim 1**

**Major Task 1: Develop chemically modified alginate (AA-MA) hydrogels and characterize material properties.**

Subtask 1: Synthesize and chemically characterize AA-MA polymer formulations.

Subtask 2: Quantify the viscosity and shear mechanical properties of AA-MA solutions and hydrogels.

Milestone(s) Achieved: An elastic AA-MA hydrogel will be fabricated with controllable degrees of methacrylation and crosslinking.

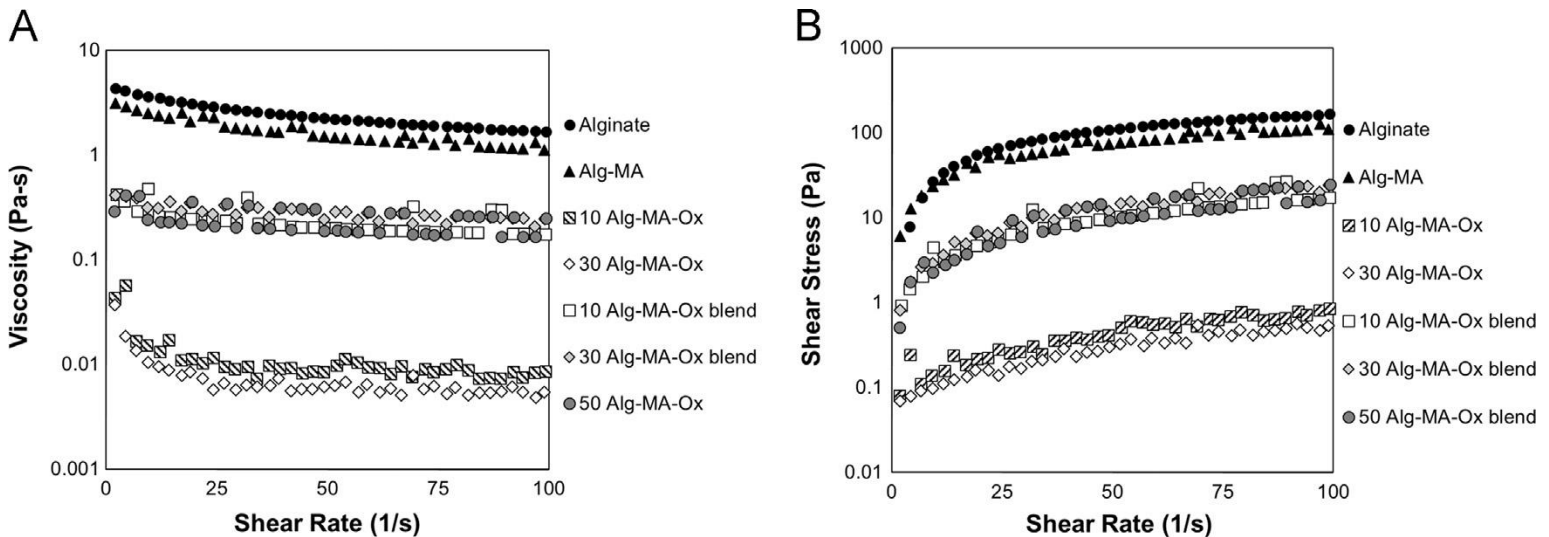
**Major Task 2: Assess the burst pressure strength and adhesiveness of AA-MA hydrogel sealants.**

Subtask 1: Measure burst pressure and analyze cohesion and adhesion of AA-MA hydrogels on collagen substrates.

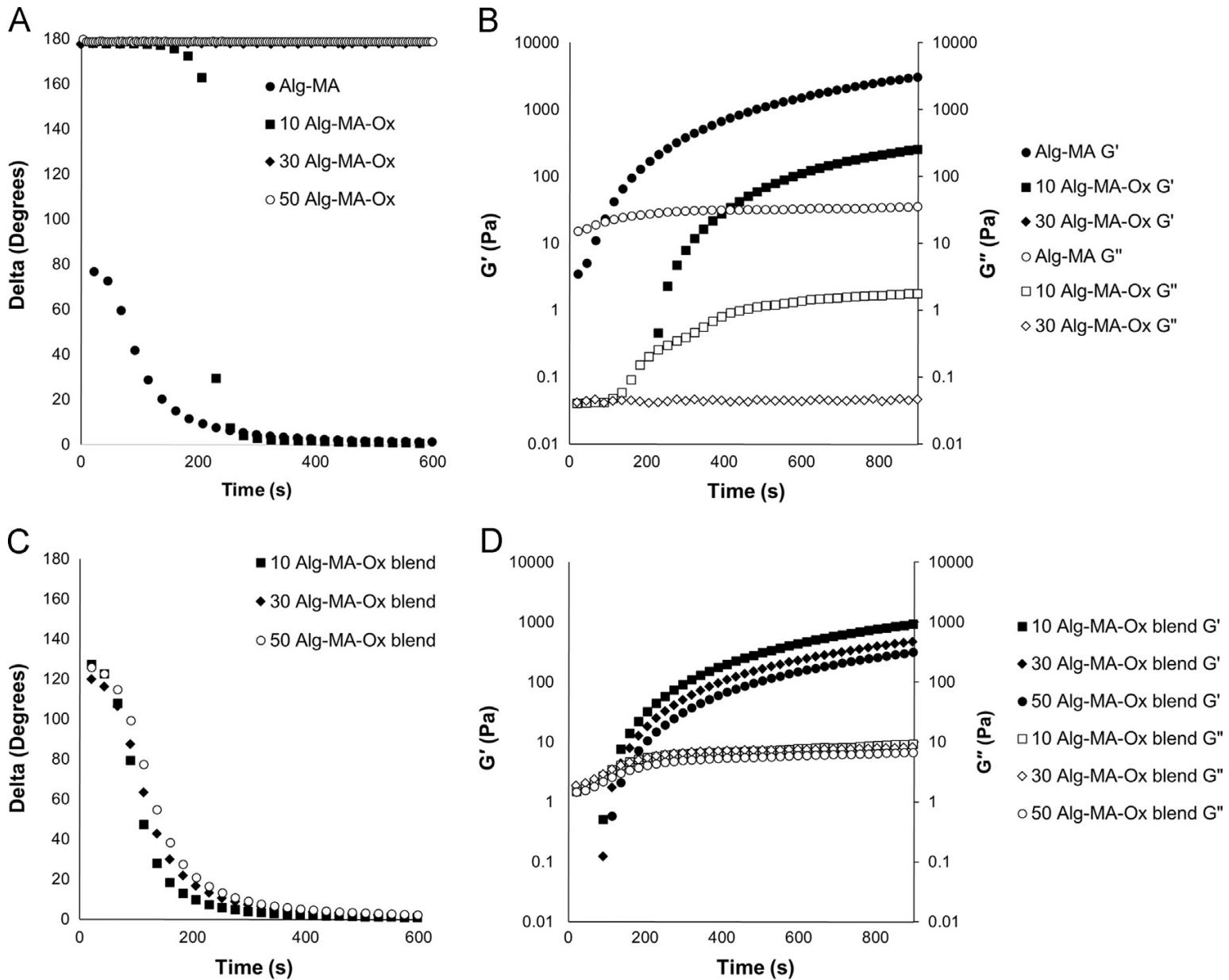
Subtask 2: Synthesize AA-MA hydrogels with the ability to covalently link to tissue proteins or create cell-material linkages.

Milestone(s) Achieved: AA-MA hydrogel sealant will exhibit burst pressures beyond the physiological range and will remain adhered to underlying substrate/tissue up to burst pressure.

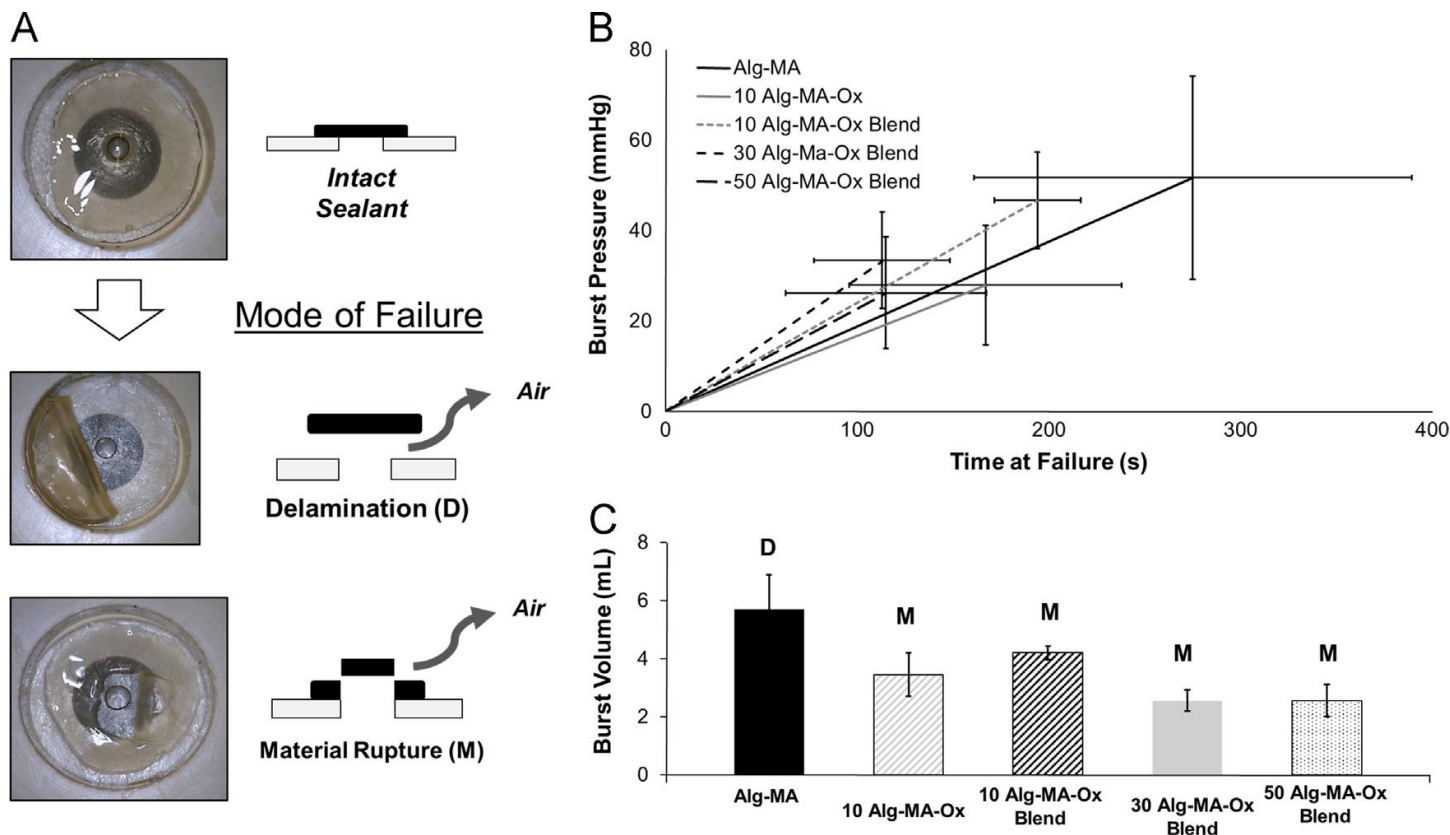
The result of the studies in **Specific Aim 1** are presented in aggregate as they are closely related materials characterizations. We comprehensively evaluated a range of AA-MA preparations with respect to both synthesis, chemical characterization (NMR spectrometry), and materials properties (viscosity, cross-linking/gelation time, shear, burst pressure). This also includes alginate and AA-MA compounds with varying degrees of oxidation (oxidized alginate; AA-OX), as well as AA-MA and AA-OX blends, as reactive aldehyde groups from oxidized formulations can react with the pleural surface providing more adhesiveness. Some of this was published in manuscript form (1) in 2016 as is outlined below in **Figures 1-3**.



**Figure 1:** (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) (1).



**Figure 2:** Gelation of alginate based sealants were assessed using oscillatory time sweeps at 10% radial strain and 1Hz during exposure to green light (525nm) over a period of 10min (A and B). Alg-MMA and oxidized Alg-MMA, and (C and D) homogenous 50:50 blends of Alg-MMA and Alg-MMA-Ox. Delta values decreased as crosslinking occurred via visible light exposure to form hydrogels (A and C). Storage moduli values,  $G'$ , and loss moduli values,  $G''$ , were collected during gelation (B and D). Representative plots for each control and experimental group are shown as average values (n=4) (1).



**Figure 4:** Failure analysis data of alginate-based tissue sealants using a burst pressure device and test system. (A) High-speed video captured the qualitative mode of failure for a sealed 3 mm diameter hole. The two modes of failure observed were delamination, D, and material failure, M. (B) Burst pressure data is shown as strain to failure with time. While the pressure input rate was constant, materials responded according to their elasticity. Burst pressure was recorded as the highest pressure achieved. Vertical error bars represent burst pressure standard deviation; horizontal error bars represent time to failure (n=4). (C) Total air volume retained in the device before failure was calculated; averages including standard deviations are reported (n=4).

Burst pressure results and mode of failure, either materials failure (M) or delamination (adhesive failure, D) are depicted below. Notably, incorporation of oxidized moieties significantly improved tissue adhesiveness. Burst pressures were measured in mm Hg and ranged from 7.09-48.04 which corresponds to 9.64-65.31 cm H<sub>2</sub>O. Using 30cm H<sub>2</sub>O as a reasonable upper limit for physiologic pressures in human lungs, the testing range thus also incorporated elevated pressures that might be observed in diseased or injured lungs.

**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.**

Group	DOM (%)	DOO (%)	G° (Pa) at t¼ 600 s	Burst pressure (mm Hg)	Principal mode of failure
Alg-MA	77	0	1560	49.41722.46	D
10 Alg-MA-Ox	70	10	113	26.11713.13	M
30 Alg-MA-Ox	64	25	0.01	7.0979.85	M
10 Alg-MA-Ox Blend	74	5	440	48.04710.64	M
30 Alg-MA-Ox Blend	71	13	235	35.40710.66	M
50 Alg-MA-Ox Blend	54	20	150	27.66712.36	M



We have subsequently carried out further extensive characterizations of materials properties of different methacrylated/oxidized alginate compounds and blends. Notably, this involved a new collaboration with a biomaterials company, Akina Inc. (West Lafayette IN) in place of the previous collaboration with Rachael Oldinski PhD at UVM. The addition of Akina Inc. and the removal of Dr. Oldinski and associated personnel (Patrick Charron) from the project was detailed in previous communications with the DOD and approved by the DOD on 5-12-16.

Detailed results of subsequent materials synthesis and testing performed by Akina Inc. is presented below.

### Materials Synthesis

Materials were synthesized chemically as detailed for each component below. Unless otherwise specified, chemicals used were of reagent grade and utilized as received from each manufacturer.

#### Alginate Low Viscosity Methacrylate (Lot# UV60630FAJ)

Alginate methacrylate was generated as a component of this system. A 2% (w/v) solution of alginate, low viscosity (Aldrich cat#A1112-100G) was reacted with a 0.170 ml of methacrylic anhydride for 24 h at room temperature with pH of approximately 8.5. The polymer was dialyzed against deionized water (MWCO 8,000Da) and freeze dried to purify. It was then collected and stored in -20°C freezer.

#### Alginate Medium Viscosity Methacrylate (Lot# UV60705FAJ)

Alginate methacrylate was generated as a component of this system. A 2% (w/v) solution of alginate, medium viscosity (Aldrich cat#A2033-100G) was reacted with a 0.170 ml of methacrylic anhydride for 24 h at room temperature with pH of approximately 8.5. The polymer was dialyzed against deionized water (MWCO 6-8kDa) and freeze dried to purify. It was then collected and stored in -20°C freezer.

#### Gelatin (300 bloom, type A) Methacrylate (Lot# UV60706FAJ)

In a 2-neck, 1000ml RBF, a 10-g gelatin solution was prepared by dissolving gelatin (Type A, 300 bloom from porcine skin, Sigma) in 100 ml phosphate buffered saline (PBS, pH 7.4, use the PBS tablets to make. No azide or tween) and warmed to 40 °C overnight under vigorous stirring. Next day it was raised to 50 °C. To this solution carefully add 1 mL of methacrylic anhydride (Sigma) was then added drop-wise to the gelatin solution (10% vol/weight gelatin). The gelatin methacrylation reaction was allowed to proceed for 1 h at 50 °C under vigorous stirring. The methacrylation reaction was then quenched by dilution of the reaction solution with 300 mL PBS warmed to 40 °C. The solution was then transferred while it was still warm into MWCO 6-8kDa membrane in a 4-L bath. Dialyze against warmed deionized water (40C) for 3-days with daily bath replacement. Remove solution and put in freeze-drier trays, freeze-dry in harvest right dryer to get fluffy product. It was then collected and stored in -20°C freezer.

#### Gelatin Methacrylate (Lot: UV61102JSG)

Into 2-neck argon purged and foil-wrapped RBF and added 100 ml of phosphate buffered saline. Stirred and heated to 60°C and slowly added 10.46 g gelatin (300 bloom, type-A, EMS catalog# 16564). Stirred to dissolve at 60 °C for 1 hour then cooled to 40 °C and added 10 ml of methacrylic anhydride dropwise with stirring overnight. Next day transferred into 40 °C pre-heated dialysis bath with MWCO 3500 Da membrane. Dialyzed for 4 days against deionized water then removed and freeze-dried.

Chitosan Methacrylation (Lot#UV60721BPR-A)

80 mesh size granular chitosan (MP Biomedicals Cat#150597) was added into a 2-neck 1L RBF 2.5g of and 200mL of DMSO was added and to make a suspension. Allowed Ar to flow over stirring suspension at 200 rpm then attached reflux column to neck and set the condenser temperature to 0°C. Then the Ar was turned off and sealed and heat was set to 85°C for one hour. Then it was allowed to cool overnight. Next day with the reflux condenser still attached 2mL of Glycidyl Methacrylate was added and allowed to stir at room temperature for one hour. Then 50µL of DEPA was added to solution and heated to 60°C for 12 hours. 8000Da MWCO tubing was used to dialysis product against 0.05M HCl solution for 2 days and was refreshed each day. Next day product was freeze dried, collected and stored in -20°C freezer.

Hydroxyethyl Starch Methacrylation (Lot# 60715FAJ)

Into a 2-neck 1L RBF 2.5g of Hydroxyethyl Starch (Sigma Cat#H-6382) was added and dissolved in 200mL of anhydrous DMSO. Allowed Ar to flow over stirring solution at 200 rpm then attached reflux column to neck and set the condenser temperature to 0°C. Then the Ar was turned off and sealed and heat was set to 85°C for one hour. Then it was allowed to cool overnight. Next day with the reflux condenser still attached 2mL of Glycidyl Methacrylate was added and allowed to stir at room temperature for one hour. Then 50µL of DEPA was added to solution and heated to 60°C for 12 hours. 500Da MWCO tubing was used to dialysis product against DIH<sub>2</sub>O for 2 days with water refreshed every day. Next day product was freeze dried, collected and stored in -20°C freezer.

PEG-co-citric acid Methacrylate (Lot#60728DWW-A)

Into 2-neck 500mL RBF put listed 13 g grams of PEG 400 and 10 grams of citric acid fit it with and a vacuum-distillation short-path outlet with collector (to collect water). Set to stir ~250RPM and heat at 160C under argon flow for 20 min. Then reduce temperature to 145 °C for 2 hours under argon flow. Close off Argon and put under deep vacuum through the short-path vacuum-distillation head for another 2 hours. After cooling, add DI water to the polymer to dissolve it and then put it in a low MWCO (500Da) dialysis filter and dialyze against DI water for 2-days. Subsequently, pull and freeze dry to obtain the PEG-CA pre-polymer. The pre-polymer was then methacrylated as follows. 13 grams of the PEG-co-CA pre-polymer was deep vacuum purged and argon back-flushed in a round bottom flask. It was dissolved in anhydrous dichloromethane. Trimethylamine (3.77 ml) and methacryloyl chloride (2.6 ml) were added to the PEG-co-CA solution. The solution was refluxed for 2 hours at 80 °C and then cooled. Afterwards it was filtered to remove trimethylamine hydrochloride and precipitated in an excess volume made of a mixture of 80:20 hexane:ethanol. Product was then collected, placed under deep vacuum until dry and stored in -20°C freezer.

Chitosan Methacrylate (set 60831\*)

Two grams of Chitosan Lot# M1310 WP Biomedical was placed in a 1L 2-neck round bottok flask (RBF) and 200 mL of anhydrous DMSO was added and stirred to make a suspension. Argon was then flushed into to RBF with a reflux condenser attached to other neck and allowed to stir at room temperature overnight. The next day 2 mL of glycidyl methacrylate was added dropwise and allowed to stir for one hour. 50µL of DPEA was added to suspension and heat set at 60°C for 12 hours. After cooling, the solution was dialized against deionized water (DIH<sub>2</sub>O) using MWCO 12kDa tubes for 3 days with water refreshed every day. Product was freeze dried using a HarvestRight freeze drier and stored in freezer.

Oxidized Alginate (set 60831\*)

Medium Viscosity Alginate Lot#SLBQ3067V Sigma (5 grams) was added in to a 500mL RBF and 250mL of DIH<sub>2</sub>O was added and allowed to dissolve overnight. The next day 500 mg of Sodium Periodate Lot# MKBN7548V Sigma was added to 10 mL of DIH<sub>2</sub>O and dissolved then added to the Alginate solution. This was allowed to stir at room temperature and protected from light for 24 hours. Solution was then dialized against DIH<sub>2</sub>O using MWCO 12kDa tubes for 3 days with water refreshed everyday. Product was then freeze dried in HarvestRight freeze drier and stored in freezer.

Methacrylated Mucin (set 61003)

Five grams of Mucin from Porcine Stomach Crude Type II Lot#54F-0154 Sigma was placed in a 2-neck 1L RBF and 500mL of DIH<sub>2</sub>O was added. Heat was set to 37°C and stirred vigorously until dissolved. RBF was then placed in a coiled copper tube that was attached to a condenser with the temperature set to 5°C. Once cooled a solution of 5M NaOH in DIH<sub>2</sub>O was added to the Mucin solution dropwise until a pH of 8 was achieved. Then 40µL of Methacrylic Anhydride was added and pH was monitored for 2 hours and allowed to stir overnight. Next day the continents were centrifuged to remove Mucin precipitates and the solution left was dialyzed against water using MWCO 12kDa tubes and bath was refreshed 2 times over the course of 3 days. Product was freeze dried in a HarvestRight freeze drier and stored in freezer

Methacrylated Hyaluronic Acid (set 61003)

Five grams of Hyaluronic Acid Sodium Salt Lot#BCBG3516V Sigma and 250mL of DIH<sub>2</sub>O was added to a 2-neck 500mL RBF and vigorously stirred with a temperature set at 50°C until dissolved. A solution of 5M NaOH was added dropwise until a pH of 8.5 was achieved. 1mL of methacrylic anhydride was added and pH was monitored for 6 hours. Solution was left to stir overnight. Next day solution was dialyzed against 4L DIH<sub>2</sub>O in 12kDa MWCO tubes and bath was refreshed 2 times over the course of 3 days. Product was freeze dried in a HarvestRight freeze drier and stored in freezer. Note: the hyaluronic acid solution was difficult to keep basic while the methacrylic anhydride was reacting.

Methacrylated Hyaluronic Acid (Lot: UV61010FAJ)

Five grams of Hyaluronic Acid Sodium Salt Lot#BCBG3516V Sigma was placed in a 2-neck 1L RBF and 250mL of Sodium Phosphate Buffered Saline (PBS) was added and stirred vigorously until dissolved. Then 37.5mL of Methacrylic Anhydride was added dropwise with an overhead dropper while pH was monitored for 6 hours. A solution of 5M NaOH was added dropwise to neutralize when solution became acidic. Solution was allowed to stir overnight. Next day the continents were filtered to remove precipitates and the solution left was dialyzed against water using MWCO 12kDa tubes and bath was refreshed 3 times over the course of 4 days. Product was freeze dried in a HarvestRight freeze drier and stored in freezer.

Oxidized High Molecular Weight Alginate (set 61003)

High viscosity Alginate Lot#G9402001 FMC Biopolymer (5 grams) was added 500 mL of stirring DIH<sub>2</sub>O and dissolved. 500 mg of Sodium Periodate Lot# MKBN7548V Sigma was added to 10 mL of DIH<sub>2</sub>O and dissolved then added to the Alginate solution. The beaker was covered with foil and allowed to stir at room temperature for 24 hours. The solution was then dialyzed against DIH<sub>2</sub>O using MWCO 12kDa tubes for 4 days with 3 water bath changes. Product was then freeze dried in HarvestRight freeze drier and stored in refrigerator.

Oxidized High Molecular Weight Alginate (Lot: UV61010SMS)

Five grams of high viscosity Alginate Lot#G9402001 FMC Biopolymer was added to 500 mL of stirring DIH<sub>2</sub>O and dissolved. 250 mg of Sodium Periodate Lot# MKBN7548V Sigma was added to 5 mL of DIH<sub>2</sub>O and dissolved, then added to the Alginate solution. The beaker was covered with foil and allowed to stir at room temperature for 24 hours. The solution was then dialyzed against DIH<sub>2</sub>O using MWCO 12kDa tubes for 3 days with 2 water bath changes. Product was then freeze dried in HarvestRight freeze drier and stored in refrigerator.

Methacrylated High Molecular Weight Alginate-aqueous method (set 61003)

Ten grams of Alginic Acid Lot #G9402001 FMC BioPolymer and 500 mL of DIH<sub>2</sub>O were added to a 2-neck 1 L RBF and vigorously stirred until dissolved. A solution of 5M NaOH was added dropwise until a pH of 8.5 was achieved. 0.5 mL of methacrylic anhydride was added and the pH was monitored for 2 hours. The solution was left to stir overnight. The next day the solution was precipitated into 4 L of chilled ethanol. The precipitate was collected and re-dissolved in DIH<sub>2</sub>O. The solution was dialyzed against 4 L of DIH<sub>2</sub>O in 12 kDa MWCO tubes and the bath was refreshed two times over the course of 6 days. The product was freeze dried in a HarestRight freeze drier and stored in the refrigerator.

Methacrylated Alginate (Lot: UV61111SMS)

5.0 g of Sodium Alginate (Manugel FMC FMC biopolymer) was dissolved in 500 mL of water. The Alginate solution was transferred to 2-neck round-bottom flask (1L) and copper pipe connected to a recirculating chiller was wrapped around the flask to control the temperature. The chiller temperature was set to 5 °C. Calcium Carbonate (5.2 g) was added to the RBF and stirring started. Using a side-arm dropper, 37.5 mL of methacrylic anhydride was added dripwise with fast stirring. After stirring for two days, RBF contained mostly solid material. The material was tested for dissolution in water and 0.1M HCl, but did not dissolve in either solution. The calcium carbonate in the RBF reacted with the alginate to form calcium alginate. This material was not purified and dried as it was insoluble and not suitable for this project.

Methacrylated Alginate (Lot: UV61116JSG-A)

Stirred 2.5 grams of Alginate (Manugel FMC biopolymer) and prepared 5M NaOH solution. Put Alginate solution in 2-neck round-bottom flask (1L) and wrapped copper pipe around the flask connected to a recirculating chiller to control the temperature. Set the temperature to 5 °C and added in 19 ml methacrylic anhydride dropwise with fast stirring. Filled 30 ml syringe with 5M NaOH and put in syringe pump. Connected by polyethylene tubing from syringe tip into RBF and used pump to control application of 5M NaOH slowly ~1-2 ml/hr. Checked pH by pH probe and it varied from 5.22 up to 9.92 over course of reaction by addition of NaOH. In total, 50 ml of 5M NaOH was used to control the pH of the reaction. After reacting for 2 days with base addition, removed solution and dialyzed against DI H<sub>2</sub>O using MWCO 3500 dialysis membrane for 4 days before lyophilization.

Methacrylated Alginate (Lot: UV61117BPR-A)

Stirred 2.5 grams of Alginate (Manugel FMC biopolymer) until it dissolved in 500mL of a 0.1M NaHCO<sub>3</sub> buffer solution. 6.37g of solid NaHCO<sub>3</sub> powder was added to the solution slowly. Temperature and pH balance was accomplished by the decomposition of bicarbonate ions into carbon dioxide and water. A dropper arm was attached to the reaction vessel and 19mL of methacrylic anhydride was added dropwise at ~1 drop/sec. The reaction proceeded overnight, and the post-reaction solution's pH of ~5.5 was balanced to ~8.5. This solution was dialyzed against deionized water using MWCO 3500 dialysis membrane for 4 days then lyophilized to recover methacrylated alginate.

Methacrylated Alginate (Lot: UV61117DWW-A)

Stirred 2.5 grams of Alginate (Manugel FMC biopolymer) until it dissolved in 500mL deionized water. 2.51g MgO powder was added to the solution slowly to form a slurry. The reaction vessel was placed in an aluminum bead bath with a refrigerant coil, and brought down to 5°C. A dropper arm was attached, and 19mL of methacrylic anhydride was added dropwise at ~1 drop/sec. The reaction proceeded overnight, and the post-reaction solution was pH balanced to ~8. This solution was dialyzed against deionized water using MWCO 3500 dialysis membrane for 4 days then lyophilized to recover methacrylated alginate.

Methacrylated Alginate (Lot: UV61118BPR-A)

Stirred 2.5 grams of Alginate (Manugel FMC biopolymer) until it dissolved in 500mL deionized water. 26g of NaHCO<sub>3</sub> powder (2.5x molar excess) was added to the reaction vessel slowly. The small excess that did not dissolve formed a slurry with the alginate solution. Temperature and pH balance was accomplished by the decomposition of bicarbonate ions into carbon dioxide and water. A dropper arm was attached to the reaction vessel and 19 mL of methacrylic anhydride was added dropwise at ~1drop/sec. The reaction proceeded for 3 days, and the post-reaction solution maintained a pH of ~7.0. This solution was dialyzed against deionized water using MWCO 12-14 kDa dialysis membrane for 7 days then lyophilized to recover methacrylated alginate.

Methacrylated Hydroxypropylcellulose (set 61003)

Four grams of hydroxypropylcellulose-H were added to a 1-neck 500 mL RBF. The HPC-H was placed under deep vacuum for 1 hour with heating at 60 °C. The HPC-H was allowed to cool to room temperature while under vacuum and then the RBF was back-flushed with argon. Anhydrous DCM was added to the HPC-H and stirred until dissolved. 1.0 mL of methacrylic anhydride was dissolved in 9 mL of anhydrous DCM. The methacrylic anhydride was added dropwise to the stirring HPC-H solution. The solution was allowed to react for two days. The solution was then precipitated into stirring hexet. The precipitate was collected and placed under vacuum to dry.

Methacrylated High Molecular Weight Alginate-anhydrous method (Lot: UV61007SMS)

10 grams of Alginic Acid Lot #G9402001 FMC BioPolymer was added to 1 L of stirring DIH<sub>2</sub>O. Once dissolved the solution was transferred to a stir plate and the stirring speed set to 1000 rpm. 20 grams of DTAB were added to 1 L of stirring DIH<sub>2</sub>O. Once dissolved the DTAB solution was slowly added to the stirring Alginate solution. The resulting precipitate was stirred for approximately 30 minutes and stored in the refrigerator overnight. The supernatant was removed and the precipitate transferred to 50 mL centrifuge tubes. The tubes were centrifuged at 3000 rpm for five minutes and the supernatant removed. The supernatant was washed with DIH<sub>2</sub>O and then placed on a freeze drier. Fifteen grams of Alg-DTA precipitate and 750 mL of anhydrous DMSO were placed in a 1 L RBF and stirred to dissolve after backflushing the RBF with argon. Once dissolved, 1.85 g of dimethylaminopyridine was added to the RBF and stirred until dissolved. 70.88 mL of methacrylic anhydride was added to the stirring solution. The RBF was covered with foil and stirred for two days. The solution was then dialyzed against DIH<sub>2</sub>O using MWCO 12kDa tubes for three days. The water bath was replaced with 0.8M sodium phosphate dibasic and dialyzed for five days with four bath changes. The pH of the bath was adjusted to 7 when the baths were refreshed. The bath was then replaced with DIH<sub>2</sub>O and dialyzed for three days with two water bath changes. The product will be freeze dried in a HarvestRight freeze drier and stored in freezer.

Methacrylated High Molecular Weight Alginate-anhydrous method (Lot: UV61012SMS)

As previously reported, 10 grams of Alginate Lot #G9402001 FMC BioPolymer was added to 1 L of stirring DIH<sub>2</sub>O. Once dissolved the solution was transferred to a stir plate and the stirring speed set to 1000 rpm. 20 grams of DTAB were added to 1 L of stirring DIH<sub>2</sub>O. Once dissolved, the DTAB solution was slowly added to the stirring Alginate solution. The resulting precipitate was stirred for approximately 30 minutes and stored in the refrigerator overnight. The supernatant was removed and the precipitate transferred to 50 mL centrifuge tubes. The tubes were centrifuged at 3000 rpm for five minutes and the supernatant removed. The supernatant was washed with DIH<sub>2</sub>O and then placed on a freeze drier. Fifteen grams of Alg-DTA precipitate and 750 mL of anhydrous DMSO were placed in a 1 L RBF and stirred to dissolve after backflushing the RBF with argon. Once dissolved, 1.85 g of dimethylaminopyridine was added to the RBF and stirred until dissolved. 70.88 mL of methacrylic anhydride was added to the stirring solution. The RBF was covered with foil and stirred for two days. The solution was then dialyzed against DIH<sub>2</sub>O using MWCO 12kDa tubes for three days. The water bath was replaced with 0.8M sodium phosphate dibasic and dialyzed for five days with four bath changes. The pH of the bath was adjusted to 7 when the baths were refreshed. The bath was then replaced with DIH<sub>2</sub>O and dialyzed for three days with two water bath changes. The product was dried in the HarvestRight freeze-drier and stored in the freezer.

Methacrylated Alginate-anhydrous method (Lot: UV611121SMS)

Using previously prepared Alg-DTA precipitate, 4.0 grams of Alg-DTA precipitate and 400 mL of anhydrous DMSO were placed in a 1 L RBF and stirred to dissolve. After several days of stirring an additional 200 mL of anhydrous DMSO was added to the RBF to aid in dissolution. Once mostly dissolved, 0.4923 g of dimethylaminopyridine was added to the RBF and stirred until dissolved. 4.77 mL of methacrylic anhydride was added to the stirring solution. The RBF was covered with foil and stirred overnight. The solution was then

dialyzed against DIH<sub>2</sub>O using MWCO 12 kDa tubes for one day. The water bath was replaced with 0.8M sodium phosphate dibasic and dialyzed for five days with two bath changes. The pH of the bath was adjusted to 7 when the baths were refreshed. The bath was then replaced with DIH<sub>2</sub>O and will be dialyzed for three days with two water bath changes. The methacrylated alginate did not appear to be re-dissolving in DIH<sub>2</sub>O during dialysis. The bath was changed to 0.8M sodium phosphate dibasic, adjusted to pH 7.0 and dialyzed for an additional day. The bath was changed to DIH<sub>2</sub>O and dialyzed for an additional three days with 2 water bath changes. The product was collected and lyophilized to recover methacrylated alginate.

Methacrylated Chondroitin Sulfate (Lot: UV61028FAJ)

As of the time that this report has been written, five grams of Chondroitin Sulfate A Sodium salt from bovine trachea lot# SLBQ0017V Sigma was placed in a 1-neck 500mL RBF and 100mL of PBS was added and vigorously stirred until dissolved. 10mL of Glycidyl Methacrylate was added to the solution and allowed to stir for 15 days. The RBF was wrapped in Aluminium foil to protect from light during reaction process. The solution was then dialyzed against water in MWCO 3.5kDa tubes over the course of 5 days with two water bath changes. After dialyzing, the solution was filtered and subsequently dried in the HarvestRight freeze-drier and stored in the freezer.

Methacrylated Alginate (Lot: UV61205SMS)

Stirred 2.5 grams of high viscosity Alginate (Lot #G9402001, Manugel FMC biopolymer) until it dissolved in 500mL deionized water. 31.27g of sodium bicarbonate powder (3x molar excess) was added to the stirring alginate solution and stirred until dissolved. The solution was transferred to a 1L 2-neck RBF containing a stir bar and placed on a reaction plate. Stirring was set to 600 rpm. A dropper arm was attached to the reaction vessel and 19 mL of methacrylic anhydride was added dropwise at ~1drop/ 5 sec. Stirring was increased to 800 rpm and the reaction proceeded overnight. The next day the pH was 7.06 and unreacted methacrylic anhydride was noted in the reaction vessel. An additional 5.2g of sodium bicarbonate was added to the reaction and it was allowed to proceed overnight. The next day the pH was 7.47 and all the methacrylic anhydride appeared to be reacted. This solution was dialyzed against deionized water using MWCO 12-14 kDa dialysis membrane for 5 days with two water bath changes, filtered, then lyophilized to recover methacrylated alginate.

Oxidized Alginate, 2.5% (Lot: UV61206SMS)

Five grams of high viscosity Alginate (Lot #G9402001, Manugel FMC Biopolymer) was added to 500 mL of stirring DIH<sub>2</sub>O and dissolved. 137.2 mg of Sodium Periodate (Lot# MKBN7548V, Sigma) was added to 2.5 mL of DIH<sub>2</sub>O and dissolved, then added to the Alginate solution. The beaker was covered with foil and allowed to stir at room temperature for 24 hours. After 24 hours of stirring 32.7 µL of ethylene glycol was added to the oxidized alginate solution. The solution was then dialyzed against DIH<sub>2</sub>O using MWCO 12kDa tubes for 6 days with 3 water bath changes. Product was then freeze dried in HarvestRight freeze drier.

Methacrylated Alginate (Lot: UV61214BPR-A)

Stirred 5.0 grams of high viscosity Alginate (Lot #G9402001, Manugel FMC biopolymer) in a reaction vessel until it dissolved in 500mL deionized water. 41.7 g of sodium bicarbonate powder (4x molar excess) was added to the stirring alginate solution and stirred. A dropper arm was attached to the reaction vessel and 19 mL of methacrylic anhydride was added dropwise at ~1drop/sec. The reaction was allowed to proceed for several days. The solution was stored in a refrigerator for several days until being dialyzed against deionized water using MWCO 12-14 kDa dialysis membrane. After dialyzing for six days with three water bath changes, the resultant solution was lyophilized in the HarvestRight freeze drier.

Methacrylated Alginate (Lot: UV70111SMS-A)

Stirred 2.5 grams of high viscosity Alginate (Lot #G9402001, Manugel FMC biopolymer) in a beaker until it dissolved in 500mL deionized water. 41.6 g of sodium bicarbonate powder (4x molar excess) was added to the stirring alginate solution and stirred until it dissolved. The solution was transferred to a 2-neck 1 L RBF and a dropper arm was attached to the reaction vessel. 38 mL of methacrylic anhydride was added dropwise at ~1drop/3 sec. The next day the pH of the solution was determined to be 6.82 and un-reacted methacrylic anhydride was noted in the vessel. Additional sodium bicarbonate (5.2 g) was added to the vessel and the pH was tested after stirring for 1 hour. The pH was determined to be 6.91. Additional sodium bicarbonate (5.2 g) was added to the vessel and it was allowed to stir for an hour. The pH was tested again and determined to be 7.11. The reaction was allowed to proceed overnight. The next day the pH was tested and determined to be 7.38 and unreacted methacrylic anhydride was noted in the reaction vessel. The reaction was allowed to proceed overnight after increasing the stir speed to 800 rpm. The solution was then dialyzed against deionized water using MWCO 12-14 kDa dialysis membrane for three days with two water bath changes. After dialysis the solution was filtered and then lyophilized in the HarvestRight freeze drier.

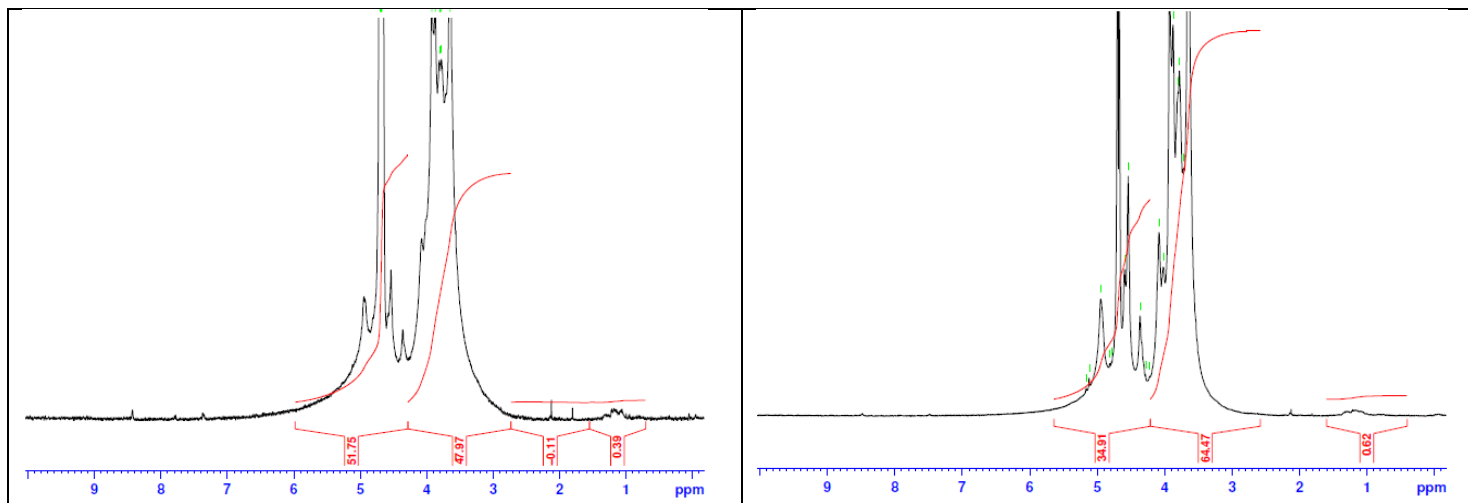
Material Characterization

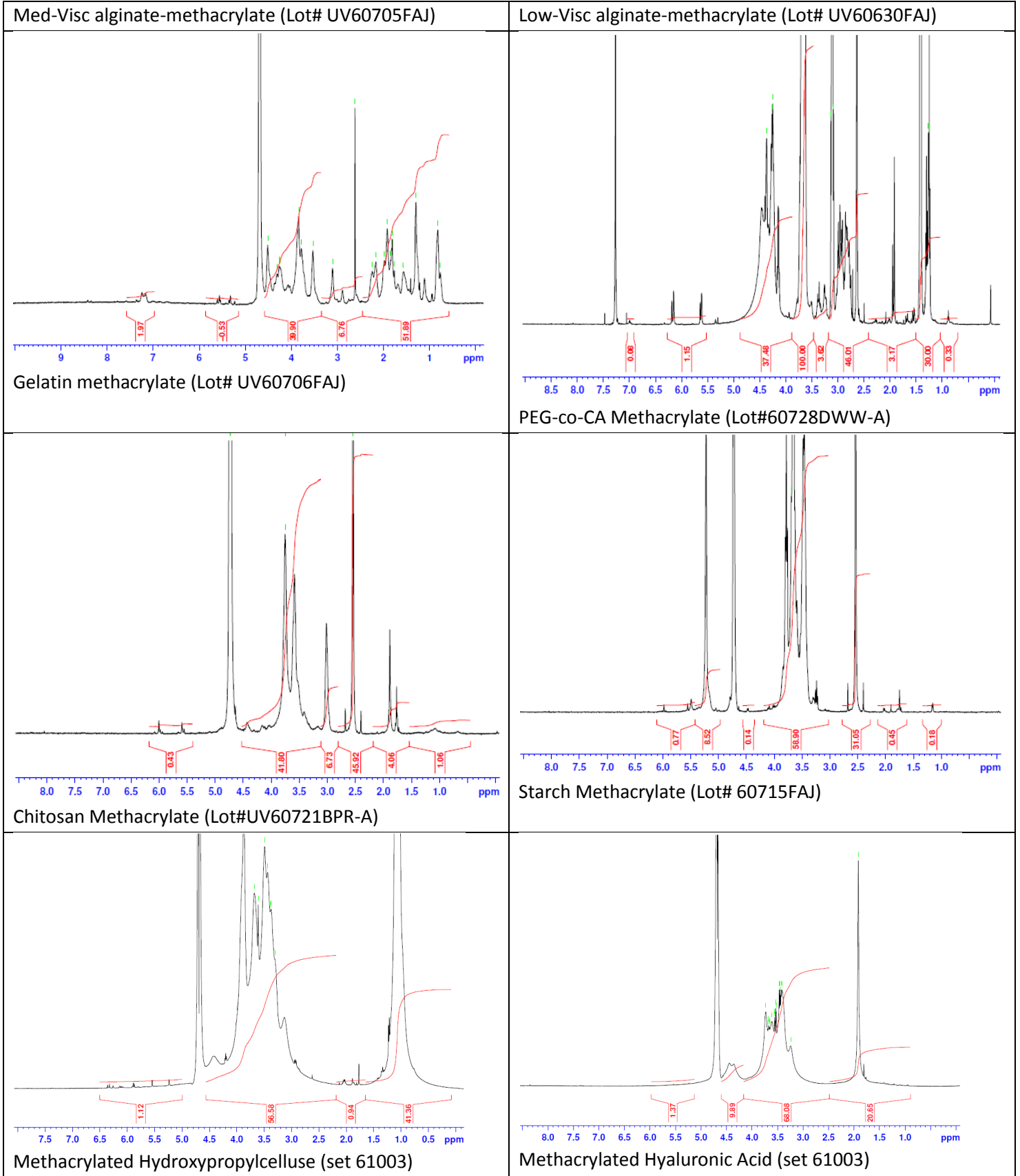
Materials synthesized by these techniques were characterized chemically using HNMR, FTIR, or GPC as appropriate. Each of the dried, methacrylated precursors was characterized with HNMR by Akina Inc. at Purdue University by the PINMRF group ([www.pinmrf.purdue.edu/](http://www.pinmrf.purdue.edu/)). Gel-permeation chromatography was performed on a waters Breeze-2 system with 1 ml/min DCM flow across three sequential GPC columns (7.6 x 300 mm, Phenomenex) and detected by refractive index. Molecular weight parameters were determined by comparison against polystyrene standards (Agilent PS2). FTIR was performed using either a cast film on KBr salt plate or by compression into a KBr pellet and scanned using a Nicolette Protégé 460 ESP FTIR spectrophotometer. Attempts at utilizing rheometry (TA instruments model AR550 rheometer) to determine molecular weight by dilute-polymer viscosity yielded unreliable results (data not shown).

HNMR

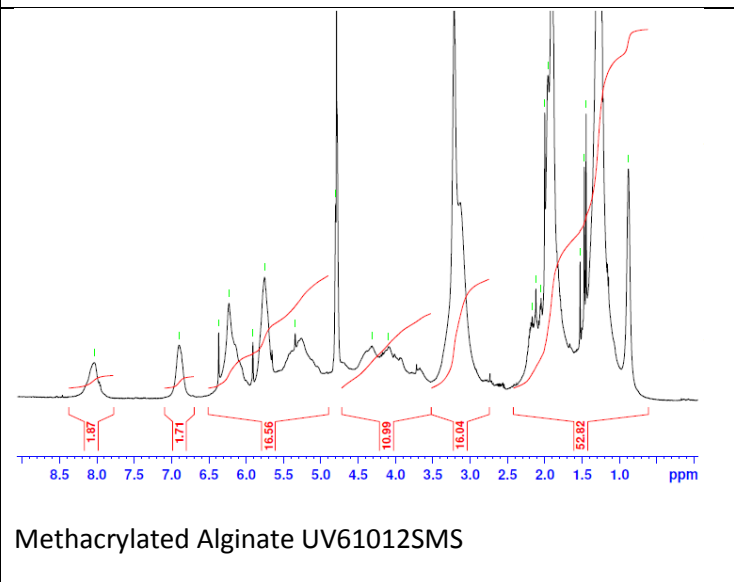
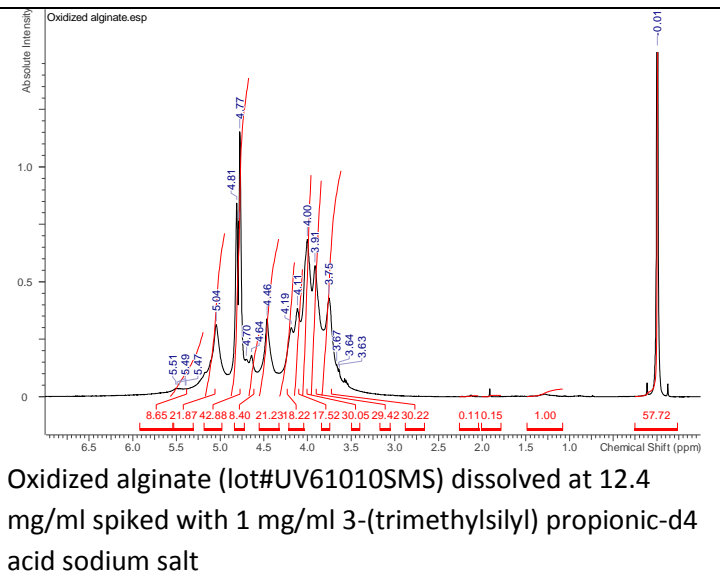
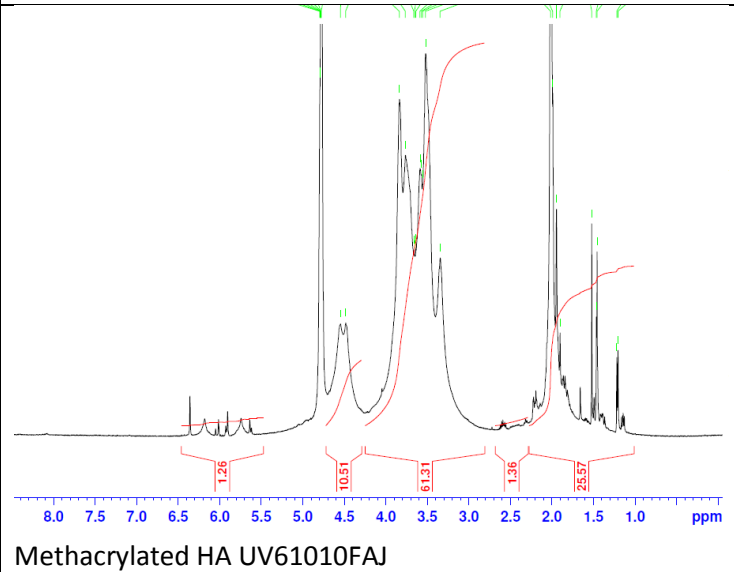
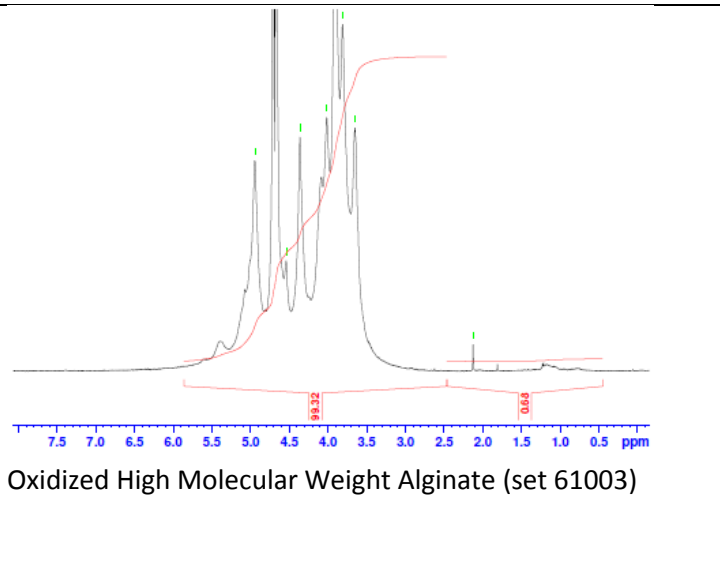
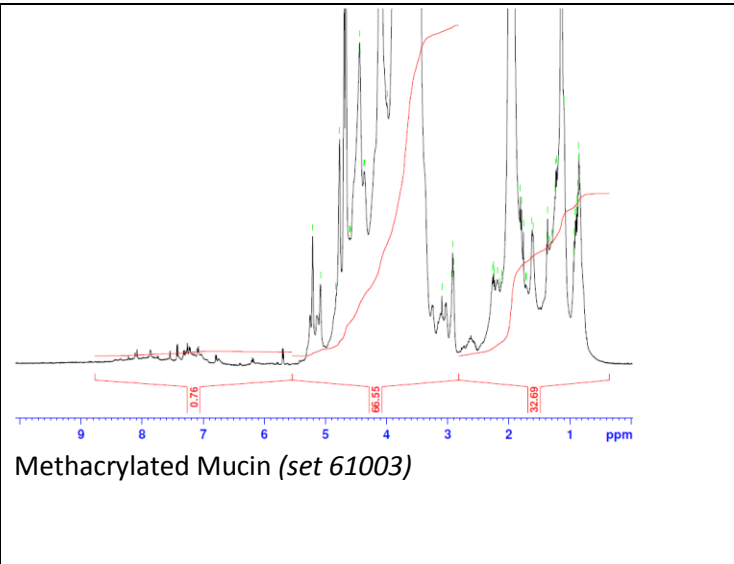
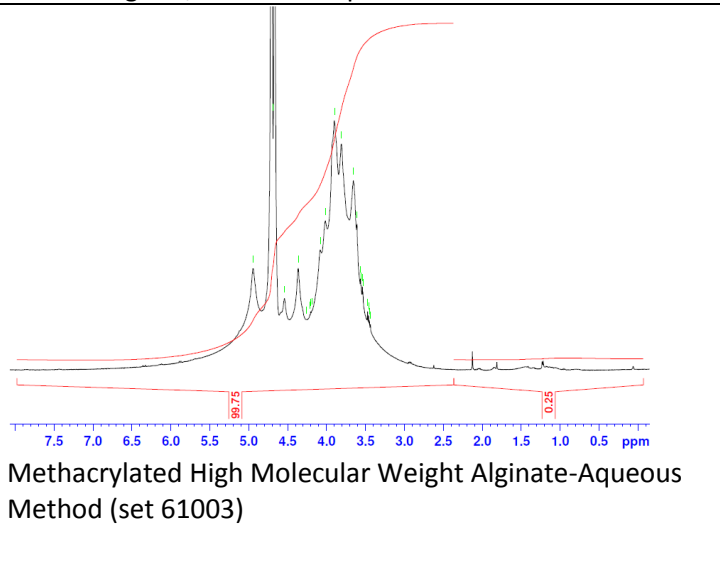
**Figure 5** below shows the resultant NMR spectra from each material as indicated. Note that the lot# is utilized to reference specific batches for repeat synthesis.

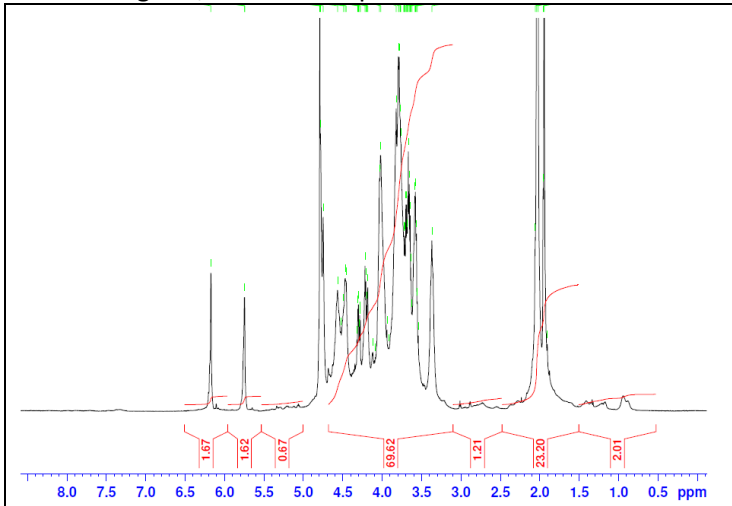
**Figure 5.** HNMR spectra collected from indicated materials and lots. Unless otherwise specified, NMR collected from solution in D2O.



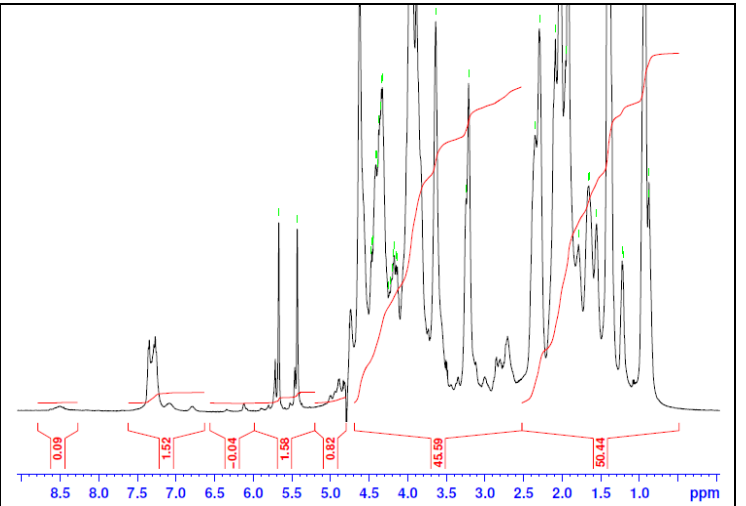




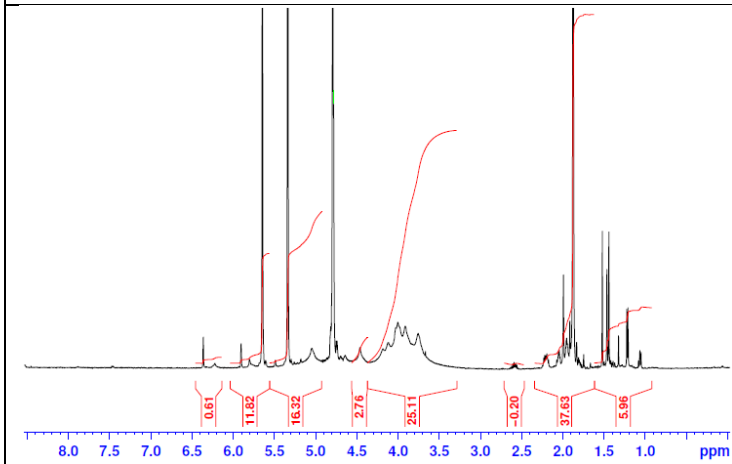




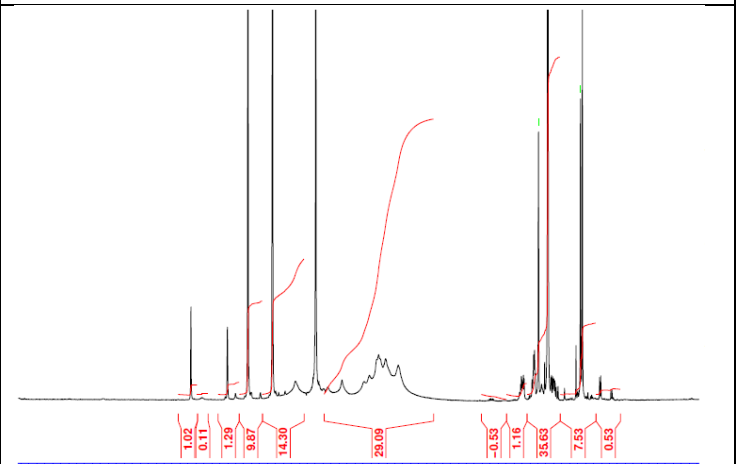
Methacrylated Chondroitin UV61028-FAJ



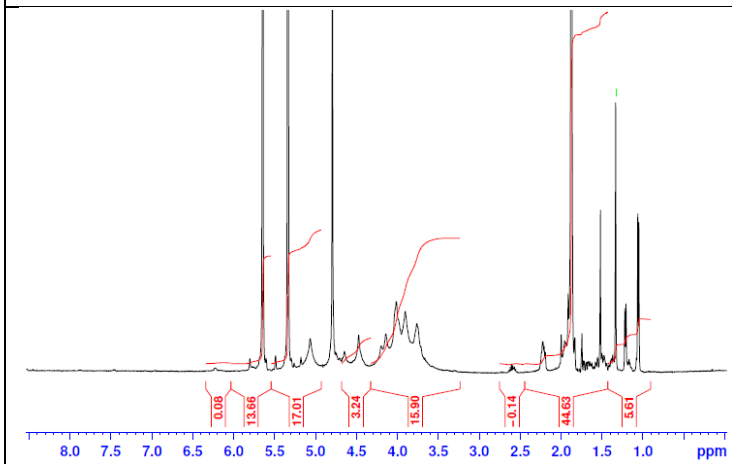
Gelatin Methacrylate UV61102JSG



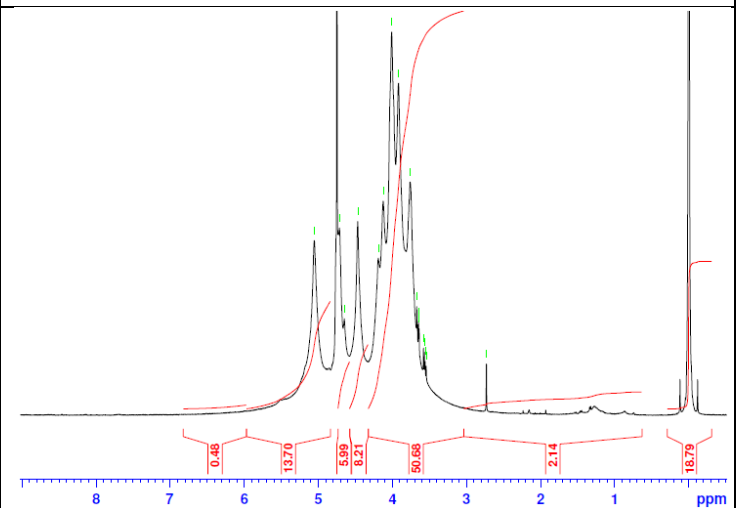
Methacrylated Alginate UV61116JSG-A



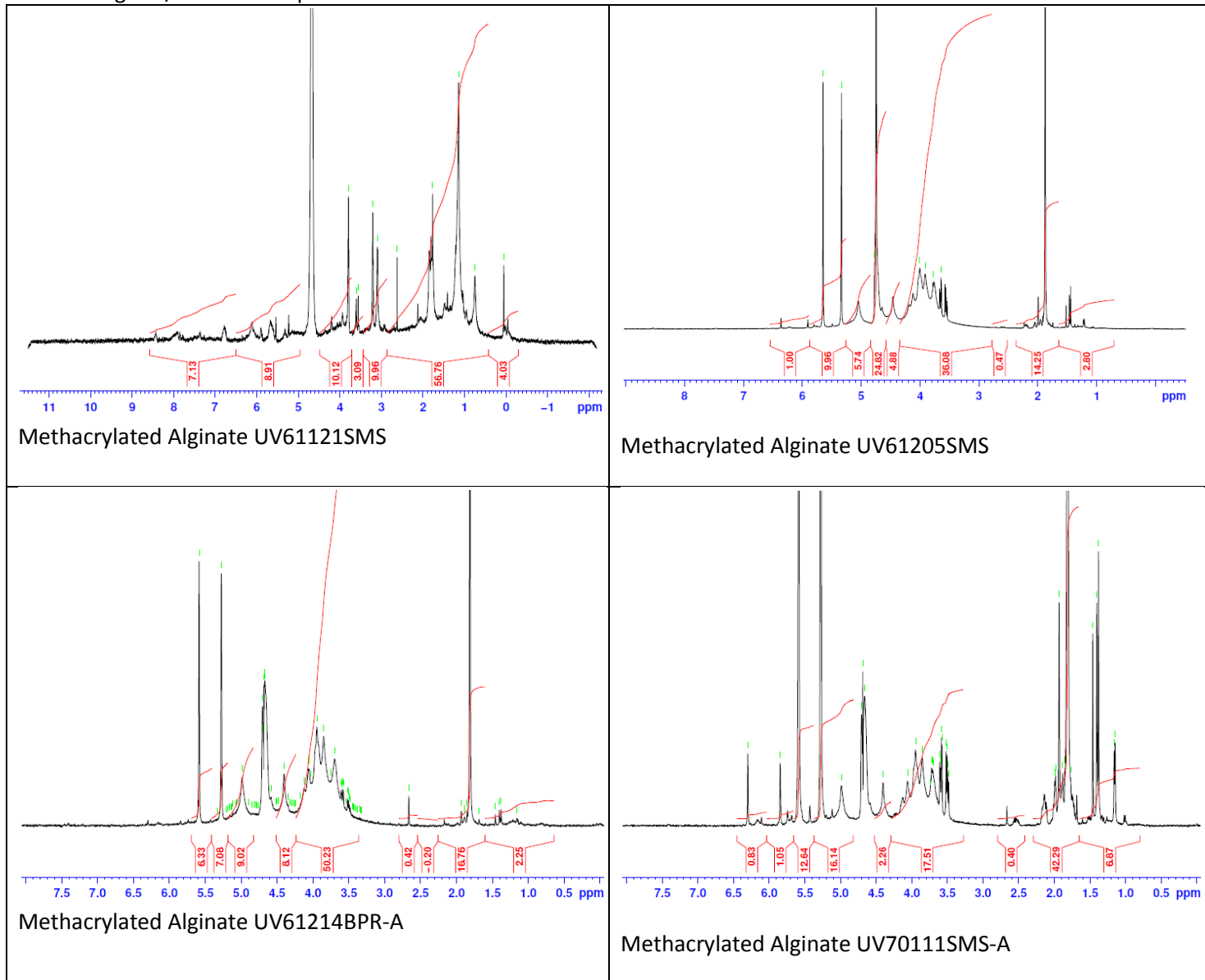
Methacrylated Alginate UV61117BPR-A



Methacrylated Alginate UV61117DWW-A



Oxidized Alginate (2.5%) UV61206SMS dissolved at 13.25 mg/ml spiked with 1 mg/ml 3-(trimethylsilyl) propionic-d4 acid sodium salt



Typically, methacrylate vinyl peaks can be observed in the 5.1-5.8 ppm range and this was used to confirm successful methacrylation of synthesized products. For degree of oxidation, the calculation from (Biomaterials. 2012 May ; 33(13): 3503–3514. doi:10.1016/j.biomaterials.2012.01.041.) was used and the degree of oxidation for using the calculations they provided (attached as separate excel file), the degree of oxidation for oxidized alginate (lot#UV61010SMS) was calculated to be 12.5%. For the Oxidized Alginate (2.5%) UV61206SMS it was determined to be 10.9%

## Functionality

### *Curing*

Preliminary testing was performed in 24 well plates. For these tests, a small amount of the respective acrylate precursor solution was put in a well and the stock solutions were mixed with it such that the final concentrations of initiators was as described before (Eosin Y 0.00125% (w/v), 125 mM triethanolamine, and 19 mM 1-vinyl-2-pyrrolidinone). This mixing process was done in the dark to prevent premature curing. The plate was illuminated using a green light source either a 5W green LED bulb or University of Vermont's custom-made green-light LED chamber for approximately 10-20 minutes. Time and condition of cure was recorded.

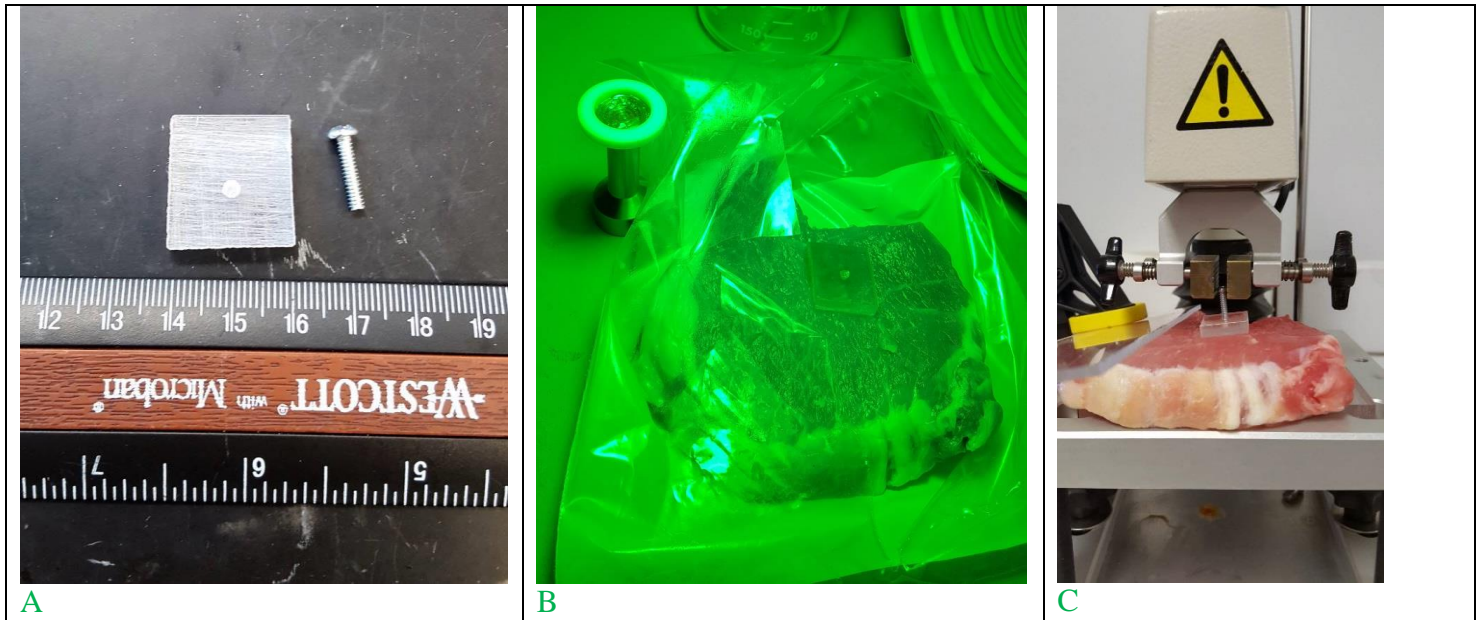
Initial tests were performed simply to determine if the crosslinking could be accomplished at all. The Starch Methacrylate (Lot# 60715FAJ) at 5% w/v solution and the PEG-co-CA Methacrylate (Lot#60728DWW-A) at a 80% w/v did not successfully crosslink after adding the photoinitiator and catalyst at a high concentration. The Medium Viscosity Alginate Methacrylate (Lot# UV60705FAJ) at a 2% w/v has had varying success at crosslinking with photoinitiator and illumination. However, when mixed with the Chitosan Methacrylate crosslinking was more successful. The Gelatin Methacrylate (Lot# UV60706FAJ) crosslinked quite nicely as well as the Chitosan Methacrylate (Lot#UV60721BPR-A) after initiator and catalyst were added. Subsequent photo-initiation tests are shown in **Table 2** below. This includes both materials custom synthesized for the project as well as commercially available products ([www.polyscitech.com](http://www.polyscitech.com))

**Table 2**

Precursor system	Exposure time	Result
PolyVivo AI102 lot# 41217FAJ-A at 20% w/v	10 min	Did not gel
PolyVivo AI103 lot# 41218FAJ at 20% w/v	10 min	Did not gel
PolyVivo AI104 lot# 5051323MJK-A at 20% w/v	10 min	Did not gel
PolyVivo AI138 lot# 60623MJK-A	10 min	Gelled
Methacrylated Mucin at 2% w/v*	10 min	Did not gel
Methacrylated HPC-H at 1% w/v*	10 min	Thickens significantly, weak gel
Methacrylated HMW alginate at 2% w/v*	10 min	Did not gel
Methacrylated hyaluronic acid at 1% w/v*	10 min	Thickens significantly, weak gel
PolyVivo AI146 60907BPR-A at 5% w/v	10 min	Gelled
Methacrylated HA UV61010FAJ at 1%	10 min	Gel
Methacrylated HA UV61010FAJ at 1%	1-2 min	Gel
Methacrylated Alginate UV61012SMS at 10%	10 min	Weak Gel
Methacrylated Alginate UV61012SMS at 20%	10 min	Weak Gel
Methacrylated Chondroitin UV61028-FAJ at 10%	10 min	Gel
Gelatin Methacrylate UV61102JSG at 5%	10 min	Gel
Methacrylated Alginate UV61116JSG-A at 2%	10 min	Weak Gel
Methacrylated Alginate UV61117BPR-A at 2%	10 min	Does Not Gel
Methacrylated Alginate UV61117DWW-A at 2%	10 min	Gel
Methacrylated Alginate UV61118BPR-A at 2%	10 min	Gel
Methacrylated Alginate UV61121SMS at 5%	10 min	No Gel
Methacrylated Alginate UV61121SMS at 10%	10 min	No Gel
Methacrylated Alginate UV61205SMS at 2%	10 min	No Gel
Methacrylated Alginate UV61205SMS at 2%	20 min	Weak Gel
Methacrylated alginate UV61214BPR-A at 2%	10 min	Weak gel
Methacrylated alginate UV70111SMS-A at 2%	10 min	No gelling

Adhesion

These tests were performed using Texture technologies model TA.XTplus mechanical analyzer equipped with a 5 kg load cell (sensitivity +/- 0.1g). As an initial biological substrate, commercially purchased pork-loin was utilized. Initial tests with a commercial muco-adhesion probe (texture technologies model A-MUC) proved the probe to be inadequate for this particular testing as the gel tended to delaminate off the probe itself and its opacity did not allow for photocuring of the gel with the probe in place. A custom probe was generated by roughing the bottom surface of a 2 x 2 cm sized acrylate sheet and threading it to attach a 4 x 40 machine screw. This adapter (named 'photo-curable rough-surfaced adhesion probe (PRAP)) allowed for the probe to be photocrosslinked onto the biological substrate and then tested using the standard tensile grasps as shown in **Figure 6**.



**Figure 6** A) Components of PRAP system individually. B) Photocrosslinking under green light. C. Testing using standard tensile

In the preliminary test test with the PRAP system, 5% w/v gelatin-methacrylate (Lot# UV60706FAJ) was green-light cured between the plate and the biosubstrate. A maximum of 761 Pascals of stress was required to remove the adherant PRAP plate from the pork piece. The cured hydrogel was observed to rupture internally leaving a portion both on the PRAP system and the pork. For this form of testing, not only is mechanical stress at rupture a critical value but also the mode of rupture (delamination from pork, internal rupture) will define the test result. **Table 3** shows data collected from this test so far for indicated materials. This test will be utilized further for screening of bioadhesion.

**Table 3: Bioadhesion test results**

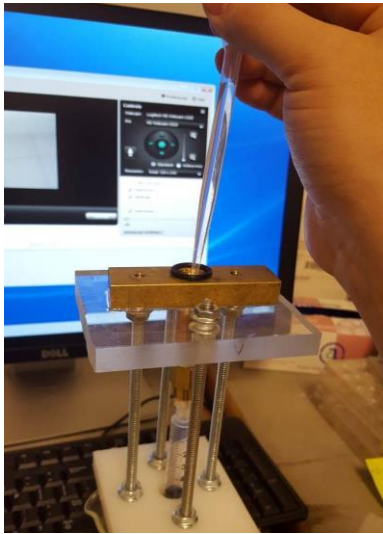
Formulation	Force of Adhesion (N)	Work of Adhesion (mJ)	Failure mode
5%GelMA	0.444 ± 0.113 (n=3)	2.249 ± 1.476	Internal rupture
2%MedVisAlg*	0.162 ± 0.014 (n=2)	0.327 ± 0.011	Internal rupture
5%4Gel1Chito	0.337 ± 0.105 (n=3)	1.909 ± 1.209	Delamination from pork
5%4Gel1Alg	0.339 ± 0.060 (n=3)	2.003 ± 0.794	Delamination from pork
Control (PBS)	0.155 ± 0.036 (n=12)	0.158 ± 0.074	NA

\* - crosslinked poorly

### *Burst pressure*

To allow for dynamic testing of burst-pressure as well as testing of burst pressure under wet conditions, a custom modified test apparatus was constructed. This tester is comprised of a 5-ml syringe attached to a 1/8 inch FIP nylon leuc adapter feeding into a 1/8" to 1/4" MIP brass adapter and a 2" piece of brass pipe which feeds into a threaded block of brass drilled to accommodate the 1/4" MIP tubing. All threaded connections are sealed with Teflon tape and have been checked for leakage.. The bottom side of the brass block is drilled to 5/8 inch (~15 mm) about 2 mm deep. It is threaded on either side to allow for attachment of a piece of steel plate (**Figure 7**), also drilled out 5/8 inch (**Figure 10**). Two, flat 3/8" x 9/8" x 1/16" rubber washers are used to accommodate a piece of collagen sheet (**Figures 8,9**) (The Sausage Maker Inc. cat# 17-1619) between the brass and steel plates.

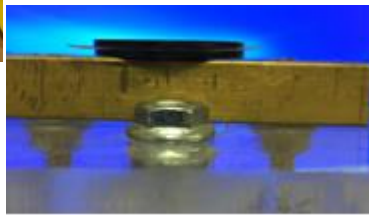
A delrin support attached to 1/4" x 20 all-thread bars allows for the syringe to be mounted upright in the tester and filled with fluid. The TA-XTplus was modified to attach a 1" acrylate probe for uniform application of force across the syringe pusher handle (**Figure 11**). Additionally, a web-cam was mounted beneath the assembly and protected by a petri-dish 'splash-shield' in order to allow for continuous imaging during the tests (**Figure 12**). The functionality of this developed system was checked using unpunctured collagen as a positive (maximum strength) control.



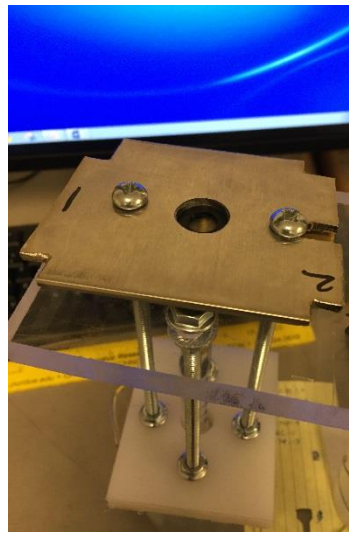
**Figure 7.** Adding water into system prior to use (note system is inverted for water loading, different o-ring in picture than used for tests).



**Figure 8 .** Placement of wet piece of collagen sheet.



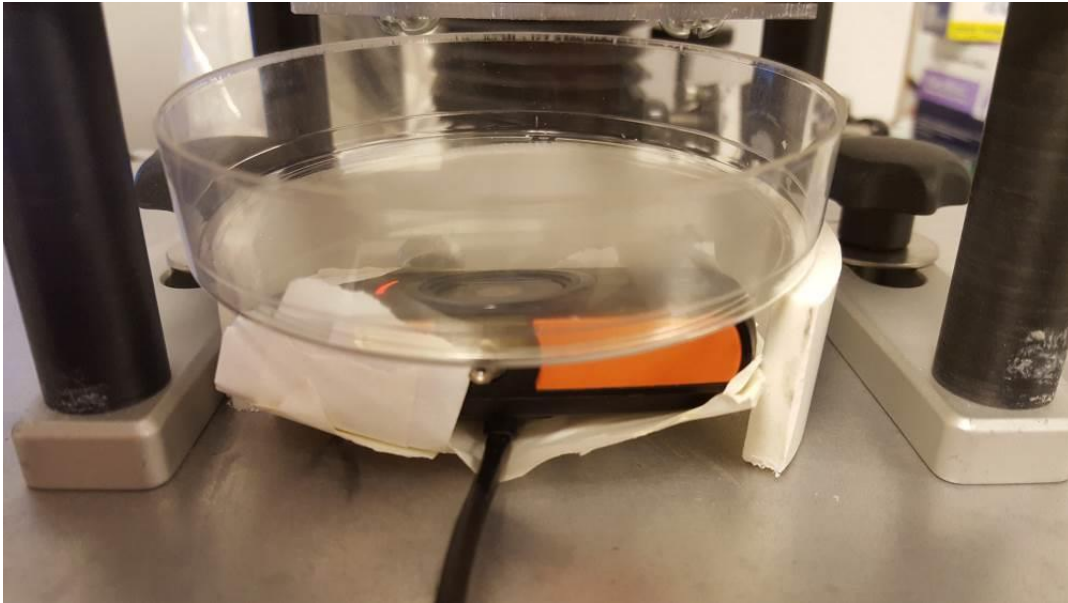
**Figure 9.** Collagen is secured between 2 rubber washers to avoid hard metal edges rupturing the sheet prematurely.



**Figure 10.** Steel plate is secured firmly on the end of the apparatus to compress the washers and ensure a tight seal.



**Figure 11.** Fully constructed assembly. Texture Analyzer is capable of compressing syringe at a stable 1 mm/s.



**Figure 12.** Camera and splash-shield for imaging condition. Splash-shield can be water-filled and raised to allow for wet testing.

The burst-testing apparatus relies on the incompressible nature of water to accurately transmit back-pressure (force data) to the syringe pusher. For initial tests, the syringe pusher was compressed at a steady rate of 1 mm/sec until the collagen ruptured. Initial tests with the un-punctured collagen indicated an average force of  $237.127 \pm 6.917$  N (note, used 30kg load cell for high pressure application) required to break the collagen. This will be used as the positive control and punctured and repaired collagen pieces will be compared against it to determine the stability of the applied seal. Later tests will focus on applying a 3 mm puncture to the collagen using a biopsy punch and then sealing this puncture using a prototype gel formulation. Additional tests may also utilize continuous oscillation of the syringe pusher by the TA.XT plus and focus on time-to-rupture. As the system holds the collagen in an inverted position, it also allows for a dish of water to be placed underneath for testing in wet condition. This apparatus will be used in subsequent tests for determining material capability to withstand high pressures or oscillating forces. A sample recent burst pressure evaluation using this new system is shown in **Table 4**.

**Table 4. Representative additional burst pressure testing**

Item	Force (Newtons)
Control (uncut collagen)	$237.127 \pm 6.917$ N (N = 3)

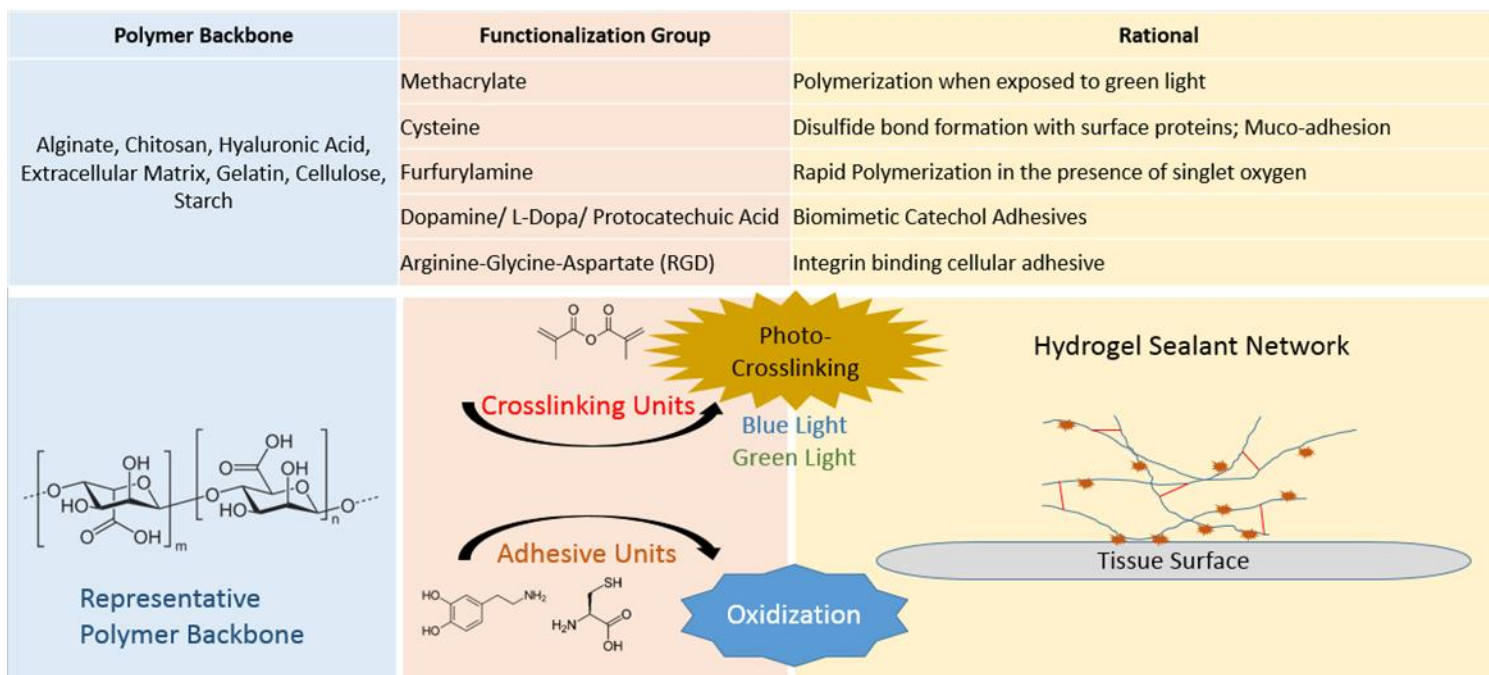
### Conclusions from materials testing of modified (methacrylated/oxidized) alginates

A number of different alginate formulations were prepared and evaluated by HNMR, gelation (curing), adhesion, and burst pressure. The materials testing demonstrated that some formulations appeared to have desirable materials properties for use in *ex vivo* testing on isolated rodent lung preparations. However, many of the compounds assessed did not exhibit these. Moreover some compounds exhibited variable performance in repeated testing. In part, this might reflect a shift in manufacturing protocol and approaches being systematically evaluated.

## Expansion of materials testing into other compounds

As further elaborated in the Other Achievements section, as data accrued with the alginate compounds, including the *ex vivo* testing described below, it became clear that additional biological compounds and/or functional modifications might provide advantages for use as sealant either in place of or in combination with the modified alginates. As such, additional biological compounds including chitosan, gelatin, hyaluronic acid, hydroxyethyl starch, hydroxypropylcellulose, and mucin were synthesized and evaluated by the materials testing approaches above (relevant data incorporated in above Tables and Figures). Notably these include biologic materials and functional modifications being investigated for other biomedical applications. As such, these are all feasible for potential clinical use. Notably, we have also broadened study of the photo-initiators and the photo-activation approach utilized. **These have produced highly promising data on a series of compounds more potent as potential pleural sealants than the originally postulated modified alginate compounds.** These have further allowed us to embark on initial studies in the pre-clinical (rat) non-survival surgery studies described in **Specific Aim 2**.

A schematic of the newly investigated range of compounds under investigation is depicted in **Figure 13**. This has further helped to optimize the materials for use as pleural sealants.



## Conclusions to date from materials testing of other compounds

Collectively, additional biologically inspired materials were investigated for their application as a pleural sealant. Notably, materials that polymerize in response to oxidation were pursued due to their rapid gelation kinetics and the presence oxidative insult in the inflammatory milieu of acute injury. At Akina, preferable bioadhesion was obtained using other biocompounds which possessed heterogeneous side moieties such as amines (chitosan) and polypeptides (gelatin). Likely, the more diverse nature of these moieties allows for more intimate attraction to biological substrates. A summary of the biological relevant properties of gelation and tissue adhesion is depicted in **Table 5** below.

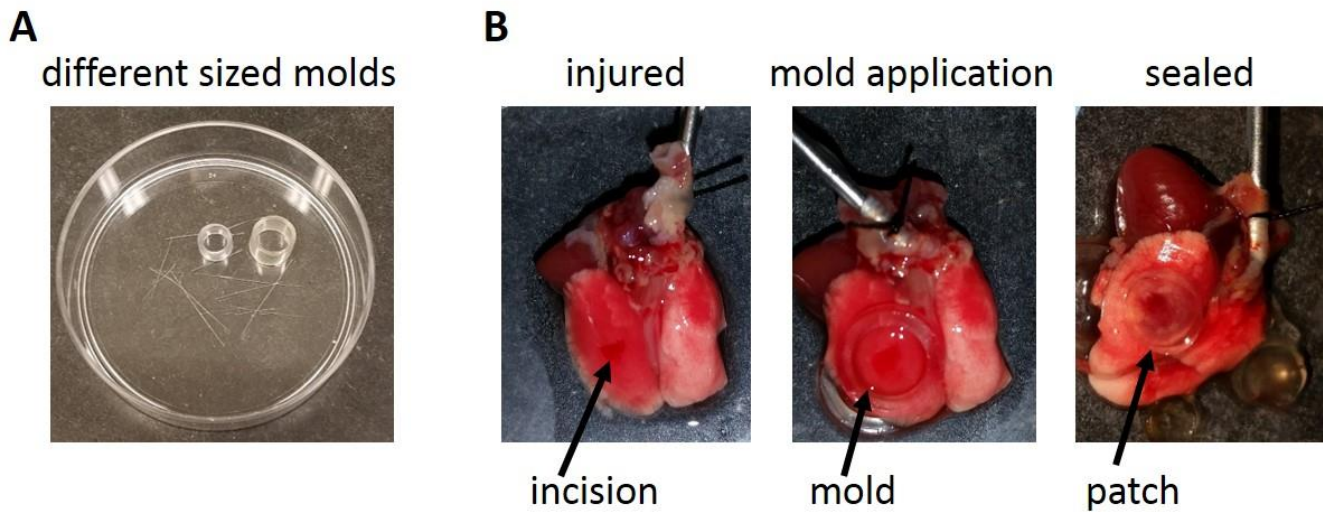


**Table 5: Summary of all materials testing: gelation and bioadhesion**

Precursor system	Exposure time	Visual Result	Bioadhesion (work in mJ to remove)
PolyVivo AI102 lot# 41217FAJ-A at 20% w/v	10 min	Did not gel	NA
PolyVivo AI103 lot# 41218FAJ at 20% w/v	10 min	Did not gel	NA
PolyVivo AI104 lot# 5051323MJK-A at 20% w/v	10 min	Did not gel	NA
PolyVivo AI138 lot# 60623MJK-A	10 min	Gelled	NA
Methacrylated Mucin at 2% w/v*	10 min	Did not gel	NA
Methacrylated HPC-H at 1% w/v*	10 min	Thickens significantly, weak gel	NA
Methacrylated HMW alginate at 2% w/v*	10 min	Did not gel	NA
Methacrylated hyaluronic acid at 1% w/v*	10 min	Thickens significantly, weak gel	NA
PolyVivo AI146 60907BPR-A at 5% w/v	10 min	Gelled	NA
Methacrylated HA UV61010FAJ at 1%	10 min	Gel	NA
Methacrylated HA UV61010FAJ at 1%	1-2 min	Gel	NA
Methacrylated Alginate UV61012SMS at 10%	10 min	Weak Gel	NA
Methacrylated Alginate UV61012SMS at 20%	10 min	Weak Gel	NA
Methacrylated Chondroitin UV61028-FAJ at 10%	10 min	Gel	NA
Gelatin Methacrylate UV61102JSG at 5%	10 min	Gel	2.249 ± 1.476
Methacrylated Alginate UV61116JSG-A at 2%	10 min	Weak Gel	NA
Methacrylated Alginate UV61117BPR-A at 2%	10 min	Does Not Gel	NA
Methacrylated Alginate UV61117DWW-A at 2%	10 min	Gel	NA
Methacrylated Alginate UV61118BPR-A at 2%	10 min	Gel	0.327 ± 0.011
Methacrylated Alginate UV61121SMS at 5%	10 min	No Gel	NA
Methacrylated Alginate UV61121SMS at 10%	10 min	No Gel	NA
Methacrylated Alginate UV61205SMS at 2%	10 min	No Gel	NA
Methacrylated Alginate UV61205SMS at 2%	20 min	Weak Gel	NA
Methacrylated alginate UV61214BPR-A at 2%	10 min	Weak gel	NA
Methacrylated alginate UV70111SMS-A at 2%	10 min	No gelling	NA
4:1 Gelatin methacrylate:Chitosan-methacrylate at 5%	NA	Gelled	1.909 ± 1.209
4:1 Gelatin methacrylate:Alginate-methacrylate at 5%	NA	Gelled	2.003 ± 0.794

**Alg-MA/Alg-Ox Evaluations**

After an incision in the lung the defect was tested for leakage and the volume necessary for leakage assessed. Lungs were then sealed using different modes of application, volumes and crosslinking times. Application inside an o-ring mold was used to prevent running off of the material from the defect location (**Figure 14**). The application without the mold was also done in multiple layers to increase material thickness and reduce material failure. In order to crosslink the individual layers, the material was only partially crosslinked before final crosslinking. The crosslinking time (x+x+y) refers to the time before removal of the mold (x) or crosslinking time between each layer (x) and final crosslinking (y). The defect was either non treated or dabbed dry before material application.



**Figure 14:** A) Different sized molds that were used for liquid application of methacrylated alginate on rodent lungs. B) Successful use of **methacrylated and oxidized alginate** applied to an experimentally injured *ex vivo* ventilated rodent lung. left: injured lobe in which an incision was made using scissors, release of bubbles from the tissue during ventilation was confirmed before mold was applied (middle) and tissue was successfully sealed (right).

Different concentrations and blends of the methacrylated and oxidized alginates were comprehensively tested in multiple iterations. Protocol details are included in the summary tables below (**Table 6**) and include:

**Material(s)**

**Formulation**

**Application method and volume**

**Cross-linking method**

**Time to failure and mode of failure**

**Location of injury in the lung**

**Ventilation parameters including tidal volume, respiratory rate (frequency), and positive end expiratory pressure (PEEP)**

**Table 6: Summaries of *ex vivo* rodent lung testing organized by dates of study**

Sept 2015	application									ventilation		
Materials	formulation	method of application	volume [ul]	crosslinking method	crosslinking time [min]	time before failure [h]	mode of failure	location of injury	defect pre-treatment	volume [ul]	frequency	PEEP
Alg-MA	3% liquid	inside mold	30	photo-crosslinking	3+2	instantly	delamination	right lower lobe (RLL)	none	200	2Hz	none
5 Alg-MA-Ox	3% liquid	inside mold	30	photo-crosslinking	3+2	7+	delamination	RLL	none	200	2Hz	none
10 Alg-MA-Ox	3% liquid	inside mold	30	photo-crosslinking	3+2	≈0.17 (10 min)	partial delamination	RLL	none	200	2Hz	none
5 Alg-Ox: Alg-MA Blend (1:1)	3% liquid	inside mold	30	photo-crosslinking	3+2	≈4.5	delamination	RLL	none	200	2Hz	none
10 Alg-Ox: Alg-MA Blend (1:1)	3% liquid	inside mold	30	photo-crosslinking	3+2	≈24	partial delamination	RLL	none	250	2Hz	none
5 Alg-MA-Ox: Alg-MA Blend (1:1)	3% liquid	inside mold	30	photo-crosslinking	3+2	≈3.17	partial delamination	RLL	none	200	2Hz	none
10 Alg-MA-Ox: Alg-MA Blend (1:1)	3% liquid	inside mold	30	photo-crosslinking	3+2	≈24, lung might have sealed itself	partial delamination	RLL	none	300	2Hz	none
10 Alg-MA-Ox: Alg-MA Blend (1:1)	3% liquid	inside mold	30	photo-crosslinking	3+2	≈0.17 (10 min)	partial delamination	RLL	none	320	2Hz	none
Jan 28 2016	application									ventilation		
Materials	formulation	method of application	volume [ul]	crosslinking method	crosslinking time [min]	time before failure [h]	mode of failure	location of injury	defect pre-treatment	volume [ul]	frequency	PEEP
10 Alg-MA-Ox	3% liquid	inside mold	50	photo-crosslinking	3 + 3	1 breath	partial delamination	RLL	none	250	2Hz	none
10 Alg-MA-Ox	3% liquid	inside mold	50	photo-crosslinking	3 + 3	instantly	partial delamination	RLL	none	250	2Hz	none
10 Alg-MA-Ox	3% liquid	inside mold	50	photo-crosslinking	3 + 4	instantly	partial delamination	RLL	none	250	2Hz	none
10 Alg-MA-Ox	3% liquid	inside mold	50	photo-crosslinking	3 + 4	instantly	partial delamination	left lung (right lung tied off) (LL)	none	250	2Hz	none
10 Alg-MA-Ox blend	3% liquid	inside mold	50	photo-crosslinking	6	1 breath	partial delamination	RLL	none	250	2Hz	none
10 Alg-MA-Ox blend	3% liquid	inside mold	50	photo-crosslinking	3 + 3	instantly	partial delamination	RLL	none	250	2Hz	none
10 Alg-MA-Ox blend	3% liquid	inside mold	50	photo-crosslinking	3 + 3	1 breath	partial delamination	LL	none	250	2Hz	none
Feb 1 2016	application									ventilation		
Materials	formulation	method of application	volume [ul]	crosslinking method	crosslinking time [min]	time before failure [h]	mode of failure	location of injury	defect pre-treatment	volume [ul]	frequency	PEEP
5 Alg-MA-Ox blend	3% liquid	inside mold	50	photo-crosslinking	4 + 3	5 breaths	partial delamination	right upper lobe (RUL)	none	250	2Hz	none
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 3	10 breaths	partial delamination	RLL	none	250	2Hz	none
5 Alg-MA-Ox blend	3% liquid	3 layers	10+10+10	photo-crosslinking	1+1+5	5 min	partial delamination	LL	none	200	2Hz	none
5 Alg-MA-Ox blend	3% liquid	3 layers	10+10+10	photo-crosslinking	1+1+4	20 min	partial delamination	RLL	none	250	2Hz	none
5 Alg-MA-Ox blend	3% liquid	3 layers	10+10+10	photo-crosslinking	1+1+4	1 breath	partial delamination	RLL	none	250	2Hz	none
5 Alg-MA-Ox blend	3% liquid	3 layers	10+10+10	photo-crosslinking	1+1+4	instantly	partial delamination	LL	none	200	2Hz	none
5 Alg-MA-Ox blend	3% liquid	3 layers	10+10+10	photo-crosslinking	1+1+4	instantly	partial delamination	LL	none	200	2Hz	none
5 Alg-MA-Ox	3% liquid	inside mold	30	photo-crosslinking	4	instantly	delamination	LL	none	200	2Hz	none
5 Alg-MA-Ox	3% liquid	inside mold	30	photo-crosslinking	5	instantly	delamination	LL	none	200	2Hz	none
5 Alg-MA-Ox	3% liquid	inside mold	30	photo-crosslinking	5	instantly	delamination	RLL	none	250	2Hz	none
5 Alg-MA-Ox	3% liquid	3 layers	10+10+10	photo-crosslinking	1+1+4	instantly	delamination	RLL	none	250	2Hz	none
5 Alg-MA-Ox	3% liquid	3 layers	10+10+10	photo-crosslinking	1+1+4	instantly	delamination	RLL	none	250	2Hz	none
5 Alg-MA-Ox	3% liquid	3 layers	10+10+10	photo-crosslinking	1+1+4	instantly	delamination	LL	none	200	2Hz	none

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Feb 3 2016	application									ventilation		
Materials	formulation	method of application	volume [ul]	crosslinking method	crosslinking time [min]	time before failure [h]	mode of failure	location of injury	defect pre-treatment	volume [ul]	frequency	PEEP
5 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	10 breaths	partial delamination	RLL	none	200	2Hz	none
5 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	instantly	delamination	RLL	none	250	2Hz	none
5 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	2 breaths	partial delamination	LL	none	225	2Hz	none
5 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	instantly	delamination	LL	none	150	2Hz	none
5 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	2 breaths	delamination	LL	dabbed dry	150	2Hz	none
5 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	1 breath	delamination	RLL	dabbed dry	250	2Hz	none
5 Alg-MA-Ox blend	1.5% liquid	3 layers	10+10+10	photo-crosslinking	1+1+5	2 breaths	delamination	LL	none	150	2Hz	none
5 Alg-MA-Ox blend	1.5% liquid	3 layers	10+10+10	photo-crosslinking	1+1+5	2 breaths	partial delamination	LL	none	200	2Hz	none
5 Alg-MA-Ox blend	1.5% liquid	3 layers	10+10+10	photo-crosslinking	1+1+5	instantly	partial delamination	RLL	dabbed dry	250	2Hz	none
5 Alg-MA-Ox blend	1.5% liquid	3 layers	10+10+10	photo-crosslinking	1+1+5	1 breath	partial delamination	LL	dabbed dry	225	2Hz	none
5 Alg-MA-Ox blend	1.5% liquid	3 layers	10+10+10	photo-crosslinking	1+1+5	10 breaths	partial delamination	LL	dabbed dry	225	2Hz	none

Feb 4 2016	application									ventilation		
Materials	formulation	method of application	volume [ul]	crosslinking method	crosslinking time [min]	time before failure [h]	mode of failure	location of injury	defect pre-treatment	volume [ul]	frequency	PEEP
5 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	1 breath	partial delamination	RLL	none	200	2Hz	none
5 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	instantly	partial delamination	LL	none	150	2Hz	none
5 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	2 breaths	partial delamination	RLL	none	225	2Hz	none
5 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	1h + 10 min	partial delamination	RLL	dabbed dry	200 / 225	2Hz	none
5 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	1 min	partial delamination	LL	dabbed dry	150	2Hz	none
5 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	2 breaths	partial delamination	RLL	dabbed dry	225	2Hz	none
5 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	5 breaths	delamination	LL	dabbed dry	150	2Hz	none
5 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	2 breaths	partial delamination	LL	dabbed dry	175	2Hz	none
5 Alg-MA-Ox	1.5% liquid	3 layers	10+10+10	photo-crosslinking	1+1+4	instantly	partial delamination	RLL	dabbed dry	225	2Hz	none

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Feb 5 2016	application										ventilation		
Materials	formulation	method of application	volume [ul]	crosslinking method	crosslinking time [min]	time before failure [h]	mode of failure	location of injury	defect pre-treatment	volume [ul]	frequency	PEEP	
10 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	instantly	partial delamination	RUL	none	200	2Hz	none	
10 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	instantly	delamination	RLL	none	200	2Hz	none	
10 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	1 min	delamination	L	none	150	2Hz	none	
10 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	10 breaths	partial delamination	RLL	dabbed dry	250	2Hz	none	
10 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	3 breaths	partial delamination	RLL	dabbed dry	250	2Hz	none	
10 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	instantly	partial delamination	L	dabbed dry	200	2Hz	none	
10 Alg-MA-Ox	1.5% liquid	inside mold	30	photo-crosslinking	3 + 3	2 breaths	partial delamination	L	dabbed dry	150	2Hz	none	
10 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	instantly	delamination	RUL	none	200	2Hz	none	
10 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	2 breaths	delamination	RLL	none	200	2Hz	none	
10 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	instantly	delamination	L	none	150	2Hz	none	
10 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	instantly	partial delamination	RUL	dabbed dry	200	2Hz	none	
10 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	instantly	delamination	RLL	dabbed dry	200	2Hz	none	
10 Alg-MA-Ox blend	1.5% liquid	inside mold	30	photo-crosslinking	3 + 4	~4h	partial delamination	L	dabbed dry	150	2Hz	none	

Feb 17 2016	application										ventilation		
Materials	formulation	method of application	volume [ul]	crosslinking method	crosslinking time [min]	time before failure [h]	mode of failure	location of injury	defect pre-treatment	volume [ul]	frequency	PEEP	
PNC MA-AA	3% liquid	inside mold	30	photo-crosslinking	3 + 6	10 breaths	delamination	RUL	dabbed dry	250	2Hz	none	
PNC MA-AA	3% liquid	inside mold	30	photo-crosslinking	3 + 6	instantly	delamination	RLL	dabbed dry	250	2Hz	none	
PNC MA-AA	3% liquid	inside mold	30	photo-crosslinking	3 + 0	-	delamination during	L	dabbed dry	175	2Hz	none	
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 4	10 breaths	partial delamination	RUL	dabbed dry	250	2Hz	2	
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 4	instantly	partial delamination	RLL	dabbed dry	250	2Hz	2	
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 4	> 20h	partial delamination at higher	L	dabbed dry	250/350	2Hz	2	
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 4	instantly	delamination	L	dabbed dry	150	2Hz	2	

Feb 18 2016	application										ventilation		
Materials	formulation	method of application	volume [ul]	crosslinking method	crosslinking time [min]	time before failure [h]	mode of failure	location of injury	defect pre-treatment	volume [ul]	frequency	PEEP	
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 4	5 breaths	partial delamination	RUL	dabbed dry	250	2Hz	2	
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 4	instantly	partial delamination	RLL	dabbed dry	250	2Hz	2	
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 0	-	delamination during	L	dabbed dry	150	2Hz	2	
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 4	5 breaths	partial delamination	RUL	dabbed dry	250	2Hz	-	
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 4	instantly	partial delamination	RLL	dabbed dry	250	2Hz	-	
5 Alg-MA-Ox blend	3% liquid	inside mold	30	photo-crosslinking	3 + 4	> 20h	partial delamination at higher	L	dabbed dry	150 / 250	2Hz	-	

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June 23 2016	application									ventilation		
Materials	formulation	method of application	volume [ul]	crosslinking method	crosslinking time [min]	time before failure [h]	mode of failure	location of injury	defect pre-treatment	volume [ul]	frequency	PEEP
5 Alg-Ox: Alg-MA Blend (1:3)	3% liquid	2 layers	30	photo-crosslinking	3+3	30s	partial delamination	L	dabbed dry	250	2Hz	-
5 Alg-Ox: Alg-MA Blend (1:3)	3% liquid	2 layers	30	photo-crosslinking	3+5	5 breaths	partial delamination	RUL	dabbed dry	230	2Hz	-
5 Alg-Ox: Alg-MA Blend (1:3)	3% liquid	2 layers	30	photo-crosslinking	2+4	instantly	partial delamination	RML	dabbed dry	200	2Hz	-
5 Alg-Ox: Alg-MA Blend (1:3)	3% liquid	inside mold	30	photo-crosslinking	6	instantly	partial delamination	RML	dabbed dry	200	2Hz	-
5 Alg-Ox: Alg-MA Blend (3:1)	3% liquid	2 layers	30	photo-crosslinking	3+3	instantly	partial delamination	L	dabbed dry	250	2Hz	-
5 Alg-Ox: Alg-MA Blend (3:1)	3% liquid	inside mold	30	photo-crosslinking	6	1 breath	partial delamination	RUL	dabbed dry	200	2Hz	-
5 Alg-Ox: Alg-MA Blend (3:1)	3% liquid	inside mold	30	photo-crosslinking	6	instantly	partial delamination	RML	dabbed dry	200	2Hz	-
5 Alg-Ox: Alg-MA Blend (3:1)	3% liquid	2 layers	30	photo-crosslinking	2+4	5 min	partial delamination	RML	dabbed dry	200	2Hz	-
10 Alg-Ox: Alg-MA Blend (1:3)	3% liquid	2 layers	30	photo-crosslinking	2+4	3 breaths	partial delamination	L	dabbed dry	250	2Hz	-
10 Alg-Ox: Alg-MA Blend (1:3)	3% liquid	inside mold	30	photo-crosslinking	6	instantly	partial delamination	L	dabbed dry	250	2Hz	-
10 Alg-Ox: Alg-MA Blend (1:3)	3% liquid	inside mold	30	photo-crosslinking	6	over night	partial delamination	L	dabbed dry	250	2Hz	-
10 Alg-Ox: Alg-MA Blend (3:1)	3% liquid	inside mold	30	photo-crosslinking	6	instantly	partial delamination	L	dabbed dry	250	2Hz	-
10 Alg-Ox: Alg-MA Blend (3:1)	3% liquid	inside mold	30	photo-crosslinking	6	instantly	partial delamination	RUL	dabbed dry	250	2Hz	-
10 Alg-Ox: Alg-MA Blend (3:1)	3% liquid	inside mold	30	photo-crosslinking	6	instantly	partial delamination	RUL	dabbed dry	250	2Hz	-
10 Alg-Ox: Alg-MA Blend (3:1)	3% liquid	2 layers	30	photo-crosslinking	2+4	instantly	partial delamination	RML	dabbed dry	250	2Hz	-

June 27 2016	application									ventilation		
Materials	formulation	method of application	volume [ul]	crosslinking method	crosslinking time [min]	time before failure [h]	mode of failure	location of injury	defect pre-treatment	volume [ul]	frequency	PEEP
5 Alg-MA-Ox: Alg-MA Blend (1:3)	3% liquid	2 layers	30	photo-crosslinking	2+4	instantly	partial delamination	L	dabbed dry	250	2Hz	-
5 Alg-MA-Ox: Alg-MA Blend (1:3)	3% liquid	2 layers	30	photo-crosslinking	2+4	instantly	partial delamination	RUL	dabbed dry	200	2Hz	-
5 Alg-MA-Ox: Alg-MA Blend (1:3)	3% liquid	2 layers	30	photo-crosslinking	2+4	instantly	partial delamination	L	dabbed dry	250	2Hz	-
5 Alg-MA-Ox: Alg-MA Blend (3:1)	3% liquid	2 layers	30	photo-crosslinking	2+4	2.25 h	partial delamination	RUL	dabbed dry	150	2Hz	-
5 Alg-MA-Ox: Alg-MA Blend (3:1)	3% liquid	2 layers	30	photo-crosslinking	2+4	3 breaths	partial delamination	RLL	dabbed dry	150	2Hz	-
5 Alg-MA-Ox: Alg-MA Blend (3:1)	3% liquid	2 layers	30	photo-crosslinking	2+4	instantly	partial delamination	RLL	dabbed dry	150	2Hz	-
10 Alg-MA-Ox: Alg-MA Blend (1:3)	3% liquid	2 layers	30	photo-crosslinking	2+4	instantly	partial delamination	L	dabbed dry	250	2Hz	-
10 Alg-MA-Ox: Alg-MA Blend (1:3)	3% liquid	2 layers	30	photo-crosslinking	2+4	instantly	partial delamination	RUL	dabbed dry	225	2Hz	-
10 Alg-AM-Ox: Alg-MA Blend (1:3)	3% liquid	2 layers	30	photo-crosslinking	2+4	>4h, failed over night	partial delamination	RLL	dabbed dry	150	2Hz	-
10 Alg-MA-Ox: Alg-MA Blend (3:1)	3% liquid	2 layers	30	photo-crosslinking	2+4	10 breaths	partial delamination	L	dabbed dry	250	2Hz	-
10 Alg-MA-Ox: Alg-MA Blend (3:1)	3% liquid	2 layers	30	photo-crosslinking	2+4	1h	partial delamination	RUL	dabbed dry	200	2Hz	-
10 Alg-MA-Ox: Alg-MA Blend (3:1)	3% liquid	2 layers	30	photo-crosslinking	2+4	instantly	partial delamination	RLL	dabbed dry	200	2Hz	-

### Summary of Alginate *Ex Vivo* Rodent Model Testing

Overall the methacrylated and oxidized alginate was not successful in long term sealing of the rodent lungs in the *ex vivo* model and primarily failed due to adhesive failure (full/partial delamination) rather than material rupture. There are multiple reasons for this. First, the oxidized alginate material may not have been sufficiently elastic as the oxidation chemistry reduces the elasticity of the material by reducing chain length of the backbone material. A lower degree of oxidation would therefore be beneficial to retain elasticity which would reduce the stress onto the attachment sites during ventilation because the material will stretch. On the contrary a lower degree of oxidation will even further reduce adhesiveness of the material. Other adhesion strategies and chemical formulations were therefore investigated as detailed below. Secondly, the area for the patch adhesion and the high curvature of the rodent lungs may simply not have been suitable testing approaches to generate data that would be comparable with human applications. **For that reason larger lungs from pigs were used to overcome these limitations and to further test alginate as well as non-alginate compounds.**

### *Ex Vivo* Pig Lung Model Sealant Testing Data: Alginate and Non-Alginate Compounds

In addition to the *ex vivo* rodent model, pig lungs were obtained from a local slaughter house and similarly utilized *in ex vivo* sealant evaluations. The advantage of the pig lungs was a larger surface area in which to apply the different sealant preparations. Representative images are shown in **Figures 15-17** and summary results organized by date of study in **Table 7**. **No IACUC approval was required to study these lungs.**

**Figure 15** (right): Successful use of **methacrylated and oxidized alginate** applied to an experimentally injured *ex vivo* ventilated pig lung. a. Left: uninjured lobe; Middle: injured lobe in which an incision was made using scissors, bubbles were released from the tissue during ventilation; Right: lobe after sealing, no air bubbles were released from the sealant during ventilation indicating successful closure.



**Figure 16** (above): A demonstration of the adhesive quality of a dopamine conjugated alginate patch applied to the pleural surface.



**Figure 17:** Composite methacrylated and oxidized alginate patches remain adherent and airtight 24 hours after application with continuous ventilation at physiologic pressures.



**Table 7: Summary of *ex vivo* pig lung testing of alginate and non-alginate compounds**  
All compounds applied as 3% solutions

July 26 2016								ventilation			
Nr	Materials	Method of Application	Volume (uL)	Crosslinking Method	Crosslinking Time (min)	Time Before Failure (h)	Mode of Failure	Tidal Volume (mL)	PIP (cmH2O)	Frequency (breaths/min)	PEEP (cmH2O)
1	10 Alg-Ox: Alg-MA Blend (1:1)	liquid	100	Photo-crosslinking	6	Instant	Complete Delamination	13	20	20	5
2	10 Alg-Ox: Alg-MA Blend (1:1)	liquid	100	Photo-crosslinking	6	2 min	Partial Delamination	13	20	20	5
3	10 Alg-Ox: Alg-MA Blend (1:3)	liquid	100	Photo-crosslinking	6	10 min	aborted	13	20	20	5
4	10 Alg-Ox: Alg-MA Blend (1:3)	liquid	100	Photo-crosslinking	6	10 min	aborted	13	20	20	5
5	10 Alg-Ox: Alg-MA Blend (1:3)	liquid	100	Photo-crosslinking	6	3 min	Partial Delamination	80	20	20	5
6	10 Alg-Ox: Alg-MA Blend (1:3)	liquid	30	Photo-crosslinking	6	Instant	Incomplete coverage	80	20	20	5

July 29 2016								ventilation			
Nr	Materials	Method of Application	Volume (uL)	Crosslinking Method	Crosslinking Time (min)	Time Before Failure (h)	Mode of Failure	Tidal Volume (mL)	PIP (cmH2O)	Frequency (breaths/min)	PEEP (cmH2O)
1	10 Alg-MA-Ox: Alg-MA Blend (3:1)	liquid	100	Photo-crosslinking	6	>24 h	aborted	80	30	20	5
2	10 Alg-MA-Ox: Alg-MA Blend (3:1)	liquid	100	Photo-crosslinking	6	>24 h	aborted	80	30	20	5
3	10 Alg-MA-Ox: Alg-MA Blend (3:1)	liquid	100	Photo-crosslinking	6	>24 h	aborted	80	30	20	5
4	10 Alg-Ox: Alg-MA Blend (3:1)	liquid	100	Photo-crosslinking	6	>24 h	aborted	80	30	20	5
5	10 Alg-Ox: Alg-MA Blend (3:1)	liquid	100	Photo-crosslinking	6	>24 h	aborted	80	30	20	5
6	10 Alg-Ox: Alg-MA Blend (3:1)	liquid	100	Photo-crosslinking	6	>24 h	aborted	80	30	20	5



8/24/2016						ventilation					
Nr	Materials	Method of Application	Volume (uL)	Crosslinking Method	Crosslinking Time (min)	Time Before Failure (h)	Mode of Failure	Tidal Volume (mL)	PIP (cmH2O)	Frequency (breaths/min)	PEEP (cmH2O)
1	7% Gelatin-MA : 10% Chitosan-MA (4:1)	liquid	100	Photo-crosslinking	6	instant	incomplete defect coverage	70	30	15	5
2	7% Gelatin-MA : 10% Chitosan-MA (4:1)	liquid	100	Photo-crosslinking	6	10 min	partial delamination	70	30	15	5
3	7% Gelatin-MA : 10% Chitosan-MA (4:1)	liquid	100	Photo-crosslinking	6	10 min	partial delamination	70	30	15	5
4	7% Gelatin-MA : 10% Chitosan-MA (4:1)	liquid	100	Photo-crosslinking	6	1 h	partial delamination	70	30	15	5
5	7% Gelatin-MA : 10% Chitosan-MA (4:1)	liquid	100	Photo-crosslinking	6	1 h	partial delamination	70	30	15	5
6	7% Gelatin-MA : 10% Chitosan-MA (4:1)	liquid	100	Photo-crosslinking	6	1 h	partial delamination	70	30	15	5
7	7% Gelatin-MA : 10% Chitosan-MA (1:1)	liquid	100	Photo-crosslinking	6	1 min	complete delamination	70	30	15	5
8	7% Gelatin-MA : 10% Chitosan-MA (1:1)	liquid	100	Photo-crosslinking	6	1 min	complete delamination	70	30	15	5
9	7% Gelatin-MA : 10% Chitosan-MA (1:1)	liquid	100	Photo-crosslinking	6	1 min	complete delamination	70	30	15	5

9/20/2016						ventilation					
Nr	Materials	Method of Application	Volume (uL)	Crosslinking Method	Crosslinking Time (min)	Time Before Failure (h)	Mode of Failure	Tidal Volume (mL)	PIP (cmH2O)	Frequency (breaths/min)	PEEP (cmH2O)
1	15% Alginate-Ox, 20% Al 138 PEG/PGLA	liquid	100	Photo-crosslinking	6	instant	complete delamination	70	30	15	5
2	15% Alginate-Ox, 20% Al 138 PEG/PGLA	liquid	100	Photo-crosslinking	6	instant	complete delamination	70	30	15	5
3	15% Alginate-Ox, 20% Al 138 PEG/PGLA	liquid	100	Photo-crosslinking	6	instant	complete delamination	70	30	15	5

10/7/16						ventilation					
Nr	Materials	Method of Application	Volume (uL)	Crosslinking Method	Crosslinking Time (min)	Time Before Failure (h)	Mode of Failure	Tidal Volume (mL)	PIP (cmH2O)	Frequency (breaths/min)	PEEP (cmH2O)
1	10% Alg-SH, 10% Chitosan	liquid	200	Oxidation	1	5 min	Did not form gel	15	20	15	5
2	10% Alg-SH, 10% Chitosan	liquid	200	Oxidation	1	5 min	Did not form gel	15	20	15	5
3	10% Alg-SH, 10% Chitosan	dried patch	200	Oxidation	1	2 h	aborted	15	20	15	5

12/14/16						ventilation					
Nr	Materials	Method of Application	Volume (uL)	Crosslinking Method	Crosslinking Time (min)	Time Before Failure (h)	Mode of Failure	Tidal Volume (mL)	PIP (cmH2O)	Frequency (breaths/min)	PEEP (cmH2O)
1	4% Alg-DA	dried patch	100 uL	Oxidation	1	2 h	material failure	70	20	15	5
2	3% Chitosan-PCA	dried patch	100 uL	Oxidation	1	24 h	aborted	70	20	15	5
3	3% Chitosan-PCA	dried patch	100 uL	Oxidation	1	24 h	aborted	70	20	15	5

### Summary of *ex vivo* pig lung testing

Testing of various sealant materials in an *ex vivo* pig lung model has demonstrated multiple promising modified alginate and non-alginate based compounds that could be used as pleural sealants. Materials such as mixed methacrylated and oxidized alginates as well as chitosan-protocatechuic acid showed durable adhesion, withstanding ventilation for over 24 hours with good adherence. While the alginate-dopamine patches underwent material failure after two hours of ventilation, they proved to be extremely adherent to pleura shortly after patch application. Ongoing testing will identify the specific formulation of materials that harnesses each these properties in one effective pleural sealant.

Notably the modified alginates performed better in *ex vivo* pig as compared to rodent lungs. We hypothesize that this reflects a larger surface area and lower radius of curvature that allows initial adhesion.

**Summary: Specific Aim 1**

<p><b>Specific Aim 1(specified in proposal)</b></p> <p><b>To optimize the modified alginate for use as a pleural sealant</b></p>
<p><b>Major Task 1: Develop chemically modified alginate (AA-MA) hydrogels and characterize material properties.</b></p>
<p>Subtask 1: Synthesize and chemically characterize AA-MA polymer formulations.</p>
<p>Subtask 2: Quantify the viscosity and shear mechanical properties of AA-MA solutions and hydrogels.</p>
<p>Milestone(s) Achieved: An elastic AA-MA hydrogel will be fabricated with controllable degrees of methacrylation and crosslinking.</p>

<p><b>Major Task 2: Assess the burst pressure strength and adhesiveness of AA-MA hydrogel sealants.</b></p>
<p>Subtask 1: Measure burst pressure and analyze cohesion and adhesion of AA-MA hydrogels on collagen substrates.</p>
<p>Subtask 2: Synthesize AA-MA hydrogels with the ability to covalently link to tissue proteins or create cell-material linkages.</p>
<p>Milestone(s) Achieved: AA-MA hydrogel sealant will exhibit burst pressures beyond the physiological range and will remain adhered to underlying substrate/tissue up to burst pressure.</p>

All milestones were met for **Specific Aim 1** although there is continued room for improvement in synthesizing compounds that have desirable burst pressure and adhesive compounds. To this end, as discussed and demonstrated above, we have embarked on a systematic exploration of other biologic compounds and also other functional modifications that have produced even more promising results in materials testing and in *ex vivo* lung model evaluations.

**Specific Aim 2: To assess the use of optimized modified alginates in an *in vivo* rat lung injury model**

Major Task 1: Assess different modified alginate hydrogels and patches in an open-chest *in vivo* rat model.

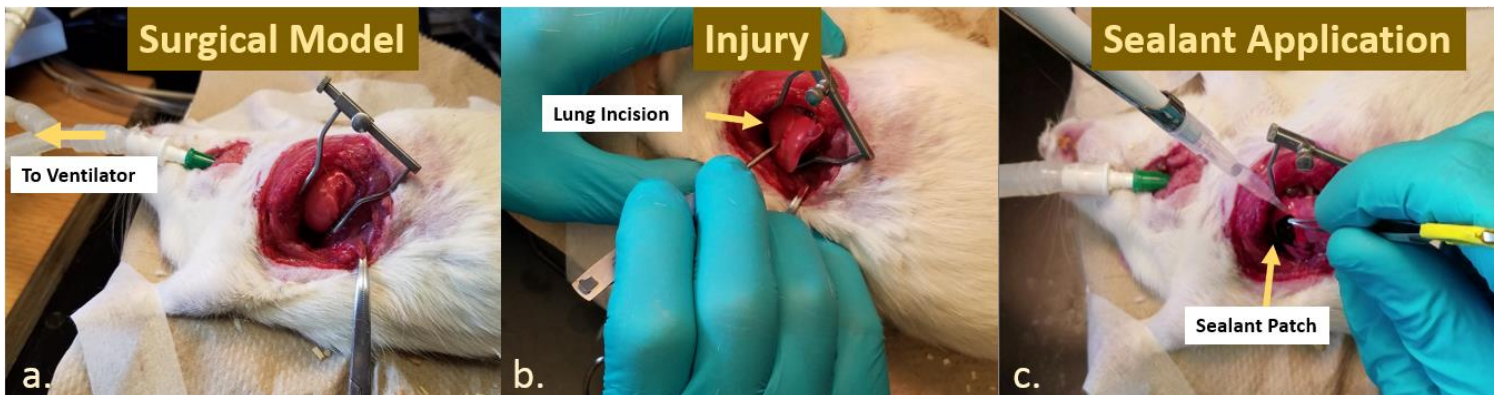
Subtask 1: Assess different alginate formulations in the non-survival rat surgery model: evaluation of lung mechanics.

Subtask 2: Assess different alginate formulations in the non-survival rat surgery model: histologic evaluation of lung tissues.

Milestone(s) Achieved: An elastic AA-MA hydrogel will be fabricated which completely seals a lung leak and is durable.

**Non-survival surgery results with new compounds**

All procedures are performed under a UVM IACUC and a DOD ACURO-approved protocol by appropriately trained laboratory personnel. **Figure 18** depicts the experimental approach. Anesthetized rats undergo tracheal cutdown and cannulation and are mechanically ventilated for a 2 hour period. Once the animal is stabilized on the ventilator under appropriate anesthesia, an incision is made in one lung lobe with resulting air leak. The sealant under study is applied and the animal observed for the remainder of the 2 hours period for development of air leaks and for potential failure (material or adhesion) of the sealant.



**Figure 18:** (above) *In vivo* model for testing pleural sealant. A thoracotomy is performed on a anesthetized ventilated live rat (a), an incision is made to induce pleural air leak (b), and sealant material [liquid, aerosolized, or solid as depicted] is applied to the defect (c)

**Table 8: Summary of initial *in vivo* non-survival surgery evaluations**

12/16/2016		Application							Ventilation				
Nr	Material	Application Method	Defect Size (mm)	Oxidant	Volume (ul)	Crosslinking Time (min)	Time Before Failure	Mode of Failure	Rat weight (g)	Tidal Volume (mL/100g)	Paralysis	Frequency	PEEP (cmH2O)
1	1:1 Alg-DA:Alg-MA	dried patch	2	Sodium Meta-periodate	100	1	1 min	material failure	500	0.29	no	100	10
2	Chitosan-PCA	dried patch	2	Sodium Meta-periodate	100	1	1 min	terial failu	495	0.29	no	100	10
3	Alg-DA	dried patch	2	Sodium Meta-periodate	100	1	instant	terial failu	485	0.29	no	100	10

### **Summary of *in vivo* non-survival rat model of pleural sealant:**

Three formulations that had proven to be promising in the *ex vivo* pig model, including alginate and non-alginate patches, were tested on anesthetized and ventilated rats in this *in vivo* rat model. Each patch initially withstood ventilation at physiologic airway pressures once applied. However, upon performing a breath hold at 30 cmH<sub>2</sub>O, we observed material failure of each patch. However, following this series of experiments we harvested the rat lungs and were able to reproduce the success observed using *ex vivo* pig lungs with the rat lungs also *ex vivo*. We therefore surmised that the application process during the three non-survival rat surgeries within the rat thorax was sub-optimal and limited by ongoing ventilation and limited exposure in the operative field. This led to poor adhesion and poor structural integrity of the applied patches. We plan to optimize this application process using ventilated cadaver rats in our lab before proceeding with further *in vivo* non-survival rat surgeries.

### **References**

- 1) Charron PN, Fenn SL, Poniz A, Oldinski RA. Mechanical properties and failure analysis of visible light crosslinked alginate-based tissue sealants. *J Mech Behav Biomed Mater.* 2016 Jun;59:314-21.

### **What opportunities for training and professional development has the project provided?**

Nothing to report.

### **How were the results disseminated to communities of interest?**

Nothing to report

### **What do you plan to do during the next reporting period to accomplish the goals?**

As detailed above and in the **Other achievements** section, we have made significant advances in the range of materials being evaluated. This now includes a wider range of biologic materials, including chitosan, hyaluronate, gelatin, and others coupled with a wider spectrum of functional modifications and functional groups as detailed in the below table. Once a comprehensive evaluation of these new compounds and new approaches has been performed, results will be submitted for peer-reviewed publication and also for presentation at relevant national/international meetings.

### **4) Other achievements. Include a discussion of stated goals not met.**

#### Stated goals not met

Although we have made significant progress, optimization of a modified (methacrylated/oxidized) alginate formulation that met all stated goals/milestones of **Specific Aims 1 and 2** is still in progress. As such, there was no relevance in pursuing **Specific Aim 3** evaluating the compounds in a pre-clinical non-survival surgery rodent model.

#### Other achievements

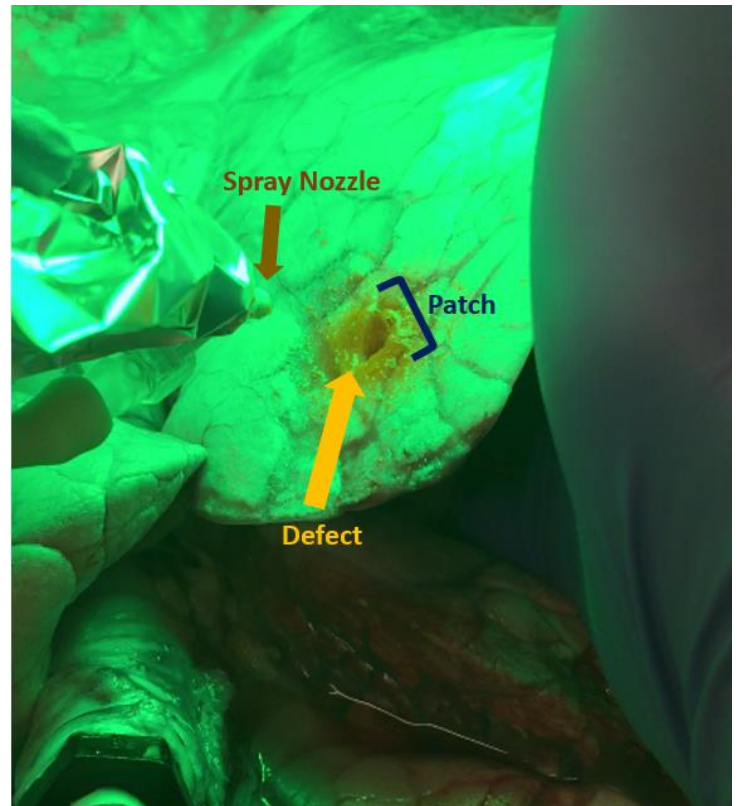
As discussed and described above, we have successfully embarked on evaluation of a larger range of biologic materials and of functional modifications with promising results. This has involved successful use of *ex vivo* lung testing using both rat and pig models to complement the materials characterization and to provide a firm basis for *in vivo* non-survival and survival testing.

As further discussed below, we have also expanded the scope of original studies to include aerosol application of proposed sealant materials to the external pleural surface and also endoscopic (bronchoscopic) administration directly to the airways. Relevant initial techniques and data are depicted below.

**Application of pleural sealants in aerosol form**

To optimize use of the proposed new pleural sealant formulations, one application method could involve administering the compound as an aerosolized liquid followed by rapid gelation on the lung surface. While not part of the original DOD proposal, this is a logical extension of those studies. We have promising pilot data depicted below (**Figure 19, Table 9**). This application method requires further experimentation to determine the optimal timing of cross-linking, whether by photo-initiation (cross-linking) or by oxidation, so that the applied material forms a homogenous liquid that quickly gels on the tissue rather than forming aerosolized gel beads prior to homogenizing on the lung surface. This method of application is promising in terms of ease of use, as well as the uniform nature of the patch it produces.

**Figure 19:** Oxidized and methacrylated alginate applied as an aerosol to a pleural defect (*ex vivo* pig lung model) and crosslinked via green light.



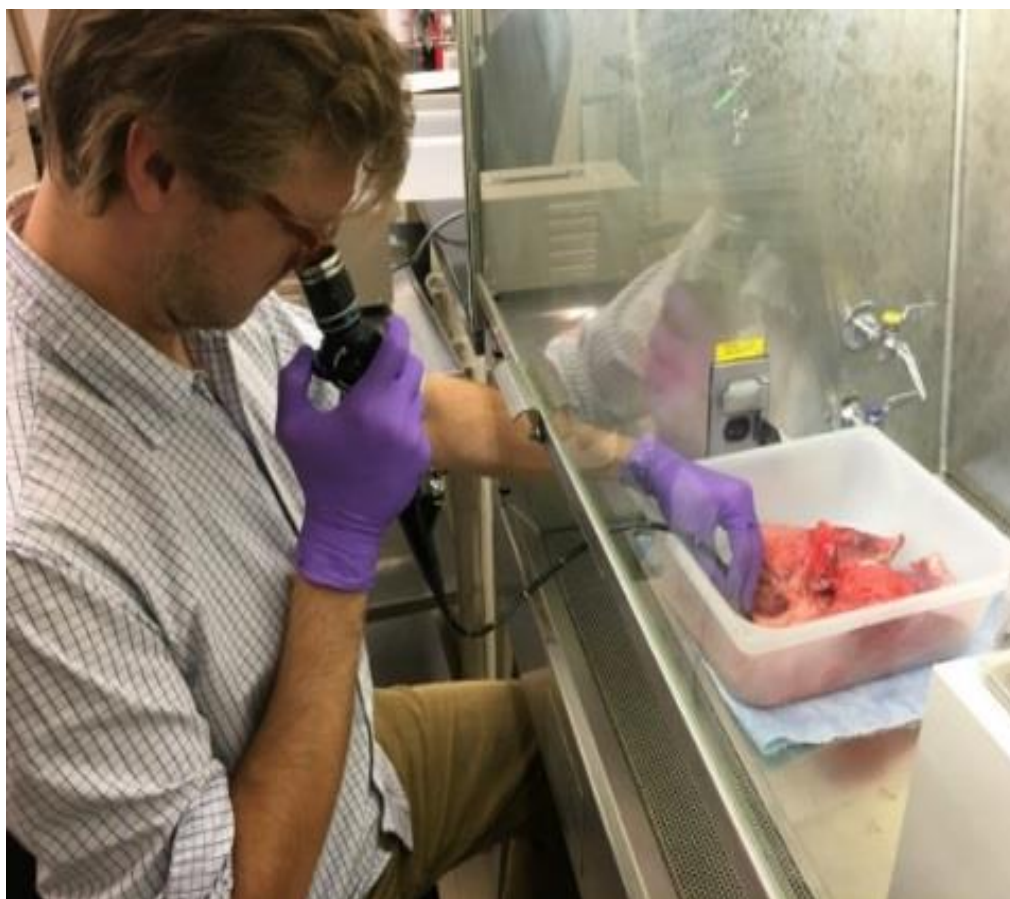
**Table 9: Summary of pilot aerosol sealant application in the *ex vivo* pig lung model**

12/2/16								ventilation			
Nr	Materials	Method of Application	Volume (uL)	Crosslinking Method	Crosslinking Time (min)	Time Before Failure (h)	Mode of Failure	Tidal Volume (mL)	PIP (cmH2O)	Frequency (breaths/min)	PEEP (cmH2O)
1	5% Alg-MA, 3% chitosan	aerosolized	1 mL	Photo-croslinking	5 min	instant	material failure	70	20	15	5
2	5% Alg-MA, 3% chitosan	aerosolized	1 mL	Photo-croslinking	5 min	instant	material failure	70	20	15	5

## Endobronchial application of pleural sealants

To further optimize potential uses of the proposed new pleural sealant formulations, endobronchial application is another route for administration. This has been tried with a variety of previous compounds without success. While also not part of the original DOD proposal, this is a logical extension of those studies. Regarding this avenue of application, we are still in the process of optimizing extrusion of viscous fluids through the narrow lumen of a bronchoscope. Once this is achieved we plan to begin testing endo-luminal application of the various sealant materials in an *ex vivo* pig lung model.

**Figure 20: Experimental set-up for endobronchial administration of sealants in the *ex vivo* pig lung model**



#### **4) Impact**

What was the impact on the development of the principal discipline(s) of the project?

We have made significant and promising progress in the overall goal of developing an effective pleural sealant. This involves developing new investigatory techniques, use of novel materials, and exploration of different application methods. We are optimistic that these will result in a clinically applicable product that can be further investigated in clinical trials.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

#### **5) Changes/Problems**

Changes in approach and reasons for change

Two significant changes occurred:

- 1) Change in personnel with removal of previous co-investigator Rachael Oldinski and associated technician Patrick Charron and replacement of services to have been rendered with a vendor to biomaterials company Akina Inc.. This was discussed with and approved by the DOD on 5-12-16.
- 2) Expanding scope of investigations to incorporate additional materials, functional modifications, and experimental approaches. As detailed above, these are all logical extensions of the original proposal and remain completely within the spirit and scope of the proposal.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing significant to report

Changes that had a significant impact on expenditures

As above, Akina Inc. was added as a vendor in place of original co-investigator Oldinski. This was a positive change and allowed more extensive and timely development and testing of materials for less overall expenditure. As such, a portion of the grant funds remained unused at the end of the original grant period and was approved by the DOD on 1-17-17 for carry forward for a 6 month period.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

#### **6) Products**

Nothing to report as yet under any category



**7) Participants and Other Collaborating Organizations**

Name: Daniel J. Weiss MD PhD
Project Role: PI
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1.2
Contribution to Project: design, implementation, analyses, reporting
Funding Support: DOD

Name: Racheal Oldinski
Project Role: co-investigator. Removed from project as of 5-16-16
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1.2 calendar months while on the award, annualized to 1.15 months
Contribution to Project: original proposal development
Funding Support: DOD

Name: Patrick Charron MS
Project Role: technician. Removed from the project as of 5-16-16
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1.2 calendar months while on the award, annualized to 1.15 months
Contribution to Project: helped develop initial preliminary data
Funding Support: DOD

Name: Keara McElroy-Yeagy
Project Role: technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 1.2 calendar months
Contribution to Project: technical assistance
Funding Support: DOD

Name: Jacob Dearborn BA
Project Role: technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 6 calendar months while on the award, annualized to 0.75 months
Contribution to Project: technical assistance
Funding Support: DOD

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

The PI, Dr. Daniel Weiss has received several additional grants since the last interim report (Quad Chart of 5-22-16). None are relevant for the current DOD proposal.

- 1) **NIH R21** (DJ Weiss, P Lee, Co-PIs) 04/01/2017-03/31/2019 1.2 calendar  
 \$125,000 annual direct for Year 1, \$150,000 annual direct for Year 2

*Decellularized Avian Lungs for Use in Pulmonary Therapeutics*

The major goals of this project are to fully characterize de- and recellularization of representative avian lungs and to develop initial technologies for novel Avian Lung Assist Devices (ALAD) incorporating recellularized avian lungs that could be potentially utilized for independent, portable, or implantable lung assist devices.

- 2) **Cystic Fibrosis Research Grant** (DJ Weiss, PI) 11/1/2016-10/31/2018 0.90 calendar  
 \$100,000 annual direct

*Mechanisms of MSC Actions that Ameliorate Bacterial Lung Infections in CF*

The major goal of this project is to further understand the mechanisms by which the mesenchymal stromal cells (MSCs) may be beneficial in treating bacterial infections in lungs of CF patients and use this to maximize any potential therapeutic approaches.

One grant has run its course

- 1) **Vermont Cancer Center Pilot Award** (Weiss, PI) 6/15/2015-6/14/2016 <0.6 calendar  
 \$50,000 direct

*Development of novel 3D bioprinted scaffolds for reconstructive use in breast cancer patients*

The goal of this project is to perform initial materials studies for use in 3D bioprinting of customized breast implants for mastectomy patients.

What other organizations were involved as partners?

Akina Inc. (West Lafayette IN) added as a vendor as approved by the DOD on 5-16-16.

**Organization Name:** Akina Inc.

**Location of Organization:** West Lafayette IN

**Partner's contribution to the project (identify one or more)**

**Financial support:** N/A

**In-kind support:** Akina manufactures and tests materials used for evaluation as pleural sealants

**Facilities:** Akina Inc. facilities are utilized for manufacture and materials evaluations of compounds to be tested at UVM in lung injury models

**Collaboration:** Akina Inc. personnel, led by John Garner, work closely and extensively with Dr. Weiss and his team at UVM

**Personnel exchanges:** N/A

**Other:** Akina in collaboration with Dr. Weiss and his team at UVM has submitted an STTR application to the NIH based on work performed under the auspice of the current DOD grant. Further applications by Dr. Weiss and Akina Inc to the DOD will be pursued.

## 8) Special Reporting Requirements

Collaborative awards: N/A

Quad Charts: see below

### PR141815 - "Development of a Novel Alginate-Based Pleural Sealant"

PI: Daniel J. Weiss MD PhD University of Vermont College of Medicine

Budget: \$200,000 Topic Area: Respiratory Health Mechanism: Medical Discovery Award



**Research Area(s):** Respiratory Health: Pleural Sealants

**Award Status:** Open; POP:

#### **Study Goals:**

The overall goal is to develop a novel, effective, and easy to use modified alginate-based pleural sealant for use in traumatic and other lung injuries

#### **Specific Aims:**

- (1) To optimize the modified alginate for use as a pleural sealant
- (2) To assess the use of optimized modified alginates in an *in vivo* rat lung injury model

#### **Key Accomplishments:**

1. Continued systematic evaluation of modified alginates and other biologic compounds in *in vitro* testing
  - A) Systematic synthesis of compounds with different forms of chemical modifications
  - B) Systematic materials testing of modified alginate formulations
    1. Burst pressure/adhesion
    2. Gelation/viscosity
2. Continued systematic evaluation of modified alginates in *ex vivo* rodent and pig lung models
3. Initial testing of promising compounds in an *in vivo* non-survival surgery rat lung model
4. Development of novel application methods: external aerosol spraying, endobronchial (bronchoscopic)

#### **Key Outcomes:**

1. Progress towards identification of optimized formulations for use in *in vivo* test models

## 9) Appendices: N/A