SYNTHESIS OF ALLYLATED ALGINATE FOR APPLICATIONS IN BIOFABRICATION

by:
SPENCER ROY SIEGEL

A THESIS

Presented to the Department of Chemistry and Biochemistry
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

May 2024

An Abstract of the Thesis of

Spencer Siegel for the degree of Bachelor of Science
in the Department of Chemistry and Biochemistry to be taken June 2024

Title: Synthesis of Allylated Alginate for Applications in Biofabrication

Approved: *Gabriella Lindberg, Ph.D.*
Primary Thesis Advisor

Organoids are *in-vitro* constructs designed to replicate *in-vivo* organs. Organoids have a wide range of potential use cases within science and medicine, but are not yet clinically relevant due to multiple factors. In order to address these factors, multiple engineering approaches have been derived to aid in organoids creation. One such approach is the creation of designer matrices, tailored and fabricated extracellular matrices for use in organoids. Designer matrices are commonly created through biofabrication techniques, techniques which use modern fabrication methods with biological components. However, the materials used in biofabrication must adhere to a strict set of design criteria. This project aims to develop a chemically modified form of alginate that can be used to form a hybrid hydrogel with allyl modified gelatin (Gel-AGE). This hydrogel would satisfy many of the biofabrication design criteria, and also contain a dual crosslinking system. Allylated Alginate (Al-AGE) was successfully synthesized, and was characterized via proton NMR. Al-AGE was tested for both ionic and covalent crosslinking abilities, and was shown to be able to undergo both forms of crosslinking. Finally, hybrid gels were assembled with Al-AGE and Gel-AGE.

Acknowledgements

First and foremost, I would like to thank my primary thesis advisor, Dr. Gabriella Lindberg, for all the mentorship, wisdom, and guidance she has provided me with throughout my time at Lindbergh Labs. Dr. Lindberg has been a grand inspiration to me with regards to both my academic and personal goals, and I am forever grateful for the knowledge and experiences she has empowered me to obtain through performing research under her watchful eye. I would also like to thank Judah Aptecker, Vinni Thoms, DeShea Chasko, and all other current and former members of the Lindberg Labs, for all their aid in training and guidance, as well as their support as both colleagues and friends.

My appreciation also goes out to the Clark Honors College Advisors Carol Paty and Miriam Alexis Jordan, for providing ample assistance in planning and keeping this thesis on track, as well as the chemistry and biochemistry department advisor Michael Koscho, for his immeasurable assistance in helping me weave together my major and my Honors College requirements.

I also want to offer a special thanks for the continued support and guidance of my parents, Rosette Manio, and Lance Siegel, as well as my older brother, Brent Siegel, for their frequent feedback and encouragement, as well as their vast array of both practical, and research-oriented knowledge. Finally, I want to thank Megan Elliott, Jacob Pockrus, Christopher Marchetti, and all of my other friends and colleagues for their support and spirit throughout my thesis writing process, keeping me attentive and helping me manage my stress levels. Without them, finishing this thesis would have been almost impossible.

This research was supported by The Knight Campus Undergraduate Scholars program.

Table of Contents

Introduction **7**
The Need for Organoids 7
Biofabrication Techniques and Strategies 8
Bioink Precursor Design Criteria and Hybrid Hydrogels 9
Project Aim and Research Goals 12
Methods **14**
Synthesis of Allylated Alginate 14
Reaction Theory and Calculations *14*
Synthesis Procedure *17*
Characterization 21
Proton NMR *21*
Spectrophotography *23*
Inversion Test *24*
Rheological Testing *25*
Hybrid Hydrogel Formation *27*
Results **29**
Synthesis of Allylated Alginate 29
Characterization of Allylated Alginate 31
Proton NMR *32*
Spectrophotography *37*
Ionic Crosslinking Tests 37
Inversion Test *37*
Covalent Crosslinking Tests 38
Rheological Testing *39*
Hybrid Hydrogel Formation *41*
Conclusion **43**
Future Goals 43
Glossary **46**
Bibliography **48**

List of Figures

Figure 1: **Epoxide Ring Opening Under Basic Conditions** 9

Figure 2: **Al-AGE Reaction Scheme** 10

Figure 3: **Toluidine Blue** 17

Figure 4: **NMR Testing Run 1** 27

Figure 5: **NMR Testing Run 2** 29

Figure 6: **NMR Testing Run 3** 30

Figure 7: **Example Inversion Test For Various Al-AGE Conditions** 32

Figure 8: **Rheological Testing: 0 and 100% DTT G1MM** 33

Figure 9: **Rheological Testing: 0 and 200% DTT Al-AGE** 34

Figure 10: **Rheological Testing: 0 and 200% DTT Plain Alginate** 34

List of Tables

Table 1: **Al-AGE Synthesis Calculations** 10

Table 2: **Reaction 1 - Varying Allyl Glycidyl Ether Quantity** 12

Table 3: **Reaction 2 - Varying NaOH Quantity** 12

Table 4: **Reaction 3 - Varying Temperature** 13

Table 5: **Control Conditions** 14

Table 6: **Al-AGE and Gel-AGE Photorheomitry Parameters** 21

Table 7: **Al-AGE + Gel-AGE Gel-Conditions** 22

Table 8: **Syntheses of Al-AGE and resulting pH** 24

Table 9: **Al-AGE/Gel-AGE Hybrid Hydrogels** 36

Introduction

The Need for Organoids

An organoid is a miniaturized *in-vitro* model system designed to replicate the function or context of an *in-vivo* organ. Organoids have immense scientific and clinical potential, and house a wide range of use cases and benefits. As an example, organoids can be designed to replicate a variety of organs, such as the gut, stomach, kidney, bones, liver, pancreas, mammary glands, prostate, airways, retina, and even the brain. Organoids also have significant use cases in drug screening, personalized medicine, and modeling tissue growth and microbe interactions. Additionally, organoids are superior to 2D cell cultures with regards to structural similarity. They are also able to more easily mimic the function of a corresponding organ, and are ideal for replicating the complex environments that many cells frequently reside in. Furthermore, the environment of organoids can be tailored to enable the replication of traditionally difficult cell types, such as untransformed stem cells (Hofer & Lutolf, 2021).

However, despite their promise, the creation of organoids is limited by multiple factors, including a limited level of complexity, inconsistency in formation, accessibility by the inladen cells, and the ability to collect data from said organoids. In order to address these limitations, multiple engineering approaches have been derived to aid in organoids creation. Hofer and Lutof describe four of them: Engineering the cell, which focuses on modifying the cells that are housed in fabricated organoids; engineering the context, which focuses on mimicking higher order inputs from surrounding tissue; engineering enhanced readouts, which focuses on enhancing and automating the way organoid data is read and collected; and engineering the niche, which focuses on biofabrication, and the creation of organoid niches that accurately replicate their in-vivo counterparts (Hofer & Lutolf, 2021).

Focusing further on engineering organoid niches, Hofer and Lutof describe that while many aspects of a complex biological niche have successfully been replicated, such as neighboring cells, and the molecules a specific niche secretes, these aspects alone are not sufficient for the creation of organoids with high enough resemblance to their in vivo counterparts, and that organoids still have limited their clinical relevance as a result. This is often due to limited cell responsiveness, and variability between batches of organoids.

Designer Matrices, manually tailored and fabricated extracellular matrices for use in organoids, are described as one of the prominent ways for niche engineering with enhanced mimicry of the *in-vivo* niche (Hofer & Lutolf, 2021). One of the frequent forms that designer matrices can take is that of custom-tailored hydrogels, the properties of which can be varied heavily depending on their planned use. Hydrogels generally tend to consist of organic polymers, often modified chemically for more tailorability and control of the matrix properties. Some prominent benefits of Designer Matrices include less batch-to-batch variance, and increased customization and tailorability for improved cell responsiveness (Hofer & Lutolf, 2021).

Biofabrication Techniques and Strategies

One of the most prominent ways of creating Designer Matrices is through the use of biofabrication techniques, specifically bioprinting. The term *biofabrication* has undergone many different definitions, but can generally be described as "a process that results in a defined product with biological function." (Groll et al., 2016). This description however, is quite broad, and as such, it is useful to emphasize some of the more unique features of biofabrication: "first, the building blocks are cells or biologics; second, the fabrication processes are bio-inspired or bio-friendly; and finally, the products are biological systems, models or devices with transformative properties" (Groll et al., 2016). Overall, biofabrication is a specialized subset of additive

manufacturing that consists of utilizing modern fabrication methods to create constructs of biological function, and acts as the umbrella term under which the disciplines of bioprinting and bioassembly sit.

Bioassembly is the process of bottom-up assembly of tissue or organoids from core components, while bioprinting consists of the use of a mechanical fabrication tool to organize biological components into a two-or three-dimensional construct. While the processes do contain some degree of overlap, one key distinction is that the smallest fabrication unit is down to the molecular level for bioprinting, while it is only down to the cellular level for bioassembly (Groll et al., 2016).

Diving further into the field of bioprinting, currently existing bioprinting technologies hold significant utility for the formation of Designer Matrices, the importance of which was described in the section above. With regards to bioprinting strategies, much research has already been done on the applications, limitations, and needs for further study. These processes of bioprinting can generally be categorized under three groups, those being laser and light based, extrusion-based, and jetting or powder based (Naghieh et al., 2021). Such bioprinting methods allow for greatly increased precision when fabricating organoids, and hold significant potential for automation. However, while available bioprinting technology has a high potential for use in the creation of organoids, there is still much research to be done, and many challenges to overcome before this technology is clinically relevant (Naghieh et al., 2021).

Bioink Precursor Design Criteria and Hybrid Hydrogels

One such challenge that organoids face lies within the base materials used to fabricate organoids themselves. These materials, known as bioink precursors, must adhere to a strict set of design criteria to be suitable for use in organoid fabrication. In the 2020 paper, From Shape to

Function: The Next Step in Bioprinting, the “Biofabrication Window" is described as “the range of material properties suitable both for printability with high shape fidelity and for the support of cell function,” indicating the need for a wide range of bioink precursors that are suitable for both cells and bioprinting, and describing some of the desirable properties a bioink precursor must have (Levato et al., 2020).

Elaborating further on the idea of the “Biofabrication Window,” some of the ideal properties of bioink precursors include cyto-compatibility, RGD sequences (sequences of amino acids that allow for cell attachment) the ability to crosslink quickly, the ability to maintain crosslinking for extended periods of time, high throughput, compatibility with current biofabrication methods, low cost, and low variability between batches.

The use of natural polymers, such as gelatin or alginate, as bioink precursors is one possible solution. Due to these meeting many of the design criteria listed above, use of natural polymers as bioink precursors is particularly enticing, with there having been over 200 publications featuring the use of gelatin, and over 50 featuring the use of alginate as bioink precursors by 2020 (Naghieh et al., 2021). Some benefits to using natural polymers include low cost and inherent cytocompatibility. However, some limitations of natural materials include additional biological complexity, variation between batch to batch, as well as shorter shelf life (Naghieh et al., 2021). Even still, the benefits of utilizing natural materials of bioinks cannot be ignored from a cell instructive and biomimicry aspect.

One such example of a natural polymer bioink precursor is a form of modified gelatin, known as Gel-AGE (Bertlein et al., 2017). Gel-AGE is synthesized by reacting gelatin with allyl-glycidyl-ether (AGE), a molecule containing both an epoxide and alkene functional group, under basic conditions. This results in a gelatin molecule with alkene groups attached, allowing for useful

crosslinking chemistry. Gel-AGE is well studied, and has a very wide range of practical properties for biofabrication. In specific, Gel-AGE is cheap, easy to synthesize, cytocompatible, and can also be crosslinked to form a stable structure for long term cell culture. This crosslinking involves mixing Gel-AGE with thiols and a source of radicals, and then exposing it to UV light, causing covalent thiol-ene crosslinks to form between the allyl groups of the Gel-AGE molecules (Northrop & Coffey, 2012). This method of crosslinking is highly efficient, very selective, and tolerant to a wide range of conditions and functional groups.

However, while Gel-AGE is useful as a bioink precursor, its main method of crosslinking is slow, occurring over the course of seconds to minutes when exposed to UV or high-energy visible light. This makes Gel-AGE impractical for certain applications that require extremely rapid crosslinking speeds such as microfluidics. One such solution for this is to form a hybrid hydrogel matrix by mixing Gel-AGE with another bioink precursor that can crosslink more rapidly.

A possible precursor that shows promise for use in hybrid hydrogels is alginate. Alginate is a natural, food grade polymer that is frequently sourced from seaweed and algae. Alginate holds great potential for use in modern biofabrication techniques, specifically within the realm of bioprinting and microfluidics, due in part to its ability to crosslink near instantly when exposed to metal ions, as well as its cyto-compatibility. However, it lacks RGD sequences, meaning cells cannot easily attach to alginate molecules. It also lacks the ability to be crosslinked for extended periods of time, as metal ion crosslinking requires a constant supply of ions to maintain matrix integrity. This limits its uses in long term culture.

In order to provide alginate with a method for long term crosslinking, alginate could be reacted with AGE in a similar fashion to gelatin, resulting in alginate with affixed alkene groups, and allowing it to photopolymerized in a way akin to Gel-AGE. These alkene groups would also

give this functionalized alginate a way to covalently crossink with Gel-AGE molecules. By combining Gel-AGE and alginate, the two polymers could make up for what the other lacks. The resulting hybrid hydrogel would contain a dual-crosslinking system, and be able to be crosslinked both rapidly, and for extended periods of time. It would also contain RGD sequences, be non-cytotoxic, easy to replicate, and low cost.

However, while Gel-AGE synthesis is well studied, the synthesis of allylated algiante is significantly less well known. While research with the aim of functionalizing alginate with various functional groups has also been performed in the past (Abulateefeh et al., 2014), and functionalization of alginate with AGE specifically has also been performed in prior literature (Du et al., 2022), it has yet to be performed with the goal of forming hybrid hydrogels, or a dual crosslinking network. Additionally, little research has been done on how varying the conditions of the Al-AGE reaction impacts the properties of resulting Al-AGE. As such, we can identify a gap in knowledge about the specifics of the synthesis of allylated alginate. It is this knowledge gap that my project aims to remedy.

Project Aim and Research Goals

This project intends to investigate the functionalization of alginate with alkene groups, or double bonded carbon atoms, by reacting unmodified alginate with allyl glycidyl ether under basic conditions. Additionally, the resulting Al-AGE will be tested for degree of modification, mechanical properties, and crosslinking kinetics. Finally, Al-AGE will be mixed with Gel-AGE, and the formation of hybrid hydrogels with a dual crosslinking system will be investigated. This dual crosslinking system will allow us to crosslink the resulting hydrogels with both metal ions (a fast crosslinking method), and thiol-ene click chemistry (a long lasting crosslinking method). This will enable increased viability with currently existing biofabrication technologies.

To summarize, this thesis was guided by the following research questions:

- Can alginate be successfully functionalized with AGE, resulting in Al-AGE?
- How can proton NMR and other characterization methods be used to confirm functionalization of Al-AGE?
- How does the functionalization of alginate affect its ability to crosslink via metal ions and thiol-ene click chemistry?
- Can a hybrid hydrogel between Al-AGE and Gel-AGE be formed and characterized?

Methods

Below details the methods utilized during this project. Said methods can be divided into two sections: synthesis of Al-AGE, and characterization of Al-AGE. Al-AGE synthesis consisted of both the theory and calculations behind the reactions within this project as well as the process of execution of said reactions. Al-AGE characterization consisted of taking the synthesized Al-AGE, and utilizing a range of analytical techniques to discern its properties, such as its degree of modification, and cross-linking ability.

Synthesis of Allylated Alginate

Allylated alginate was synthesized by reacting food-grade alginate with allyl glycidyl ether (AGE) under basic conditions. Both the procedure detailed below and the calculations done prior were based on previously established methods for synthesizing Allylated Gelatin (Bertlein et al., 2017).

Reaction Theory and Calculations

Before beginning synthesis, knowledge of organic chemistry, and previously established research was used to calculate and plan the reaction conditions used during this project. Said calculations were based on previously established methods for synthesizing Allylated Gelatin (Gel-AGE) (Bertlein et al., 2017). Specifically, the method for functionalization of Gel-AGE involves what is known as an epoxide ring opening reaction (Figure 1). When performed in basic conditions, a strong nucleophile will attack the epoxide ring at the least substituted site, causing the ring to open and resulting in the formation of a new bond.

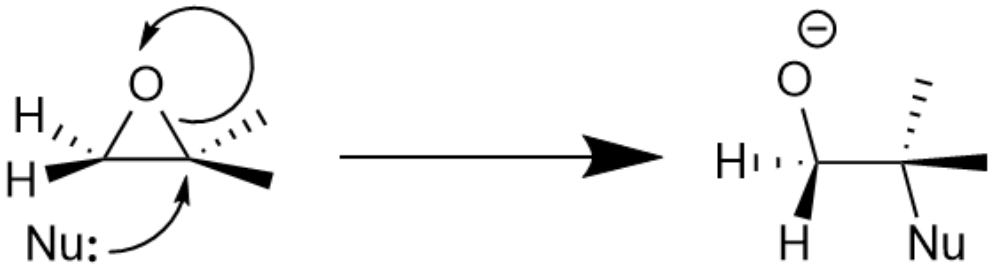


Figure 1: **Epoxide Ring Opening Under Basic Conditions**

This reaction results in the formation of a bond between the nucleophile and the less substituted carbon. Following workup, the alkoxide anion (O-) is converted into a hydroxyl group via protonation.

Previously mentioned methods have established that primary amine groups on gelatin act as the main nucleophile in this reaction, with carboxylic acid and hydroxyl groups acting as secondary nucleophiles. For the following synthesis, we assumed that a similar reaction would occur to the one that occurs with gelatin, with the carboxyl groups on alginate acting as the primary nucleophile. This assumption was made on the premise that the pKa of alginate hydroxyl groups would be too high for them to act as effective nucleophiles. As such, the following reaction scheme was used moving forward:

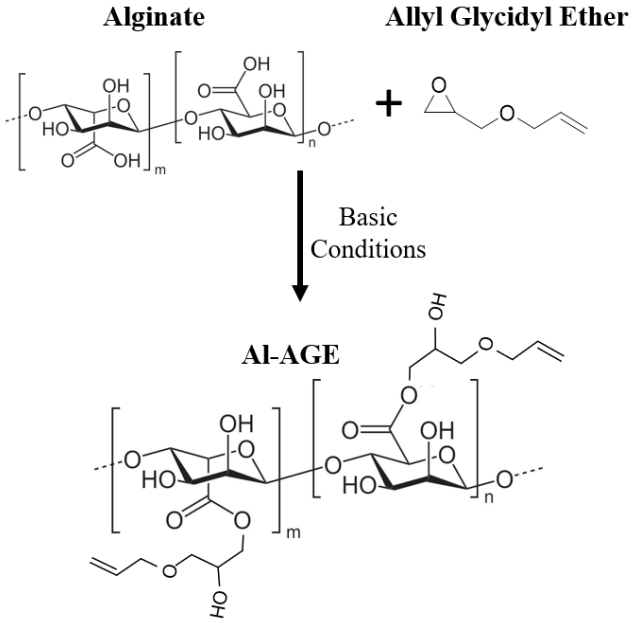


Figure 2: **Al-AGE Reaction Scheme**

The resulting Al-AGE molecule has its AGE groups attached to the deprotonated carboxylic acid groups of the alginate molecule.

For the previous work done with Gel-AGE, three AGE quantities were used during synthesis, Low (L), Medium (M), and High (H), corresponding to values of mmol AGE per gram of gelatin equivalent to 2.4, 12, and 60 respectively. In order to maintain consistency during our synthesis, we utilized these numbers to calculate corresponding values for alginate instead of gelatin, as is detailed in the table below:

Calculating Functional Groups Per Gram of Alginate		
- *Sodium Alginate - Repeating Unit - $C_6H_9NaO_7$:*	216.12	g/mol
- *Sodium Alginate - Chain:*	2660000	g/mol
- *OH Groups Per Gram of Alginate:*	13.881	mmol/g
- *Carboxyl Groups Per Gram of Alginate:*	4.627	mmol/g
Viable Nucleophile Amino Acid Composition of Gelatin		
- *Lysine*:	0.280	mmol/g
- *Hydroxylysine*:	0.062	mmol/g
- *Histidine*:	0.058	mmol/g
- Total:	0.400	mmol/g
Mmol AGE per Gram of Gelatin		
- *Low (L)*:	2.4	mmol/g
- *Med (M)*:	12	mmol/g
- *High (H)*:	60	mmol/g
Ratio of AGE per Mmol of Gelatin Functional Groups		
- *Low (L) - 2.4 mmol to 0.4 functional groups*:	x6	excess
- *Med (M) - 12 mmol to 0.4 functional groups*:	x30	excess
- *High (H) - 60 mmol to 0.4 functional groups*:	x150	excess
Mmol AGE Needed Per Gram of Alginate		
- *Low (L) = 4.627 * 6*:	**27.762**	mmol/g
- *Med (M) = 4.627 * 30*:	**138.812**	mmol/g
- *High (H) = 4.627 * 150*:	**694.059**	mmol/g

Table 1: **Al-AGE Synthesis Calculations**

This table contains the resulting values for calculating the proper ratios of alginate to allyl glycidyl ether.

The process of calculating the quantities of AGE needed for our reactions was as follows (Table 1): First, basic information regarding alginate was gathered from the manufacturer. This included the number of carboxyl groups per gram of alginate, as well as the repeating units of alginate. Second, the total number of applicable functional groups for a gelatin-based synthesis were calculated. In this specific case, only primary amines were factored in. This value was sourced from the Gelatin Manufacturers Institute of America Gelatin Handbook. Third, the ratios of AGE/gelatin were recorded from the supplemental material of Bertlein et al., 2017. Fourthly, these ratios were divided by the quantity of applicable functional groups to get the ratio of mmol of AGE to mmol of gelatin functional groups. Finally, these values were multiplied by the number of carboxyl groups per g of alginate to get the values we use for the synthesis parameters below.

Synthesis Procedure

Food grade sodium alginate (FujiFilm, Osaka, Japan) was mixed with deionized water at 40 °C to form a stock with a concentration of 1wt%. Alginate stock was divided into 50mL conditions for a total of 0.5g alginate per condition.

In order to observe the effects of varying reaction conditions during Al-AGE synthesis, a total of three separate types of reactions were run, with each reaction type corresponding to a different parameter varied (AGE, NaOH, Temperature). A varying quantity of AGE (Sigma-Aldrich, St. Louis, Missouri), and sodium hydroxide (NaOH) (Sigma-Aldrich, St. Louis, Missouri) were added to each alginate condition, and the temperature was set. All three of these properties varied depending on the reaction (Table 2, 3, 4). The conditions used for each reaction are detailed in the tables below.

	Ratios		Material Measurements		
#	Mmol NaOH per Gram of Alginate	Mmol Allyl Glycidyl Ether per Gram of Alginate	uL NaOH	mL AGE	Temperature (°C)
1	1	12.38	250	0.7620952219	40 °C
2	**1**	**27.76**	**250**	**1.524190444**	**40 °C**
3	1	138.81	250	7.620952219	40 °C
4	1	694.05	250	38.10476109	40 °C

Table 2: **Reaction 1 - Varying Allyl Glycidyl Ether Quantity**

For this set of reactions, the quantity of AGE added was varied, while base and temperature were kept constant. Condition #2 is highlighted, as said condition was utilized as a "standard" point of comparison for other resulting synthesis during characterization.

	Ratios		Material Measurements		
#	Mmol NaOH per Grams of Alginate	Mmol Allyl Glycidyl Ether per Grams of Alginate	uL NaOH	mL AGE	Temperature (°C)
1	0.5	27.76	125	1.524190444	40 °C
2	**1**	**27.76**	**250**	**1.524190444**	**40 °C**
3	2	27.76	500	1.524190444	40 °C
4	5	27.76	1250	1.524190444	40 °C

Table 3: **Reaction 2 - Varying NaOH Quantity**

For this set of reactions, the quantity of NaOH added was varied, while AGE and temperature were kept constant. Condition 2 was omitted from this synthesis, as it had the same conditions as condition 2 from reaction 1, and was thus redundant.

	Ratios		Material Measurements		
#	Mmol NaOH per Grams of Alginate	Mmol Allyl Glycidyl Ether per Grams of Alginate	uL NaOH	mL AGE	Temperature (°C)
1	1	27.76	250	1.524190444	4 °C
2	1	27.76	250	1.524190444	21 °C
3	**1**	**27.76**	**250**	**1.524190444**	**40 °C**
4	1	27.76	250	1.524190444	85 °C

Table 4: **Reaction 3 - Varying Temperature**

For this set of reactions, the temperature of the reaction was varied, while AGE and NaOH were kept constant. Condition 3 was omitted from this synthesis, as it had the same conditions as condition 2 from reaction 1, and was thus redundant.

Additionally, a series of control reactions were run for each reaction, with said controls containing identical temperature and NaOH, but no AGE. The properties of each control are as follows (Table 5).

		Ratios	Material Measurements		
Reaction #	Condition #s	Mmol NaOH per Grams of Alginate	uL NaOH	mL AGE	Temperature (°C)
1 - Varying AGE	**1,2,3,4**	**1**	**250**	**0**	**40 °C**
2 - Varying NaOH	1	0.5	125	0	40 °C
	2	**1**	**250**	**0**	**40 °C**
	3	2	500	0	40 °C
	4	5	1250	0	40 °C
3 - Varying Temperature	1	1	250	0	4 °C
	2	1	250	0	21 °C
	3	**1**	**250**	**0**	**40 °C**
	4	1	250	0	85 °C

Table 5: **Control Conditions**

For this set of reactions, conditions were the same as their corresponding non-control reactions. However, all reactions had no AGE. Bolded conditions were omitted from these synthesis, as they had the same conditions as other controls, and were thus redundant.

All NaOH used had a stock concentration of 2 M. Each condition was allowed to react for 2 hours, with this time starting immediately following the addition of NaOH. After, the volume of each condition was doubled using additional deionized water, and any remaining base in each condition was neutralized via the addition of Hydrochloric Acid (HCl) (Sigma-Aldrich, St. Louis, Missouri), until a pH of ~7 was obtained. The pH for each condition was measured at the start of reaction time, end of reaction time, and after neutralization to a neutral pH.

Following successful completion and neutralization, each condition was placed into 14kd cellulose membrane dialysis tubes, and submerged in deionized water to allow unreacted AGE to diffuse out of solution.

The water in each condition was changed every 2-4 hours for 4-6 days, or until a total of 10 or more water changes had elapsed. Conditions were then placed into storage cups, frozen, and then lyophilized for a minimum of 10-14 days, resulting in a porous alginate macromer ready for use in characterization and experiments.

Characterization

Following the synthesis of Al-AGE, multiple forms of characterization were utilized to discern properties of our resulting product.

Proton NMR

Synthesized Al-AGE was analyzed via proton nuclear magnetic resonance (H-NMR). Nuclear magnetic resonance is an analytical technique that relies on the inherent magnetic spin that all atoms have. By exciting these spins and letting them drop back to their base energy level, a molecule can be made to emit radio waves, which can then be recorded and analyzed to infer information about that compound's structure and properties. For this project, H-NMR was utilized to determine the degree of functionalization of our synthesized Al-AGE.

Quantities of synthesized al-AGE ranging in mass from 5-10 mg were mixed in 500ml deuterium oxide (D2O) to yield several Al-AGE stocks ranging from 0.5-2 wt%. Techniques for analyzing alginate-based samples via H-NMR were adapted from previously established literature (Jensen et al., 2015). Samples were pipetted into NMR tubes and H-NMR was run.

For this project, three separate sets of H-NMRs were taken, with each set varying the

parameters at which our samples were analyzed. For the first set, H-NMRs of all samples were run with no additional modification.

For the second set, H-NMRs of all samples were taken with the addition of 100mL Dimethylformamide into the NMR tube. The aim of this addition was that Dimethylformamide could act as a "proton standard," or a signal that would occur on the NMR that could act as a baseline for NMR analysis later down the line. Techniques for the inclusion of DMF within alginate NMR characterization, as well as the analysis of the DMF peak itself were also adapted from established literature (Ooi et al., 2018).

Finally, for the third set, certain alginate conditions were hydrolyzed prior to being prepared for NMR, in accordance with previously established literature (Jensen et al., 2015). 100mg of alginate (either synthesized Al-AGE, a synthesized alginate control, or plain alginate) was dissolved in 100mL deionized water to yield a 0.1 wt% Al-AGE stock, and the pH of solution was adjusted to 5.6 using 1 and 0.1 M HCL. The mixture was then refluxed for 1 hour at 100 °C while stirring. Following reflux and cooling to room temperature of the reaction, pH was once again adjusted to 3.8, and reflux was repeated for 30 mins. The second reflux was halted via cooling on ice. pH of the reaction was then re-adjusted to pH 7-8 using NaOH, before being frozen and lyophilized. Following hydrolysis, the resulting alginate was prepped for NMR identically to what was detailed prior.

All H-NMR samples were run at 80 °C, and alongside a control sample consisting of plain, unreacted alginate. Resulting NMR spectra were analyzed using the NMR analytics tool MestReNova v14.0.0-23239.

Spectrophotography

We also used spectrophotometry tests as another method to determine the degree of modification of our synthesized Al-AGE.

Spectrophotometry is a technique that allows us to discern properties of molecules based on the quantity of light they absorb. Specifically regarding our Al-AGE, we attempted to utilize a colorimetric assay, which was a compound that would change color when in the presence of certain functional groups. Using a spectrophotometer, we can measure this color change by recording a baseline measurement of the absorptivity of our assay, then adding the compound we want to quantify, and using beer's law $A = \varepsilon bC$ where A is absorption, epsilon is molar absorptivity, b is light path length, and C is concentration, to determine the concentration of compound that has changed color. We can then use the resulting value to infer properties of our compound.

For our Al-AGE tests, we used Toluidine Blue O (TBO) (Figure 3) (Sigma-Aldrich, St. Louis, Missouri), a colorimetric assay that changes color when exposed to certain nucleophiles, such as carboxylate ions. By comparing the color change of our functionalized Al-AGE to the color change of unreacted alginate, we could measure how many carboxylic acid groups had reacted with AGE, and thus determine a degree of modification.

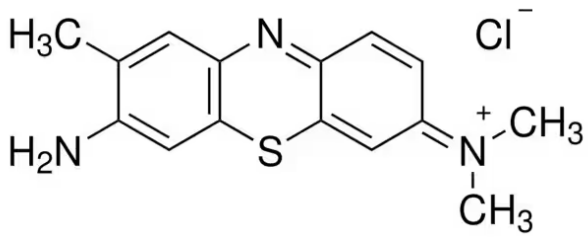


Figure 3: **Toluidine Blue**

The chemical structure of toluidine blue. A mixture of this compound in water appears a deep purple at high concentrations, and a bright blue at lower ones. It turns a bright red when exposed to strong nucleophiles.

First, we had to determine the ideal absorption wavelength of TBO. Using previously established literature (Ben Fradj et al., 2014), we determined this to be 630nm normally, and 540nm when TBO is under the effects of metachromatism (after it has reacted with a carboxylic acid). Following this, we wanted to establish a calibration curve by measuring how the absorption changed when mixed with cyclopentane carboxylic acid (CPA) as a carboxylic acid standard. The ratio of moles of TBO to CPA varied from 0.2-15 per singular mol of TBO, and a total of 10 tests were run for this curve, including a blank with zero CPA.

Following attempts at establishing a calibration curve with cyclopentane carboxylic acid, no further work was done utilizing this colorimetric assay.

Inversion Test

In order to determine the ionic crosslinking properties of our synthesized al-AGE, inversion tests were performed on all conditions. Alginate and deionized water were mixed at a ratio of 10mg per 1ml to yield a 1wt% alginate stock. Calcium Chloride (CaCl2) (Sigma-Aldrich, St. Louis, Missouri) was mixed with 10% EtOH (Decon Labs Inc, Montgomery County, Pennsylvania) to yield a 0.2M CaCl2 solution. Alginate and CaCl2 stocks were then combined in equal parts in a microfuge tube, and mixed via vortex at room temperature. The microfuge tube was then inverted, and the resulting mixture was visually analyzed for the formation of a hydrogel. Successful formation of a hydrogel was characterized by observing whether the resultant mixture would settle to the bottom of the tube or remain at the top of the tube as a crosslinked polymer. This characterization method was performed on all synthesized Al-AGE conditions, as well as unreacted alginate, and all al-AGE controls.

Rheological Testing

In order to determine both the rheological properties of our alginate, as well as its crosslinking kinetics, we performed rheology and photorheology on our synthesized Al-AGE.

Rheology measures the mechanical properties of a material. Specifically, the measure of deformation when stress is applied. This is measured primarily through two values: storage modulus (G'), and the loss modulus (G''). Storage modulus represents the contribution of elastic rigidity to the overall resistance of a material, while loss modulus represents the contribution of inelastic, or liquid rigidity to the overall resistance of a material. Both of these factors can also be measured together, and their vector contributions can be merged to form complex modulus (G*). By running rheology on a crosslinkable substance (for these tests, our hydrogel precursors containing Al-AGE, DTT, and LAP) that is being exposed to crosslinking conditions (365 nm light), we can measure the kinetics of crosslinking and material property changes caused by said crosslinking in a process known as photorheology. For this project, photorheology acted both as a means of characterizing our Al-AGE's G' and G'', and also as a means of confirming functionalization via a successful crosslinking reaction.

First, baseline conditions for running rheological tests on alginate were established by running preliminary tests. This procedure for establishing rheological conditions was based on previously established literature (Zuidema et al., 2014). All rheological tests involve placing samples in between two plates, and then rotating the plates and measuring the resistance. Specifically, there were three types of tests run to establish our baseline parameters. The first was a frequency ramp, which keeps the total distance the plates rotate consistent, but varies the speed at which the plates rotate back and forth. The second was an amplitude ramp, which does the inverse, varying the total distance the plates rotate, but keeping the speed at which the plates rotate

back and forth constant. And the third was a temperature ramp was run, which kept the amplitude and frequency consistent, but varied temperature.

For all of these tests, the data was analyzed to find a region in which the properties of the hydrogel stay relatively consistent (i.e. the linear region), and that data was used for our photorheology tests, which keep both amplitude and frequency consistent, and measure how G' and G'' change over time.

To establish baseline rheology conditions, plain, unmodified alginate and deionized water were mixed at a ratio of 10mg per 1ml to yield a 1wt% alginate stock. Calcium Chloride (CaCl2) was mixed with 10% EtOH to yield a 0.2M CaCl2 solution. 500 uL of both alginate and CaCl2 stocks were then combined in a microfuge tube, and mixed via vortex. The resulting hydrogel was then loaded onto the rheometer, and any excess was trimmed off. For all tests, 20mm plates were used, with a 30000um loading gap, 250um trim gap, and a 200 um geometry gap. Additionally, all tests were run at 22 °C with a 30s soak time to allow any samples to reach room temperature. First, a frequency ramp was run at 10% strain, from 0.1-50 Hz. Second, an amplitude ramp was run at 1 Hz from 0.1-100% strain. Third, another frequency ramp was run at 0.3% strain and 0.1-100 Hz. And finally, one final frequency ramp was run at 0.03% strain and 0.1-100 Hz. Through these tests, we settled on the conditions of 0.03% strain, and 1Hz for our Al-AGE photorheometry.

Following the establishment of our baseline conditions, we performed photorheology on our Al-AGE samples. First, the rheometer was assembled so that the bottom plate was replaced with a quartz plate that connected to a UV light source, to allow for photocrosslinking of our Al-AGE. Hydrogel precursors containing Al-AGE, LAP, and DTT were then assembled in accordance with the table below (Table 6). Additionally, conditions containing Gel-AGE were also assembled.

Cond #	Macromer Type	DTT (% Functional Groups)	DTT : Assumed Functional Groups	DTT (Mm)
1	5wt% Gel-AGE (G1MM)	0	0:1	0
2	5wt% Gel-AGE (G1MM)	100%	1:1	0.01775
3	1wt% Unmodified Alginate	0	0:1	0
4	1wt% Unmodified Alginate	200%	2:1	0.133812
5	1wt% Al-AGE, vAGE 2	0	0:1	0
6	1wt% Al-AGE, vAGE 2	200%	2:1	0.133812

Table 6: **Al-AGE and Gel-AGE Photorheomitry Parameters**

Al-AGE from varying AGE condition 2 was used for these tests. When calculating DTT to add to each condition, Al-AGE was assumed to have alginate at maximum possible functionalization. DTT (% Functional groups) represents the number of applicable thiol groups to the number of assumed crosslinking sites on our Al-AGE. All hydrogel precursor conditions contained 1 wt% of their respective macromer (aside from the Gel-AGE condition, which had 5 wt%) 0.1 wt% LAP, and were a total of 200ml in volume.

Finally, hydrogel precursors were loaded onto the rheometer, and a time sweep was performed on each condition in triplicate. The properties of this time sweep were a 30 second soak, and 6 minute test time, with 1 minute of idle time before UV light was turned on. Our 0.03% strain and 1Hz conditions were also used for these tests. The resulting data was recorded and then graphed using GraphPad Prism v10.0.2.

Hybrid Hydrogel Formation

In order to test the ability of the Al-AGE to form thiol-ene bonds, and thus be polymerized into a hydrogel with covalent bonds for long term use, we fabricated hybrid hydrogels utilizing both Al-AGE and Gel-AGE.

First the following stock solutions were mixed for use in hydrogel creation: a 30 wt% stock of G1MM Gel-AGE (Bertlein et al., 2017), a 3 wt% stock of reaction 1, condition 2 Al-AGE, a 1M stock of Dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, Missouri), and finally, a 1 wt% stock

of Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) (Sigma-Aldrich, St. Louis, Missouri). All stock solutions were mixed in deionized water.

In order to vary the properties of our hybrid hydrogels, the quantity of stock solution in each condition was also varied. This formed hydrogel precursors with the following final ratios of material according to the table below (Table 7).

Gel #	Gel-AGE (wt%)	Al AGE (wt%)	LAP (wt%)	DTT (mM)
1	2	0	0.1	7.1
2	0	1	0.1	69.4
3	2	1	0.1	7.1
4	5	1	0.1	17.7
5	5	1.5	0.1	17.7
6	5	0	0.1	17.7

Table 7: **Al-AGE + Gel-AGE Gel-Conditions**

When calculating DTT to add to each condition, conditions with no Gel-AGE were assumed to have alginate at maximum possible functionalization. For conditions with both Gel-AGE and Al-AGE, only the Gel-AGE AGE groups were considered when calculating the quantity of DTT to add, in order to ensure that any crosslinking was the result of the addition of alginate, and not excess DTT.

For these experiments, only condition 2 of reaction #1 (Varying AGE) was used, due to it being the standard for which all other synthesis conditions were based off of. All hydrogel precursors had a total volume of 150 mL.

Hydrogel Precursors were then pipetted into disc shaped silicone molds with a height of 1mm, a diameter of 5mm, and a volume of 33mL, and crosslinked via exposure to UV light (365nm) for 180 seconds. Resulting hydrogels were then removed from molds and analyzed for successful crosslinking.

Results

Below details the results of the synthesis and characterization of Allylated Alginate. Al-AGE was successfully synthesized in accordance with the established conditions, and functionalization was confirmed via proton NMR. Ionic crosslinking was confirmed through the use of inversion tests, and covalent crosslinking was confirmed through the use of photorheology and hydrogel formation tests.

Synthesis of Allylated Alginate

Synthesis of Al-AGE was completed without issue. Reaction procedure was followed as is detailed above in the methods section of this thesis. Synthesis of Al-AGE was divided into 5 individual syntheses over the span of multiple months, each 2 hours long, and with 2-4 conditions per synthesis. pH values of each synthesis were recorded, as well as any notable observations. Reactions performed in each synthesis, as well as start and end pH values of each reaction are noted in the table below (Table 8).

Synthesis #	Condition	pH (Initial)	pH (Final)	pH (Neutralized)
1	Varying AGE, 2	11.0	10.9	7.0 * *(4.6)*
	Varying AGE, 3	11.0	10.9	7.2
	Varying AGE Control 1,2,3,4	11.6	10.9	6.7
2	Varying Base, 1	11.6	11.4	7.8
	Varying Base, 3	12.0	11.8	7.7
	Varying Base, 4	12.4	12.3	7.8
3	Varying Base Control, 1	11.7	11.5	7.7
	Varying Base Control, 3	12.1	12.0	6.1 * *(5.0)*
	Varying Base Control, 4	12.4	12.2	6.5 * *(5.0)*
4	Varying Temperature, 1	12.0	12.0	7.8
	Varying Temperature, 2	11.9	11.9	6.6
	Varying Temperature, 4	11.5	11.1	7.5
5	Varying AGE, 1	11.8	11.5	6.7
	Varying AGE, 4	11.8	10.0	8.15

Table 8: **Syntheses of Al-AGE and resulting pH**

Reaction 3, Varying Temperature's controls 1,2, and 4 were omitted from synthesis due to time constraints. Conditions whose ph contains an asterisk (*) denotes an overshoot when adding acid and thus resulted in a lower than desired pH that had to be corrected with NaOH, with the number in parenthesis being the lowest pH the condition reached before additional base was added.

During the addition of HCl, some reactions had their pH brought to lower than desirable levels due to experimental error, and had to be re-neutralized via NaOH. However, none of these

pH fluctuations prevented functionalization altogether, as is detailed in the characterization section below. Additionally, for reaction 1, addition of 1M HCl resulted in the formation of a slightly visible white precipitate, that was presumed to be alginate becoming less soluble due to the rapid change in pH. This precipitate was especially noticeable in conditions where too much acid was added, and base had to be added to re-neutralize. For the following reactions, a weaker stock of HCl was used (0.25M), and the precipitate was either much less visible or not observable at all as a result.

Midway through synthesis, an important piece of literature was procured that suggested that epoxide ring opening reactions in basic conditions were more likely to occur on hydroxyl groups than they were carboxyl groups (Reis et al., 2009). This placed our previous assumption that the carboxyl groups were the nucleophile of the reaction under question. Due to the fact that alginate molecules contain twice as many hydroxyl groups as they do carboxylic acid groups, then all of our reacted conditions contained twice as many applicable functional groups as predicted, and that the chosen Al-AGE quantity for each synthesis was likely half as high as it should have been.

Nonetheless, synthesis proceeded, and was later characterized to be successful in functionalizing the alginate, as is detailed below. All synthesized alginate conditions successfully underwent dialysis for 10 washes over the span of 5-7 days, and were successfully lyophilized over the span of 10-14 days. Said conditions were then bottled and stored for future use.

Characterization of Allylated Alginate

Al-AGE was successfully characterized in terms of both success of functionalization, as well as dual crosslinking abilities, using the following characterization techniques.

Proton NMR

Functionalization of Al-AGE was successfully confirmed via NMR, however precise values for the degree of functionalization (that is to say, the exact number of functional groups that reacted with AGE) of each condition was not able to be confirmed. Prior attempts to analyze alginate via NMR at room temperature were attempted, but yielded very low resolution spectra that did not correspond with literature, so NMRs were attempted again at an increased temperature. Proton NMRs were taken of all Al-AGE conditions, excluding Varying Temperature 2 and 4 due to time constraints. Following previously established literature (Jensen et al., 2015), the first condition HNMRs were recorded at 80 °C, with no DMF. Only NMRs of select varying AGE and Base conditions were utilized for this first NMR run (Figure 4).

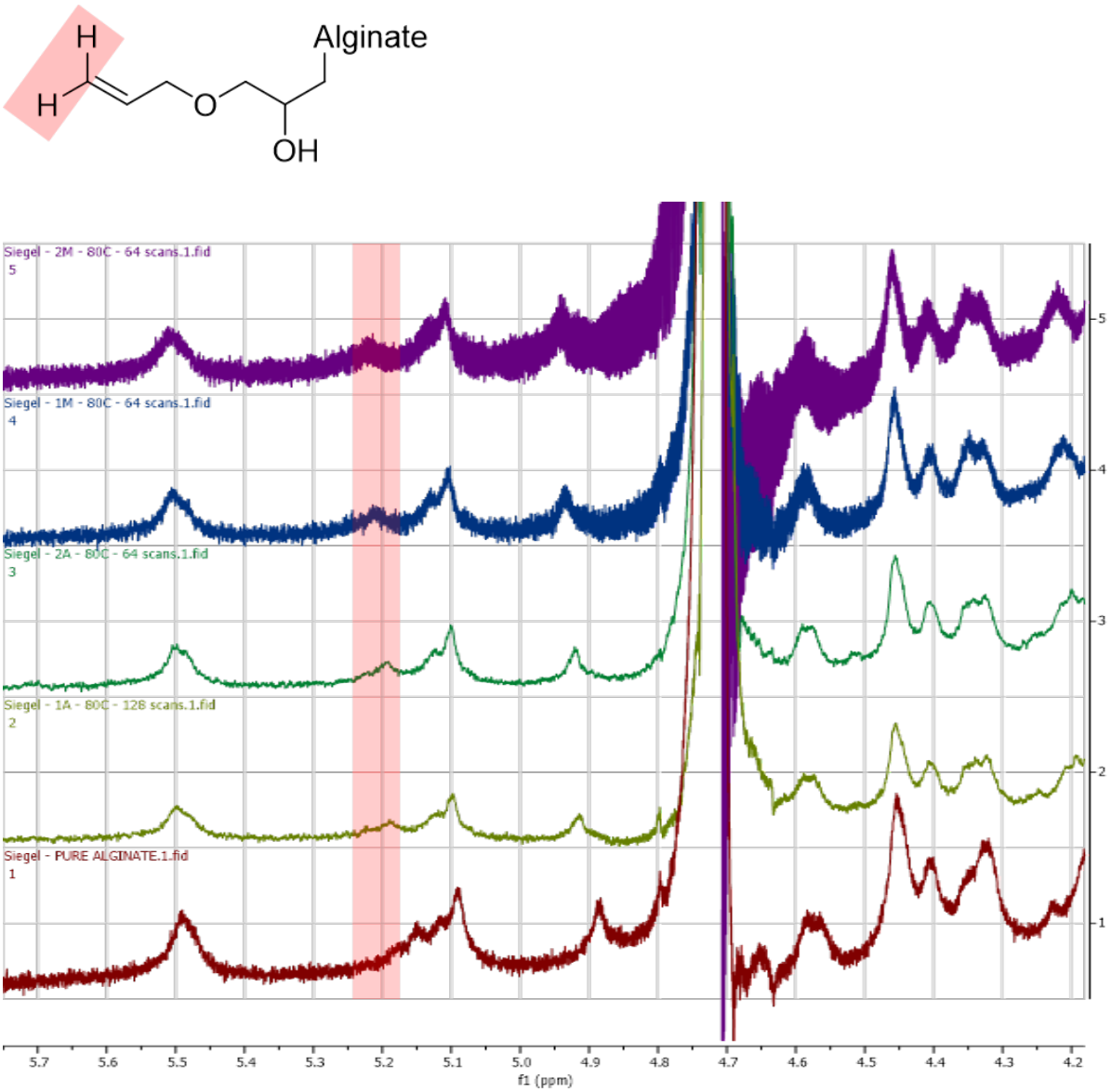


Figure 4: **NMR Testing Run 1**

1A, 2A, 1M and 2M correspond to varying AGE condition 2, varying AGE condition 3, varying base condition 1, and varying base condition 4 respectively. The AGE peak as well as the protons it represents are highlighted in red.

The existence of a subtle but noticeable peak at around 5.2 pmm that is absent on the plain alginate NMR indicates that functionalization was successful, as the location of this peak correlates with it's established appearance on the NMR spectra of functionalized gelatin in previous literature (Bertlein et al., 2017).

However, the resolution of this first run was far too low to be used as a means of calculating the degree of modification, as significant zoom was required to process the spectra, resulting in a significant quantity of noise from the final data. Additionally, even with higher resolution, it would still be very difficult to use these results to quantify the exact degree of functionalization of our synthesized alginate. While gelatin has a phenylalanine peak that can be used as a standard for quantifying functionalization, alginate has no such peak, and functionalized groups must either be calculated using a separate proton standard, or by factoring in the entire alginate spectra itself through calculating the general composition of the alginate being used.

In order to remedy the issue of a lack of a standard for functionalization, a second batch of H-NMRs were taken with the addition of DMF as a proton standard (Ooi et al., 2018.), and with more attentiveness to ensuring the NMR samples were taken at the proper temperature to hopefully improve resolution. All conditions minus varying temperature 1 were analyzed within this set of NMR (Figure 5).

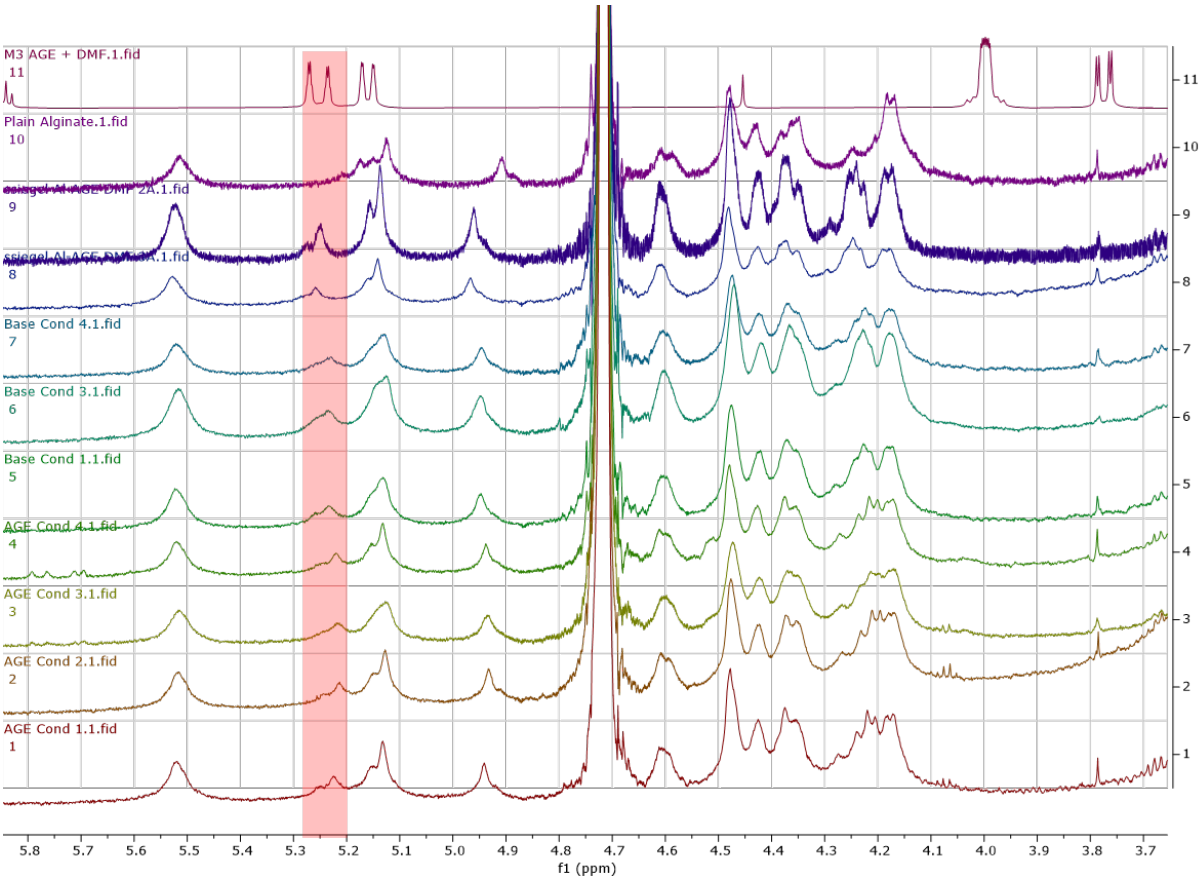


Figure 5: **NMR Testing Run 2**

2A and 3A correspond to varying temperature conditions 2 and 4 respectively. The AGE peak has once again been highlighted in red. Furthermore, the corresponding peak of interest on a pure AGE NMR spectra has been included for comparison's sake.

Once again, a peak at around 5.2 ppm confirms successful functionalization. This indicates to us that we were able to successfully functionalize our Al-AGE, regardless of experimental conditions. While the resolution of the second batch of NMRs was better than the first, it was still too low to accurately characterize the exact degree of modification. An attempt was made to compare the integral of the highlighted AGE peak using DMF as a proton standard, and then gather a concentration of reacted functional groups using previous methods established in literature (Ooi et al., 2018.) to limited success.

In order to remedy the issue of low resolution within NMRs, a third batch of tests were run on unmodified alginate in an attempt to generate a more viable procedure for running alginate H-NMRs. In accordance with previous literature, alginate was hydrolyzed with acid beforehand, and NMR spectra were taken (Figure 6).

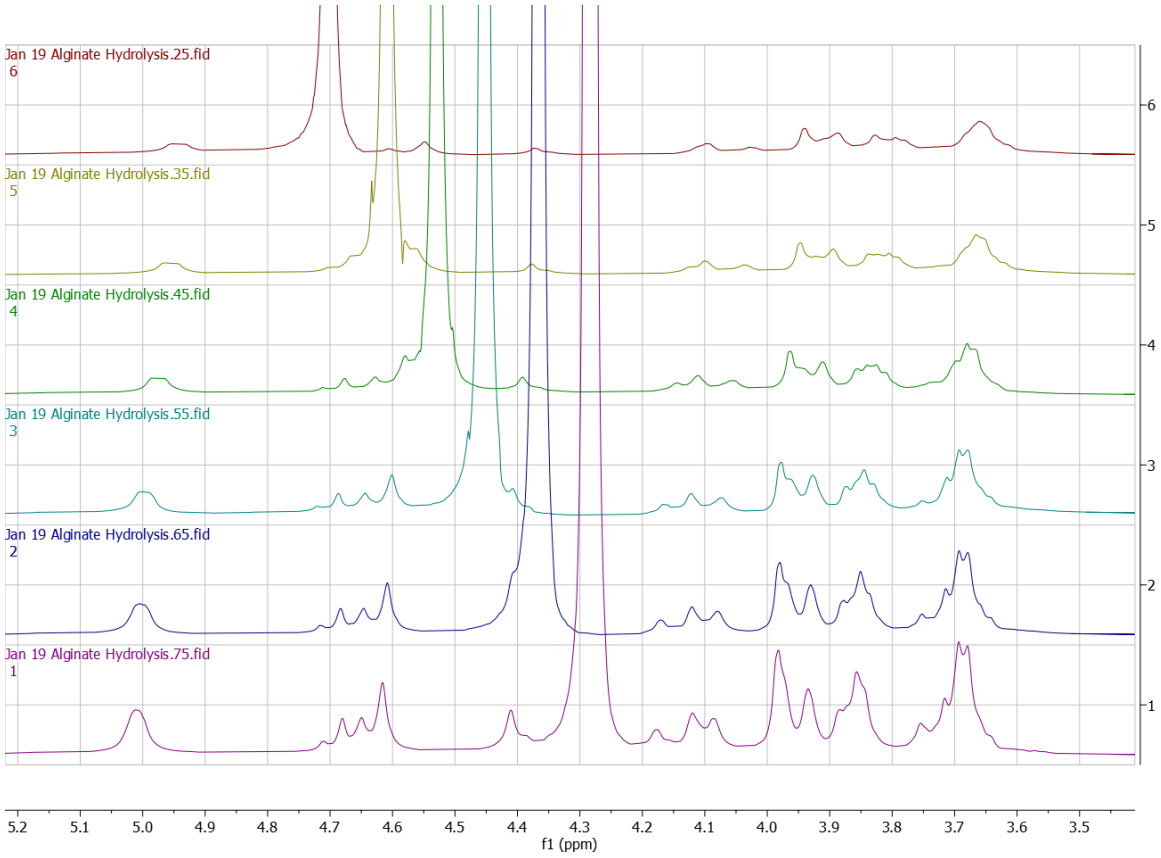


Figure 6: **NMR Testing Run 3**

A series of plain alginate proton NMRs taken at varying temperatures (from 25°C to 75°C). Significantly increased peak definition can be observed as temperature increases, confirming that high temperature paired with hydrolysis yields significantly clearer alginate NMR spectra.

It was observed that the resolution of our alginate spectra was dramatically increased following hydrolysis, and thus highlighted that high temperature and hydrolysis techniques were ideal for improving the resolution of our alginate NMRs. However, we were unable to run NMR tests on our functionalized Al-AGE conditions due to time constraints. Further plans involve

utilizing this hydrolysis method on our functionalized Al-AGE samples, in order to hopefully generate spectra with sufficient resolution for accurate degree of modification characterization.

Spectrophotography

Due to being unable to establish a proper calibration curve using cyclopentane carboxylic acid, results from spectrophotometry tests were limited. Additionally, due to it being likely that the carboxylic acids of alginate are not the primary functionalization sites for epoxide ring opening reactions, and toluidine blue only being sensitive to carboxylic acids, the usefulness of this assay was further diminished. No clear results could be drawn from the utilization of spectrophotography.

Ionic Crosslinking Tests

Following successful confirmation of functionalization, tests were performed in order to confirm that our Al-AGE could crosslink both ionically and covalently. We started by confirming ionic crosslinking utilizing inversion testing.

Inversion Test

Inversion tests were successfully performed on all Al-AGE conditions in order to confirm that reaction conditions did not remove our product's ability to crosslink ionically (Figure 7).

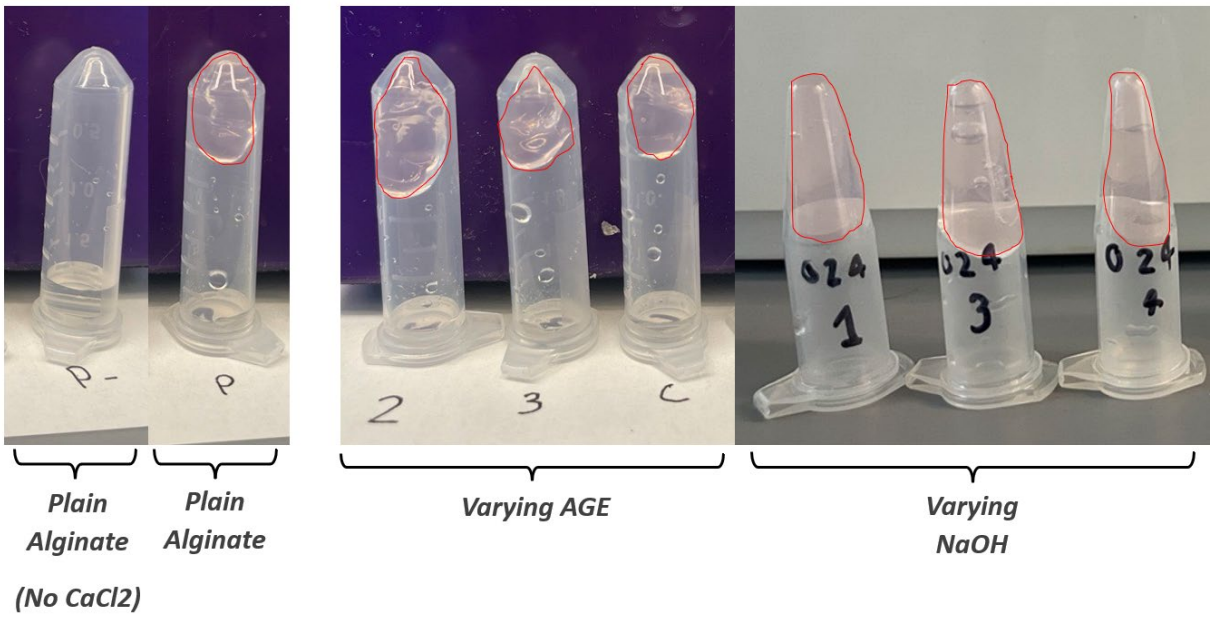


Figure 7: **Example Inversion Test For Various Al-AGE Conditions**

Photos of inversion tests for plain alginate with and without calcium chloride, alongside synthesized Al-AGE for the varying AGE and varying NaOH conditions. Varying temperature synthesis and controls for all reactions have been omitted for brevity, however all omitted conditions did gel successfully. Resulting hydrogels have been highlighted in red in order to enhance visibility. All tubes were 4.25cm tall, and 1.25cm in diameter at their widest point.

All synthesized Al-AGE conditions were able to crosslink into a hydrogel, confirming that functionalization did not diminish ionic crosslinking ability. Seeing as one of the main potential benefits of Al-AGE is a dual crosslinking system, confirmation of ionic crosslinks further elevated the prospects of utilizing Al-AGE for further projects, and allowed us to proceed to testing the covalent crosslinking abilities of our synthesized Al-AGE.

Covalent Crosslinking Tests

Following confirmation of ionic crosslinking, we then performed tests to confirm covalent crosslinking within our Al-AGE. Varying AGE condition 2 was utilized for all covalent crosslinking tests, due to it being a standard condition that all other conditions were based off of.

Rheological Testing

Following the establishment of conditions for measuring the rheological properties of alginate (as is detailed in methods), we were able to successfully confirm the covalent crosslinking ability of synthesized Al-AGE.

First, rheological tests were performed on Gel-AGE. Previous work done in the lab has shown that 5wt% G1MM (A specific type of Gel-AGE) will not crosslink to a sufficient degree for use in hydrogels alone. This was confirmed via photorheology (Figure 8).

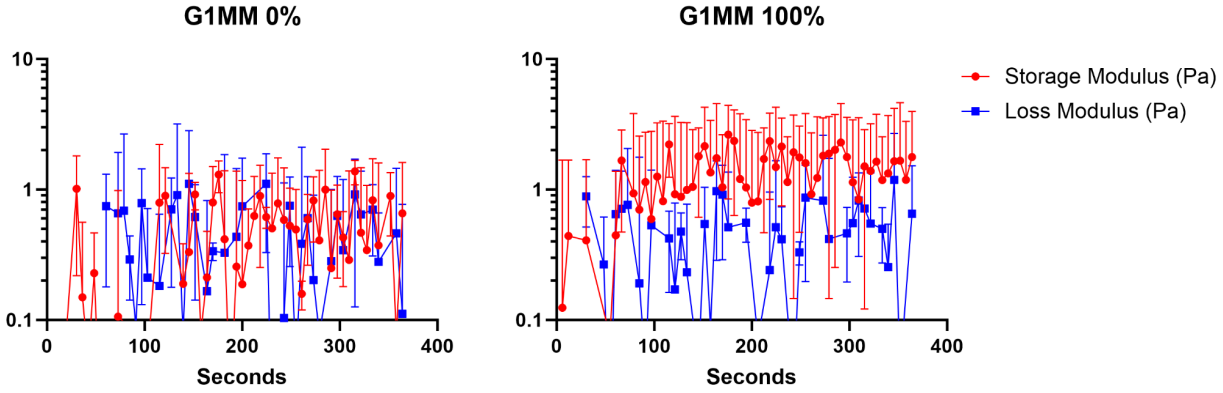


Figure 8: **Rheological Testing: 0 and 100% DTT G1MM**

All graphs are scaled logarithmically on the Y axis. Exposure to UV light began after the 60 second mark of each condition.

A graph showing a logarithmic increase in both G' and G'' as exposure to crosslinking light increases indicates that covalent crosslinking is undergoing. The first Gel-AGE graph resembles noise, indicating little crosslinking has occurred. However, the second graph displays limited but noticeable crosslinking, which is in line with expected results.

Following this, rheological tests were performed on Al-AGE in order to confirm its ability to undergo covalent crosslinking (Figure 9).

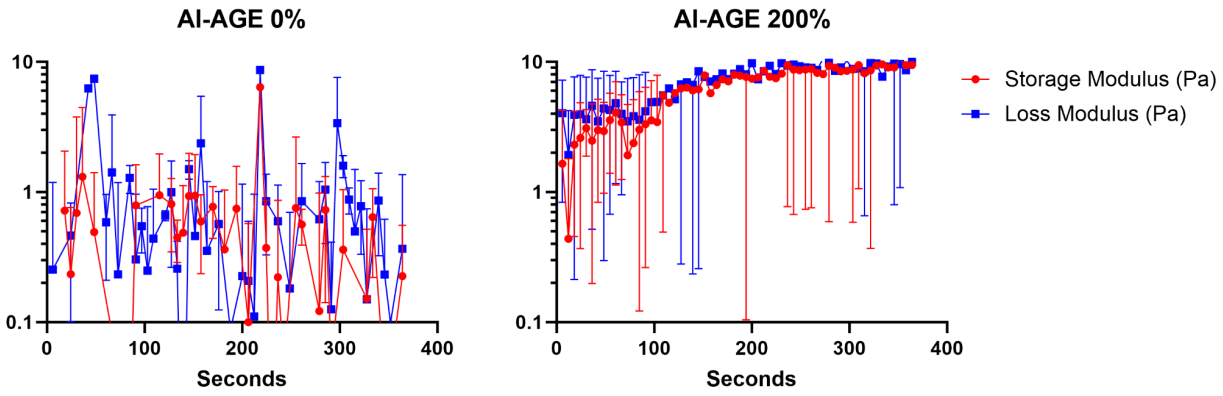


Figure 9: **Rheological Testing: 0 and 200% DTT Al-AGE**

All graphs are scaled logarithmically on the Y axis. Exposure to UV light began after the 60 second mark of each condition.

Once again, the 0% DTT condition displayed only noise, indicating no substantial crosslinking. The 200% DTT condition however, displayed visible and significant logarithmic growth, strongly indicating that the Al-AGE was undergoing covalent crosslinking.

Finally, rheological tests were performed on unmodified alginate as a control in order to confirm that covalent crosslinking was not an inherent property of alginate (Figure 10).

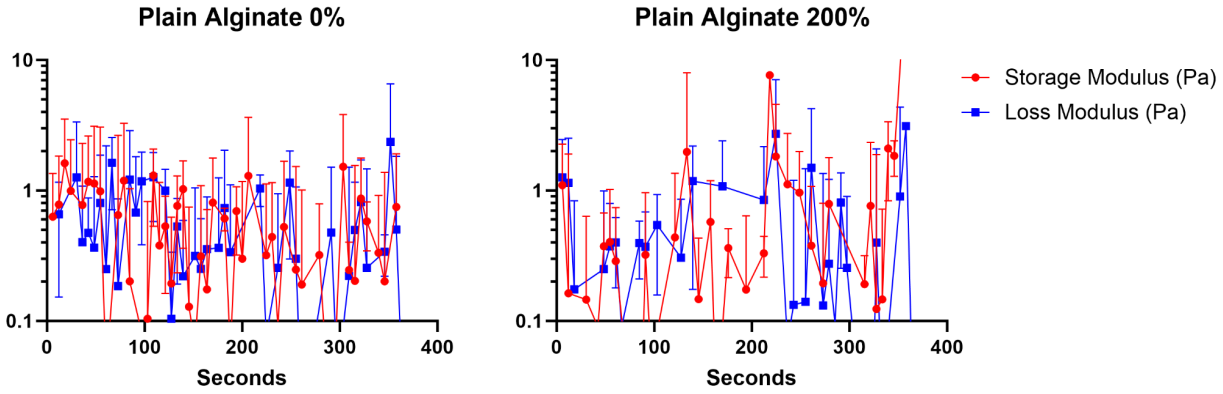


Figure 10: **Rheological Testing: 0 and 200% DTT Plain Alginate**

All graphs are scaled logarithmically on the Y axis. Exposure to UV light began after the 60 second mark of each condition.

Due to being just noise, these graphs indicate that either no crosslinking was undergoing, or that any crosslinking that was occurring was too subtle for the rheometer to measure, which aligns with expected results. However, it should further be noted that no statistical difference was confirmed between the Al-AGE plain alginate, due to high variability and low sample size. Therefore, additional experiments to confirm covalent crosslinking were performed with hybrid hydrogels.

Hybrid Hydrogel Formation

In order to further confirm Al-AGE's ability to covalently crosslink, we attempted to mix it with Gel-AGE to enhance its covalent crosslinking ability. Due to Al-AGE and Gel-AGE both containing allyl groups, a hydrogel consisting of both materials joining together could theoretically be polymerized. Hybrid hydrogels were successfully formed from a mix of Al-AGE and Gel-AGE. Hybrid hydrogel formation procedure was followed as was detailed in the above methods section, resulting in six conditions of varying levels of gelation (Table 9).

Only conditions containing both Al-AGE and Gel-AGE successfully gelled. This indicated that Al-AGE was viable for use in biofabrication techniques due to its ability to crosslink covalently when combined with Gel-AGE. It also indicated that the combined quantity of functional groups in both the Al-AGE and Gel-AGE have a synergistic effect when forming a solid matrix, that low weight percentages of Al-AGE or Gel-AGE alone lacked.

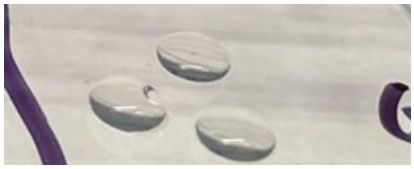
#	Gel-AGE (wt%)	Al-AGE (wt%)	Image	Viscosity
1	2	0		Liquid
2	0	1		Liquid
3	2	1		Viscous Liquid
4	5	1		Gel
5	5	1.5		Gel
6	5	0		Viscous Liquid

Table 9: **Al-AGE/Gel-AGE Hybrid Hydrogels**

A table of the resulting hybrid hydrogels.

Conclusion

At the start of this thesis, the current methods for fabricating organoids, *in-vitro* models designed to mimic *in-vivo* organs, through the use of biofabrication and hydrogels were reviewed. Although major progress in this field has been achieved, organoids still lack clinical relevance due to multiple issues. One of the possible solutions to help alleviate these issues is the utilization of biofabrication techniques. These techniques however, require a library of bioink precursors that fit a wide range of design criteria. This thesis aims to synthesize and characterize a form of alginate that has been functionalized with allyl glycidyl ether. This resulting Al-AGE fits many, if not all of the design criteria desired for a bioink precursor, and also has the ability to undergo a dual crosslinking system, making it extremely useful for biofabrication applications.

Al-AGE was successfully synthesized by combining unmodified Alginate and AGE under a wide range of conditions. Functionalization of said Al-AGE was confirmed via Proton NMR. However, the precise degree of modification was unable to be confirmed. Ionic crosslinking ability of Al-AGE was confirmed via inversion tests, and was found to be unhindered independent of reaction conditions. Covalent crosslinking ability was confirmed via photorheology and hybrid hydrogel formation.

While the overall results of the project as it currently stands have been successful, there are still many different avenues of exploration for both the characterization and function of Al-AGE.

Future Goals

Due to the confirmation of functionalization within Al-AGE, and a dual crosslinking network within synthesized Al-AGE, further plans can be divided into three categories.

The first category involves continuing the characterization of Al-AGE, by investigating its degree of modification and crosslinking kinetics more thoroughly. Plans involve repeating proton

NMR tests using Al-AGE that has undergone hydrolysis in order to hopefully generate spectra with higher resolution. With these spectra, we can ideally calculate the exact quantity of allyl functional groups on our Al-AGE, and use this information to develop conclusions on how the reaction conditions we established prior impacted the overall functionalization of our Al-AGE. A prior research goal for this thesis that later had to be postponed due to time constraints was to investigate if there was correlation between degree of modification, and varying either the AGE, base, or temperature of our Al-AGE reactions, something made difficult by the lack of resolution in our current NMR spectra. As such, there are plans to re-investigate this research goal in the future following the acquisition of better NMR spectra. There are also plans to perform repeat rheological experiments with more thorough conditions in order to better characterize the covalent crosslink ability of our Al-AGE. These rheological experiments will involve tests on both plain Al-AGE, and hybrid Al-AGE/Gel-AGE mixtures. Aside from further confirming the functionalization of our Al-AGE, these tests will also help establish information about the mechanical properties of crosslinked Al-AGE, which is useful for experiments involving biofabrication techniques in the future.

The second category involves testing the compatibility of Al-AGE with biofabrication techniques, primarily microfluidics. Future experiments will utilize in house fabrication equipment, with the goal of generating monodispersed hydrogels, or hydrogel microspheres, with a wide range of stiffness and sizes. By varying parameters of our experiments, such as pressure, weight percent of alginate, and calcium chloride concentration, we want to generate a library of these microspheres for use in biological applications. Additionally, the formation of these spheres is initially initiated via ionic crosslinking. However, testing will also be performed to see if resulting spheres can be successfully crosslinked covalently as well. This will be achieved by

mixing components necessary for thiol-ene click chemistry (DTT, a photo-initiator like LAP, and possibly Gel-AGE for extra crosslink ability) into our Al-AGE mixture, and then exposing any microspheres to UV light after they have formed. If successful, we will be able to utilize this to generate microspheres that can hold their form for an extended period of time, something not possible with unmodified alginate.

The third category involves testing the compatibility of Al-AGE with cells. Due to the inherently biological nature of biofabrication, it is essential that Al-AGE can be used to culture live organisms. In theory, while nothing about the final Al-AGE product on its own should be cytotoxic, it is still paramount to confirm this assumption through actual tests. While details on this future goal were never expanded upon in great detail, the exact plans for this moving forward will be established following the completion of the first two areas of future research.

Glossary

Absorption Wavelength : The wavelength of light for which a compound absorbs photons.

Alkene : A carbon-carbon (C=C) double bond.

Allyl : A functional group consisting of an alkene followed by a carbon-carbon single bond (C=C-C).

Allyl Glycidyl Ether : A molecule containing both an epoxide ring and an allyl group joined by an ether.

Amino Acid : The building blocks of proteins used by all living things.

Biomimetic : Mimicking of something biological.

Carbonyl : A functional group composed of a carbon oxygen double bond (C=O).

Carboxylic Acid : A functional group comprised of an OH group bound to a carbonyl carbon.

Crosslinking : the formation of a bond or a short sequence of bonds that links one polymer chain to another.

Crosslinking Kinetics : The rate at which a material undergoes crosslinking.

Deuterium Oxide : Water consisting of two deuterium atoms and oxygen, with deuterium being a heavier isotope of water.

Dialysis : A technique that separates particles in a liquid based on differences in their ability to pass through a membrane.

Epoxide : A functional group consisting of a three-membered ring involving one oxygen and two carbon atoms.

Ether : A functional group consisting of a Carbon-Oxygen-Carbon bond (C-O-C).

Functional Group : A group of atoms responsible for the characteristic reactions of a particular compound.

Functionalization : The process of adding additional functional groups to a molecule or compound.

Hydrogel : A gel with a water based liquid component.

Hydroxyl : A functional group consisting of a carbon-oxygen-hydrogen bond (C-O-H)

In-vitro : A process occurring outside a living organism, such as in a beaker or test tube.

In-vivo : A process occurring inside a living organism.

Ion : A positively or negatively charged atom or molecule.

Least Substituted Site : A carbon atom that has the highest quantity of small groups (such as hydrogens) bonded to it.

Lyophilize : To freeze dry.

Microsphere : A small spherical particle consisting of biocompatible material.

Nucleophile : A molecule or atom that has a tendency to donate electrons or react at electron poor sites.

pH : A measure for how acidic or basic a substance is, with 1 pertaining to high acidity, 12 pertaining to high basicity, and 7 pertaining to a neutral solution.

Phenylalanine : An amino acid with a benzyl group.

pKa : The acidic dissociation constant. A lower pKa pertains to increased acidity.

Polymer : A substance with a molecular structure consisting of a large chain of similar subunits.

Ppm : Parts per million.

Primary Amine : A functional group consisting of a carbon-nitrogen-hydrogen bond ($C\text{-}NH_3$).

Reflux : A chemistry technique for which you heat a solution in a controlled manner at a constant temperature.

Thiol : A functional group consisting of a carbon-sulfur-hydrogen bond (C-S-H).

Thiol-ene : Containing both a thiol, and an alkene.

Bibliography

Abulateefeh, S. R., Khanfar, M. A., Al Bakain, R. Z., & Taha, M. O. (2014). Synthesis and characterization of new derivatives of alginic acid and evaluation of their iron(III)-crosslinked beads as potential controlled release matrices. *Pharmaceutical Development and Technology*, *19*(7), 856–867. https://doi.org/10.3109/10837450.2013.836222

Ben Fradj, A., Lafi, R., Gzara, L., Hamzaoui, A. H., & Hafiane, A. (2014). Spectrophotometric study of the interaction of toluidine blue with poly (ammonium acrylate). *Journal of Molecular Liquids*, *194*, 110–114. https://doi.org/10.1016/j.molliq.2014.01.008

Bertlein, S., Brown, G., Lim, K. S., Jungst, T., Boeck, T., Blunk, T., Tessmar, J., Hooper, G. J., Woodfield, T. B. F., & Groll, J. (2017). Thiol-Ene Clickable Gelatin: A Platform Bioink for Multiple 3D Biofabrication Technologies. *Advanced Materials*, *29*(44), 1703404. https://doi.org/10.1002/adma.201703404

Du, W., Zhao, Z., & Zhang, X. (2022). Sodium alginate crosslinker engineered UCST hydrogel towards superior mechanical properties and controllable dye removal. *Carbohydrate Polymers*, *285*, 119232. https://doi.org/10.1016/j.carbpol.2022.119232

Groll, J., Boland, T., Blunk, T., Burdick, J. A., Cho, D.-W., Dalton, P. D., Derby, B., Forgacs, G., Li, Q., Mironov, V. A., Moroni, L., Nakamura, M., Shu, W., Takeuchi, S., Vozzi, G., Woodfield, T. B. F., Xu, T., Yoo, J. J., & Malda, J. (2016). Biofabrication: Reappraising the definition of an evolving field. *Biofabrication*, *8*(1), 013001. https://doi.org/10.1088/1758-5090/8/1/013001

Hofer, M., & Lutolf, M. P. (2021). Engineering organoids. *Nature Reviews Materials*, *6*(5), 402–420. https://doi.org/10.1038/s41578-021-00279-y

Jensen, H. M., Larsen, F. H., & Engelsen, S. B. (2015). Characterization of Alginates by Nuclear Magnetic Resonance (NMR) and Vibrational Spectroscopy (IR, NIR, Raman) in Combination with Chemometrics. In D. B. Stengel & S. Connan (Eds.), *Natural Products From Marine Algae* (Vol. 1308, pp. 347–363). Springer New York. https://doi.org/10.1007/978-1-4939-2684-8_22

Levato, R., Jungst, T., Scheuring, R. G., Blunk, T., Groll, J., & Malda, J. (2020). From Shape to Function: The Next Step in Bioprinting. *Advanced Materials*, *32*(12), 1906423. https://doi.org/10.1002/adma.201906423

Naghieh, S., Lindberg, G., Tamaddon, M., & Liu, C. (2021). Biofabrication Strategies for Musculoskeletal Disorders: Evolution towards Clinical Applications. *Bioengineering*, *8*(9), 123. https://doi.org/10.3390/bioengineering8090123

Northrop, B. H., & Coffey, R. N. (2012). Thiol–Ene Click Chemistry: Computational and Kinetic Analysis of the Influence of Alkene Functionality. *Journal of the American Chemical Society*, *134*(33), 13804–13817. https://doi.org/10.1021/ja305441d

Ooi, H. W., Mota, C., & Baker, M. B. (n.d.). *Supplemental Information: Thiol-ene alginate hydrogels as versatile bioinks for bioprinting*.

Reis, A. V., Fajardo, A. R., Schuquel, I. T. A., Guilherme, M. R., Vidotti, G. J., Rubira, A. F., & Muniz, E. C. (2009). Reaction of Glycidyl Methacrylate at the Hydroxyl and Carboxylic Groups of Poly(vinyl alcohol) and Poly(acrylic acid): Is This Reaction Mechanism Still Unclear? *The Journal of Organic Chemistry*, *74*(10), 3750–3757. https://doi.org/10.1021/jo900033c

Zuidema, J. M., Rivet, C. J., Gilbert, R. J., & Morrison, F. A. (2014). A protocol for rheological characterization of hydrogels for tissue engineering strategies. *Journal of Biomedical Materials Research Part B: Applied Biomaterials*, *102*(5), 1063–1073. https://doi.org/10.1002/jbm.b.33088