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14. ABSTRACT
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. The current report details progress made since the inception of DOD grant support.

15. SUBJECT TERMS
Lung, lung health, respiratory health, lung disease, pneumothorax, pleural sealant

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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 previous studies, in part supported by a Peer Reviewed Medical Research Program Discovery Award, "Development of a Novel Alginate-Based Pleural Sealant" (W81XWH-15-1-0107, Principal Investigator DJ Weiss, 2014-2017), we had identified several promising compounds, notably dopamine-conjugated methacrylated alginate (ALG-MA-DA) and also dopamine-conjugated methacrylated gelatin (GEL-MA-DA) that are being further evaluated as part of the current DOD support. This includes extensive materials characterization, initial evaluations in small (rodent) and large (pig) *ex vivo* lung models, initial *in vivo* evaluations of several compounds in a non-survival and survival surgery rat and pig lung injury models, initial evaluations in survival surgery models of rat and pig tracheal injuries, and initial evaluations in *ex vivo* human lungs. Results of these initial investigations have recently been published (Gasek et al. Development of Alginate and Gelatin-Based Pleural and Tracheal Sealants, Acta Biomaterialia, in press 2021, UI: 34245891).

The goal of the continuing studies is to continue materials optimization, continue small animal (rat) pleural and pig pleural injury survival studies, initiate the survival pig pleural and tracheal studies, and initiate the *ex vivo* human lung studies. The overall goal is to provide a firm platform for initial discussions with the FDA about new INDs 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 (see next pages)
Participating Sites:
1) University of Vermont and State Agricultural College
85 South Prospect Street, Burlington, VT 05405

2) University of Connecticut
Department of Pediatrics, CT Children's Surgical Research Laboratory, 263 Farmington Avenue, Farmington, CT 06030-1319

3) Akina, Inc. 3495 Kent Avenue, West Lafayette, IN 47906

PI: Daniel J. Weiss MD PhD UVM (DW)
Partnering PI: Christine Finck MD UConn (CF)
Partnering Contracted Vendor: John Garner PhD Akina Inc. (JG)
Post-Doctoral Associates: Robert Pouliot PhD UVM (RP), Ishna Sharma MD UConn (IS)
Laboratory Technicians: Evan Hoffman UVM (EH), Todd Jensen UConn (TJ), Nirav Daphthary (ND)
Animal Technicians: Stephen Bell UVM (SB), Sheila Russell UVM (SR)
Thoracic Surgical Consultant: Bruce Leavitt MD UVM (BL)

Additional personnel since last progress report: Lori Asarian PhD: technician UVM (LA), Keara McElroy-Yaggy: part-time animal technician UVM (KY), Amie Tyler: technician Akina (AT), Fuyuki Hirashima MD: thoracic surgery consultant UVM (FH).

Specific Aim 1(specified in proposal) **To optimize manufacturing, sterilization, preservation, and storage conditions of pre-formed ALG-MA-DA patches**	**Timeline** 1-36	**Sites 1,3**
Major Task 1: Standardize synthesis and characterization of ALG-MA-DA patches	Months	
Subtask 1: Develop large scale synthesis approach for ALG-MA-DA patches	1-6	JG
Subtask 2: Standardize characterization of ALG-MA-DA patches: NRM, FTIR	1-12	JG
Milestone(s) Achieved: Standardized large scale ALG-MA-DA patch synthesis with controllable, reliable, and reproducible degrees of methacrylation and dopamine conjugation.	By Month 12	JG
Major Task 2: Develop quality control approaches for rheologic and mechanical characterization of ALG-MA-DA patches.	Months	
Subtask 1: Burst pressure and analyze cohesion and adhesion assessments on collagen substrates.	1-12	JG
Subtask 2: Degradation of standardized patches Subtask 3: Cytotoxicity of standardized patches	1-12	JG JG, DW, EH
Milestone(s) Achieved: Reliable and reproducible degradation and lack of cytotoxicity.	1-24	JG, DW, EH
Major Task 3: Define optimal sterilization, and storage conditions.	Months	
Subtask 1: Define optimal sterilization approach for optimized patch	1-24	JG
Subtask 2: Clarify need for addition of preservatives to optimized patch Subtask 3: Define optimal long term storage (packaging, temperature, humidity, etc) conditions	1-36 1-36	JG
Milestone(s) Achieved: Optimal sterilization and storage conditions	By month 36	JG

Specific Aim 2(specified in proposal) **To define long term efficacy and safety in longitudinal small (rat) and large (pig) models of adult pleural injury and of adult and pediatric tracheal injuries.**	**Timeline** 1-36	**Sites 1,2**
Major Task 1: Assess longitudinal efficacy and safety in adult pleural and tracheal injury models	Months	
Subtask 1: Small animal (rat). Includes post-operative behavioral observation, chest radiographs (CT), lung mechanics evaluation, histologic and toxicologic evaluations.	1-36	DW, BL, EH, SB, SR, CF, TJ, IS
Subtask 2: Large animal (pig). Includes post-operative behavioral observation, chest radiographs (CT), lung mechanics evaluation and toxicologic evaluations.	6-36	DW, BL, EH, SB, SR, CF, TJ, IS
Milestone(s) Achieved: Define longitudinal efficacy and safety in adult rat pleural injury models	By month 36	DW, BL, EH, SB, SR, CF, TJ, IS, ND
Milestones Achieved Local IRB/IACUC and HRPO/ACURO approvals	Will be obtained prior to institution of animal studies	DW, CF
Major Task 2: Assess longitudinal efficacy and safety in pediatric tracheal injury models	Months	
Subtask 1: Small animal (rat). Includes post-operative behavioral observation, chest radiographs (CT), lung mechanics evaluation, histologic and toxicologic evaluations.	1-36	CF, TJ, IS
Subtask 2: Large animal (pig). Includes post-operative behavioral observation, chest radiographs (CT), lung mechanics evaluation and toxicologic evaluations	6-36	DW, BL, EH, SB, SR, CF, TJ, IS
Milestone(s) Achieved: Demonstration of ALG-MA-DA patch longitudinal efficacy and safety in pediatric tracheal injury models	By month 36	DW, BL, EH, SB, SR, CF, TJ, IS

Specific Aim 3 (specified in proposal) **To assess short term efficacy in a pleural injury model in ex vivo ventilated normal and diseased (COPD/emphysema) human lungs**	Timeline 1-36	Site 1
Major Task 1: Assess short term ALG-MA-DA patch efficacy in ex vivo ventilated human autopsy lungs	Months	
Subtask 1: Normal lungs	1-36	DW, EH
Subtask 2: COPD lungs Milestone(s) Achieved: Demonstration of patch adherence and absence of air leak over a 24 hour period	By month 36	DW, EH

What was accomplished under these goals?

1) Major activities

We have continued to make significant progress towards milestones for all three Major Tasks in **Specific Aim 1**. Further, despite restrictions on laboratory research at the participating sites in the setting of the COVID-19 pandemic, we have initiated and made significant progress in **Major Task 1** in **Specific Aim 2**. This in particular involves utilizing the improved materials developed through **Specific Aim 1**. COVID restrictions have also limited progress for **Major Task 2** in **Specific Aim 2** and for **Specific Aim 3** but we anticipate initiating these studies by late summer/early Fall 2021. The accomplishments are listed in brief below and fully detailed in subsequent sections.

Specific Aim 1

- Developed improved synthesis methods for ALG-MA-DA and GEL-MA-DA: increased reproducibility and reliability
- Developed improved chemical and mechanical characterizations of both materials
- Developed improved manufacturing of ready-to-go patches of both materials
- Further characterization of potential cytotoxicity
- Continuing evaluations of sterilization methods

Specific Aim 2

- Continued *ex vivo* studies on rat and pig lungs with new materials: enhanced performance
- Nearly completion of non-survival pleural injury surgery studies in rats: pressure volume curve analyses demonstrate efficacy and reliability of both sealant materials
- Initiated long term survival surgery for pleural injuries in adult rats: animals for 1 year, 6 month, and 3 month analyses currently under way.
- Initiated long term survival surgery for tracheal injuries in adult rats: animals for 1 year, 6 month, and 3 month analyses currently under way
- Initial adult pig pleural and tracheal injury survival surgeries to commence August-September 2021

Specific Aim 3

- Initial human lung studies to commence August 2021

2) Specific objectives

The major objective of the proposal is to develop a pleural and tracheal 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 data at the time of proposal submission, the proposal was initially focused on dopamine-conjugated methacrylated alginate (ALG-MA-DA), however, we have expanded to incorporate investigations of another promising material, dopamine-conjugated methacrylated gelatin (GEL-MA-DA). Continued study of the ALG-MA-DA and GEL-MA-DA formulations in **Specific Aim 1** has focused on optimizing manufacturing and quality control with focus on eventual large scale manufacturing. These studies have identified a number of issues including but not restricted to control of manufacturing conditions (oxidation, pH), optimization of the degree of dopamine conjugation, and identification of alternative more effective cross-linking reagents (Major Task 1).

We have further continued with burst pressure and other mechanical evaluations of the new preparations of both sealants, including tensile, peel, and shear strength evaluations and in particular have developed enhanced properties compared to and sometimes exceeding those of the only currently available lung sealant, Progel™ (Major Task 2). We have also developed a novel oscillatory burst pressure technique that is more applicable for tissue sealants for dynamic tissues such as lung compared to current ASTM static burst pressure testing (Major Task 2). We have also generated initial data on sterilization, storage and preservation conditions for the ALG-MA-DA patches (Major Task 3).

The objectives for **Specific Aim 2** are to demonstrate efficacy and safety in *in vivo* models of both pleural and tracheal injuries in adult small (rat) and large (pig) animal models. An additional goal is to evaluate sealant efficacy in a large animal model (pig) of juvenile tracheal injury in order to assess potential use in repair of congenital tracheal defects. We have now completed a series of *in vivo* studies in adult rats (non-survival) in which measurements of pressure-volume loops have demonstrated the effectiveness and short-term durability of ALG-MA-DA for pleural injuries. A parallel series of studies with GEL-MA-DA will be completed by end of summer 2021. Given this, and also the advances in manufacturing in **Specific Aim 1**, we initiated long term survival studies of ALG-MA-DA in adult rat models of both pleural (UVM) and tracheal (UConn) injuries (Major Task 1). These are progressing well and cohorts of 1 year, 6 month, and 3 month animals are well underway for both types of injuries. Addition of one month cohorts for both types of injuries will take place in August-September 2021 and parallel studies for all cohorts with GEL-MA-DA will also be initiated.

We have now also carried out successful parallel studies in non-survival pig pleural injuries with the new ALG-MA-DA formulation. These have provided the platform for initiating survival surgery studies for pleural injuries in pigs to initiate in August 2021 (Major Task 1). Once these are under way, we will initiate parallel studies with tracheal injuries in both adult and juvenile pigs (Major Task 2). Overall, these studies are anticipated to demonstrate both effectiveness and safety for use in both pleural and tracheal injuries.

The objective for **Specific Aim 3** is to extend studies to *ex vivo* preparations of both normal and diseased, ie chronic obstructive pulmonary disease (COPD), human lungs. This is a logical planned next step, in particular for study of diseased human lungs as this will often be the clinical scenario in which the sealants might be utilized. These studies were on hold over the past year given both COVID-19 limitations in obtaining human lungs (autopsy at UVM) as well as the materials optimization described for **Specific Aim 1**.

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

Specific Aim 1

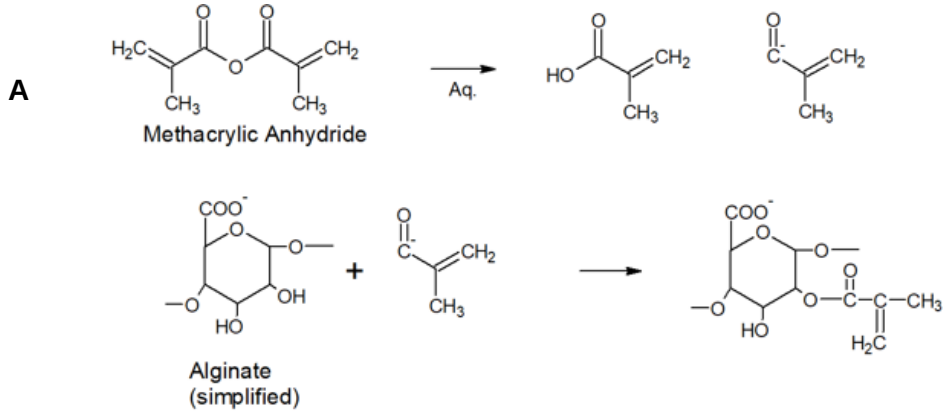
Major Task 1: Standardize synthesis and characterization of ALG-MA-DA and GEL-MA-DA

Subtask 1: Develop large scale synthesis approach for ALG-MA-DA patches

The previous annual progress report presented data regarding the improved methodologies around material production and synthesis as it relates to the generation of both ALG-MA-DA and GEL-MA-DA polymeric materials. The synthetic production of these materials continued over the past year and throughout this course the scale at which the material was generated was adjusted to adapt for larger quantities. For visibility on the specific methodologies and data, the batch manufacturing processes are listed below arranged by type of material with different lots presented as examples.

Chemical Reaction Overview

The chemical reactions involved in the production of ALG-MA-DA are shown schematically in **Figure 1** and similar reaction strategies are applied for the production of GEL-MA-DA.



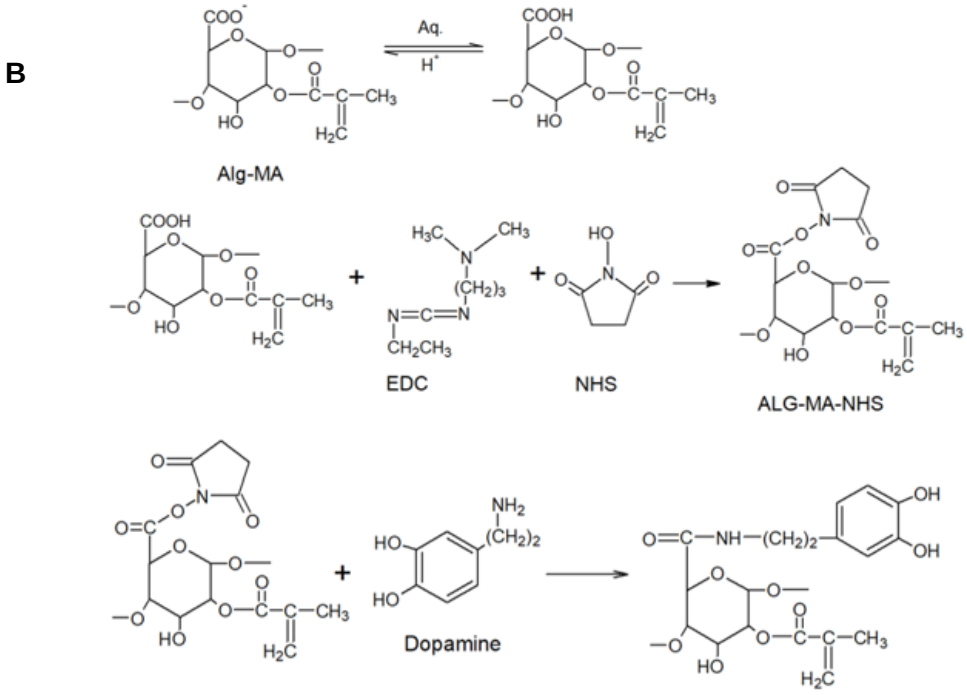


Figure 1. Reaction schematics for polymer production. A) the reaction of sodium alginate with methacrylic anhydride anhydride to form ALG-MA precursor. **B)** EDC/NHS mediated conjugation of dopamine onto the ALG-MA precursor.

It should be noted that the majority of reactions which occur during this do not create the product, rather side-reactions consume the vast majority of the reagents. Notably, methacrylic anhydride reacts with water to simply form methacrylic acid. Additionally, the alginate-methacrylate hydrolyzes in aqueous solutions to revert to alginate and methacrylic acid. For dopamine conjugation, there are three primary side reactions which occur during the manufacturing state. Notably, dopamine readily reacts to self-polymerize forming the dark-colored solutions. Alginate-methacrylate hydrolyzes to break down to alginate and methacrylic acid. Alginate-methacrylate-NHS also hydrolyzes in water to release the conjugated NHS leaving group. The primary means to deal with these side reactions currently is to use an extremely high molar equivalent of the reagents (methacrylic anhydride, dopamine, NHS/EDC) relative to the alginate to compensate for the relative inefficiency of these reactions.

Gelatin-Methacrylate

Gelatin-MA General Recipe

A 2-neck 500 ml round bottom flask was prepared with a stopcock and oval stirbar and wrapped in aluminum foil. The flask was flushed with argon and into the flask 1 phosphate buffered saline tablet (PBS, Aldrich P4417, dilute in 200 ml water according to MFG instructions) and 200 ml of deionized water was added and stirred at 550 RPM at 60ºC to dissolve. To this solution, gelatin (Type B from bovine skin) was added via funnel and stirred at 60ºC with argon flush for 15 minutes. Afterwards the argon flush was stopped and the solution was stirred for an additional 45 min at 60ºC to dissolve to a clear yellowish-tan solution. Afterwards the solution was cooled to 40ºC and a dropper arm was attached. Into the dropper arm, methacrylic anhydride (Aldrich Cat# 276685) was measured in by graduated cylinder. The dropper was partially opened and the methacrylic anhydride added dropwise to the stirring solution. The reaction was allowed to proceed overnight. Subsequently the reaction was transferred into dialysis tubing (MWCO 12-14kDa, SpectraPor P/N: 132680) and dialyzed against purified water at 40ºC in a heated incubator (Quincy Labs). Water bath refreshed over the course of 3-5 days and material removed to freeze-dry (HarvestRight). Freeze-drying yielded a solid white, fluffy, styrofoam-like material.

Table 1. Quantities, resultant yield, and notes from different reaction lots of **GEL-MA** production.

Lot#	**Gelatin**	**Methacrylic anhydride**	**Yield (g) (D)**
200721AHT-A121	10.01 g gelatin (Type B from bovine skin, Aldrich Cat# G6650)	10 ml	7.75
200727AHT-B121	10.01 g Type A, 300 bloom (Aldrich)	10 ml	7.64 g
210126JSG-A	20.18g Type B (Aldrich)	20 ml	9.79 g

Gelatin-Methacrylate-Dopamine

Gelatin-MA-DA General Recipe

0.01 M KH_2PO_4 made by weighing out ~1.1 g KH_2PO_4 (Fisher) and added 800 mL argon sparged DIH_2O. Stirred to dissolve. Added 0.1M NaOH dropwise to pH solution to 5.5. Argon sparged. Into 2-neck RBF with stirbar and stopcock, wrapped in aluminum foil, measured GEL-MA (different lots utilized in different reaction series).

Placed a septum onto 2nd neck of RBF, vacuum purged/argon flushed several times. Added 250 mL KH_2PO_4 by 60 mL syringe through septum. Stirred to dissolve about 2 hours. Removed septum while argon flowing, quickly added in EDC (Aldrich) and NHS (Aldrich) (these are 75% of the calculated amount due to a shortage of EDC at the time). Stirred to react EDC/NHS solution with GEL-MA for about 45 min. In argon filled glove box, added Dopamine HCl (Aldrich) and ~50 mL KH_2PO_4 and stirred to dissolve ~5 min. Argon sparged the dopamine solution and then attached a septum to carry it to the reaction hood. Then attached a double-tipped cannula between Dopamine solution RBF and main reaction RBF with GELMA (EDC/NHS) in it. Used argon pressure to push Dopamine solution up and over into GEL-MA/NHS solution RBF. Reacted overnight under slow argon flush. Placed argon sparged ethanol (~3L) into refrigerator overnight to cool. On ethanol bottle placed cap with hole drilled in it to feed cannula needle and tubing (16 ga) attached to argon source. Placed other end of cannula into septum on reaction RBF with GELMA-DA solution and needle from argon source. Flushed ethanol with argon and then attached argon to reaction flask to push the GEL-MA-DA up and over into the sparged ethanol bottle. Capped off ethanol and precipitate and transferred to glove box with argon continuously flowing. Filtered ethanol/precipitate through coffee filter and Buchner funnel. Collected precipitate off of coffee filter and placed in 1500 mL beaker with about 500 mL fresh ethanol and stirred for about 5 minutes. Then filtered through coffee filter again and collected precipitate and placed in small tared containers to be placed in freeze drier (about 4 hours for filtering). Placed containers into Harvest Right freeze drier over night to dry. Argon flushed container and placed in freezer for storage. Material when processed in this manner was observed to have a white to light grey powdery appearance and had to be stored carefully as exposure to room air over time darkened the material.

Table 2. Quantities, resultant yield, and notes from indicated reaction lots of **GEL-MA-DA** production.

Lot#	**GEL-MA (Lot#)**	**EDC**	**NHS**	**Dopamine**	**Yield (g)**
200914AHT-A121	2.00 g (200727AHT-B121)	5.743 g	4.26 g	3.5 g	1.94 g
201118AHT-A121	1.71 g (200727AHT-B121)	4.915 g	3.65 g	3.0 g	1.73 g
210208AHT-A121	3.0237g (200127JSG-A121)	8.672 g	6.433 g	5.30 g	2.83 g
210223AHT-A121	3.0066g (200127JSG-A121)	8.636 g	6.402 g	5.28 g	3.14 g
210302AHT-A	2.49 g (200127JSG-A121)	5.40 g	3.978 g	3.28 g	2.37 g

Alginate-Methacrylate

Alginate-MA General Recipe

Weighed out Alginate (BioPolymer) and added 500-1000 mL DiH_2O (100X mass of Alginate) under stirrer for ~2 hours in a 4-neck 2000mL (clean/dried in 100ºC oven) RBF and placed in a jacketed cooling bath (set at 5ºC) which was placed on a ring stand with an overhead stirrer and plastic stir rod with plastic paddle and placed in middle neck of RBF. This in turn was placed on top of wooden stand with pH pump (Hanna instruments BL 7916) attached. Dosing tube (inlet) on pH pump in 2M Na_2CO_3 solution. Outlet tube connected to a stopper attachment arm which fit into another neck of the RBF. A dripper arm with methacrylic anhydride (Sigma-Aldrich) was connected to another neck of the RBF and added to alginate solution dropwise. A pH probe (attached to the pH pump) was placed in the 4th neck of the RBF (with rubber adapter and O-ring around the probe). The pH pump was set to 8, so as to add Na_2CO_3 when pH droped below 8. Pump turned on after methacrylic anhydride had been added. Let stir overnight at ~150 - 250 rpm with pH pump on to control pH. This batch was dialyzed in a 12-14 kDa cutoff dialysis membrane for 3-5 days, changing out the DIH_2O once per business day). Next day, poured ALG-MA solution onto trays and placed in Harvest Right freeze drier to dry overnight. The ALG-MA was collected from the freeze-dryer and is white and "insulation-like" in texture.

Table 3. Quantities, resultant yield, and notes from indicated reaction lots of **ALG-MA** production.

Lot#	**Alginate**	**Methacrylic anhydride**	**final pH**	**Yield (g)**
201020AHT-A121	5.0013 g	15 ml	8.10	2.15
210309AHT-A121	5.0036 g	15 ml	8.10	4.26
210421AHT-A121	10.0016 g	30 ml	7.90	9.06

Alginate-Methacrylate-Dopamine

Alginate-MA-Dopamine (Generic Description)

Buffer Preparation: Weighed out ~ 0.55 g KH2PO4 (Fisher) and added 400 mL argon sparged DIH2O. Stirred to dissolve. Added 0.1M NaOH dropwise to pH solution to ~5.5. Into 2-neck RBF with stirbar and stopcock, wrapped in aluminum foil, ALG-MA (different lots utilized in different reaction series). Placed a septum onto 2nd neck of RBF, vacuum purged/argon flushed several times. Added 150-350 mL KH2PO4 by 60 mL syringe through septum. Stirred to dissolve about 3 hours. Stored in refrigerator overnight with argon. Removed septum while argon flowing, quickly added in EDC (SA #SLCJ0701) and NHS (SA #MKCM6740). Stirred to react EDC/NHS solution with ALG-MA for about 45 min. In argon filled glove box, added Dopamine HCl (SA lot BCCD6811) and ~ 55-75 mL KH2PO4 and stirred to dissolve ~5 min. Argon sparged the dopamine solution and then attached a septum to carry it to the reaction hood. Then attached a double-tipped 20 ga cannula between Dopamine/BHT solution RBF and main reaction RBF with ALG-MA (EDC/NHS) in it. Used argon pressure to push Dopamine solution up and over into ALG-MA/NHS solution RBF dropwise. Reacted overnight under slow argon flush. Placed argon sparged ethanol (~3L) into refrigerator overnight to cool. On ethanol bottle placed cap with hole drilled in it to feed cannula needle and tubing (16 ga) attached to argon source. Placed other end of cannula into septum on reaction RBF with ALG-MA-DA solution and needle from argon source. Flushed ethanol

with argon and then attached argon to reaction flask to push the ALG-MA-DA up and over into the sparged ethanol bottle (about 3 hours). Capped off ethanol/precipitate and sealed with parafilm then transferred to refrigerator overnight. Next day transferred to glove box with argon continuously flowing. Filtered ethanol/precipitate through coffee filter and Buchner funnel. Collected precipitate off of coffee filter and placed in 1500 mL beaker with about 500 mL fresh ethanol and stirred for about 5 minutes. Then filtered through coffee filter again and collected precipitate and placed in small tared containers to be placed in freeze drier (about 4 hours for filtering). Placed containers into Harvest Right freeze drier over night to dry. Material when processed in this manner was observed to have a white to light grey powdery/fleece appearance and had to be stored carefully as exposure to room air over time darkened the material.

Table 4. Quantities, resultant yield, and notes from indicated reaction lots of **ALG-MA-DA** production.

Lot#	**ALG-MA (Lot#)**	**EDC**	**NHS**	**Dopamine**	**Yield (g)**
200727AHT-A121	1.11 g (200108AHT-A121)	7.014 g	5.197 g	1.948 g	1.38 g
200824AHT-A121	1.44 g (200108AHT-A121)	4.027 g	2.989 g	2.46 g	1.62 g
200924AHT-A121	1.77 g (200108AHT-A121)	5.083 g	3.77 g	3.11 g	2.15 g
201110AHT-A121	1.96 g (200108AHT-A121)	5.428 g	4.024 g	3.30 g	2.97 g
Lot# 210329AHT-A121	2.09 g (210309AHT-A121)	6.00 g	4.45 g	3.66 g	2.79 g
210413AHT-A121	1.814 g (210309AHT-A121)	5.200 g	3.857 g	3.18 g	2.75 g
210505AHT-A121	2.79 g (210421AHT-A121)	8.013 g	5.947 g	4.9 g	4.27 g
210609AHT-A121	2.37 g (210421AHT-A121).	6.807 g	5.044 g	4.16 g	3.13 g

Gelatin-Dopamine

Gelatin-Dopamine (Lot# 201207AHT-A121)

Weighed out 0.55 g KH_2PO_4 (Fisher lot #1166582) and added 400 mL argon sparged DIH_2O. Stirred to dissolve. Added 0.1M NaOH dropwise to pH solution to 5.5. Into 2-neck RBF with stirbar and stopcock, wrapped in aluminum foil, measured 1.71 g Gelatin Type A (Sigma Aldrich SLCF1596). Placed a septum onto 2nd neck of RBF, vacuum purged/argon flushed several times. Added ~170 mL KH_2PO_4 by 60 mL syringe through septum.

Stirred to dissolve about 2 hours. Removed septum while argon flowing, quickly added in 4.91 g EDC (SA #SLCF8591) and 3.64 g NHS (SA #MKCJ1481). Stirred to react EDC/NHS solution with GEL-MA for about 45 min. In argon filled glove box, added ~3.01 g Dopamine HCl (SA lot #BCCC7857) and ~ 45 mL KH_2PO_4 and stirred to dissolve ~5 min. Argon sparged the dopamine solution and then attached a septum to carry it to the reaction hood. Then attached a double-tipped cannula between Dopamine solution RBF and main reaction RBF with Gel (EDC/NHS) in it. Used argon pressure to push Dopamine solution up and over into Gel/NHS solution RBF. Reacted overnight under slow argon flush. Placed argon sparged ethanol (~3L) into refrigerator overnight to cool. On ethanol bottle placed cap with hole drilled in it to feed cannula needle and tubing (16 ga) attached to argon source. Placed other end of cannula into septum on reaction RBF with GelMA-DA solution and needle from argon source. Flushed ethanol with argon and then attached argon to reaction flask to push the GelMA-DA up and over into the sparged ethanol bottle. Capped off ethanol and precipitate and transferred to glove box with argon continuously flowing. Filtered ethanol/precipitate through coffee filter and Buchner funnel. Collected precipitate off of coffee filter and placed in 1500 mL beaker with about 500 mL fresh ethanol and stirred for about 5 minutes. Then filtered through coffee filter again and collected precipitate and placed in small tared containers to be placed in freeze drier (about 4 hours for filtering). Placed containers into Harvest Right freeze drier over night to dry. Yield = 1.66 g. Argon flushed container and placed in freezer for storage.

Kinetics Assay

One of the steps to understanding the chemical nature of the study was to investigate the rate of the reaction. For this work, a series of kinetic studies were performed.

Gelatin-MA-DA (Lot# 210210AHT-A121) Kinetic Study

Into 2-neck RBF with stirbar and stopcock, wrapped in aluminum foil, measured 1.02 g Gel-MA (200127JSG-A121). Placed a septum onto 2nd neck of RBF, vacuum purged/argon flushed several times. Added 100 mL KH_2PO_4 by 60 mL syringe through septum. Stirred to dissolve about 2 hours. Removed septum while argon flowing, quickly added in 2.933 g EDC (SA #SLCF8591) and 2.174 g NHS (SA #MKCJ1481). Stirred to react EDC/NHS solution with GEL-MA for about 45 min. In argon filled glove box, added 1.79 g Dopamine HCl (SA lot #DCCC0493) and ~ 30 mL KH_2PO_4 and stirred to dissolve ~5 min. Argon sparged the dopamine solution and then attached a septum to carry it to the reaction hood. Then attached a double-tipped cannula between Dopamine solution RBF and main reaction RBF with GEL-MA (EDC/NHS) in it. Used argon pressure to push Dopamine solution up and over into GelMA/NHS solution RBF. Reacted overnight under slow argon flush. Sample for kinetic study taken after all of the dopamine had been added (T0). Samples taken every hour. Solution started turning black after third sample. Samples added to ~45mL cold ethanol (argon sparged) each in centrifuged tubes (T1-5). Took one more sample from reaction solution the next day (T6). Centrifuged samples for 10 minutes at 3000 rpm. Transferred to glove box, poured off supernatant and placed each sample into tared containers. Samples were placed into HarvestRight freeze drier overnight to dry.

However, the collected samples were observed to be heavily oxidized and also failed to dissolve in the D2O for further assay. The process of collecting samples at discrete time points appears to interfere with the product and is infeasible for readily obtaining kinetic understanding. These results highlight the importance of maintaining good oxygen discipline when working with these sensitive and unstable compounds.

Due to the difficulties obtaining useful reaction kinetics using NMR analysis from preselected time points, a separate assay was performed studying heat-flow using differential scanning calorimetry (DSC). The typical DSC aluminum pan design is setup to hold a few milligrams of a solid sample and then the DSC heats the sample through a range of temperatures to determine various transitions. Aluminum pans (TA instruments tzero) were initially measured for volume by weighing both tared and after filling with room-temperature RO purified water to determine the pans can hold about 48 µL (48 mg of water). Initially the pans were attempted to be modified by using silicone adhesive (GE advanced, clear) to attach circles of 1/16 inch thick rubber cut out using a 5/16 inch circular punch (Mayhew) (**Figure 2B**). These modifications failed however as the dried adhesive didn't keep the rubber attached to the DSC pan. Instead of this the DSC pans were covered after loading using 5/16 inch cut adhesive-backed Duralar (Grafix Plastics, 0.005 inch thick, clear) (**Figure 2C**). Air-sensitive ingredients were prepared in a small glass vial (2-dram) which was sealed using a Rubber Serum Stopper, D-88 (Preiser Scientific, Cat# 10-4798-02) (**Figure 2A**) and wrapped with parafilm to create an air-tight septa seal.

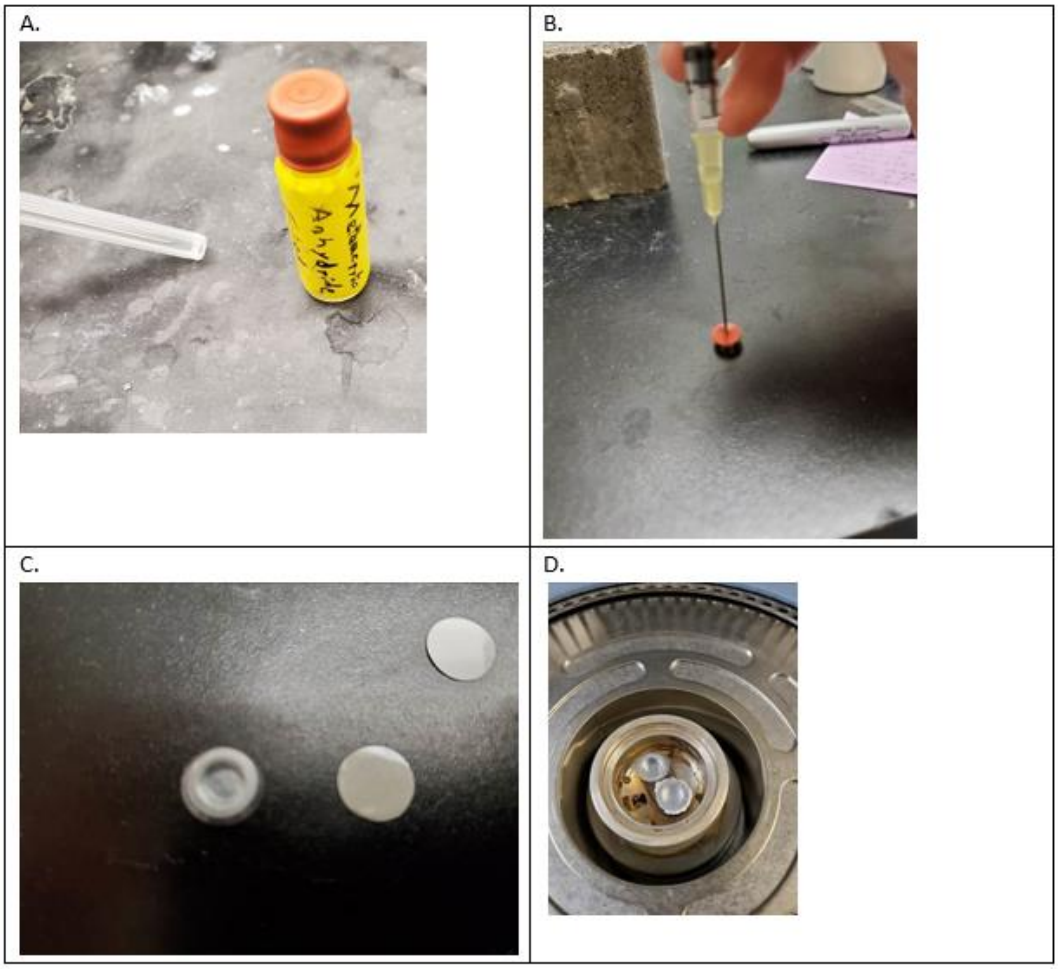


Figure 2. Image series from DSC kinetics assay of ALG-MA-DA reaction: **A)** Septa-sealed 2-dram bottle used for transfer/handling of small volumes of air-sensitive reagents, **B)** rubber-septa sealed DSC pan, ultimately abandoned after adhesional failure, **C)** DSC pan sealed with adhesive Duralar, **D)** DSC loaded with empty reference (upper) and ALG-MA reacted (lower) pans after testing had completed and pan was ready for removal.

Using the DSC approach, we have now been able to successfully assess kinetics of both the alginate and gelatin-based compounds. Representative detailed protocols are presented followed by the respective results.

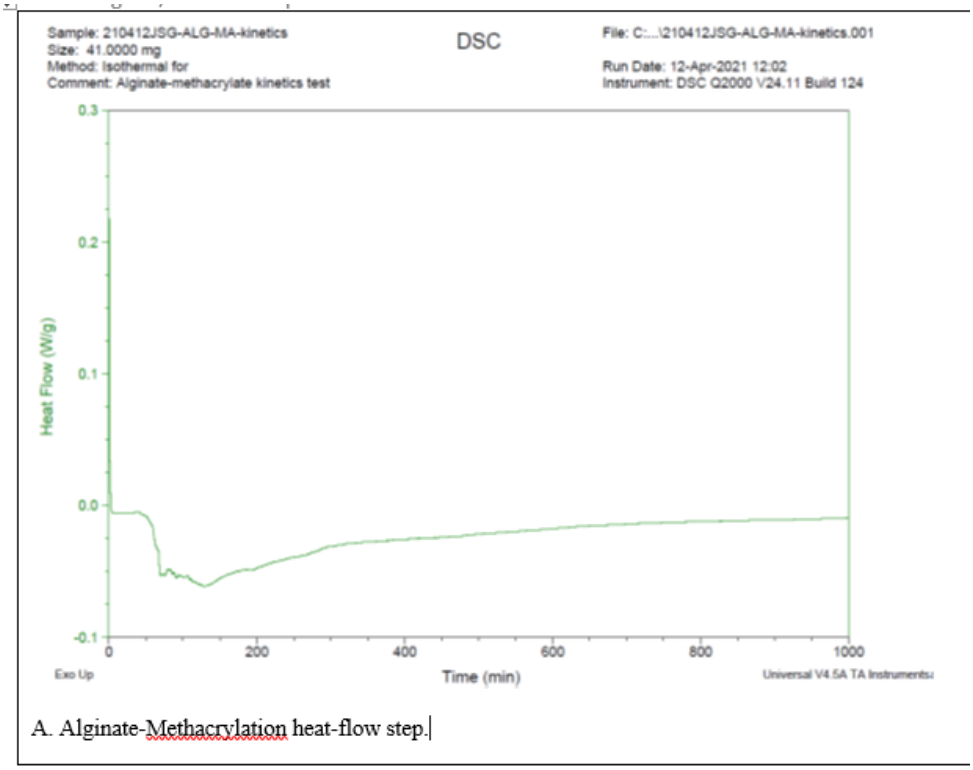
Alginate-Methacrylate Kinetics reactive solution preparation

Into glass vial (20 ml scintillation vial) added 0.2113 grams of sodium carbonate (Fisher, Cat# S263-500), 8.1 mg of sodium alginate (FMC Biopolymer, Manugel GMB), and 1 ml of deionized water. For this reaction, the alginate solution was prepared slightly dilute with additional sodium carbonate solution since addition of extra sodium carbonate solution during the reaction would not be feasible. Vial incubated at 30ºC with 100 RPM orbital agitation (Incushaker Mini, Southwest Scientific) for three days followed by incubation at 37ºC and 10 minutes of sonication (Bransonic 5510) to dissolve. Separately, a 2-dram vial was sealed with a rubber septa and connected to a gas/vacuum manifold by 20 gauge needle. The bottle was vacuum purged and argon flushed three times. Subsequently, methacrylic anhydride (Aldrich Cat# 276685, lot# STBH5178, received 6/28/2019) was injected (~ 0.5 ml) into the vial by syringe and backflushed with argon. A reference cell was prepared by covering over an empty DSC pan with adhesive backed duralar seal. This pan was weighed empty (60.0 mg) and this mass was input to the DSC software as the empty mass of both reference and test pan.

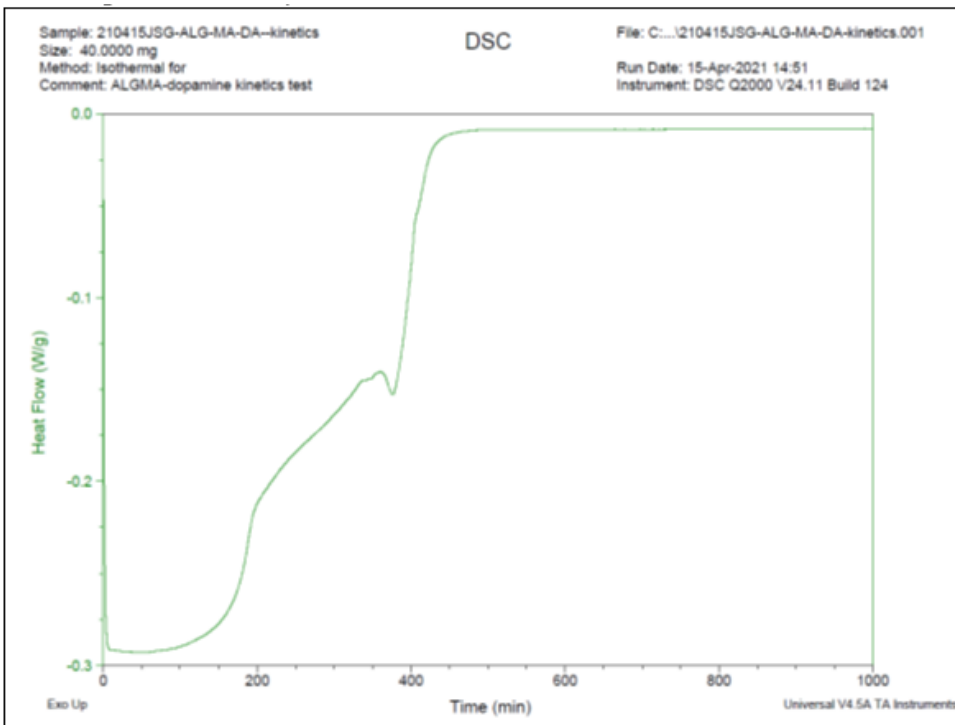
Prepared DSC (TA instruments Q2000) by running it through a cell-conditioning cycle (heat 100ºC for 30 minutes). After cooling to standby temperature (40ºC) the reference pan was placed in the reference platform and an empty pan was placed on the test platform. Used pipette (Eppendorf) to add 40 µL of the prepared 8 mg/ml Alginate/2M sodium carbonate solution into the pan. Afterwards used syringe (3/10 cc, 30Ga needle, Kendall Co.) to transfer 1 µL of methacrylic anhydride into the pan. Covered with Duralar seal, closed DSC and ran isothermal program to reduce temperature to 5ºC and hold it constant for 1000 minutes (16 hrs, 40 minutes) (**Figure 2D**) under 20 cc/min argon flush.

Alginate-Methacrylate-Dopamine conjugation Kinetics testing solution preparation

Into scintillation vial put 11.2 mg of Alginate-Methacrylate (210309AHT-A) and added 1.15 ml of previously prepared phosphate buffer (210413NDR, pH = 5.5, resparged for 20 minutes at 100 cc/min Argon) along with a magnetic stirbar. Stirred at 300 RPM/30ºC on stirring hotplate (Fisher Scientific, Isotemp) to dissolve then cooled. Into the alginate-methacrylate stirring solution added 25 mg of NHS and 32 mg of EDC and stirred for 45 minutes to prepare ALG-MA-NHS. Prepared DSC (TA instruments Q2000) by running it through a cell-conditioning cycle (heat 100ºC for 30 minutes). Into 2-dram vial put 12 mg dopamine HCl (Aldrich, cat# H8502) and sealed with septa. Vacuum purged and argon flushed dopamine 3 times then added 0.19 ml PBS via syringe shaken to dissolve. Into DSC pans added 31 ul of ALG-MA-NHS solution and 9 µL of Dopamine solution. Left pan open to have good exposure to argon flush for reducing oxidation. DSC held isothermal at 25ºC under 20 cc/min argon flush for 1000 minutes and heat-flow monitored. The DSC heat-flow data for both methacrylation and dopamine conjugation is shown in **Figure 3** below.



A. Alginate-Methacrylation heat-flow step.



B. Alginate-Methacrylate Dopamine Conjugation step.

Figure 3 Collected heat-flow data from each experiment. DSC performed on TA instruments Q2000 system with isothermal setting and observing heat-flow (Y-axis) versus time (X-axis) indicating progression of chemical reaction by enthalpy change.

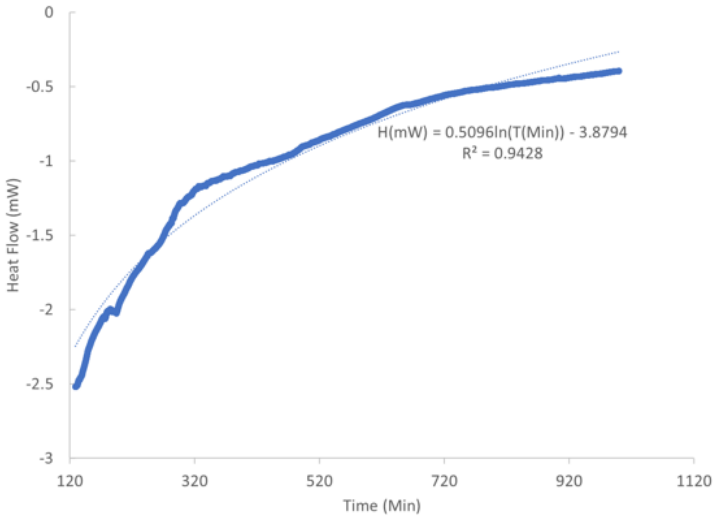
Small-scale kinetic testing of methacrylate conjugation of alginate

In addition to the main reaction of methacrylate conjugation the thermal measurement also indicates the pH stabilizing reaction of sodium carbonate reacting with the formed methacrylic acid and decomposing to carbon dioxide and water as a mechanism to prevent acidification of the solution which would inhibit the reaction from proceeding. During production runs, a pH-triggered pump is fixed to the reaction flask such that when the reaction solution becomes too acidic it adds additional sodium carbonate solution to the reaction. This mechanism can not be readily achieved during the DSC run so the initial reaction solution was prepared with additional sodium carbonate. On the DSC scan, there is a substantial initial drop within the first few minutes, however a portion of this is an artifact caused by the instrument cooling the solution to 5ºC from 40ºC. This is followed by a stair-step drop around 50-70 minutes followed by a roughly steady-state thermal status until ~ 130 minutes. It is considered that these portions represent the initial methacrylic anhydride reaction, as well as, the process of mixing the hydrophobic methacrylic anhydride in with the aqueos solution. Afterwards, the heat-flow transitions in a logarithmic pattern according to equation (H(mW) = 0.5096ln(T(Min)) - 3.8794) (**Figure 4A**) though does not quite fully return to 0 heat-flow nor completely stop transitioning. This indicates that, at the completion of a typical reaction run, the methacrylation is not quite complete.

Small-scale kinetic testing of dopamine conjugation of alginate-methacrylate

The ALG-MA-DA reaction is rather complicated with multiple overlapping steps occurring simultaneously. By heat flow, the reaction presents as two very different kinetics with a relatively slow reaction after cooling from ~ 100 to about 350 min which occurs according to kinetics of H(mW) = 5.7115ln(T(Min)) - 39.081. This is followed by a somewhat complicated transition and a relatively rapid reaction from about 375 – 450 minutes which occurs according to kinetics of H(mW) = 35.935ln(T(Min)) - 218.86. The rapid leveling of the curve at 450 minutes indicate that the reaction is substantially finished by this point with no further thermal transitions. Kinetically these experiments indicate that it may be beneficial to let the methacrylation reaction occur longer than the normal 16 hour overnight run while the dopamine conjugation reaction is effectively finished by 8 hours.

A. Alginate-Methacrylate plotting (after 129 minutes of initial phase)



B. Alginate-Methacrylate dopamine conjugation (2-stage)

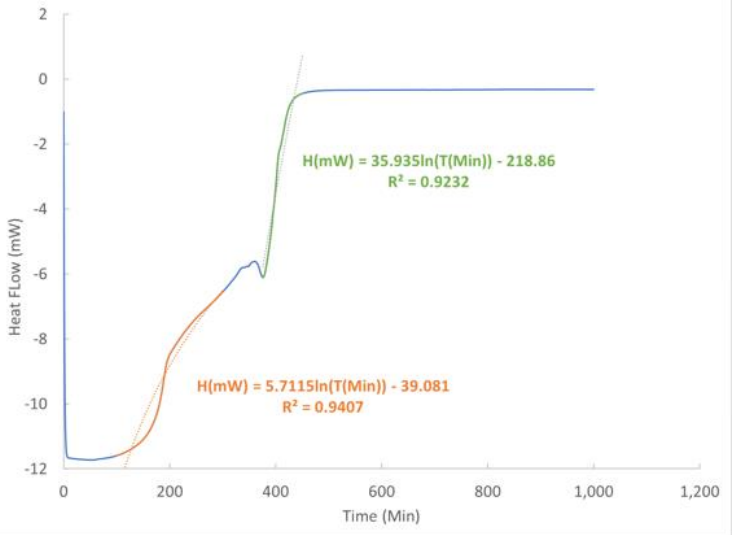


Figure 4 Heat flow and thermal transitions which occur during the progression of A) Alginate reaction with methacrylic anhydride to form ALG-MA precursor and B) EDC/NHS mediated conjugation of dopamine to ALG-MA precursor.

Overall Summary for Major Task 1: Subtask 1

Through the course of working on Subtask 1, several features of the reaction chemistry were elucidated. The systematic application of methacrylate to the alginate requires careful pH control through the use of a less-caustic basifying agent in order to both achieve good degree of methacrylation as well as minimalize any degradation of the alginate backbone. The formation of ALG-MA by this methodology is robust and routinely reproducible.

Dopamine in liquid state is extraordinarily oxygen sensitive and requires protection beyond the normal practices of typical laboratory conditions. Performance of the dopamine conjugation requires absolute air (oxygen)-free conditions in order to minimize the rapid and spontaneous oxidation of this reagent. To feasibly achieve this, several modifications were made to the general method in terms of how certain steps are performed to focus on steps which are more amenable to air-free reaction measures. Under the right conditions, the formation of ALG-MA-DA is reproducible however the degree of care required in the reaction makes the process onerous and subsequent steps will focus on simplifying the methodology so that manufacture, particularly large-scale manufacture, can be routine and efficient.

Recent work has focused on the dopamine conjugation methodology. The applied dopamine reagent is in 4X molar excess. In normal chemistry the only motivating factor to reduce reagent usage is typically cost, however, given dopamine's capacity to self-polymerize, oxidize, and participate in other reactions, it may improve the quality of the material to not use more than the requisite minimum amount of dopamine in these conjugation reactions. Current efforts are focused on minimizing the dopamine for conjugation to only the absolute minimum quantity necessary.

Subtask 2: Standardize characterization of ALG-MA-DA and GEL-MA-DA patches: NMR, FTIR

Characterization of ALG-MA-DA and GEL-MA-DA by NMR and comparable methods is a critical tool for ensuing batch to batch consistency and reproducibility. Previous testing established methods for analysis by NMR and FTIR. Briefly, Fourier-Transform Infrared Spectroscopy (FTIR) is a spectroscopic process by which the sample is illuminated with infrared light across the wavelength range of 2500 nm (4000 cm-1) to 25,000 nm (400cm-1). Electromagnetic radiation at this frequency interacts with chemical moieties to cause bending, rotation, stretching and other transitions along the covalent bonds within a chemical and this interaction leads to absorption of the light at that wavelength. Due to the inherent noise in this process, Fourier-Transform is used to expedite the measurement of multiple frequencies of IR light simultaneously providing for larger number of scans per test. This assay gives qualitative information regarding the presence of common chemical moieties (alkenes, carboxylic acids, alcohols, aromatics, etc.) and is considered a routine test for use in organic chemistry. Nuclear magnetic resonance, specifically proton (H) nuclear magnetic resonance relies on the electrical charge of the hydrogen nuclei and its inherent spin. Under a strong magnetic field energy transfers occur at specific radio-frequencies related to the relative shielding/deshielding condition of the nucleus which is related to the electronegativity of the atoms nearby. This technique allows for collection of discrete and specific peaks which can be compared for their integration areas/intensities to obtain relative content of specific chemical moieties (a process known as "proton counting"). The proton counting must be done against a known peak with a specific quantity which is then used to compare other peaks. As such, spiking an internal standard into the NMR sample which has a peak that is removed from other, common chemistries, is routine practice. This is briefly reviewed below along with subsequent assays and information from indicated batches.

NMR – Internal Standard Method

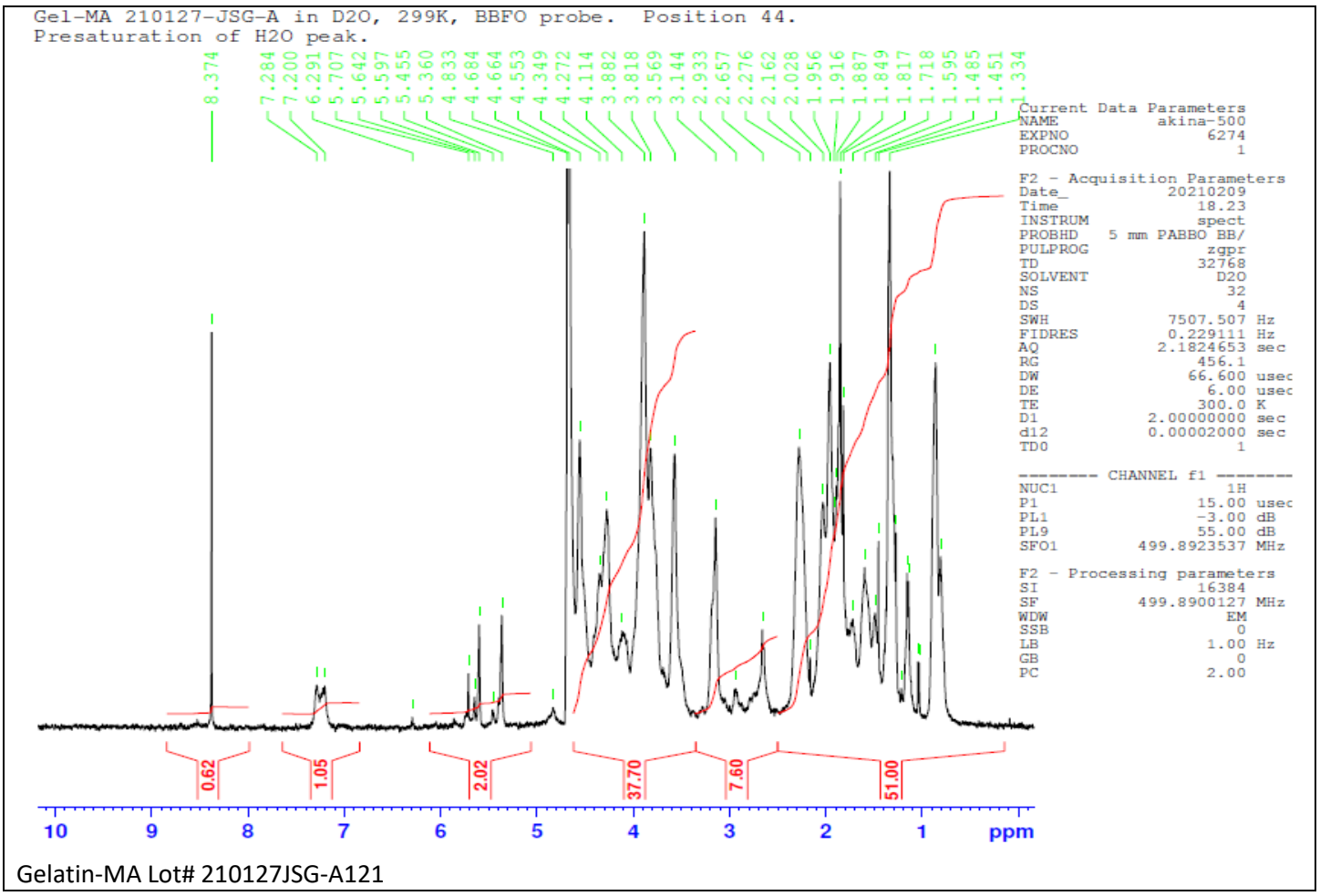
An internal standard stock solution was prepared by dissolving 10.4 mg calcium formate (Aldrich cat# 03826-1G) in with 10.408 g of D_2O (Cambridge Isotopes). Tests were performed using this solution with addition of 0.10 µl of stock solution to prepared samples of indicated mass (~ 5-10 mg) diluted up to 0.8 ml using D_2O. These samples were transferred into a NMR tube (Wilmad glass). The NMR spectrum was collected from indicated solution by Purdue Interdepartmental NMR Facility (PINMRF http://www.pinmrf.purdue.edu/). By comparing

the peak intensity at ~ 8.4 ppm (calcium formate, **2H**) and the methacrylate peak at 5.7 ppm (**1H**) the quantity of methacrylate can be determined as follows:

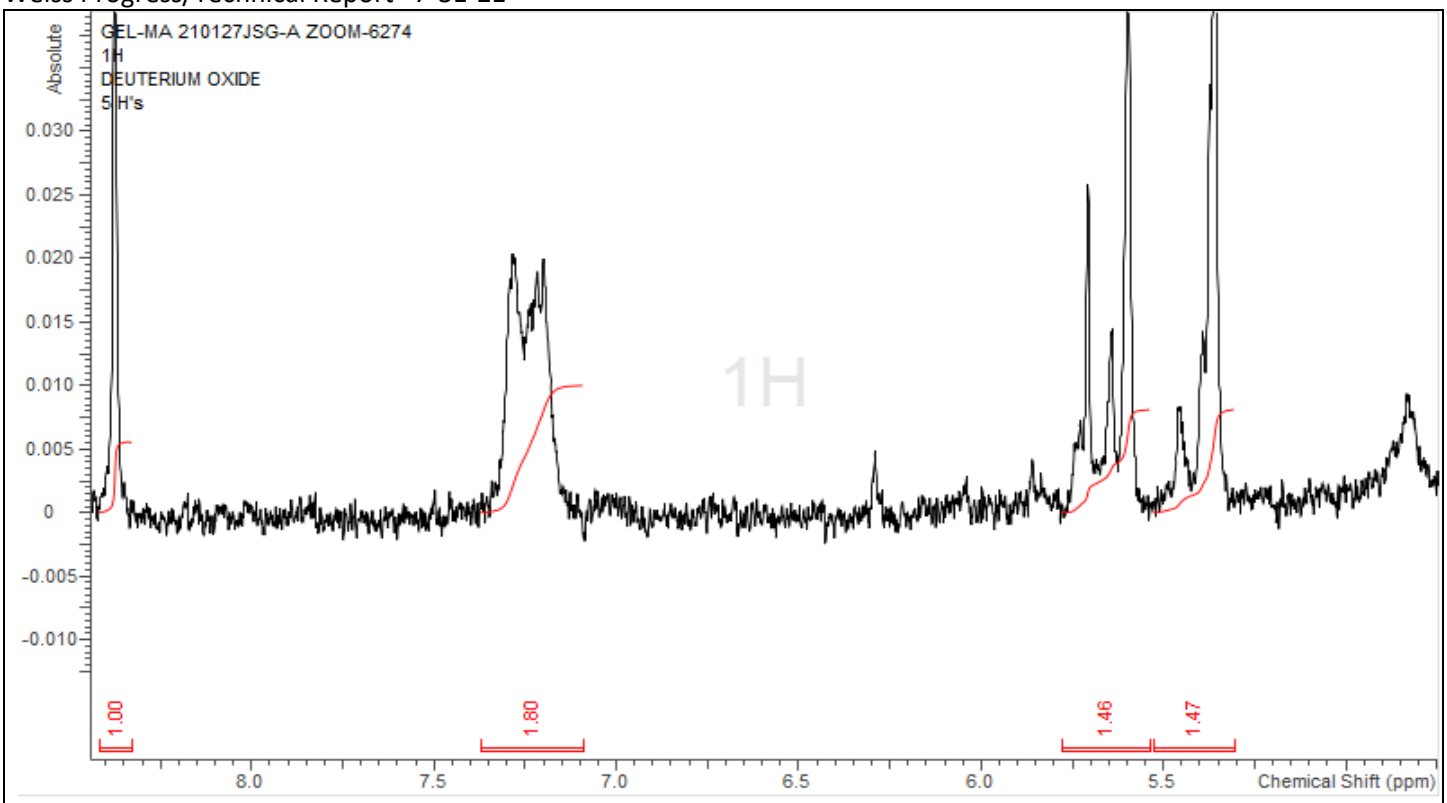
Mc(g) = (Vs(ml) x Cs(mg/ml))/1000
Pm/(Pc/2) x (Mc(g)/130.113) = mole MA
mole MA/mg sample * 1000000000 = micromoles MA/g Alg-MA

Where Pm is peak integration of methacrylate peak at 5.7 ppm, Pc is peak integration of calcium formate at ~ 8 ppm. Mc(g) is mass of calcium formate in grams calculated using volume of stock "Vs(ml)"and concentration of stock "Cs(mg/ml)". Similarly, the value for dopamine was determined according to the peaks for 3H of the benzyl chain from 6.4 – 6.8 ppm The only change for this calculation was dividing the peak integration for the DOPA signal. Here "Pd" is the dopamine peak around 6.5 ppm. (Pd/3)/(Pc/2) x (Mc(g)/130.113) = mole MA. The other calculations are the same as performed for methacrylate determination. This calculation was applied both to ALG-MA-DA and GEL-MA-DA materials.

Figure 5. Representative, example NMR Spectra (whole-scale, generic auto-integration) and region of interest (Zoom, 4 – 9 ppm of indicated peak with integration area indicated) collected for chemical analysis of indicated product. Presented in order of GEL-MA (Precursor), GEL-MA-DA (product), ALG-MA (precursor), ALG-MA-DA (product), GEL-DA (product).



Gelatin-MA Lot# 210127JSG-A121



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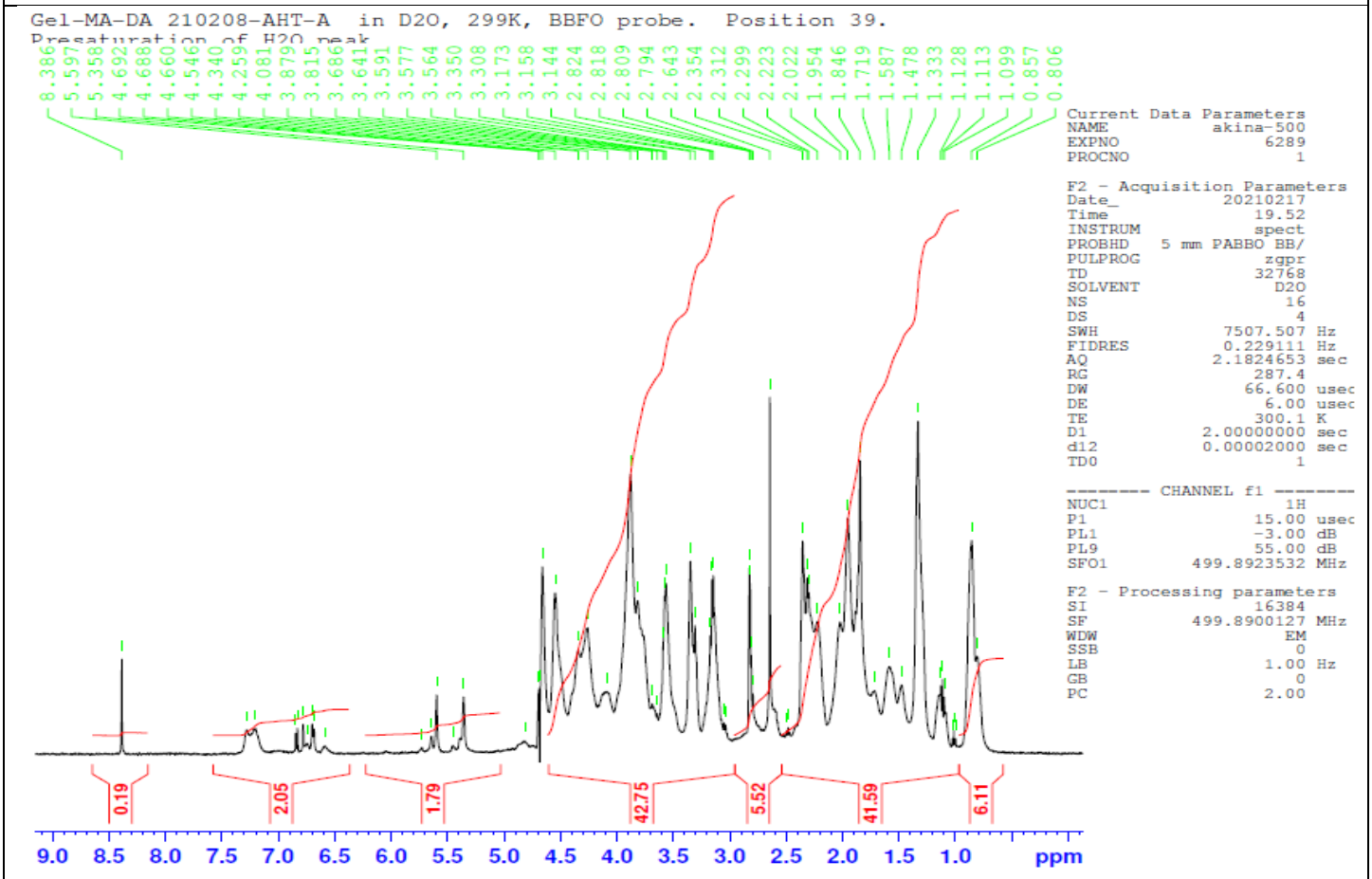

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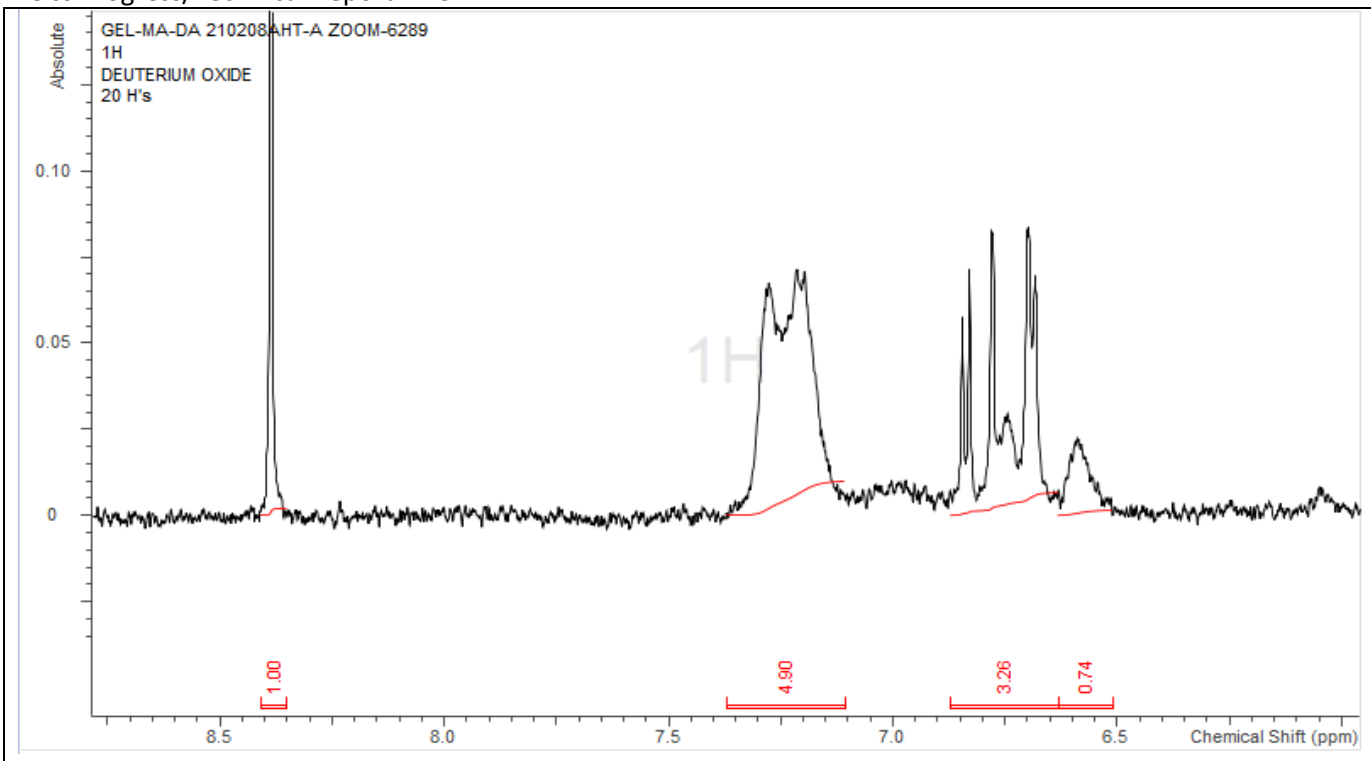

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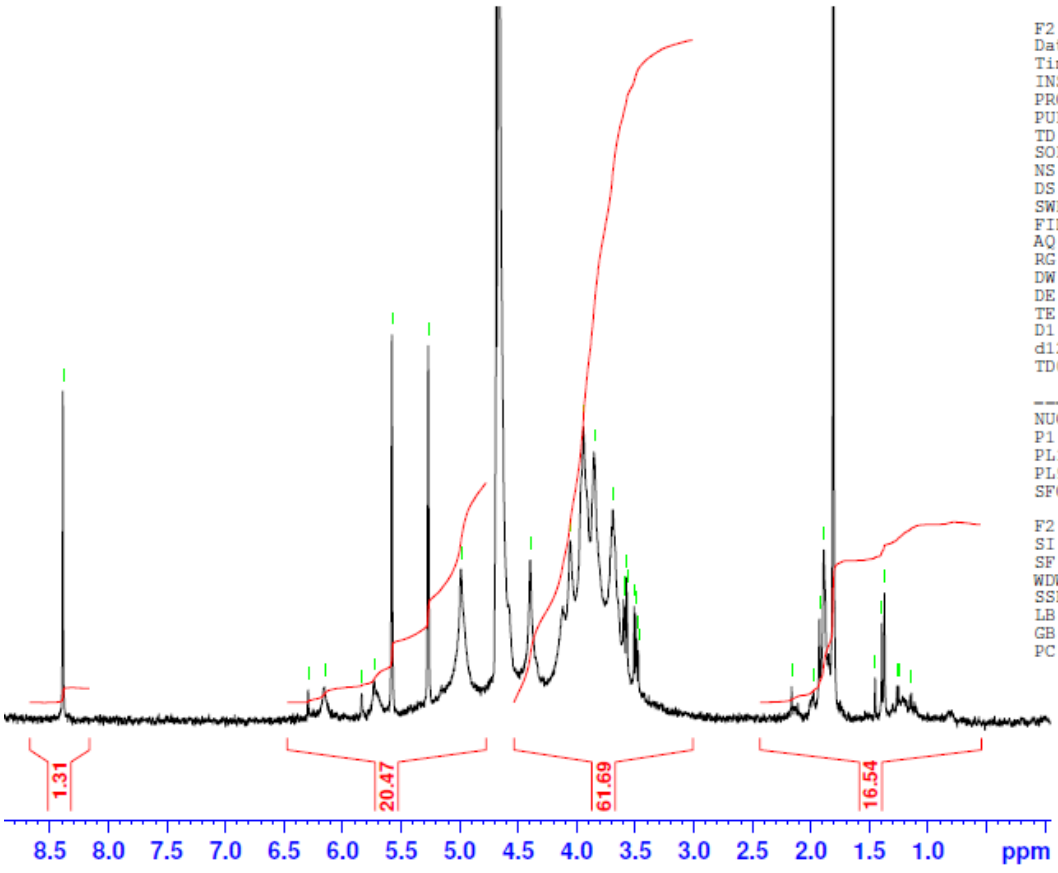


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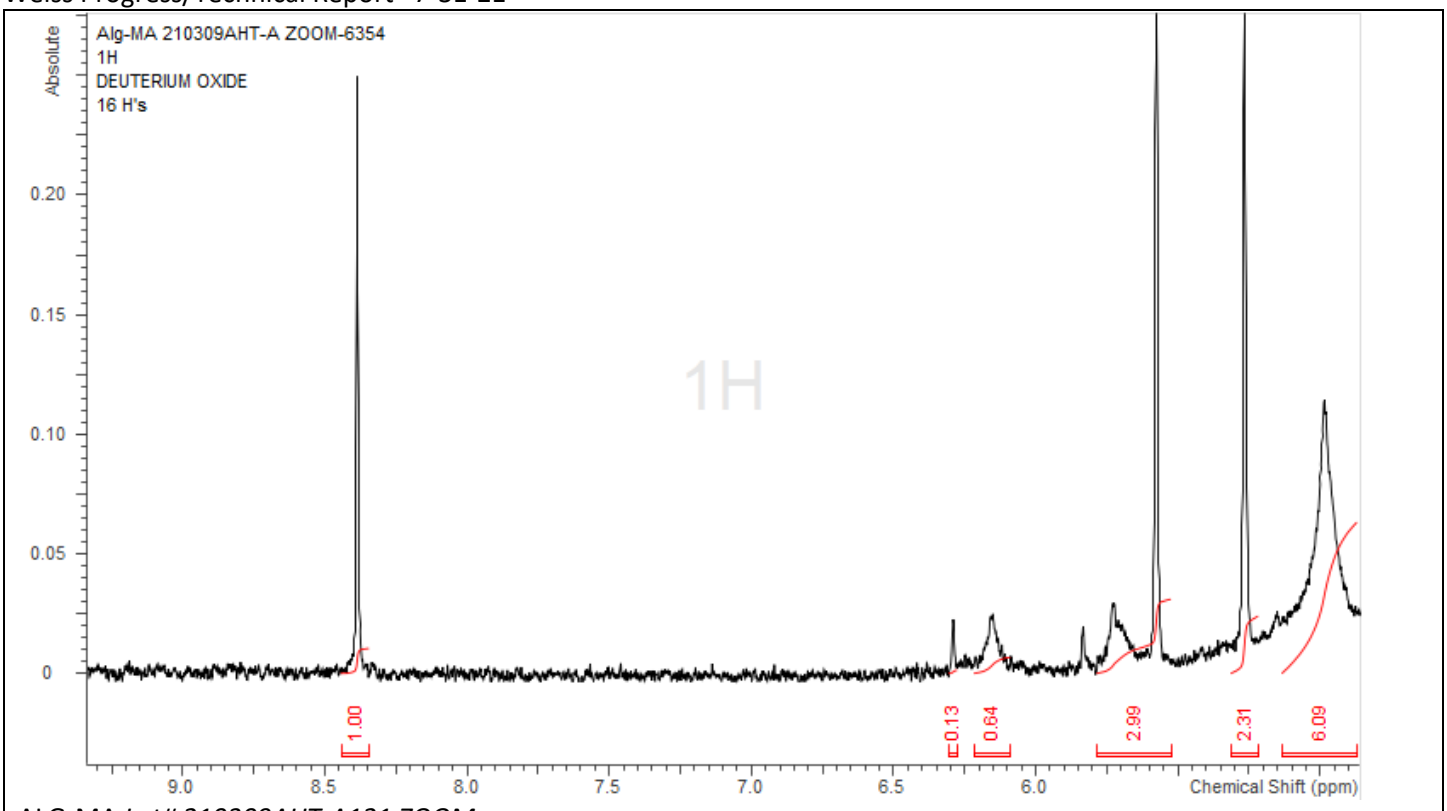
ALGMA Lot# 210309AHT-A121



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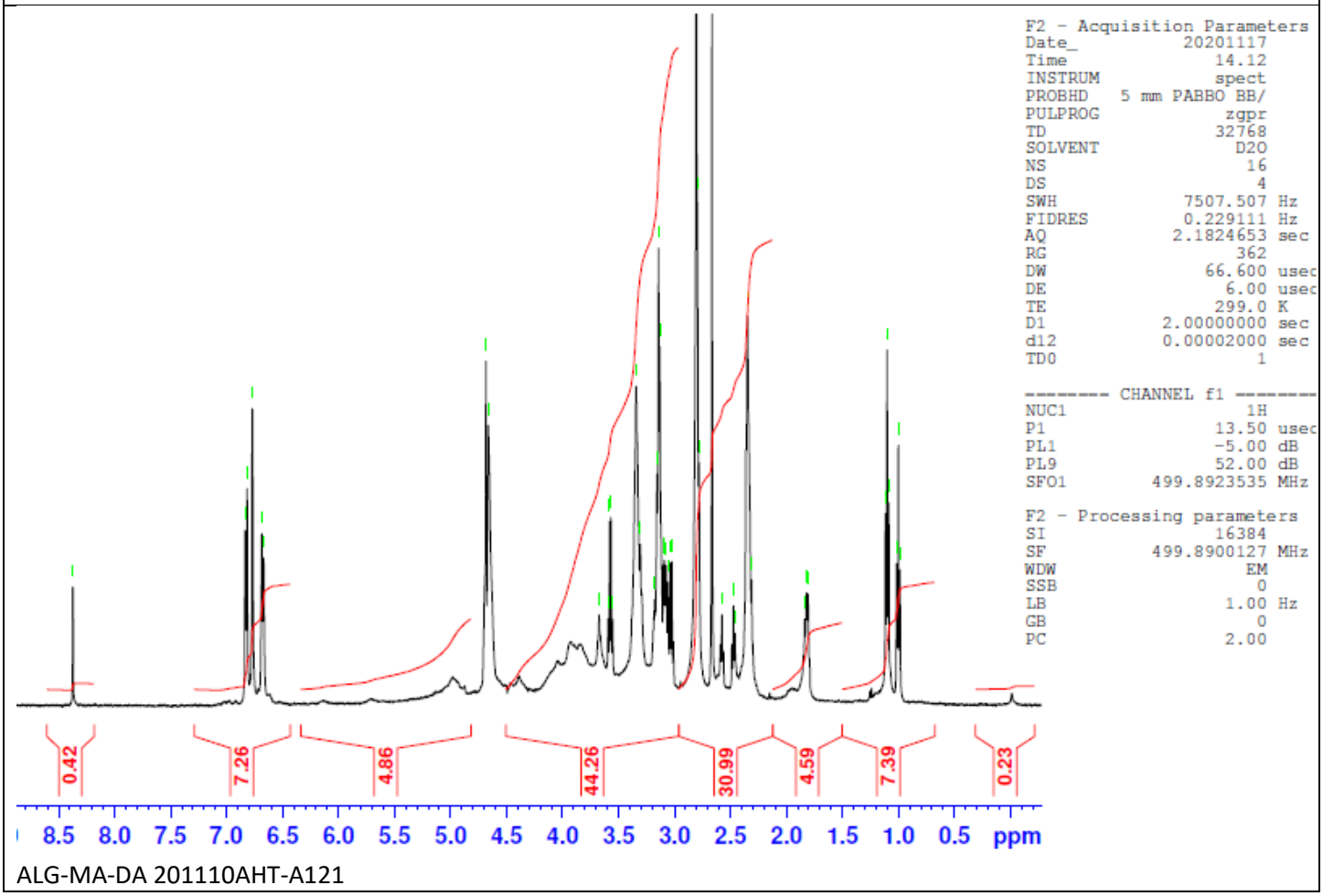

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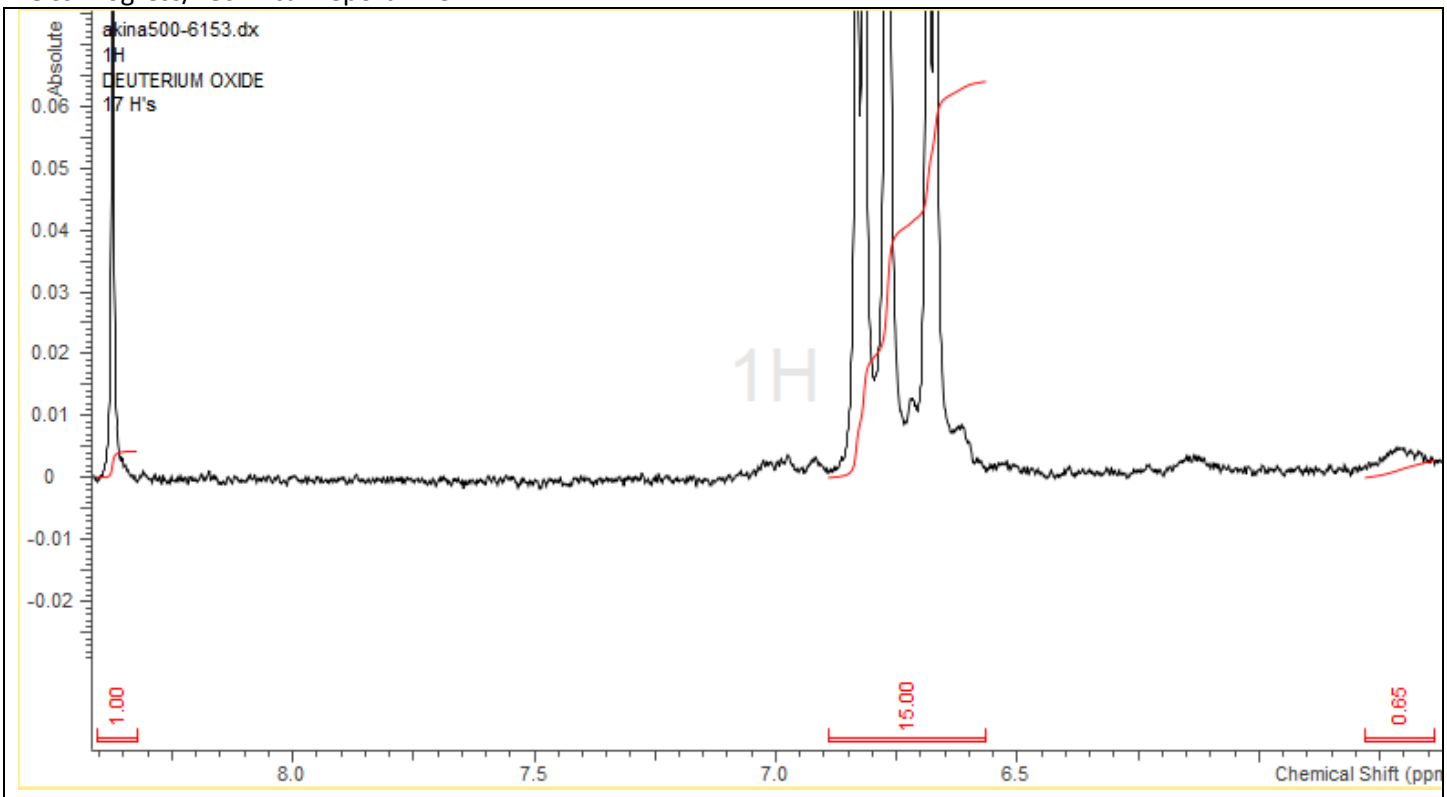

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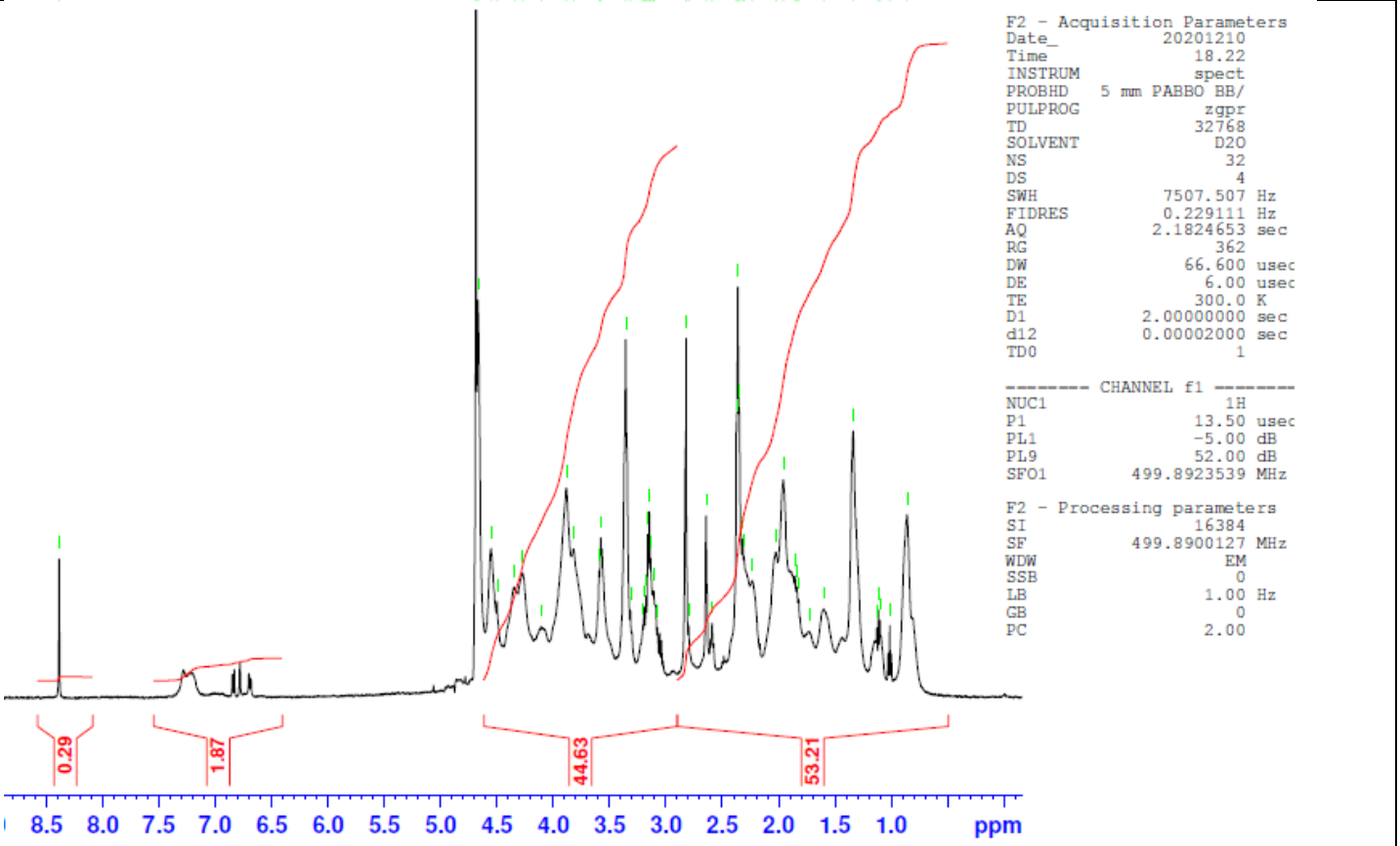


GEL-DA 201207AHT-A

The calculated dopamine and methacrylate quantities of each indicated sample is provided in the table. The calculated methacrylate addition was average of 73 ± 61 micromoles of methacrylate per gram of ALG-MA-DA (N = 8) and 878 ± 198 micromoles of methacrylate per gram of GEL-MA-DA (N=5). The calculated addition for dopamine was 1564 ± 898 micromoles of dopamine per gram of ALG-MA-DA (N = 8) and 342 ± 159 micromoles of methacrylate per gram of GEL-MA-DA (N=5). The higher efficiency of methacrylate addition to gelatin is likely caused by the presence of amine units (lysine, etc.) along the gelatin backbone which are significantly more reactive towards methacrylic anhydride than the alcohol units of alginate. Similarly, the higher conjugation of dopamine on alginate is likely due to the higher number of carboxylic acid units on the alginic acid molecule.

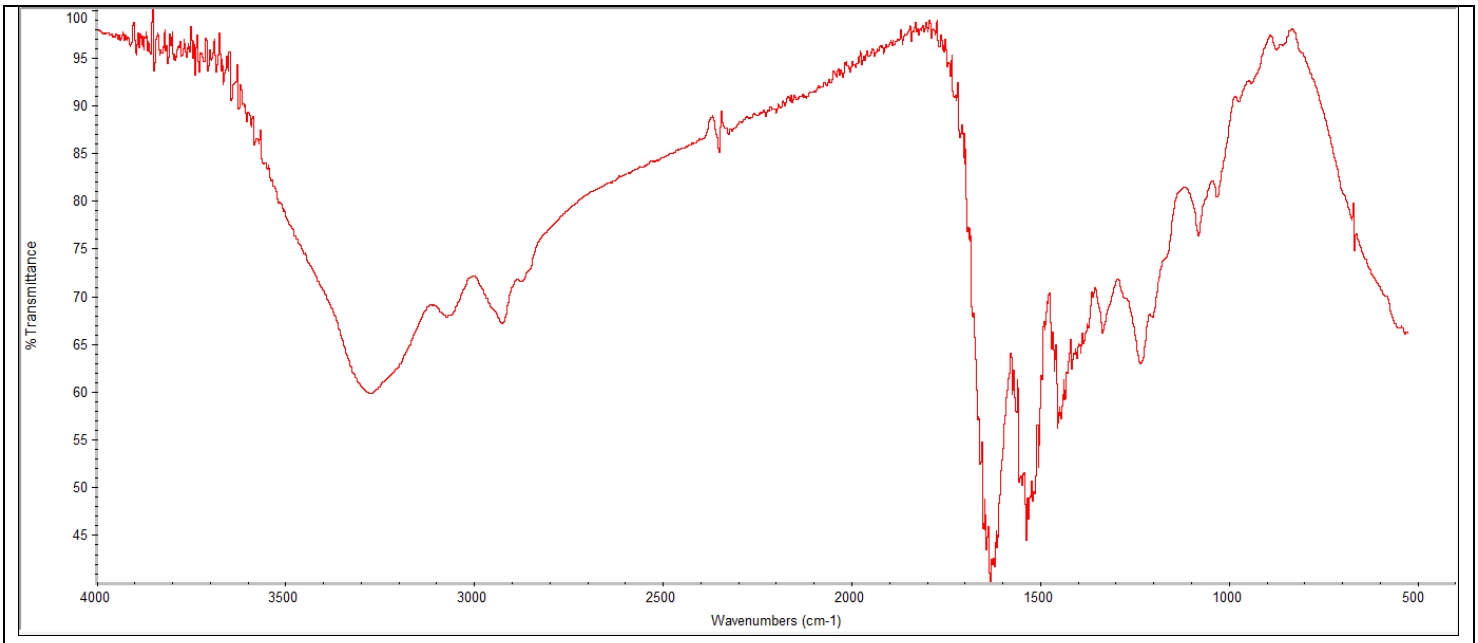
Table 5 Methacrylate and dopamine conjugation quantities for indicated samples as determined by HNMR.

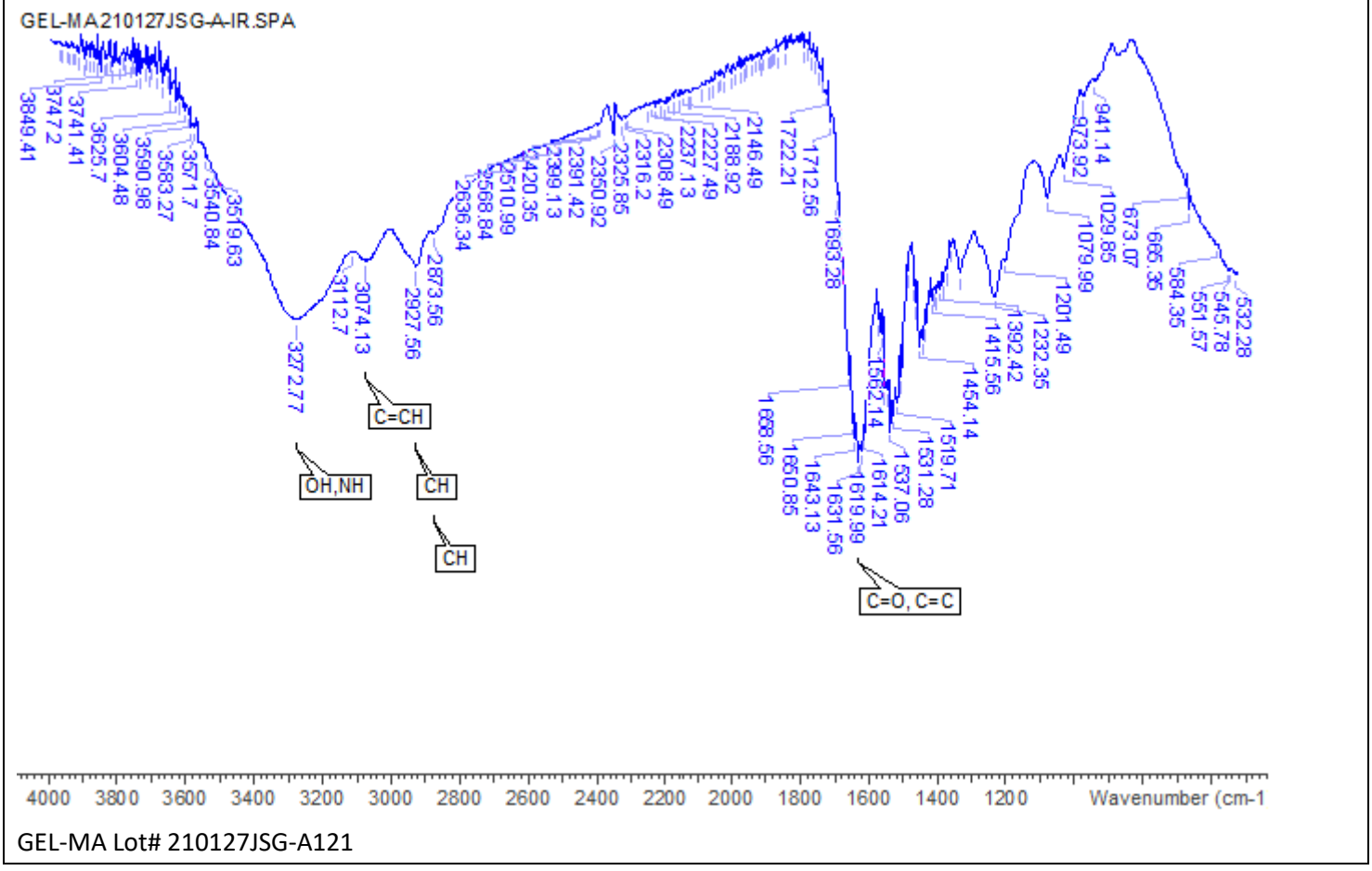
Samples	Methacrylate	Dopamine
Alg-MA-Dopa	**uM MA/g ALG-MA-DOPA**	**uM DOPA/g ALG-MA-DOPA**
ALG-MA-DOPA (Lot200727AHT-A) ZOOM	12	1458
ALG-MA-DOPA (Lot200824AHT-A) ZOOM	2	838
ALG-MA-DOPA (Lot200924AHT-A) ZOOM	38	1335
ALG-MA-DOPA (Lot 201110AHT-A) ZOOM	188	1164
Alg-MADA 210329-AHT-A (akina 500-6379 ZOOM)	105	166
AlgMADA 210413AHT-A121 ZOOM	84	2703
AlgMADA 210505AHT-A121 ZOOM	108	2141
AlgMADA 210609AHT-A121 ZOOM	49	2706
ALG-MA-DOPA (Average ± STDEV, N = 8)	**73 ± 61**	**1564 ± 898**
Gel-MA-Dopa		
GEL-MA-DOPA (Lot200914AHT-A) ZOOM	1023	235
GEL-MA-DOPA (Lot 201118AHT-A) ZOOM	829	351
GEL-MA-DA 210208AHT-A (ZOOM)	1056	523
GEL-MA-DA 210223AHT-A ZOOM	561	138
GelMA-DA 210302AHT-A ZOOM	920	463
GEL-MA-DOPA (Average ± STDEV, N = 5)	**878 ± 198**	**342 ± 159**
Other Type Samples		
Gelatin-Dopamine (201207AHT)	-NA- (0)	569

Fourier-Transform Infrared Spectroscopy (FTIR)

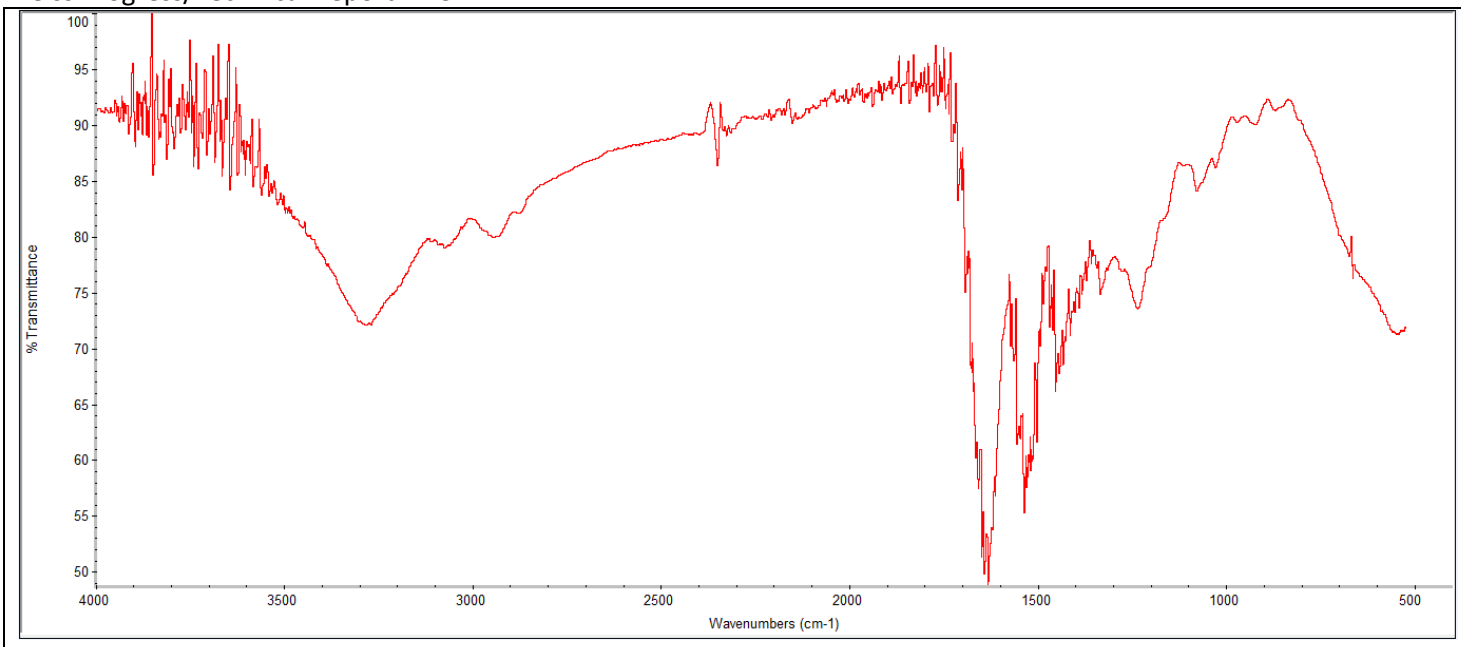
Similar to how visible light is absorbed by conjugated dienes or other specific chemical moieties which interact with the light, infrared light is absorbed by specific chemical moieties which interact with that particular wavenumber of the infrared light. The interactions of specific chemical moieties and their type of motion (rotation, wagging, stretching, etc.) are ascribed to specific wavenumbers (another way to present frequency) and can be used to gain general chemical understanding of a sample as well as compare materials to each other.

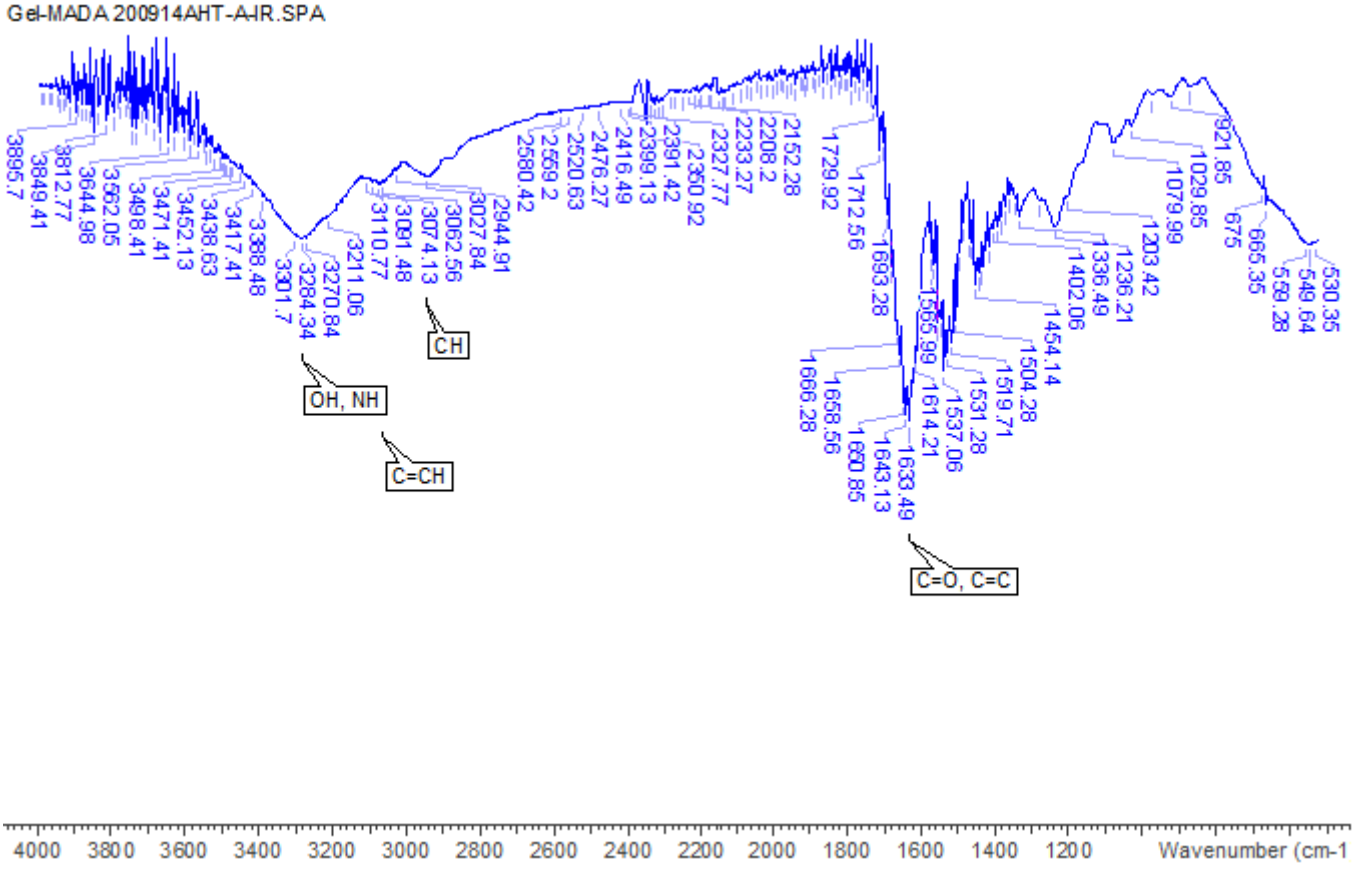
After background collection, samples were compressed against the window of the diamond ATR attachment using the manufacturer provided sample holder. Spectra were collected by a Nicolet Avatar model 380 from 400 – 4000 cm-1 and processed using Omnic Software. The peaks were further interpreted and marked using ACDLabs Spectrus Processor (2015, pack 2).



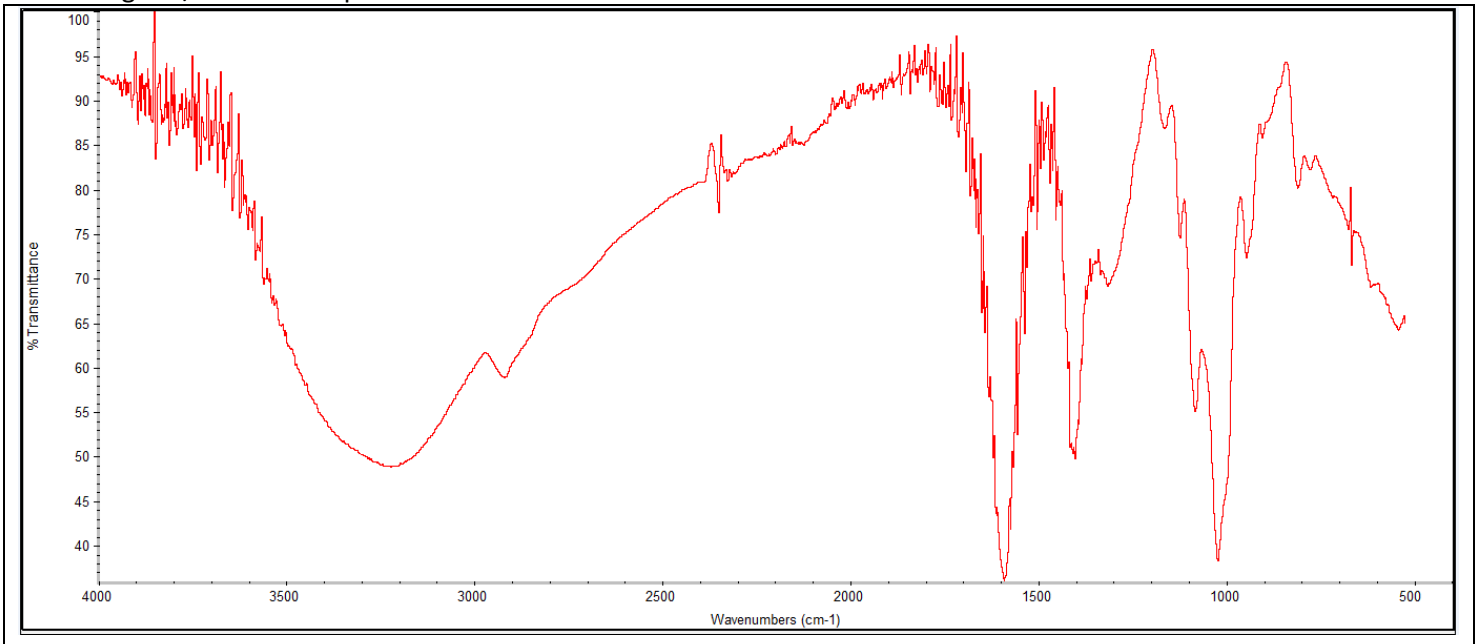


GEL-MA Lot# 210127JSG-A121

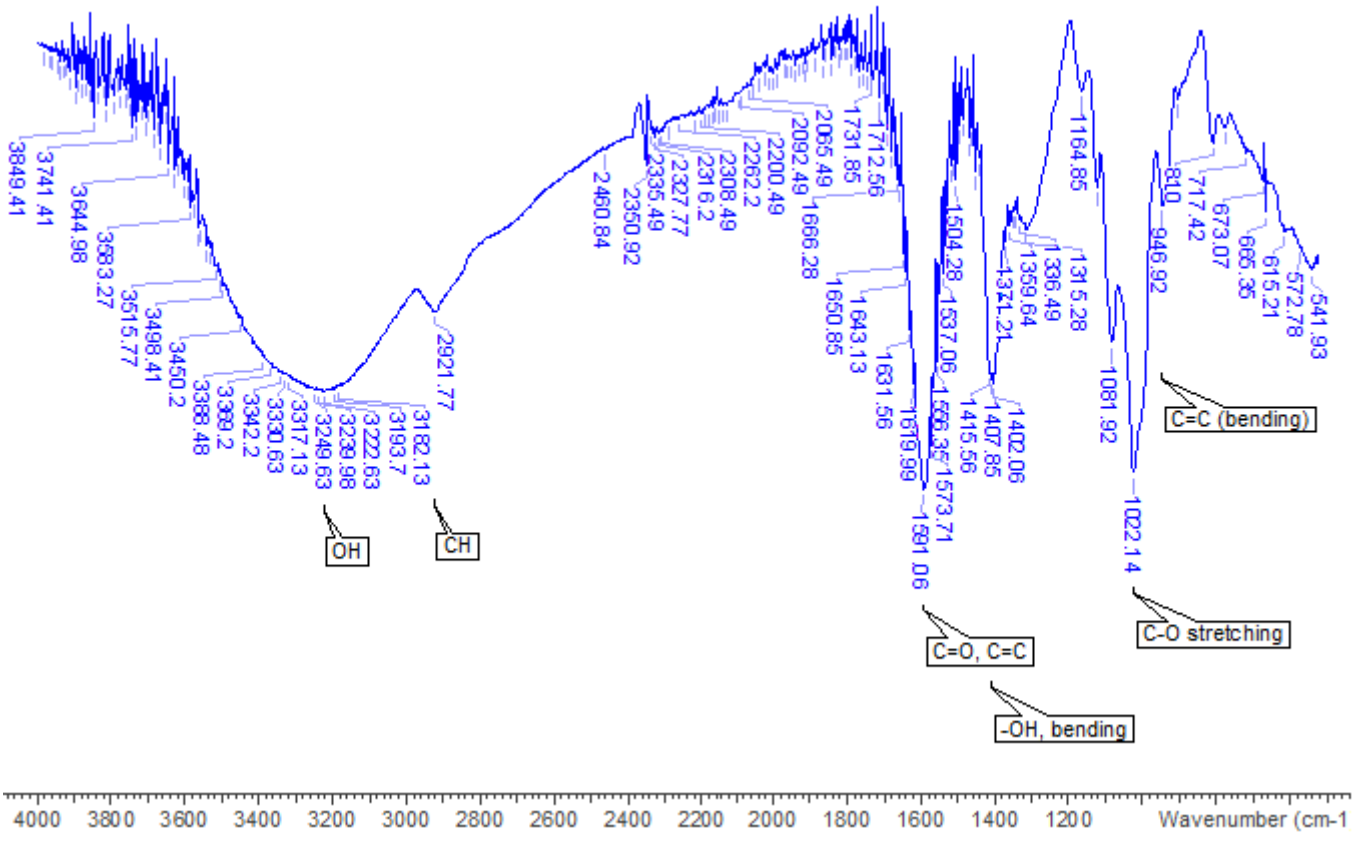




GEL-MA-DA 200914AHT-A



Alg-MA 201020AHT-A-IR.SPA



ALG-MA 201020AHT-A

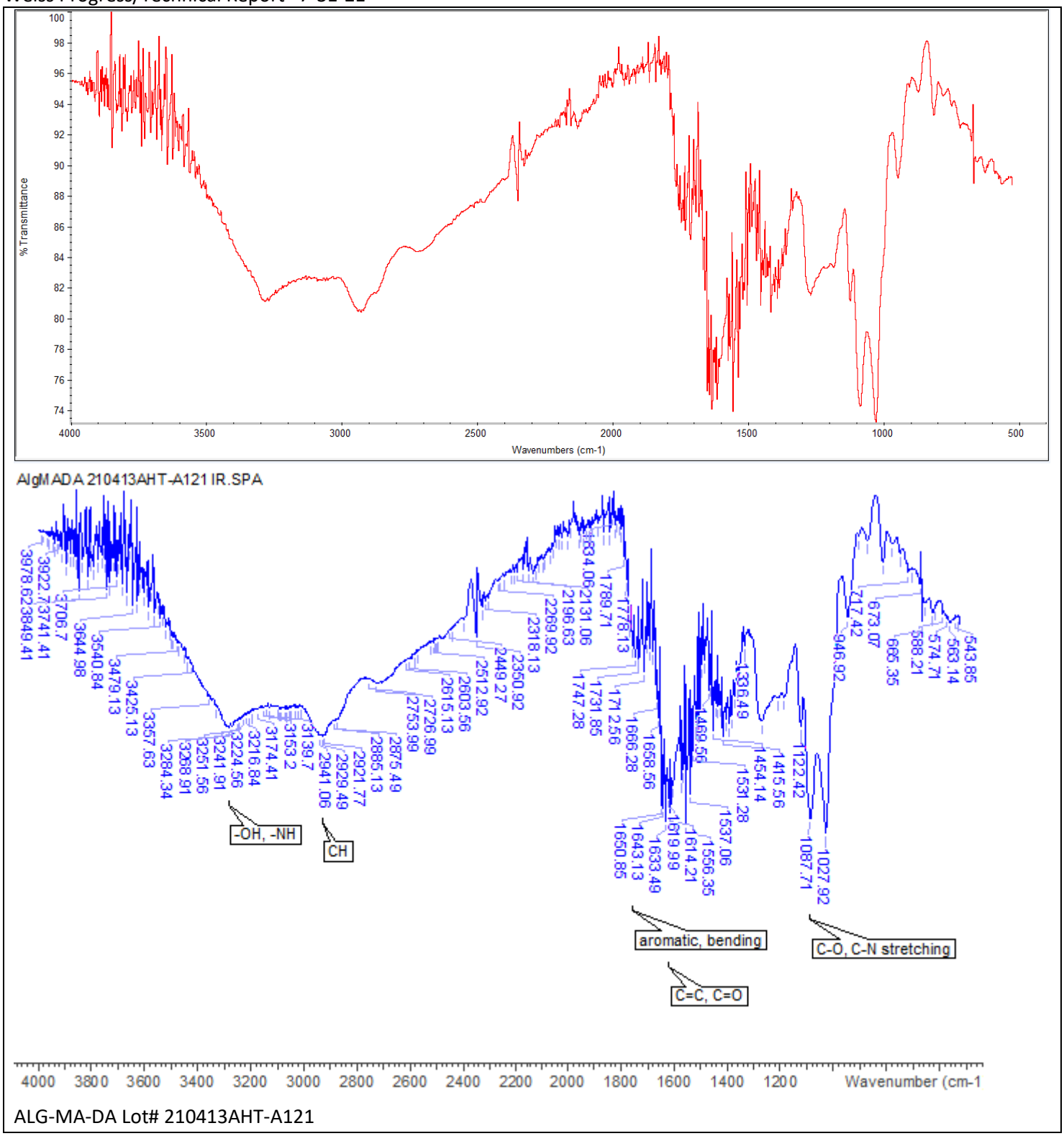


Figure 6. FTIR spectra collected from indicated sample using diamond-ATR (Nicolette 380). For each sample indicated both the overview of the spectra and also a detailed listing of wavenumbers with certain peaks of interest denoted with their moiety and absorption type (bending, stretching, etc.).

Briefly, most scans were confirmed as possessing strong peak at 3000-3500 correlates to hydroxyl and amine stretching from a multitude of hydroxyl (O-H) and some amine (N-H) bonds. Peak at 1600-1700 corresponds to carbonyl stretching (C=O) from esters and amides. Peak around 1400 ppm corresponds to C-H bending (alkane), 1300-1200 ppm to C-O stretching, and peak around 1000-1200 to C-N stretch. It should be noted that several of these peak regions overlap and so the assignments are not conclusive. GEL-MA-DA provides many more peaks due to gelatins more chemically heterogenous nature as compared to alginate. Regardless, the absorption peaks observed in the sample correspond to expected moieties in the product. More importantly, the FTIR data was observed to generally be reproducible from one batch to the next.

Gelation Test

Gel testing was performed as previously described. Briefly, a 4% methacrylated polymer solution in DiH2O was generated and placed on shaker to dissolve. A 0.5% Eosin Y (Aldrich MKBW0030V) in 1-Vinyl-2-Pyrrolidinone (1V2P) (Aldrich MKCC6000) was generated and dissolved on the shaker. A 5M triethanolamine (initiator, TEOA) (Aldrich MKCC8886) in DiH2O solution was created and dissolved with shaking. To test, 5µL of Eosin Y/1V2P solution and 50 µL TEOA solution was added to 2 ml of the 4% w/v alginate-methacrylate solution and mixed thoroughly with the vortexer. Parafilm was wrapped around both ends of a microscope slide to create space between the top and bottom slides. Alginate-methacrylate solution mixed with photoinitiator (100 µL) was pipetted onto the space between these slides. Exposed the slide to visible green light of wavelength 510 nm (provided by UVM) for specified minutes as indicated in results to crosslink hydrogel.

Table 6A Gelation

SAMPLE NAME	**AMOUNT (mg)**	**DIH_20 ADDED (mL)**	**GEL/DID NOT GEL**
2% Alg-MA-DA 200727AHT-A	78.4	3.92	Did not gel
2% Alg-MA-DA 200824AHT-A	78.6	3.93	Did not gel
4% Gel-MA 200721AHT-A	160.6	4.01	Gelled
4% Gel-MA 200727AHT-A	158.3	3.95	Gelled
2% Alg-MA-DA 200924AHT-A	80.9	4.04	Did not gel
4% Gel-MA-DA 200914AHT-A	80.4	2.00	Did not gel

To review the impact of using different compounds, gel testing was performed using 0.5% Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, Aldrich Cat# 900889-1G) or higher concentration as listed. The listed amounts and volumes were vortexed and shaken to dissolve. They were then Left under blue light (405 nm) for 5 min and then another 5 min and checked for gelation by poking with a spatula.

Table 6B Gelation

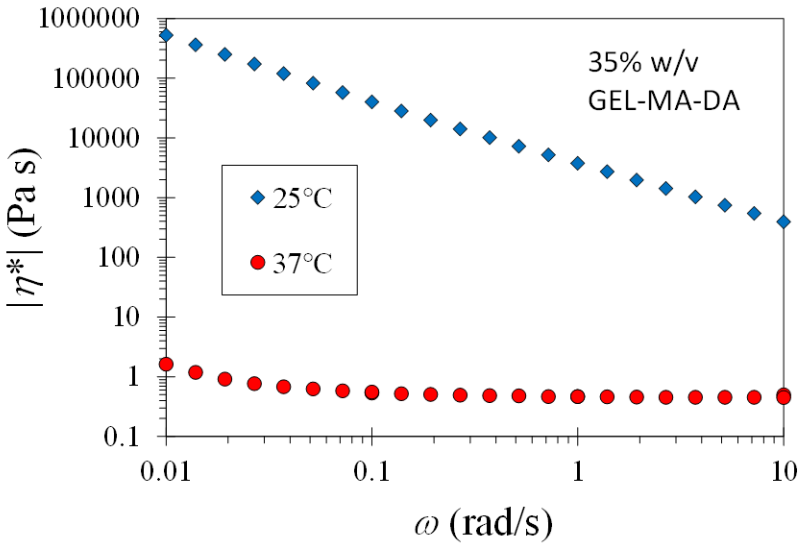
SAMPLE NAME	AMOUNT (mg)	0.5% LAP ADDED	GEL/DID NOT GEL
3% Alg-MA 201020AHT-A	81.4	2.71	Did not gel
SAMPLE NAME	AMOUNT (mg)	1.0% LAP ADDED	GEL/DID NOT GEL
3% Alg-MA-DA 201110AHT-A121	44.0	1.47	Did not gel

Further discussions with UVM indicated that the gelation process was encountering more success at UVM. There were several reasons for this primarily related to preparation facilities and light supply. The need for material to be used for creation of crosslinked patches in PTFE mold led to the decision to halt further gel-testing at Akina and leave this work for UVMT to continue.

Gelation kinetics

Gelation properties (shear rheometry) of the ALG-MA-DA and GEL-MA-DA formulations is being assessed on an ongoing basis. Representative data for GEL-MA-DA depicting the magnitude of the complex viscosity versus frequency for 35% w/v solution of GEL-MA-DA/DI water at room and body temperatures, respectively 25 and 37°C, is depicted in **Figure 7** below. The magnitude at 25°C is 103 to 106 higher than that at 37°C. Gelatin derivatives go through sol-gel (physical gelation) transition below 30°C while alginate derivatives do not, and thus understanding the flow property of GEL-MA-DA is important for applications. Further clarification of gelation characteristics will be pursued over the next year.

Figure 7: Shear rheometry. The magnitude of complex viscosity versus frequency for 35% w/v solution of GEL-MA-DA/DI water at 25 and 37°C. The magnitude at 25°C is significantly higher than that at 37°C due to physical gelation.



Specific Aim 1, Major Tasks 1 and 2: Additional studies on producing ready-to-go pre-crosslinked patches.

In the initial design of the ALG-MA-DA and GEL-MA-DA sealants, the protocol was to perform photo-crosslinking of the sealant materials by addition of an FDA-approved photo-initiator, Eosin Y (0.00125% w/v), along with 125 mM triethanolamine (Sigma) and 19 mM for 1-vinyl-2-pyrrolidinone. While this is a successful approach, as depicted in the recently published manuscript, we had already begun refining the photo-initiator approach with use of an alternative FDA-approved photoinitiator, 0.5% Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, Aldrich Cat# 900889-1G). This has proven to be a more reliable and also easier approach.

To this end, both ALG-MA-DA and GEL-MA-DA materials, produced at Akina, are solubilized with addition of 1% LAP, poured into a custom-designed custom-built Teflon mold of 150 mm x 150 mm x 1 mm and frozen at -80°C resulting in a 1 mm thick polymer sheet. The frozen polymer sheet was allowed to briefly thaw and was subsequently photo-crosslinked under blue light (405 nm) emitting LEDs with solid patch formation occurring within 5 minutes before being refrozen at -80°C. After discharging the polymer sheet from the mold, the sheet was lyophilized, and individual patches were prepared from the lyophilized sheet using a 6mm diameter circular biopsy punch. (**Figure 8**).

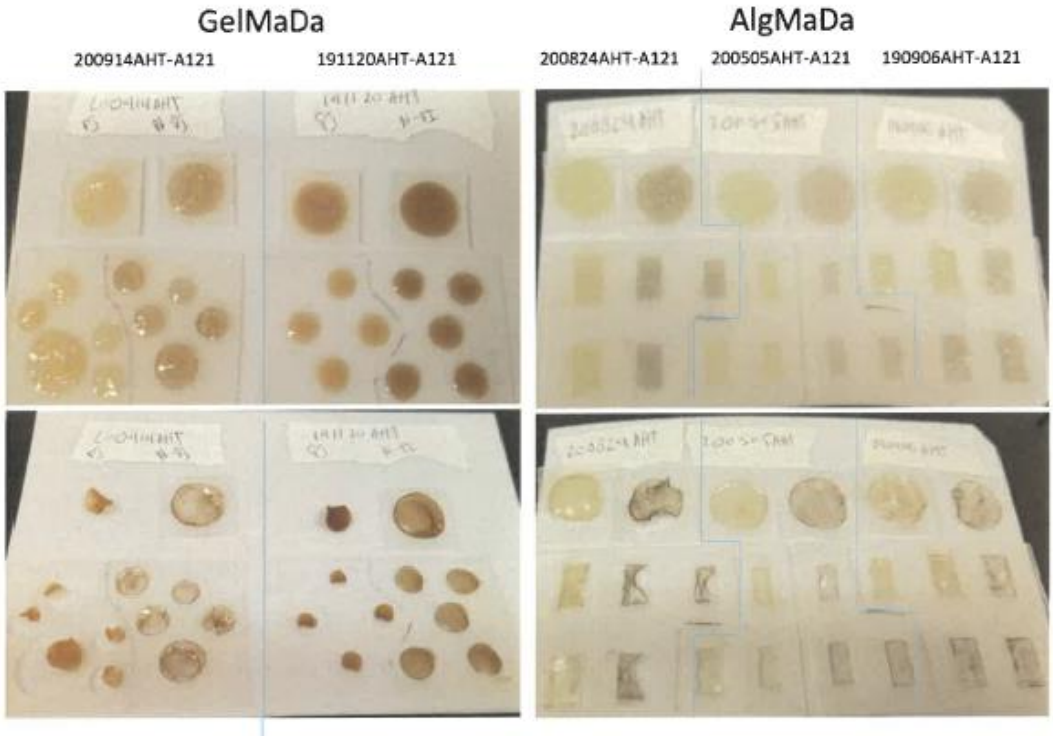


Figure 8 Representative examples of ready-to-use freeze-dried lyophilized ALG-MA-DA and GEL-MA-DA patches

This approach works well for both ALG-MA-DA and GEL-MA-DA materials. In addition, we have further explored oven drying the patches rather than freeze-drying and lyophilization. To this end, both patch formulations were oven dried for 30' at 40°C. This also produced viable patch materials. However, as further depicted below under **Specific Aim 2**, the oven-dried ALG-MA-DA and GEL-MA-DA patches were not as reliably adherent to tissue surfaces.

Overall Summary for Specific Aim 1, Major Task 1: Subtasks 1 and 2
Through the course of working on Subtask 2, several features of the HNMR and also FTIR analytical methods were refined. These will continue to be powerful tools for continuing material characterizations. In parallel, further refinements of ready-to-go patch production have provided insights into optimizing large scale, reproducible and reliable production.

Significant Milestones Achieved:
Progress towards large scale ALG-MA-DA and GEL-MA-DA patch synthesis with controllable, reliable, and reproducible degrees of methacrylation and dopamine conjugation.

- Scale-up of synthesis of ALG-MA-DA and GEL-MA-DA performed at twice the scale.
- Synthesis efficiency improved to require less quantity of dopamine.
- Achieved ranges of methacrylate and dopamine identified and characterized.
- Reaction kinetics and rate information obtained.
- Shear rheometry/gelation kinetics data obtained
- Successful incorporation of an alternative photo-initiator for cross-linking
- Development of 2 parallel means for processing and producing cross-linked materials into ready-to-go patches: freeze-drying/lyophilization and oven-drying.

Specific Aim 1, Major Task 2: Develop quality control approaches for rheologic and mechanical characterization of ALG-MA-DA patches.

Subtask 1: Burst pressure and analyze cohesion and adhesion assessments on collagen substrates.
We have made significant progress in mechanical testing of the ALG-MA-DA and GEL-MA-DA patches. This includes assessments of burst pressure, tensile testing, lap-shear testing, and peel testing. As detailed in the previous progress report and also in the recently published manuscript, all testing was done according to appropriate ASTM standards. The specific methodologies employed are detailed as well in those two documents.

Initial proof of concept results with the original formulations of ALG-MA-DA and GEL-MA-DA (Eosin Y photo cross linker) are included in the published manuscript and are shown below (**Figure 9**). Mechanical testing was performed on ALG-MA-DA and GEL-MA-DA, comparing both to each other and to ALG-MA and GEL-MA, respectively. Notably, the ALG-MA-DA hydrogel formulation tended to gel quickly while being applied with a dual lumen syringe, comparable to what occurs during clinical use of ProGel™. Conversely, the GEL-MA-DA hydrogel formulation was easily applied without clogging of the dual lumen application syringe. Both ALG-MA-DA and GEL-MA-DA were easily formulated into pre-formed freeze-dried lyophilized patches. Both ALG-MA-DA and GEL-MA-DA demonstrated enhanced tensile, shear, and peel strengths compared to their non-conjugated counterparts (**Figure 9a-c**). Dopamine conjugation enhanced burst pressure performance of both ALG-MA and GEL-MA with values 16 times higher in ALG-MA-DA compared to ALG-MA, although with higher variance in ALG-MA-DA, and 3 times higher in GEL-MA-DA compared to GEL-MA (**Figure 9d**). Notably, the recorded burst pressure of 250 cm H_20 surpasses the 218 cm H20 burst pressure reported for Progel™.

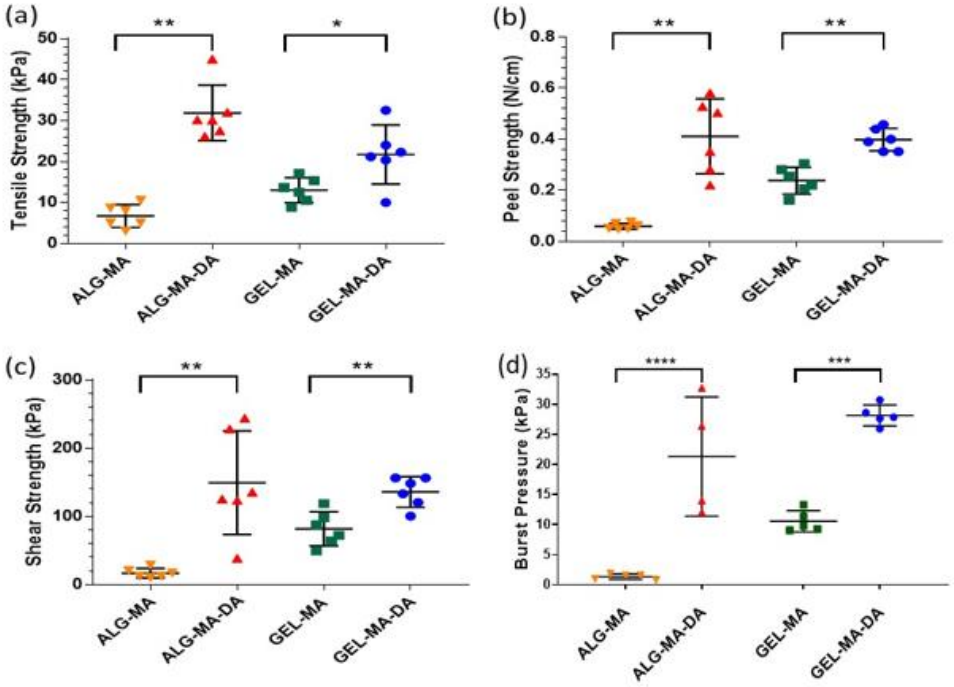


Figure 9: Mechanical testing. Patches of alginate derivatives and solutions of gelatin derivatives were used. (a) Tensile strength determined by tensile tests. Values represent mean ± standard deviation (SD, error bars) and n = 6. (b) Peel strength by T-peel tests. Values represent mean ± SD (error bars) and n = 6. (c) Shear strength by lap-shear tests. Values represent mean ± SD (error bars) and n = 6. (d) (d) Burst pressure versus time to burst (failure). Values represent mean ± SD (error bars) and n = 5. Values represent mean ± SD (error bars) of n = 4 from one of two representative experiments. * $p<0.05$, ** $p<0.01$, *** $p = 0.0001$, and **** $p<0.0001$.

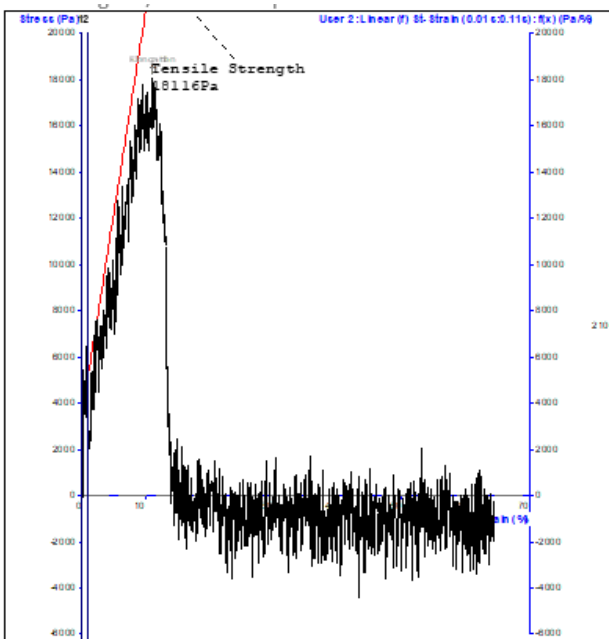
We are now performing comparable assessments with the new formulations of ALG-MA-DA and GEL-MA-DA described in **Specific Aim 1**. Mechanical testing is also now being done at both UVM and at Akina. For testing done at Akina, patches made at UVM with the new formulations utilizing both freeze dried/lyophilized and oven baked patches are sent back to Akina and tested for mechanical properties using a TA.XTplus mechanical tester equipped with 5 kG load cell operated by exponent software. In parallel with comparable methods utilized at UVM, the specific methodologies utilized at Akina are depicted here. Samples (20 x 10 mm rectangle) have cut out 5 mm holes on each side to make a dogbone configuration for optimal tensile, shear, and peel testing. 10 ul of 1 w/v% sodium metaperiodate is added to the middle of the dogbone allowed to oxidize for 5 minutes prior to loading onto the testing device. Importantly, we have now also incorporated direct head to head testing with ProGel™ in addition to comparing to previously published data. For comparison purposes ProGel™ is reconstituted and prepared according to manufacturer instructions, samples cast inside of a polystyrene 6-well plate and then cut to a dog-bone for testing with no applied oxidizer. The specimen thickness and width for all samples were measured with calipers and these values were multiplied to obtain a cross sectional area. This parameter was then input to the Exponent software for calculating mechanical stress applied. The initial distance between the clamps is recorded by the software and deformative strain is calculated as % change in distance. The samples are held by tensile clamps and upper fixture pulled at 1 mm/sec at room temperature until mechanical rupture.

Calculations obtained using Exponent software include:
Elastic modulus: the slope of the stress-strain curve between ~0.1 – 2% strain (small-strain) was taken in kPa as the elastic modulus. Due to noise in the relatively low-force applied to a heterogenous material, the exact location of the slope varied to match the most linear portion of the sample.

Tensile Strength: The stress at break (kPa) was recorded as the tensile strength.

Elongation: The strain (% length change) at break was recorded as maximum elongation.

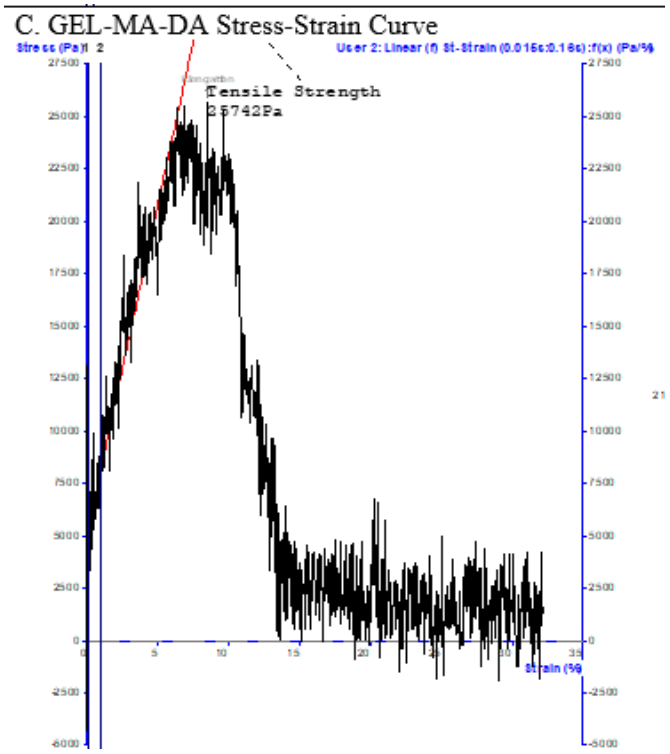
Figure 10. Photos of tensile testing samples (pretest) at Akina including ALG-MA-DA, GEL-MA-DA, and Progel as well as their respective stress-strain curves used for determining their mechanical properties. Note middle portion of ALG-MA-DA and GEL-MA-DA is darkened by the oxidizing solution added prior to testing while Progel has an optically clear appearance.



A. ALG-MA-DA Stress-strain curve

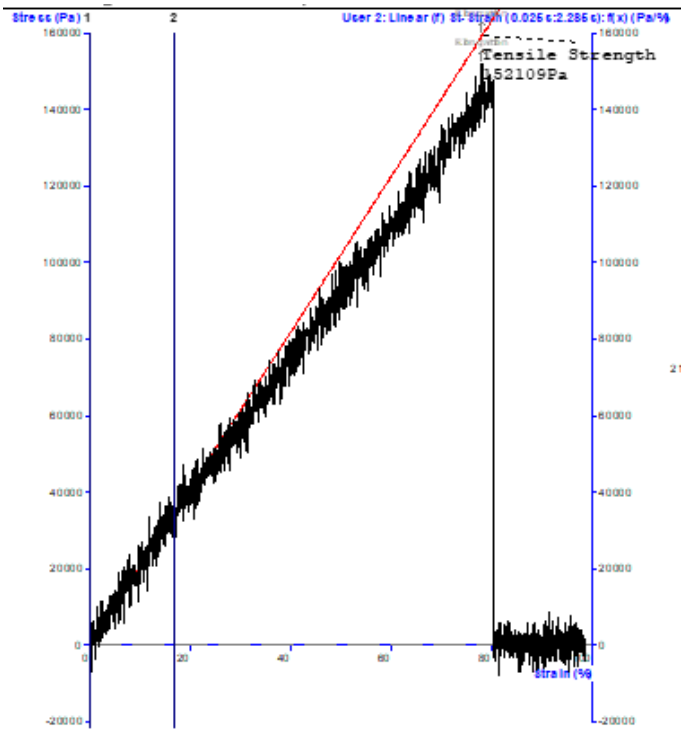
B. ALG-MA-DA sample

C. GEL-MA-DA Stress-Strain Curve



D. GEL-MA-DA sample

E. Progel Stress-Strain Curve



F. Progel Sample

Representative results from are depicted in **Table 7** below. Notably the tensile strength of the new material formulations is comparable to that achieved with the previous formulations.

Table 7. Tensile Testing Results

Sample (N, Mean ± STDEV)	Elastic Modulus (kPa)	Tensile Strength (kPa)	Elongation (%)
ALG-MA-DA 110, 4% (N=3)	1.9 ± 0.9	21.7 ± 9.8	10.2 ± 1.1
GEL-MA-DA 223 15% (N = 1)	5.3	33.7	5.7
GEL-MA-DA 302 15% (N = 3)	4.5 ± 1.3	35.5 ± 13.8	8.6 ± 3.0
GEL-MA-DA 208 (15% (N = 2)	3.2 ± 3.9	18.3 ± 16.7	2.3 ± 3.1
PROGEL 210621 (N = 3)	0.9 ± 1.0	83.7 ± 62.7	55.6 ± 20.1

Burst Pressure Assessments

Representative burst pressure evaluations with the new ALG-MA-DA formulations are shown in **Figure 11**. Four different batches are shown (labeled as Alg 110, 413, 505, and 924, respectively). Notably, all formulations have strong adherence with burst pressures exceeding increased pleural and tracheal pressures found under different disease conditions, for example peak and plateau pleural pressures of 30-50 cm H_2O characteristic of the acute respiratory distress syndrome (ARDS). These are comparable to both published data on ProGel™ and also to our previous experience with ProGel™. Ongoing burst pressure assessments are in progress with both ALG-MA-DA and with GEL-MA-DA and will continue to directly compare to ProGel™.

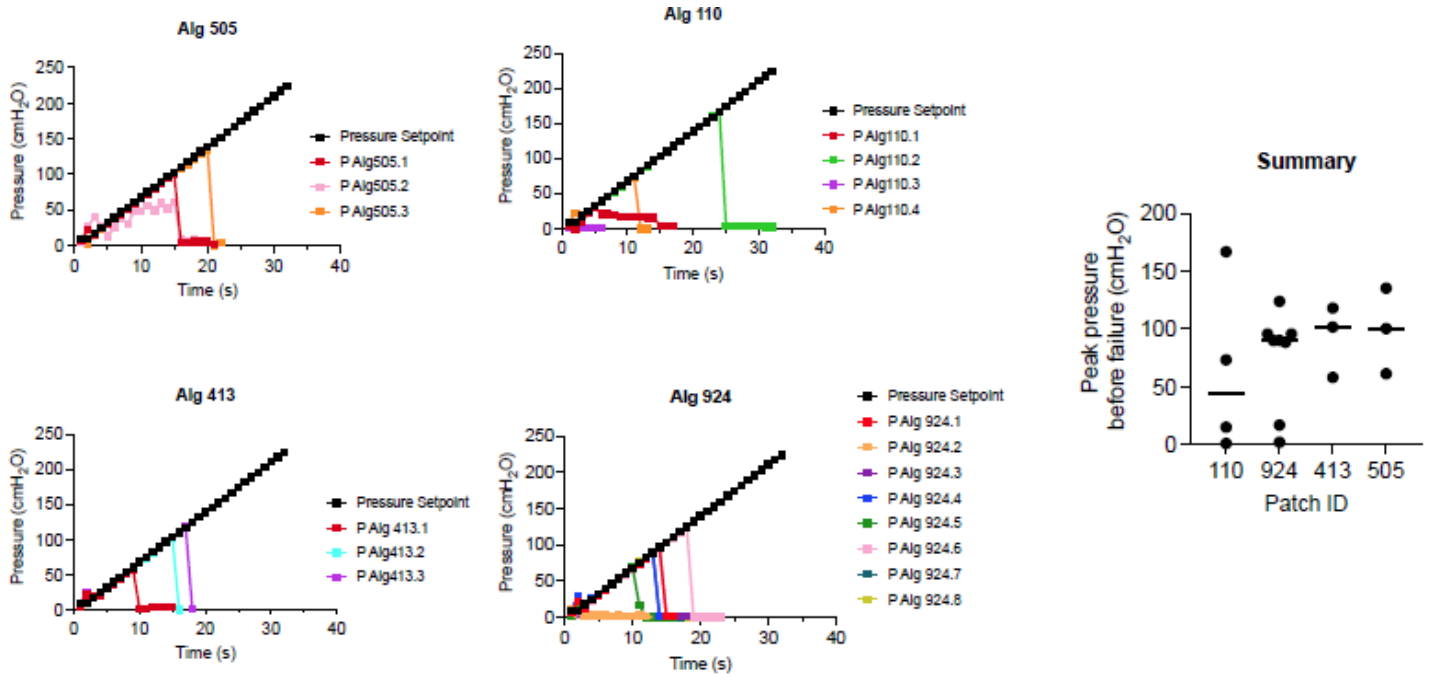


Figure 11 Burst pressure evaluations. Pressures are indicated on the Y-axis in cm H2O and time in seconds on the X-axes. Pressure setpoint (black line) indicates applied ramped pressures and pressure feedback (different colored lines) indicates measured pressures for repeat assessments of 4 separate ALG-MA-DA preparations. The divergent point is the burst pressure. The burst pressures for each preparation are summarized in the accompanying table showing the range of repeat measures. Of note is good consistency with the 3 later batches: 924, 413, and 505 with mean pressures well above the values optimal for use as a pleural sealant.

Oxidation and Sealant Adhesion to standard ASTM sausage casing (collagen) for burst pressure testing
The original oxiding agent utilized with the Eosin Y/TEOA cross-linking agent was 0.1% sodium metaperiodate. which also has some intrinsic cross-linking activity. Now that the cross-linker has been changed to LAP, which has no appreciable effect on dopamine oxidation, further refinement of the concentration and timing of metaperiodate application has being systematically evaluated. 1% w/v appears to cause significant and functional oxidation and adherence of both ALG-MA-DA and GEL-MA-DA patches within approximately 2 minutes. As further discussed below, 1% metaperiodate appears to have minimal toxicity on representative lung cell types (**Subtask 3**) and has further been successfully utilized in *ex vivo* and *in vivo* animal studies (**Specific Aim 2**)

Major Task 2: Subtask 1 Milestone(s) Achieved:

a. Successful materials testing (burst pressure, tensile, shear, peel) with values in physiologic ranges appropriate for use in lung injuries and also comparable to values obtained with the only commercially available pleural sealant, ProGel™.
b. Modifications in protocols for optimizing use of the oxidizing agent during patch application

Subtask 2: Degradation of standardized patches
Initial studies are in progress with the new ALG-MA-DA and GEL-MA-DA formulations and we anticipate completion by end of calendar year 2021.

Subtask 3: Cytotoxicity of standardized patches
Initial studies were performed utilizing the original ALG-MA-DA and GEL-MA-DA formulations and the original photo initiator cross linking approach (Eosin Y). Neither ALG-MA-DA nor GEL-MA-DA demonstrated obvious toxic effects on growth of a range of relevant lung cell types including human lung airway epithelial (HBE), alveolar carcinoma (A549), human lung fibroblast (HLF), and human pleural mesothelial cells (HPM) (**Figure 12A**). This include both photo-cross-linked (polymerized) as well as non-polymerized compounds despite significant effects on cell viability observed with the photo-initiator cocktail or oxidant (0.1 % sodium periodate) alone, particularly with HBE, HLF and HPM cells (**Figure 12B**). This demonstrates that although the photo-initiator compounds or oxidants utilized can be toxic toxicity is removed as the compounds are used up or washed out.

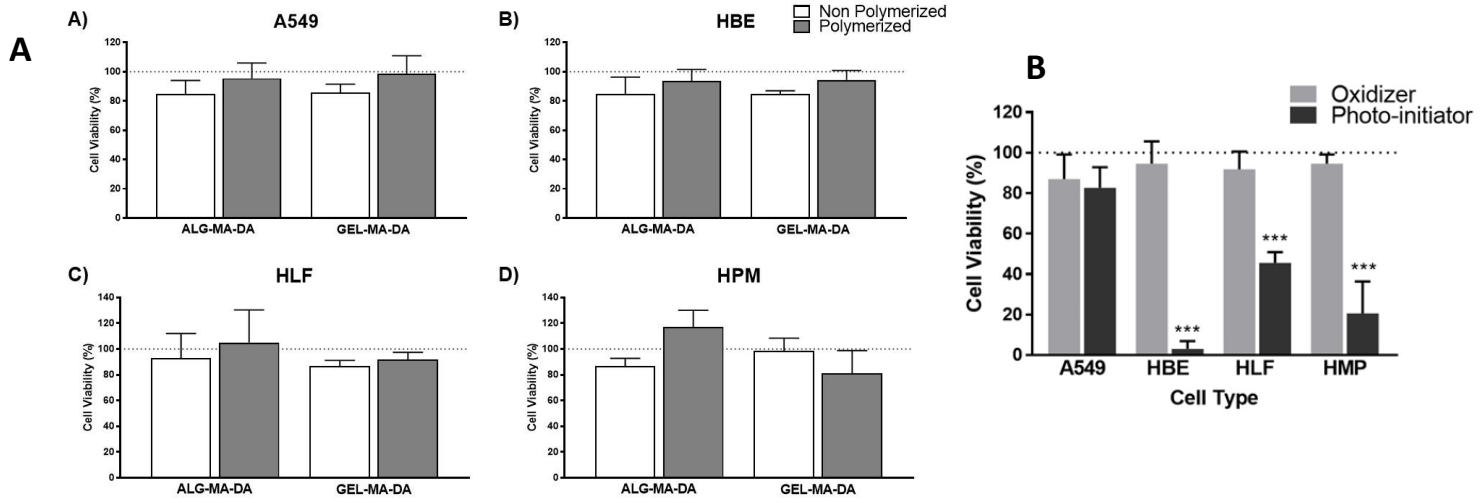
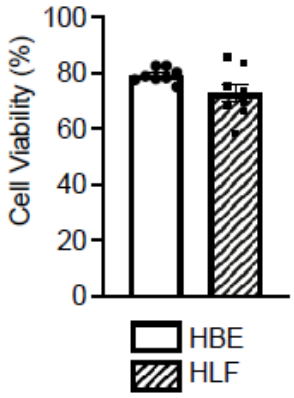


Figure 12: Cytotoxicity screening of ALG-MA-DA and GEL-MA-DA sealant materials without (non-polymerized) or following photocrosslinking (polymerized) demonstrate no siginificant cytotoxicity for human bronchial epithelial (HBE) or transformed alveolar epithelial cells (A549), human pleural mesothelial cells (HPM), or human lung fibroblasts (HLF). (a) In contrast, both the photo-initiator compound and oxidant alone each had significant toxicity on HBE, HPM, and HLF cells. (b) Data is presented as means + SD (n= 4 for each cell line) of similar results from one of two separate experiments. *** $p<0.001$.

In ongoing studies, we have now performed initial cytotoxicity assessments comparing the new photo initiator LAP with the previously utilized Eosin Y. As depicted in **Figure 13**, LAP is significantly less toxic for HBEs and also less toxic for HLFs. Ongoing studies will better define toxicity of the LAP alone, as well as the higher periodate concentration, both alone and after reacting with the ALG-MA-DA and GEL-MA-DA sealant materials.

Figure 13: Cytotoxicity screening of LAP on human bronchial epithelial (HBE) or human lung fibroblasts (HLF). Data is presented as means + SE (n= 8 for each cell line). * $p<0.0001$ compared to control viability as per 1-way ANOVA.



Major Task 2: Subtask 3 Milestone(s) Achieved:

a. Comprehensive cytotoxicity testing with old ALG-MA-DA and GEL-MA-DA formulations demonstrates no significant toxicity for a range of relevant lung cell types.
b. 0.1% sodium periodate alone has no significant toxicity on these cell types. Further testing will determine any potential toxicity of 1% sodium periodate.
c. Eosin Y alone is profoundly toxic to lung epithelial cells, fibroblasts, and pleural mesothelial cells. LAP alone is significantly less toxic to epithelial cells and fibroblasts. Continued testing is ongoing.

Major Task 3: Define optimal sterilization, and storage conditions.

Subtask 1: Define optimal sterilization approach for optimized patch

For viable production of clinical material, the generated formulation must be either aseptically handled or terminally sterilized. Due to the cost of aseptic manufacture, terminal sterilization is preferred. Prior work presented in the previous progress report indicated that the old formulation of ALG-MA-DA survived sterilization by ethylene oxide (ETO) with minimal chemical degradation or interference applicability of ethylene oxide sterilization in general.

We have subsequently assessed hydrogen peroxide vapor and found this to be unsuitable, in part as it oxidizes the patches and renders them less adherent when applied to tissue. Standard autoclaving is not an option as the materials will be degraded. Gamma radiation remains an option and we will be exploring this and also ethylene oxide with the new formulations

Interestingly, we have also found that the 1% sodium periodate utilized for oxidation and enhancement of sealant adherence also has a sterilizing effect. Patches that have been sprayed with 1% sodium periodate or with sterile saline were cultured in Brain Heart Infusion (BHI) medium or Lysogeny Broth (LB) on a shaker at 37 C for 48 h. Any turbidity is indicative of microorganism growth. BHI is a nutrient-rich medium, and can therefore be used to culture a variety of fastidious organisms, such as bacteria, in particular streptococci, pneumococci and meningococci, as well as fungi, whereas LB promotes growth of a wide spectrum of bacteria. Both media are widely used in water and food safety as well as antibiotic resistance tests. As depicted in **Figure 14**, in the case of BHI, turbidity does not occur when patches are sprayed with sodium periodate. No turbidity was visible with LB incubation, whether or not patches were treated with sodium periodate.

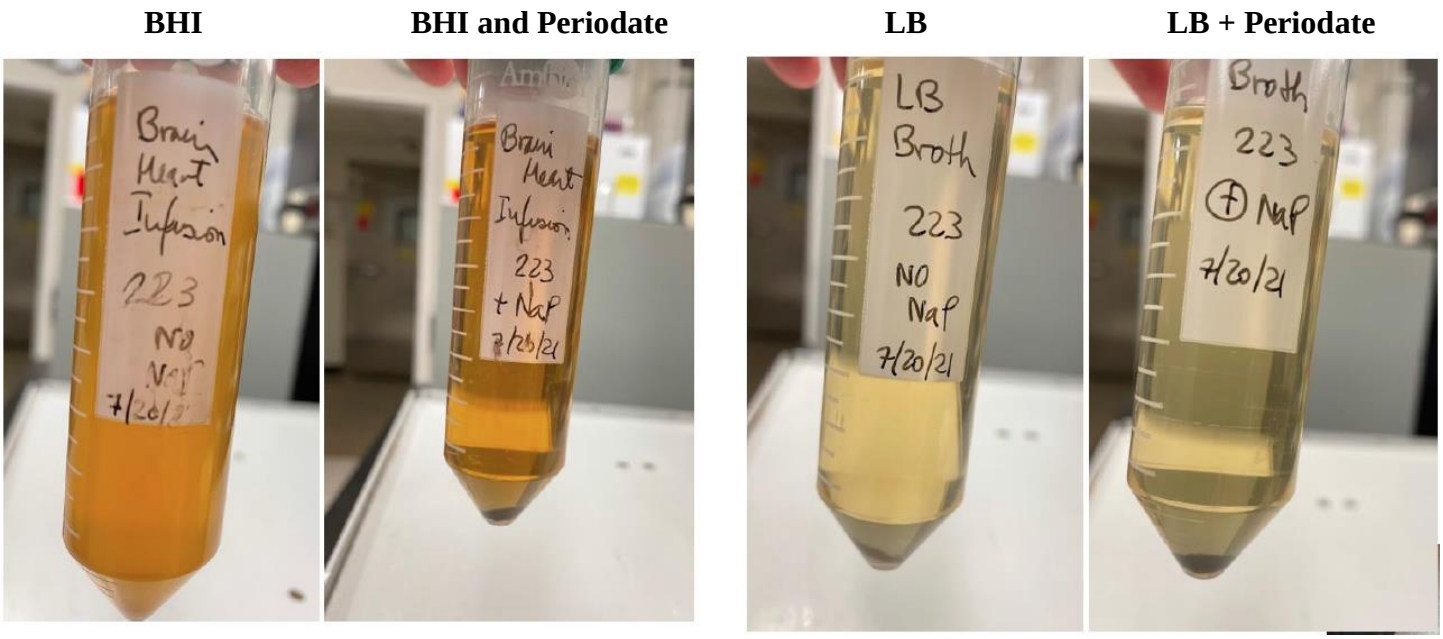


Figure 14: Addition of the oxidizing agent sodium periodate (1% v:v) to ALG-MA-DA patches decreases turbidity when incubated in brain heart infusion medium (BHI). No turbidity was observed in lysogeny broth (LB) medium, with or without sodium periodate addition.

2: Clarify need for addition of preservatives to optimized patch

Subtask 3: Define optimal long term storage (packaging, temperature, humidity, etc) conditions

Extensive data was presented in the previous progress report on storage stability of the old formulations of ALG-MA-DA and GEL-MA-DA tested under a variety of conditions at Akina including forced degradation (40°C), light degradation, room temperature degradation, and freezing-induced (-20°C) degradation. These were all performed utilizing standard testing methodologies.

In parallel, a range of FDA-approved preservatives and preservation conditions were assessed including Dark (closed, amber-glass vial in normal air); Argon-Dark (closed, amber-glass vial, argon-flush); Argon-Dark-Cys (closed, amber-glass vial in argon with Cysteine); Argon-Dark-EDTA (closed, amber-glass vial in argon with EDTA); Argon-Dark-CA (closed, amber-glass vial in argon with Citric acid); and Argon-Dark-ASA (closed, amber-glass vial in argon with Ascorbic Acid) Preservatives were assessed at 1% v/v in a forced degradation chamber at 40ºC/75% relative humidity for one month.

These initial studies demonstrated that the dopamine and methacrylate are quickly damaged under 40ºC conditions as well as significant self-reaction. Most samples self-crosslinked to become insoluble. NMR on the soluble portions indicated that the use of argon and dark-sided glassware slightly reduces damage under some conditions but the effect is not consistent. The use of preservatives actually made the storage conditions worse and introduced other problems as some materials presented a change in color (green). Raw data and additional spectra are available on request.

A subsequent study was performed in which ALG-MA-DA (old formulation) was ETO-sterilized prior to the storage stability and the preservative agents were more thoroughly mixed in with the ALG-MA-DA followed by freeze drying to ensure more thorough incorporation. A higher concentration of preservative agents was used relative to the ALG-MA-DA and the degradation time was reduced. In addition to the preservative agents listed above (cysteine, EDTA, citric acid, and ascorbic acid), another FDA-approved preservative, butylated hydroxytoluene (BHT), was also evaluated.

In brief summary, unlike the prior testing, the ALG-MA-DA samples did not spontaneously crosslink. However, the addition of preservatives was at a much higher concentration and the shelf-life test drastically shortened. These results do generally indicate the potential for longer-term storage however highlight the importance of careful packaging. The preservative agents did not provide substantial improvements in material stability relative to vacuum-packed control indicating that these materials, under the tested conditions, may not provide much advantage. Additionally, the process may be simplified by simply converting the material to the patch first and then storing the patch instead of the ALG-MA-DA precursor. This is the subject of current investigations with the new ALG-MA-DA and GEL-MA-DA formulations.

Specific Aim 1: Major Task 3: Milestone(s) Achieved: Optimal sterilization and storage conditions

- Confirmed capacity of ALG-MA-DA to be sterilized using commercially available and conventional ethylene oxide gas exposure.
- Eliminated other potential sterilization methods (autoclave, hydrogen peroxide
- Identified hydrogen peroxide as a potential additional sterilizing agent
- Storage stability testing and use of preservatives/antioxidants under accelerated degradation conditions evaluated for old ALG-MA-DA formulations. Confirmed need for air and light protection.
- Recognition that the best approach may to be initial production of the patches rather than long-term storage of the precursor materials.
- Subsequent studies in progress with new ALG-MA-DA and GEL-MA-DA formulations and pre-formed patches

Specific Aim 2: To define long term efficacy and safety in longitudinal small (rat) and large (pig) models of adult pleural injury and of adult and pediatric tracheal injuries.

We have now initiated these studies and in some cases brought to completion. A detailed presentation follows.

Major Task 1: Assess longitudinal efficacy and safety in adult pleural and tracheal injury models
To initiate these studies, it was necessary to demonstrate efficacy in actual lung or tracheal tissue itself. This was accomplished utilizing three approaches: a) Assessments with *ex vivo* ventilated rat and pig lungs; b) *In vivo* application to rat and pig pleural surfaces accompanied by assessment of repair of experimentally-induced pleural injuries; c) Similar *ex vivo* and *in vivo* approaches for tracheal injuries (rats).

Utilizing the original formulations of ALG-MA-DA and GEL-MA-DA, we had demonstrated effectiveness in sealant adherence to both rat and pig lungs and notably repair of the experimentally-induced leaks using as determined by cessation of air bubbling from the leaks following placement of either ALG-MA-DA or GEL-MA-DA patches (**Figure 15**). Using this type of modeling, we were able to demonstrate persistence of patch repair for the usable longevity of these models (generally 24 hrs).

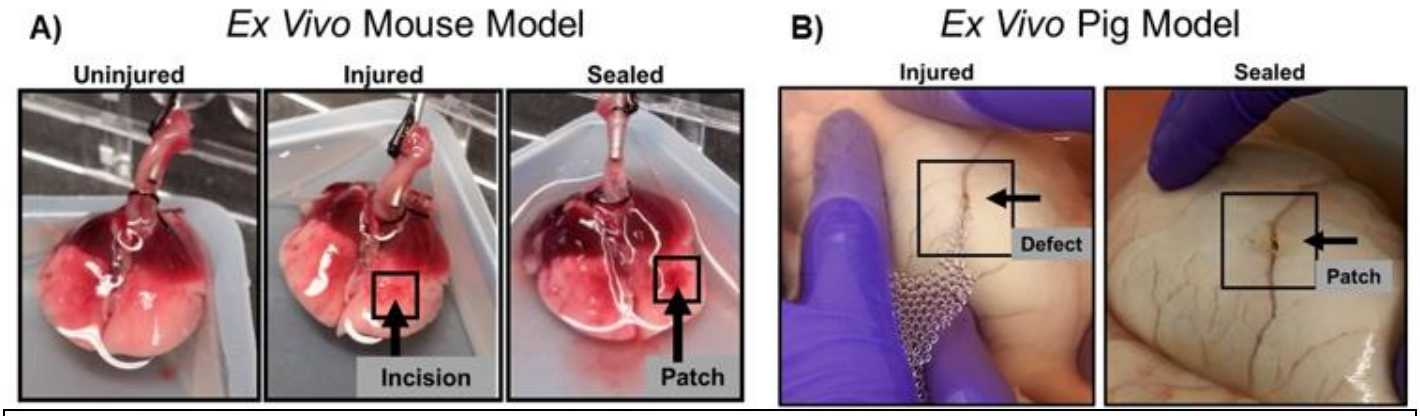
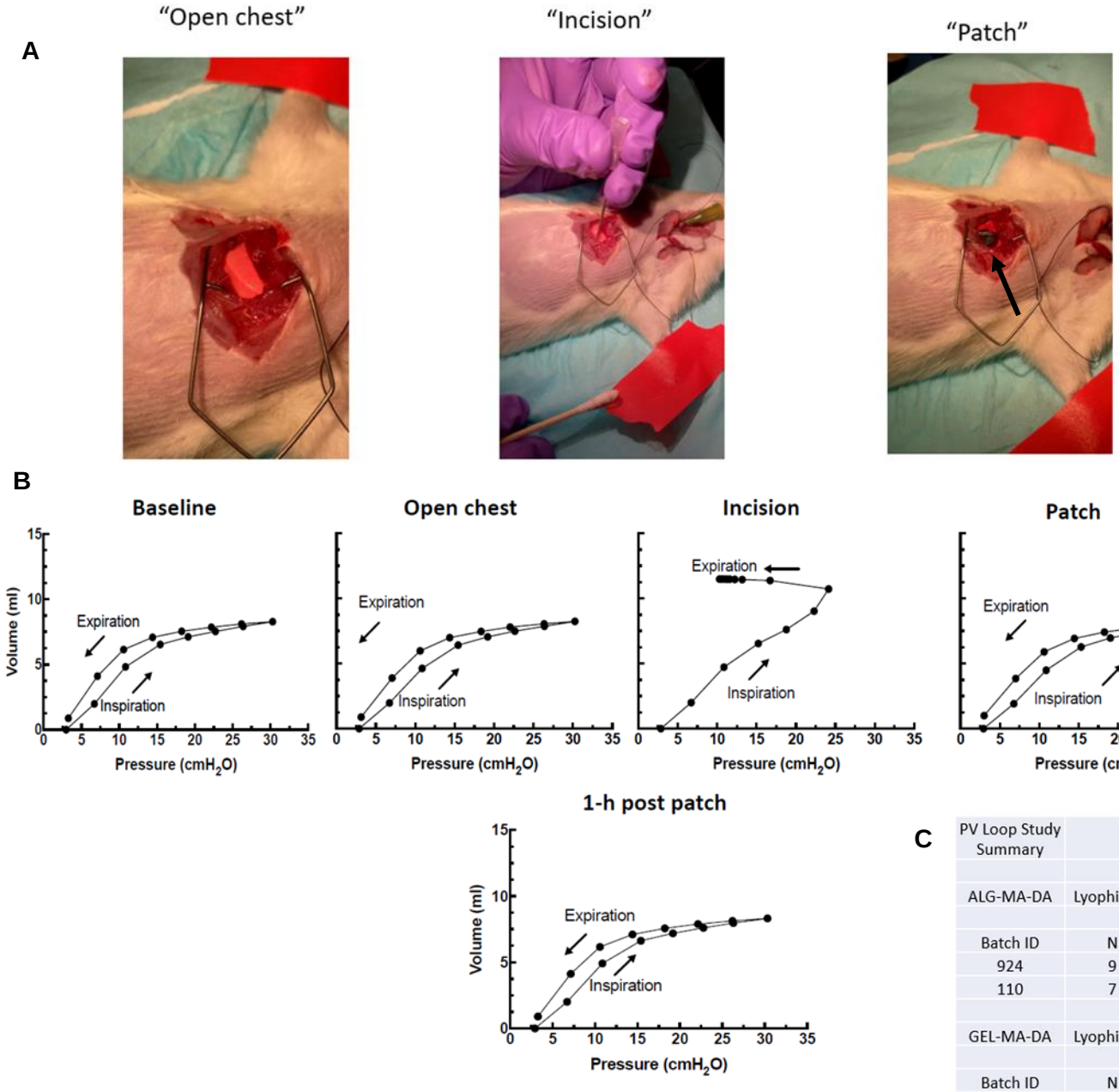


Figure 15: *Ex vivo* lung injury models. Initial testing of patch adherence and efficacy to seal the defect were performed in *ex vivo* rat lung (A) and pig lung (B) models. Arrows determine the position of the defect and the patch. Lungs were inflated and immersed in 1X PBS to visualize the air leak and the seal after patch application.

Short-and long term assessments in *in vivo* rat lung injury models with new formulations
We next assessed the new lyophilized ALG-MA-DA and GEL-MA-DA as well as oven-dried GEL-MA-DA formulation in short term *in vivo* models of rat and pig pleural injuries. Utilizing institutional IACUC and ACURO-approved protocols, rats were anesthetized, intubated, and mechanically ventilated with a FlexiVent ventilator (Scireq, Montreal). The Flexivent device has programmable modules which can measure lung mechanics and other respiratory parameters including pressure volume loops. The power of this approach is that, in addition to qualitative assessments of repair on bubble leaks as in the *ex vivo* models above, quantitative assessments of altered PV loops following injury, reflecting air escaping through the experimentally-induced leaks, and restoration of normal PV loops following patch application can be made. As demonstrated in **Figure 16**, following exposure of the lung with thoracotomy, institution of an experimental leak resulting from puncture of the ventilated lung with an 18 gauge angiocatheter resulted both in qualitative bubble leak as well as disruption of the PV loop. Application of a representative lyophilized/freeze-dried ALG-MA-DA patch results in both qualitative and quantitative leak repair that lasts up to one hour (IACUC/ACURO-allowed duration of these studies).



C

PV Loop Study Summary		
ALG-MA-DA	Lyophilized	
Batch ID	N	Sex
924	9	3M/6F
110	7	2M/5F
GEL-MA-DA	Lyophilized	
Batch ID	N	Sex
914	3	3F
208 @ 10%	3	3M
208 @ 15%	3	1M/2F
223	8	4M/4F
GEL-MA-DA	Oven Dried	
223	7	5M/2F

Figure 16: *In vivo* model for testing pleural sealant. **A)** Anesthetized, intubated and mechanically ventilated rats underwent thoracotomy to expose the right lung. Injury is induced by puncture with an 18g needle and leak of air bubbles observed to confirm injury. Following patch placement (arrow), absence of visible leaks is observed. **B)** Representative pressure volume curves. **C)** Summary of studies to date.

As shown, baseline PV loops obtained on the anesthetized intubated rat prior to ("baseline") and then after thoracotomy ("open chest") demonstrated normal hysteresis loops. Following injury ("incision") with visible leak of air bubbles, the curve is disrupted indicating continued leak of air from the wound. Following patch application and adherence, no further air bubbling is observed and the PV loop returns to baseline. The animal was then maintained on the ventilator for 1 hour (longest time allowed by IACUC/ACURO) at which time PV loops remained normal demonstrating persistent effective seal.

Given the success of the *ex vivo* and short-term *in vivo* rat pleural injury studies, we have now embarked on long term assessment of efficacy and safety. Original proof of concept of this was provided with use of the original ALG-MA-DA and GEL-MA-DA formulations. Assessing rats either 1 week or 1 month after injury and sealant application demonstrated good adherence of the sealants both grossly (**Figure 17**) and histologically and no obvious significant air leak or pneumothorax by fluoroscopy or CT scanning. Histologic analyses demonstrated juxtaposition of the sealant with underlying lung tissue with no obvious inflammatory cell infiltrates in hematoxylin and eosin stained sections (**Figure 18**). Residual sealant was observed in 1 week GEL-MA-DA-treated animals and in both 1 week and 1 month ALG-MA-DA-treated animals.

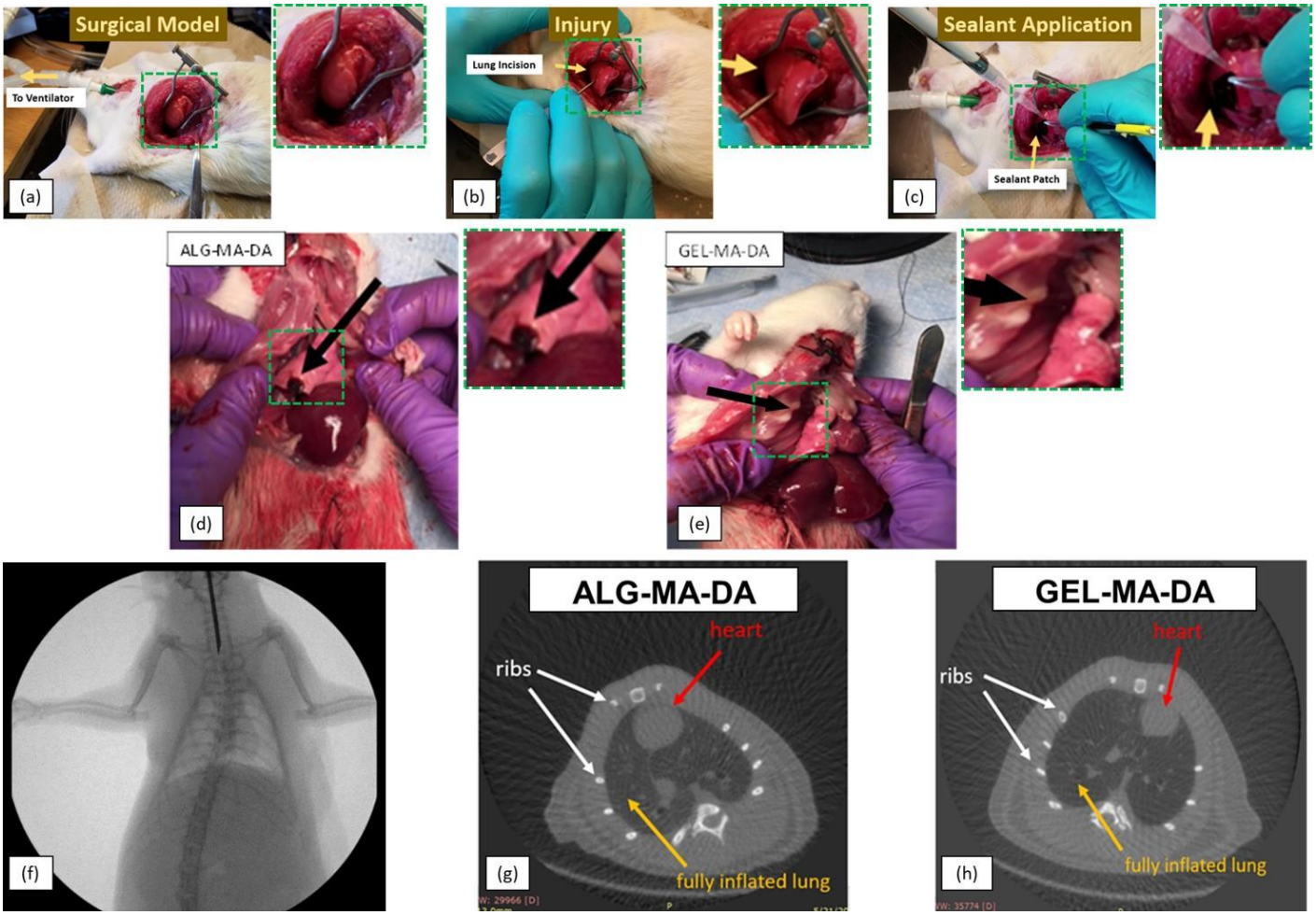


Figure 17: *In vivo* model for testing pleural sealant. (a) Anesthetized, intubated and mechanically ventilated rats underwent thoracotomy to expose the right lung. (b) Injury is induced by puncture with an 18g needle and leak of air bubbles observed to confirm injury. (c) The sealant is applied with cessation of air leak. (d) Necropsy of rat 1 week or month post operatively. Residual ALG-MA-DA patch material was observed in rats receiving ALG-MA-DA patch (black arrow). No GEL-MA-DA material was grossly visible in rats receiving GEL-MA-DA in situ formed hydrogel application. In neither case was obvious inflammatory reaction or evidence of sealant observed on the parietal pleura/chest wall. High power images are included for panels a-e. (f) Representative fluoroscopy after 1 week demonstrates clear lungs and no obvious pneumothorax. (g) and (h) Representative CT scans at 1 month demonstrates clear lungs and no pneumothorax. White arrows point the ribs; yellow arrows demonstrate a fully inflated lung and red arrows show the position of the heart.

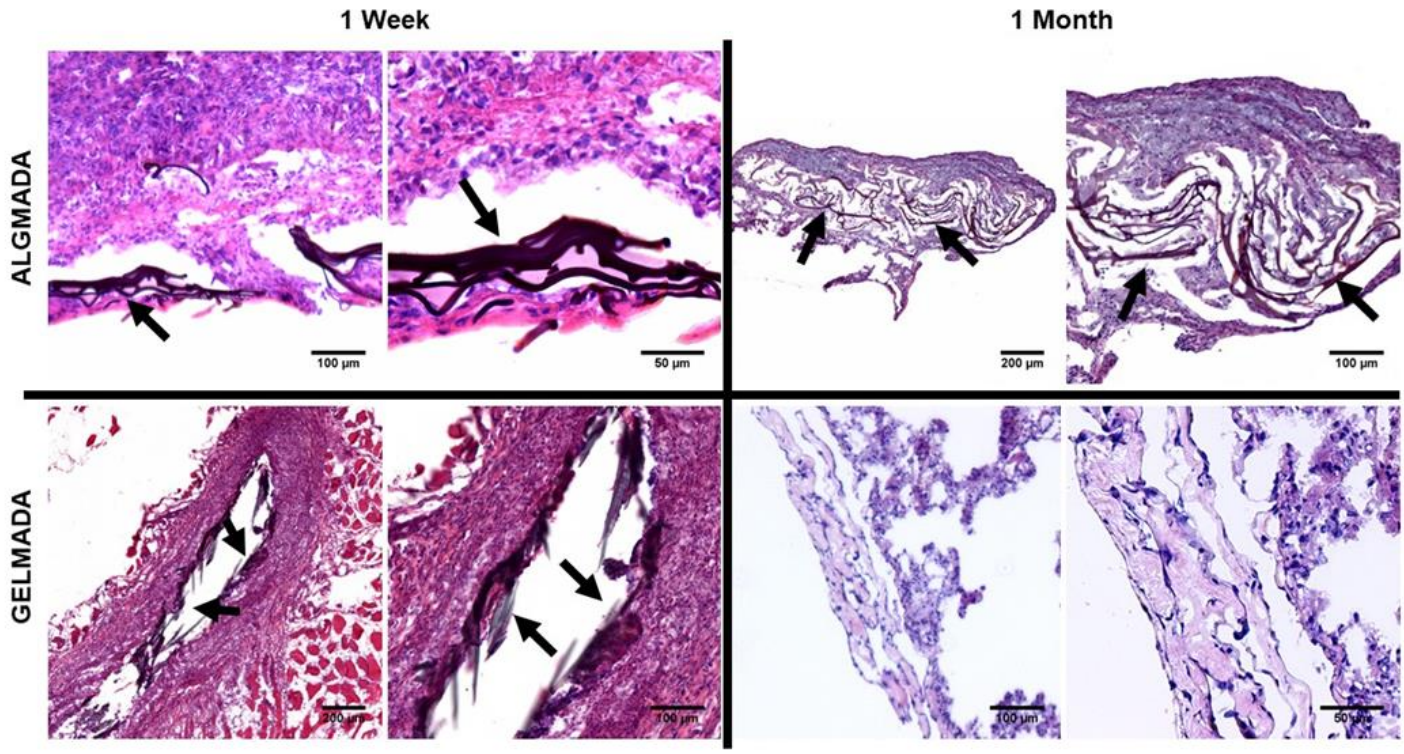


Figure 18: H and E stained sections demonstrate good wound repair, no obvious histologic inflammation, and residual sealant (arrows) for 1 week GEL-MA-DA and both 1 week and 1 month ALG-MA-DA. The one week GEL-MA-DA image depicts residual sealant in what appears to be the needle injury tract. Inset depict higher power magnifications of the respective designated areas.

Table 8: Summary of Long Term Rat Survival Pleural injury Studies

Summaries			
In vivo rat surgeries			
ALG-MA-DA	Lyophilized		
Batch ID	N	Sex	Time to sacrifice
924	6	4M/2F	1 yr
110	4	3M/1F	1 yr
924	2	2M	6 mo
110	8	3M/5F	6 mo
GEL-MA-DA	Lyophilized		
Batch ID	N	Sex	Time to sacrifice
914	2	2M	1 yr

We are currently conducting a more extensive series of long term efficacy and safety studies utilizing the new ALG-MA-DA formulation (freeze-dried/lyophilized) in adult rats with experimentally-induced pleural injuries. As depicted in **Table 8**, cohorts of animals to be assessed at 3, 6, and 12 months are well underway and one month cohort animals will be added. In addition to similar endpoint analyses of fluoroscopy, gross lung appearance, and histologic appearance/assessment of inflammation, serial blood samples will undergo toxicologic analyses. Parallel studies are being initiated with both oven-dried and freeze-dried GEL-MA-DA preparations. Parallel studies will also be initiated with both ALG-MA-DA and GEL-MA-DA patches that have been stored for up to 6 months to assess shelf life stability.

A representative fluoroscopy image one month after injury and application of an ALG-MA-DA patch is shown in **Figure 18**. No obvious pneumothorax is apparent.

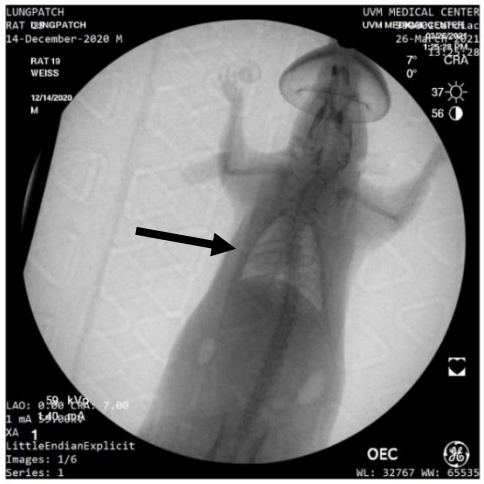


Figure 18 Fluoroscopic image of anesthetized rat 1 month after thoracotomy, institution of lung injury, and repair with ALG-MA-DA patch. Arrow highlights fully inflated lungs.

Short-and long term assessments in *in vivo* pig lung injury models

Similar approaches have been now utilized with the new formulations of ALG-MA-DA and GEL-MA-DA patches in both *ex vivo* pig lung preparations and in short-term non-survival surgery studies of anesthetized, intubated, mechanically ventilated pigs with experimentally induced pleural injuries. As depicted in **Figure 19**, both types of patches successfully adhere to *ex vivo* ventilated pig lungs for up to 24 hrs and for up to 1-2 hours in anesthetized, mechanically ventilated pigs (maximal time allowed by IACUC/ACURO).

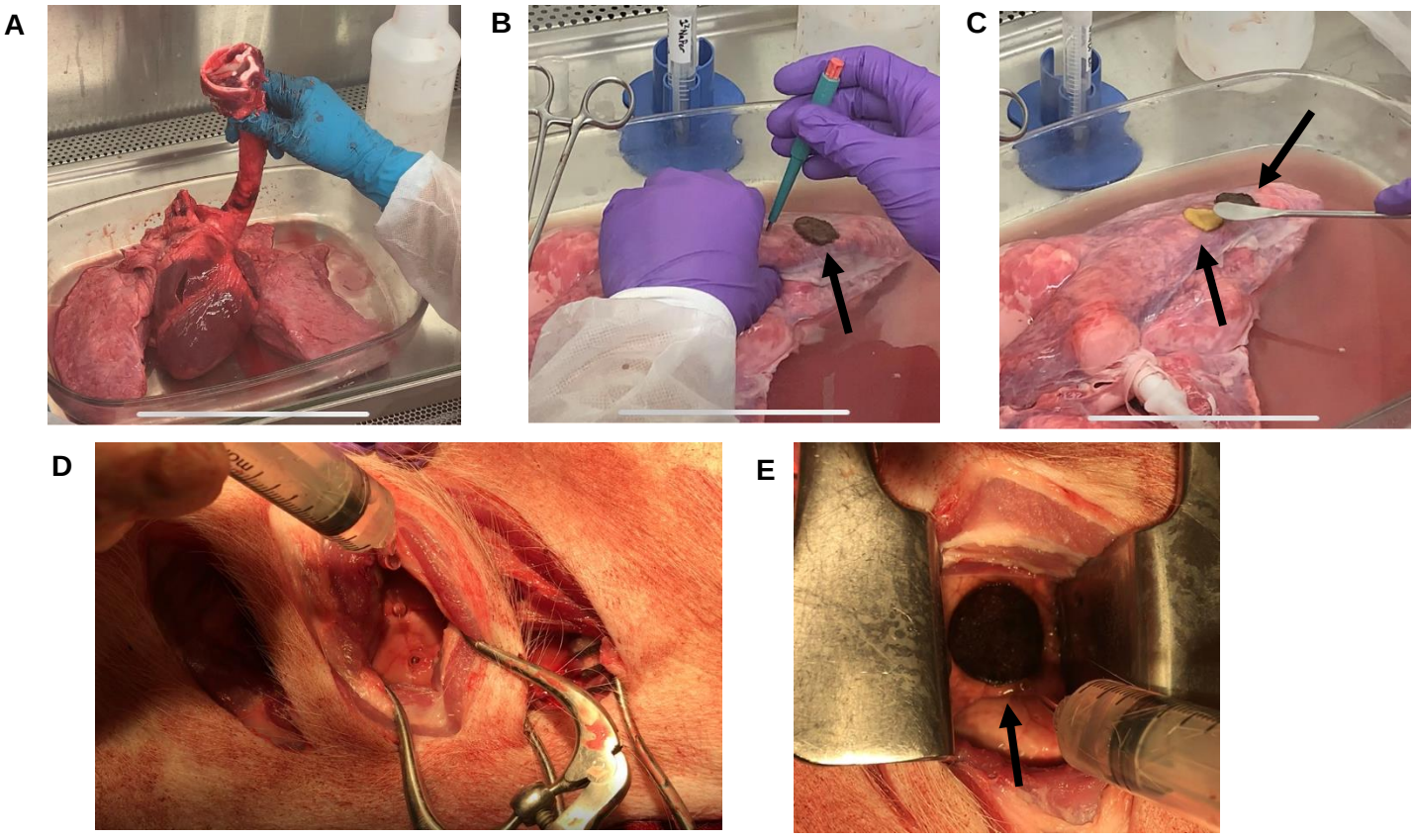
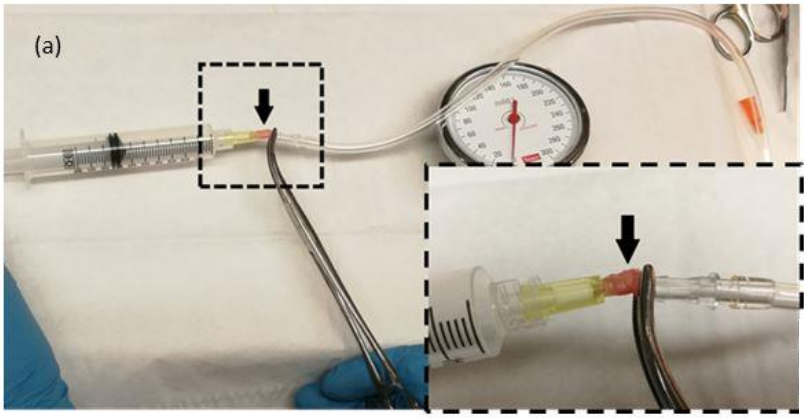
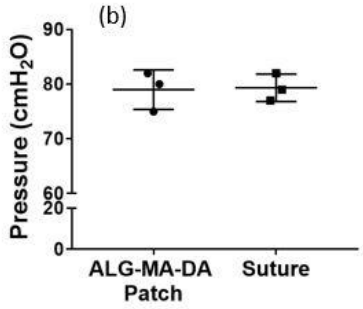


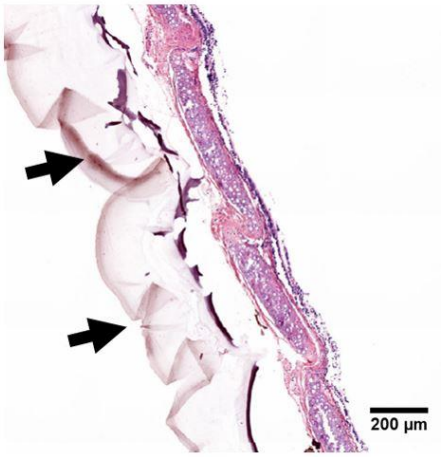
Figure 19: Pig lung pleural injury studies: Upper row depicts from left to right **A)** *ex vivo* pig trachea-heart-lung bloc; **B)** induction of puncture injury next to a previous puncture injury (brown patch); **C)** Ventilating lung with side-by side patches and no obvious bubble leak. Lower row depicts in vivo surgery model (short-term survival) of thoracotomy and lung exposure with **D)** injury and bubble leak; and **E)** patch placement and no bubble leak. Patches indicated by arrows

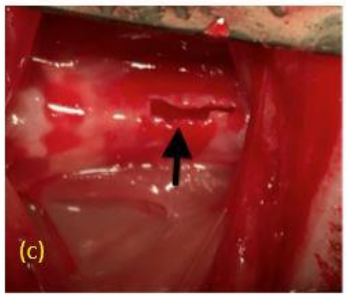
Short-and long term assessments in *ex vivo* and *in vivo* rat tracheal injury models

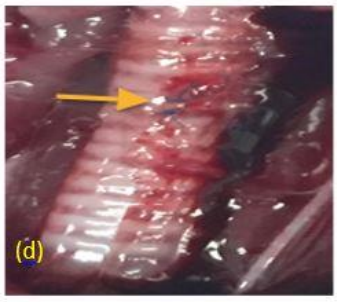
Tracheal injury studies in adult rats have now been successfully implemented by co-Investigator Christine Finck, Chief of Pediatric Surgery at Connecticut Children's Hospital. As proof of concept utilizing the original ALG-MA-DA formulation and using an *in vivo* rat model of tracheal injury induced with scalpel laceration, ALG-MA-DA was easily applied and formed a durable seal, comparable to suture repair, as assessed over a two week period. Burst pressure testing on 3 isolated rat tracheas harvested from patch and suture repair groups, respectively, demonstrated comparable strength to that provided with conventional sutures (**Figure 20**). Gross and histologic analyses demonstrated juxtaposition of the sealant with underlying lung tissue with no obvious inflammatory cell infiltrates (**Figure 20**).











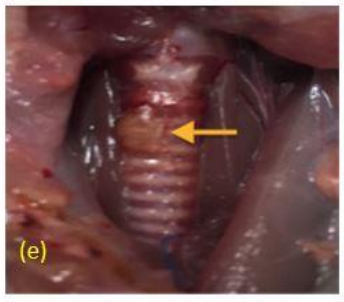
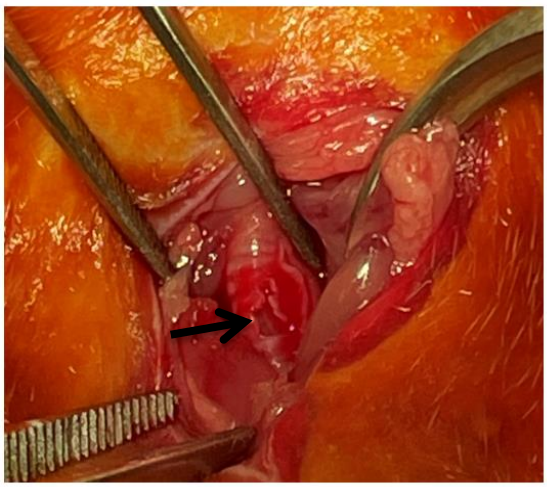
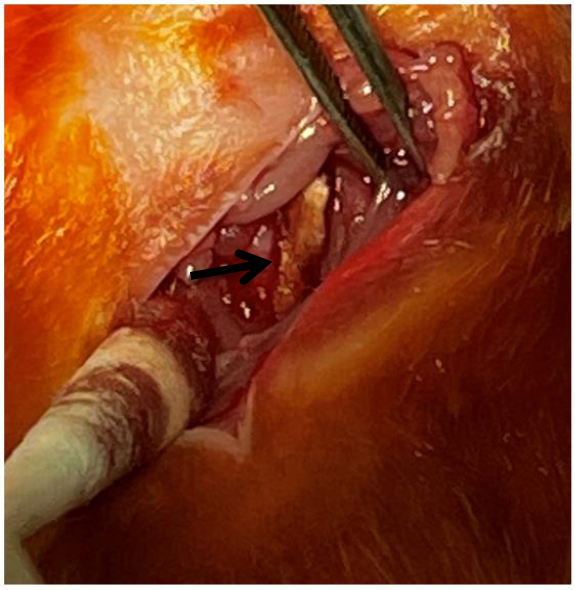


Figure 20: (a) Set-up for measuring burst pressures in isolated rat trachea. Arrow indicates cannulated rat trachea. (b) Application of the ALG-MA-DA sealant yielded comparable burst pressures to suture control. (c) Exposed rat trachea with scalpel incision (black arrow). (d) Repaired leak with suture at 2 weeks. Yellow arrow indicates residual suture. (e) Repaired leak with ALG-MA-DA patch at 2 weeks. Yellow arrow indicates residual patch. (f) H& E stained cryosection following ALG-MA-DA patch application demonstrates good repair and residual patch material along the tracheal wall (black arrows) Original Mag 20X.

Dr. Finck has now initiated a larger cohort of long term studies of the new ALG-MA-DA formulation in tracheal injuries in adult rats. Representative images of injury and repair following ALG-MA-DA patch application are depicted in **Figure 21** and a compilation of animals to date in **Table 9**. As with the rat pleural injury studies, parallel studies will be initiated with oven baked and freeze-dried GEL-MA-DA and with both types of patches that have been stored for up to 6 months to assess shelf life stability.



Tracheal Defect 3W x 3L (3 rings)
Tracheal Diameter= 4 mm



3.5% Rectangular Alg-Ma-Da patch applied
Batch #: 924

Figure 21: Depiction of adult rat tracheal injury and repair with ALG-MA-DA patch. Arrows indicate injury (L) and patch (R), respectively. Original Mag 20X).

Table 9: Summary of Survival Rat Tracheal Injury Studies to Date

Date	Defect Size (mm)	Experimental Group	Average Patch Size	Harvest Date
3/15/2019	10%	Control	N/A	12 months
2/12/2021	10%	Control	N/A	12 months
3/3/2021	0.2W x 0.8L	3.5% Alg-Ma-Da-Rectangle	3.0 mm W x 5.0 mm L	12 months: No ring resected
3/3/2021	0.2W x 0.8L	3.5% Alg-Ma-Da-Rectangle	3.0 mm W x 5.0 mm L	12 months
3/3/2021	0.2W x 0.82L	3.5% Alg-Ma-Da-Rectangle	3.0 mm W x 5.0 mm L	12 months: No ring resected
3/16/2021	2 rings : 1.25L x1.25W	3.5% Alg-Ma-Da-Circle patch	Diameter= 4 mm	12 months
3/16/2021	2 rings: 2.0L x1.0W	3.5% Alg-Ma-Da-Circle patch	Diameter= 4 mm	12 months
3/16/2021	2.0W x 2.0W	3.5% Alg-Ma-Da-Circle patch	Diameter= 4 mm	12 months
6/11/2021	1 ring: 1.0W x 2L	3.5% Alg-Ma-Da-Rectangle patch	3.0 mm W x 5.0 mm L	6 months
6/11/2021	1 rings: 2.0W x 2.0L	3.5% Alg-Ma-Da-Rectangle patch	3.0 mm W x 5.0 mm L	6 months
6/11/2021	1 rings: 2.0W x 2.0L	3.5% Alg-Ma-Da-Rectangle patch	3.0 mm W x 5.0 mm L	6 months
6/18/2021	3 rings: 3.0W x 3.0L	3.5% Alg-Ma-Da-Rectangle patch	3.0 mm W x 5.0 mm L	6 months
6/18/2021	3 rings: 2.0W x 3.0 L	3.5% Alg-Ma-Da-Rectangle patch	3.0 mm W x 5.0 mm L	6 months
6/18/2021	3 rings: 2.0Wx 4.0L	3.5% Alg-Ma-Da-Rectangle patch	3.0 mm W x 5.0 mm L	6 months
6/30/2021	3 rings: 2.0W x 3.0L	3.5% Alg-Ma-Da-Rectangle patch	3.0 mm W x 5.0 mm L	3 months
6/30/2021	3 rings: 1.0W x 2.0L	3.5% Alg-Ma-Da-Rectangle patch	3.0 mm W x 5.0 mm L	3 months
6/30/2021	3 rings: 1.0W x 3.0L	3.5% Alg-Ma-Da-Rectangle patch	3.0 mm W x 5.0 mm L	3 months

*Batch #924 Alg-Ma-Da 3.5% patches used for all experiments above

Major Task 2: Assess longitudinal efficacy and safety in pediatric tracheal injury models
These will be initiated August-September 2021.

Specific Aim 3
To assess short term efficacy in a pleural injury model in ex vivo ventilated normal and diseased (COPD/emphysema) human lungs.
These will be initiated August-September 2021.

What opportunities for training and professional development has the project provided?
Nothing to report.

How were the results disseminated to communities of interest?
Two relevant manuscripts are now published. The first of these, (Gasek et al. Development of Alginate and Gelatin-Based Pleural and Tracheal Sealants, Acta Biomaterialia, in press 2021, UI: 34245891), details the experience and knowledge gained with the initial formulations of ALG-MA-DA and GEL-MA-DA. These provide a firm basis for the ongoing studies detailed in this report. The second manuscript, Park HE, Gasek N, Hwang J, Weiss DJ, Lee PC. Effect of temperature on gelation and cross-linking of gelatin methacryloyl for biomedical applications. Physics of Fluids.32:3, DOI: 10.1063/1.5144896. 2020, describes more detailed information on the materials properties of the methacrylated gelatin compound. Further manuscripts are planned with respect to the ongoing studies, particularly the long term efficacy and safety studies. These results will also be submitted for presentations at relevant national/international Pulmonary and Bioengineering scientific meetings.

What do you plan to do during the next reporting period to accomplish the goals?
As detailed above, we will systematically continue all of the major tasks in **Specific Aims 1** and **2** and will initiate **Specific Aim 3**. The methodologic and materials advances achieved so far give us confidence that we will be continue to achieve major milestones with specific goal of developing a large scale manufacturing process capable of producing reliable high quality ALG-MA-DA and GEL-MA-DA for continued in vivo assessments. The longer term goal is to have sufficient and appropriate data with which to engage in conversation with the FDA for potential clinical investigations.

Other achievements
In parallel, we are continuing to validate a new oscillatory burst pressure approach that we will eventually look to file for IP.

Stated goals not met
Although we have made significant progress in all major tasks of **Specific Aim 1**, there is still further optimization of the ALG-MA-DA and GEL-MA-DA materials to be accomplished for this aim. The specific plans for doing so have been articulated throughout the above progress sections. Similarly, Major Task 1 in **Specific Aim 2** is ongoing and as described above, Major Task 2 of **Specific Aim 2** and **Specific Aim 3** are to be shortly initiated.

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 effective ALG-MA-DA and GEL-MA-DA based pleural sealants. This involves developing new technical approaches techniques 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. Continuance and completion of these studies and initial engagement with the FDA will be to the focus of further grant submissions to the DOD and other funding agencies.

What was the impact on other disciplines?
Nothing to report as yet

What was the impact on technology transfer?
Nothing to report as yet

What was the impact on society beyond science and technology?
Nothing to report as yet

5) Changes/Problems

Changes in approach and reasons for change
The expanding scope of investigations to incorporate technical advancements, functional modifications, and experimental approaches, detailed above, 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
Nothing to report

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

PI: Daniel J. Weiss MD PhD UVM
Partnering PI: Christine Finck MD UConn
Partnering Contracted Vendor: John Garner PhD Akina Inc.
Post-Doctoral Associates: Robert Pouliot PhD UVM, Ishna Sharma MD UConn
Laboratory Technicians: Evan Hoffman UVM, Todd Jensen UConn, Nirav Daphthary
Animal Technicians: Stephen Bell UVM, Sheila Russell UVM
Thoracic Surgical Consultant: Bruce Leavitt MD UVM

Robert Pouliot, Todd Jensen (UConn) and Bruce Leavitt (UVM) have each left their previous positions. Otherwise, there is no change in effort for any of the above listed study participants

Additional personnel over the last year:
Lori Asarian PhD: laboratory technician UVM (funded in place of Robert Pouliot)
Keara McElroy-Yaggy: part-time animal technician UVM (funded from another source)
Amie Tyler: laboratory technician, Akina
Fuyuki Hirashima MD: Thoracic surgery consultant UVM.

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 grant submission. These are listed below. None are relevant for the current DOD proposal.

New grants and update on existing grants since last year's progress report.

OTHER SUPPORT

WEISS, DANIEL J.

Active:

This project is now in a no-cost extension & there has been effort change since last report:
1) WEISS18P0 (Weiss) 04/01/2019-03/31/2022 0.12 calendar months
Cystic Fibrosis Foundation annual direct costs
Role of Glycoproteins in Lung Recellularization
The goals for this proposal are to systematically analyze key matrikine binding and activation patterns to specific individual HS, CS and DS in decellularized human lungs, to systematically determine matrikine dependent and independent effects of individual HS, CS and DS chains on representative lung cell growth and differentiation and to determine matrikine-dependent and independent effects of systematic repletion of decellularized normal or diseased human lung ECM with key HS, CS and/or DS functional groups on cell growth and differentiation.

Specific Aims:
1: Systematically analyze key matrikine binding and activation patterns to specific individual HS, CS and DS in decellularized human lungs
2: Systematically determine matrikine dependent and independent effects of individual HS, CS and DS chains on representative lung cell growth and differentiation
3: Determine matrikine-dependent and independent effects of systematic repletion of decellularized normal or diseased human lung ECM with key HS, CS and/or DS functional groups on cell growth and differentiation.

Name & Address of funding agency's procuring Contracting/Grants Officer:
CFF Grants and Contracts Office
email: grants@cff.org
phone: 301-841-2614
Cystic Fibrosis Foundation
Office of Grants and Contracts
4550 Montgomery Avenue, suite 1100
N. Bethesda, MD 20814

2) VLC Pilot Project 2019 (Weiss/van der Velden) 04/01/2020-03/31/2022 0.12 calendar months
Institutional source annual direct costs
Effects of human lung derived hydrogel organoid culture on lung progenitor cell behaviors
The goals of the project are to determine composition and differential effects of bulk lung or regional specific (airway vs alveolar) human lung dECM hydrogels on growth and functional behaviors of iAEC2 alveolospheres and the effects of combinatorial addition of relevant GAGs (heparan, chondroitin, and dermatan sulfates) to dECM hydrogels on growth and functional behaviors of iAEC2 alveolospheres.

Specific Aims:
1) To determine composition and differential effects of bulk lung or regional specific (airway vs alveolar) human lung dECM hydrogels on growth and functional behaviors of iAEC2 alveolospheres.
2) To determine the effects of combinatorial addition of relevant GAGs (heparan, chondroitin, and dermatan sulfates) to dECM hydrogels on growth and functional behaviors of iAEC2 alveolospheres.

Name & Address of funding agency's procuring Contracting/Grants Officer:
Carolynn Hatin
Pulmonary Business Manager
Address: 89 Beaumont Ave
Given Building D213, University of Vermont
Burlington, VT 05405
Email: Carolynn.Hatin@uvmhealth.org
Phone: 802-656-3506

New award since last report:

3) ETRA 736479 (COVID & Emerging Threat Award) (Weiss) 07/01/2020-06/30/2022 0.72 calendar months
American Lung Association annual direct costs

Mechanisms of SARS-CoV-2 induced type 2 alveolar epithelial cell pathogenesis

The goals of this project are to characterize 1) SARS-CoV-2 S-glycoprotein-mediated ACE2 downregulation and the corresponding impact on iAT2 regeneration (i.e. hyperplasia) and function (i.e. surfactant production) and 2) the impact of TNFa-mediated ADAM-17-dependent ACE2 downregulation and cleavage and the corresponding impact on iAT2 regeneration and function.

Specific Aims:
1) Characterize SARS-CoV-2 S-glycoprotein-mediated ACE2 downregulation and the corresponding impact on iAT2 regeneration (i.e. hyperplasia) and function (i.e. surfactant production)
2) Characterize the impact of TNFa-mediated ADAM-17-dependent ACE2 downregulation and cleavage and the corresponding impact on iAT2 regeneration and function.

Name & Address of funding agency's procuring Contracting/Grants Officer:
Alexandra Sierra
National Director, Research
American Lung Association
55 W Wacker Dr., Suite 1150
Chicago, IL 60601
Email: alexandra.sierra@lung.org
Phone: 312-940-7616

New award since last report:

4) Path for a Cure (Feinberg) 11/01/2020-10/31/2022 no measurable effort
Carnegie Mellon University (CFF) annual direct costs

ECM Shrinkwrapped Airway Epithelial Cells Towards Function Engraftment

Dr. Weiss' laboratory will be responsible for producing and supplying ECM derived from normal human lungs over the two years of the study period. Dr. Weiss' laboratory will also conduct studies evaluating incorporation of shrink-wrapped basal epithelial cells into human lung airway organoid cultures. These will mostly take place in the 2nd year of the study period.

Specific Aims:
Aim 1. Develop and validate the nanofabrication process to shrink-wrap AECs in an engineered ECM nanoscaffold with defined composition and structure.
Aim 2. Characterize the *in vitro* integration and function of shrink-wrapped hBEs into ALI and organoid cell cultures.

Name & Address of funding agency's procuring Contracting/Grants Officer:
CFF Grants and Contracts Office
email: grants@cff.org
phone: 301-841-2614
Cystic Fibrosis Foundation
Office of Grants and Contracts
4550 Montgomery Avenue, suite 1100
N. Bethesda, MD 20814

New award since last report:

5) W81XWH-20-PRMRP-DA (Jakus/Weiss Co-I) 05/01/2021-04/30/2023 0.48 calendar months
Dimensions Inx. (DOD/PRMRP) annual direct costs

Bi-Phasic, Regenerative Tracheal Implants From 3D-Printed Advanced Biomaterials with Integrated Vascularizing and Epithelializing Factors

The goals of this project are to 1) establish processes for synthesizing growth factor-containing 3D-Paints and 3D-Printing those 3D-Paints into bi-phasic cylindrical structures for in vitro and small animal testing, 2) evaluate the mechanical, compositional, and growth factor release characteristics of the bi-phasic structures and 3) evaluate biocompatibility and activity of human trachea endothelial cells on bi-phasic structures.

Specific Aims:
Aim 1: Establish processes for synthesizing growth factor-containing 3D-Paints and 3D-Printing those 3D-Paints into bi-phasic cylindrical structures for in vitro and small animal testing,
Aim 2: Evaluate the mechanical, compositional, and growth factor release characteristics of the bi-phasic structures
Aim 3: Evaluate biocompatibility and activity of human trachea endothelial cells on bi-phasic structures.

Name & Address of funding agency's procuring Contracting/Grants Officer:
Abigail Strock
Grants Officer
USA Med Research ACQ Activity
820 Chandler St, Fort Detrick, MD 21702-5014. Phone: 301-619-2342

Overlap Statement: This new DOD award is conceptually similar to the current DOD award. However, the Specific Aims, materials, and approach being investigated are different.

New award since last report:

6) R21 HD104069 (Finck) 04/01/2021-03/31/2023 0.48 calendar months
University of Connecticut Health Center (NIH) annual direct costs

Ex vivo bioengineering of functional biomimetic airways for treatment of neonatal and pediatric respiratory conditions

A specific goal of the studies is to develop sophisticated methods of 3D bioprinting airways using bioinks derived from decellularized lungs.

Specific Aims:

1. Mechanical optimization of human dECM 3D bioprinted airway structures
2. Optimization of cell viability and phenotype in 3D bioprinted dECM airway structures

Name & Address of funding agency's procuring Contracting/Grants Officer:

Chantell Stevenson-Brown
Eunice Kennedy Shriver National Institute of Child Health & Human Development
Chantell.Stevenson-Brown@nih.gov
301-594-2258

New award since last report:

7) R13 HL160043 (Weiss) 07/23/2021-06/30/2022 0.24 calendar months
NIH

Stem Cells, Cell Therapies, and Bioengineering in Lung Biology and Diseases

The purpose of this funding is to help support the 2021 Vermont Stem Cell conference, "Stem Cells, Cell Therapies, and Bioengineering in Lung Biology and Diseases".

Specific Aims:

1) To convene national and international experts in stem cells, lung biology and bioengineering to discuss and debate recent advances in the roles of stem cells and cell therapies in lung biology and lung diseases

2) To address critical issues in this field, including the identity and role of endogenous lung progenitor cells, induced pluripotent stem cells, stem cells and lung cancer, and bioengineering approaches to lung regeneration

3) To address breaking advances in the field including endogenous lung progenitor cells, induced pluripotent stem cells, stem cells and lung cancer, and bioengineering approaches to lung regeneration

4) To provide a forum for junior investigators, graduate students, postdoctoral associates, and pulmonary research fellows to participate and present data in oral or poster format

5) To discuss the alarming rate of growth of unregulated and unproven stem cell and cell therapies for lung diseases in both the US and abroad and how to best combat this growing problem

6) To craft a series of recommendations for the NHLBI, FDA, and other relevant organizations to utilize for guiding basic and translational research in stem cells and cell therapy approaches in lung diseases. These same guidelines will also be designed to help investigators pursue NIH and other funding opportunities.

Name & Address of funding agency's procuring Contracting/Grants Officer:
Jasmine Nichole Johnson
National Heart, Lung and Blood Institute
jasmine.johnson@nih.gov
Phone: (301) 827-8177

New award since last report:
8) (Weiss) 07/01/2021-07/30/2021 no measurable effort
Alpha-1 Foundation
2021 Stem Cells, Cell Therapies, and Bioengineering in Lung Biology and Diseases Conference
The purpose of this funding is to help support the 2021 Vermont Stem Cell conference, "Stem Cells, Cell Therapies, and Bioengineering in Lung Biology and Diseases".
Specific Aims:
Conference support as iterated in the above NHLBI R13 award.

Name & Address of funding agency's procuring Contracting/Grants Officer:
David E. Fernandez
Director of Research Administration
Alpha-1 Foundation
3300 Ponce de Leon Blvd.
Coral Gables, Florida 33134
Phone: (305) 567-9888

New award since last report:
9) (Weiss) 07/09/2021-07/08/2022 1.80 calendar months
United Therapeutics Corporation
Modeling Pulmonary Hypertension Drug Mechanisms of Actions in Pulmonary Vascular Smooth Cells: Correlation of in silico and in vitro studies
The goal of this project is to utilize human pulmonary artery smooth muscle cells and test several permutations of agonist and antagonists of 3 pharmacological pathways (endothelin, prostacyclin and nitric oxide pathways).

Specific Aims:
To correlate *in silico* modeling with *in vitro* biological effects of human pulmonary artery smooth muscle cells of 3 pharmacological pathways currently investigated in pre-clinical studies and in clinical trials, namely the endothelin, prostacyclin, and nitric oxide pathways

Name & Address of funding agency's procuring Contracting/Grants Officer:
Joe Bender
United Therapeutics Corporation
1040 Spring Street
Silver Spring, MD 20910

Completed:

This award has ended since the last report:

1) R13 HL149436 (DJ Weiss, PI) 07/04/2019-06/30/2020
NIH/NHLBI annual direct costs
The principle goals of the proposed meeting, "Stem Cells, Cell Therapies, and Bioengineering in Lung Biology and Diseases" are to convene the relevant experts and up and coming junior investigators to share and debate recent developments in this rapidly moving and exciting field with a specific goal to formulate basic, translational, and clinical research directions.

Specific Aims:
1) To convene national and international experts in stem cells, lung biology and bioengineering to discuss and debate recent advances in the roles of stem cells and cell therapies in lung biology and lung diseases
2) To address critical issues in this field, including the identity and role of endogenous lung progenitor cells, translational studies, and clinical cell therapy approaches for lung diseases
3) To address breaking advances in the field including embryonic stem cells, induced pluripotent stem cells, stem cells and lung cancer, and bioengineering approaches to lung regeneration
4) To provide a forum for junior investigators, graduate students, postdoctoral associates, and pulmonary research fellows to participate and present data in oral or poster format
5) To discuss the alarming rate of growth of unregulated and unproven stem cell and cell therapies in both the US and abroad and how to best combat this growing problem
6) To craft a series of recommendations for the NHLBI, FDA, and other relevant organizations to utilize for guiding basic and translational research in stem cells and cell therapy approaches in lung diseases. These same guidelines will also be designed to help investigators pursue NIH and other funding opportunities.

Name & Address of funding agency's procuring Contracting/Grants Officer:
Grant Management Specialist: Alyse Burton
Email: burtonam@mail.nih.gov
Phone: 301-827-8019
Address: NIH/NHLBI
BG RKL1 RM 201-J
6705 ROCKLEDGE DR
BETHESDA MD 20817

This award has ended since the last report:

2) SPARK UVM (DJ Weiss/Adam Jakus, Dimensions Inc.) 07/01/2018-06/30/2020
Development of novel lung bioinks total direct
Project goals are to test the mechanical and biochemical properties of lung-specific ECM-derived materials and evaluate optimized lung ECM-origin gels and patches in pre-clinical models of lung injury.

Specific Aims:
Specific Aim 1: To test the mechanical and biochemical properties of lung-specific ECM-derived materials.
Specific Aim 2: Evaluate optimized lung ECM-origin gels and patches in pre-clinical models of lung injury.

Name & Address of funding agency's procuring Contracting/Grants Officer:
Dan Harvey
Director of Operations to the Vice President for Research
Address: 85 South Prospect Street
330 Waterman Building, University of Vermont
Burlington, VT 05405
Email: Dan.Harvey@uvm.edu
Phone: 802-656-4566

This award has ended since the last report:

3) S10 OD026976 (Weiss) 08/01/2019-07/31/2020
NIH annual direct costs

Zeta View TWIN Laser Nanoparticle Tracking Analyzer

The goal of this proposal is to obtain a state-of-the-art ZetaView TWIN Laser Nanoparticle Tracking Analyzer (NTA) device at the University of Vermont (UVM).

Specific Aims:
1) Evaluate the accumulation, penetration depth, and efficacy of MSNs within the lung during an asthmatic response.
2) Test the ability of anti-IL-6-MSN to reduce an asthmatic response in vivo.

Name & Address of funding agency's procuring Contracting/Grants Officer:
Grants Management Specialist: Donna M James
Email: jamesd@mail.nih.gov
Phone: 301-496-7484
Address: NIH/Office of the Director
BG RKL1 RM 202-J
6705 ROCKLEDGE DR
BETHESDA MD 20817

This award has ended since the last report:

4) P20 GM125498 (Kirkpatrick) 07/01/2020-06/30/2021
NIH/NIGMS
Translational research to prevent and control global infectious diseases (Translational Global Infectious Diseases Research Center, TGIR)
Pilot Award (Thali/Weiss) annual direct costs
Alveolar epithelial cell-based air-liquid interface to study SARS-CoV-2 biology and pathogenesis

The goals of this pilot project are to validate an iAT2-based ALI model for analyses of distinct SARS-CoV-2 functions and to start investigating SARS-CoV-2 spreading and host responses.

Specific Aims:
1) To validate an iAT2-based ALI model for analyses of distinct SARS-CoV-2 functions.
2) To start investigating SARS-CoV-2 spreading and host responses.

Name & Address of funding agency's procuring Contracting/Grants Officer:
Grants Management Specialist: Courtney Tardd-Wright
Email: tarddwrightc@mail.nih.gov
Phone: 301-496-9441
Address: NIH/NIGMS, BG 45 RM 2AN32A
45 CENTER DR, BETHESDA MD 20814

What other organizations were involved as partners?

A. 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: N/A

B. Organization Name: University of Connecticut (UConn)

Location of Organization: Farmington CT

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

Financial support: N/A

In-kind support: N/A

Facilities: Full laboratory and animal facility resources for planned animal testing

Collaboration: Collaborators at UConn will perform some of the studies in **Specific Aim 2**

Personnel exchanges: N/A

Other: N/A

8) Special Reporting Requirements
Collaborative awards: N/A

Quad Chart

PR181641 - "Clinical Development of a Novel Pleural and Tracheal Sealant"

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

Budget: $1,724,027 **Topic Area: Respiratory Health** **Mechanism: PRMRP Expansion 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 manufacturing, sterilization, preservation, and storage conditions of pre-formed ALG-MA-DA patches
(2) To define long term efficacy and safety in longitudinal small (rat) and large (pig) models of adult pleural injury and of adult and pediatric tracheal injuries
(3) To assess short term efficacy in a pleural injury model in ex vivo ventilated normal and diseased (COPD/emphysema) human lungs

Key Accomplishments for Year 2:

Detailed systematic evaluation of ALG-MA-DA large scale manufacturing

A) Continued progress towards large scale ALG-MA-DA and GEL-MA-DA synthesis with controllable, reliable, and reproducible degrees of methacrylation and dopamine conjugation.
B) Continued progress towards large scale reliable and reproducible production of pre-crosslinked ready-to-go ALG-MA-DA and GEL-MA-DA patches
C) Successful mechanical testing of ALG-MA-DA and GEL-MA-DA patches with favorable behaviors compared to the one commercially available product
D) Successful demonstration of patch efficacy in ex vivo and short term in vivo rat and pig models of pleural injuries
E) Initial demonstration of long term efficacy in rat models of pleural and tracheal injuries
F) Initiation of multiple long term rat pleural and tracheal injury studies
G) Planned initial long term pig pleural injury, juvenile pig tracheal injury and human lung studies

Key Overall Outcome for Year 2:

Progress towards reliable, reproducible, and clinically effective large scale sealant manufacturing

9) Appendices: N/A