Final Lab Report

Alginate Hydrogels

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Table of Contents

1. Introduction

Intervertebral disc (IVD) degeneration, caused by degenerative disc disease, progresses faster than any other rate of connective tissue degeneration in the body.[1] With over 3 million US cases per year, IVD degeneration is a common disease that occurs due to wear-and-tear on the spinal disc.[2] In particular, it affects the nucleus pulposus (NP), a gelatinous center, which is surrounded by a tough collagenous exterior [annulus fibrosus (AF)] (Figure 1).

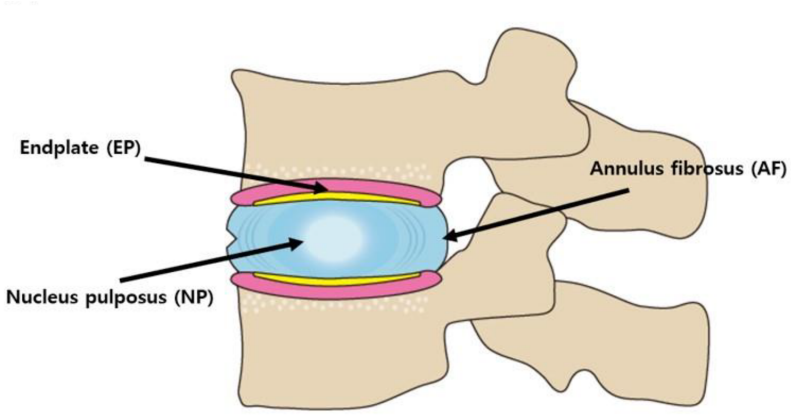

Figure 1. Cross-section diagram of intervertebral disc.[3]

As the NP degrades from shock absorption over time, it wears down, reducing the separation between the discs. This can cause compression of the spinal nerves, weakness in the arms and/or legs, bladder complications, and even impaired movement and nerve damage.[3] IVD degeneration has sparked the need for tissue engineering strategies to provide a viable alternative to current treatments, such as spinal fusion due to its recovery time of upwards of six months and permanent physical restrictions. A promising alternative treatment for IVD degeneration is a biomaterial scaffold.

Alginate hydrogels are common biomaterials in wound healing, drug delivery, and tissue engineering applications due to their ability to retain structural similarity to extracellular matrices (ECM) in tissues. Naturally found within brown seaweed in cold waters along the coast, alginate

is a polysaccharide polymer that assists cellular structure while being used as a vehicle to transport biological cues, such as hormones and neurotransmitters. Alginate has numerous applications in biomedical science and bioengineering, such as wound dressing and protein and cell delivery, due to its favorable properties, including biocompatibility and ease of gelation.[4] Hydrogels of any material are formed by using crosslinkers through cell crosslinking, free radical polymerization, phase transition, and ionic crosslinking. Crosslinking is the process by which microscopic alginates are molecularly linked together to form macromolecule structures. Ionic crosslinking, employed in this study, is commonly used because alginate is easily soluble in water or culture media and cations can form ionic bonds between alginate polymer strands. Previous studies demonstrated that calcium ions, Ca^{2+}, were the best cations and calcium chloride ($CaCl_2$) was the optimal source, as it readily dissociates in water.[5] In this study, calcium carbonate ($CaCO_3$) and glucono-δ-lactone (GDL, formula: $C_6H_{10}O_6$) were used as crosslinkers. $CaCO_3$ is a potential improvement to $CaCl_2$ during hydrogel synthesis as it functions as a highly accessible calcium ion substitute and dissociates when there is a presence of excess hydrogen atoms. GDL is a complementary dissociating agent for the calcium in alginate. It hydrolyzes to gluconic acid at a pH and temperature dependent rate, which is important for maintaining environmental pH during hydrogel formation.

Determining the optimal time before gelation occurs is critical to creating a useful NP replacement. In the context of clinical treatment, the hydrogel solution, while still in its liquid state prior to gelation, would first be injected into the spinal column and then form a hydrogel. There must be enough time prior to gelation in order for clinicians to have sufficient time to both mix and inject the solution; however, gelation time must be rapid enough so that patients do not have to wait a long time with the liquid solution in their spine, as well as ensuring that the

partially gelled solution does not become misplaced. The ideal gelation time, given this context, was determined to be 5 to 10 minutes.[4]

Prior research completed by scientists at Saint Louis University have looked into what creates the various highly adjustable properties of the polymer that allow for higher specificity to various tissues.[6] Based on their conclusions, we hypothesize that the 2% gel with 60:120 mM concentrations of $CaCO_3$:GDL will be the most viable solution for further studies with a gel time between 10 and 30 minutes. Factors such as pH, ionic versus covalent linkages, and temperature can change the physical and mechanical properties of the gel. The project sought to look into the optimized combination of cross-linking that best resembles what is required for patients by varying the concentration of cross-linker and testing the gels through various physical and mechanical tests in the scope of treating IVD degeneration with injectable alginate hydrogels.

2. Experimental Procedures

2.1.1 - Alginate Stock Preparation

Stock solutions of 1% and 2% weight to volume (w/v) alginates were made by combining alginic acid sodium salt powder and 1x phosphate buffered solution (PBS). The solutions were left to dissolve on a stir plate for approximately 6 hours. PBS (pH of 7.4) was used instead of deionized (DI) water (pH of roughly 6.2), to better mimic the pH of the human body (pH of 7.4). The alginate was kept in a 4 ℃ refrigerator when not in use so as to preserve the gel and remade weekly.

2.1.2 - Crosslinker Solution Preparation

Stock solutions of the crosslinkers were made by combining calcium carbonate and glucono-δ-lactone in the following molar ratios: 60:100, 60:120, 60:180, 80:160, and 120:120 (millimolar $CaCO_3$:millimolar GDL). The previous ratios were chosen as they provide many different ratios at many different concentrations in order to determine the relevant relationships between how much crosslinker is added and the effects on gelation, poresize, and strength. A 10% (w/v) solution of $CaCl_2$ was also prepared as a control.

2.1.3 - Hydrogel Preparation

Once the reagents were made, qualitative tests were initially performed to discover the best way in which to plate the gels in 24-well plates. Of the four attempted protocols, the best method was to first plate 1.5 mL of alginate and then pipette 1.5 mL of the desired crosslinker on top. The large differences in viscosity between the alginates and the crosslinker solutions allowed the solutions to rest nicely on top of the alginate and preserve a flat surface, while the

settling time allowed the crosslinker to fully permeate through the gel and allow for full gelation. Table 1 summarizes the qualitative tests run and Figure S1 in Appendix 6.1 (page 18) illustrates a completed gel from Procedure 4 below.

Table 1. Qualitative procedures and results from gel creation trials.

Procedure	Summary	Results
1	Addition of 1 mL crosslinker proceeded by 1 mL alginate into well plates	Gels maintained the same shape they came out of the pipette with (long, stringy, not flat)
2	Addition of 1 mL alginate proceeded by 1 mL of crosslinker, and subsequently stirred to allow for homogenous mixtures	Gels became amorphous blobs
3	Addition of 1 mL alginate proceeded by 1 mL of crosslinker	Gels flat but bottom of gel not crosslinked properly. Also, extra room remaining in the well plate
4	Addition of 1.5 mL alginate and then 1.5 mL crosslinker, but allowed to sit for 12 hours minimum	Gels flat, fully crosslinked, and filled the entire well!

All the gels were prepared using Procedure 4 from the table and then allowed to crosslink overnight in a 4 ℃ refrigerator.

2.2 - Gelation Time

Gelation time was determined by adding 1.5 mL of crosslinker solution to 1.5 mL of alginate in a 15 mL conical tube. The solution was briefly vortexed (2 seconds), then inverted every 15 seconds until one homogenous gel was visible or there was no change at the next inversion (implying that all crosslinker was consumed). This “inverted tube method” was validated in literature with parallel sonication methods, which precisely determined gelation time by analyzing phase composition over time.[7] Gelation time was originally planned with 30 second

inversions, but this data revealed few differences between samples; the shorter 15 second inversion time was adapted to better elucidate crosslinker impact on gelation time.

2.3 - Strain Testing

Samples were removed from the well plates and washed with DI water to remove excess crosslinker. Strain testing was performed on the instron shown in Table S1 of Appendix 6.2 (page 19), with a maximum load of 500 N and compression rate of 5 mm/minute. Three samples of each composition were placed in the instron, one sample at a time, and were intended to be compressed until failure; however, many of the compositions slipped out of the instron prior to failure due to a lack of support around the gel to prevent movement. The data was then analyzed by dividing the extension of the instron by the starting height of each sample (to find the percent change in height) and plotting against the force applied, giving the strain curve. Each of the three curves for each composition were modeled with a line of best fit and then averaged to find the model for that particular composition. The models were then plotted on one graph against each other.

2.4 - Lyophilization

In order to take scanning electron microscope (SEM) photographs of the samples to analyze pore size and swell testing, the samples had to be fully dried to remove all water from them and leave behind just the crosslinked alginate. To accomplish this, the gels were removed from the well plates and rinsed with DI water to remove excess crosslinkers and then frozen for an hour in a -80℃ freezer. After an hour, the samples were removed from the freezer and placed onto a lyophilizer (Labconco). The lyophilizer creates a vacuum around the samples at an

extremely low pressure which allows for any water in the sample (now solid from freezing) to sublime directly into vapor, bypassing the liquid layer. This allows the samples to maintain their structure.

2.5 - Swell Testing

Lyophilized samples were weighed to determine initial weight and then placed into 12 well plates and submerged in PBS (again PBS was chosen over DI water to better mimic the body's natural pH). Sample weights were then measured at the following intervals: 5, 10, 20, 30, 60, 90, 120, 300, and 1440 mins. The purpose of this was to determine water retention as it was hypothesized that faster crosslinking gels should be able to hold less water than slower crosslinking gels.

2.6 - Pore Analysis

Lyophilized samples were imaged using SEM to provide information on the gel microstructure. Samples were cut in half to take a cross sectional photo and were also sputter coated with gold in order to obtain a clearer picture. After the images were taken (Table S3 of Appendix 6.2.2, page 22), ImageJ was used to select 30 pores from each picture. The freehand tool was utilized in order to determine pore area. This was because the polygon tool did not capture the curves of the pores accurately while the diameter/line tool did not account for how oblong the pores could be. The average pore area and standard deviation of n=30 were then calculated, plotted, and compared.

3. Results & Discussion

Four tests were performed to determine the overall impact of crosslinker composition on alginate gel performance. Two of the tests - gelation time and swell testing - pertain to the storage and manufacturing of the gels while another two - pore size and compression tests - are implications of the biological impact of the material once inside a living organism. On a weighted scale from 1 (worst) to 5 (best) for each test, a collective score was summed for each crosslinker solution, further separated by percent alginate (Table 2). The gel containing 2% alginate with 60:180 mM CaCO3:GDL performed best in all tests except swell testing. As the optimal millimolar ratio varies for each test, the best expected performer and molecular *why* is discussed in each test subsection below.

Table 2. Compiled test results of various crosslinkers with 1% and 2% alginate solutions. 'Best' (5) was defined for each test as follows: gelation time - slowest, swell testing - greatest percent change, pore size - area greater than 0.1 mm, compression test - percent change in height with 70 N of force.

Crosslinker	Alginate	Gelation Time	Swell Testing	Pore Size (x2)	Compression Test (x2)	TOTAL	
10% $CaCl_2$	1%	2	1	5	1	15	11.5
	2%	1	1	1	2	8	
60:100 (mM $CaCO_3$:GDL)	1%	1	5	4	3	20	16.5
	2%	1	4	1	3	13	
60:120 (mM $CaCO_3$:GDL)	1%	3	4	4	1	17	16.5
	2%	2	4	2	3	16	
60:180 (mM $CaCO_3$:GDL)	1%	5	3	3	2	18	22.5
	2%	5	2	5	5	27	
80:160 (mM $CaCO_3$:GDL)	1%	3	3	5	2	20	20
	2%	3	3	2	5	20	
120:120 (mM $CaCO_3$:GDL)	1%	3	5	3	4	22	21
	2%	2	4	5	2	20	

3.1 - Gelation Time

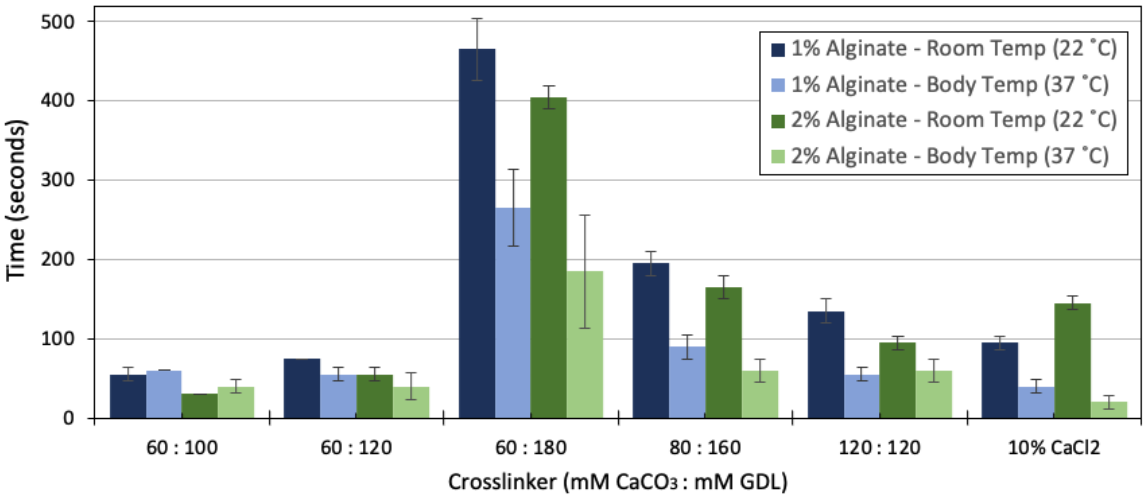

Figure 2. Gelation times for various crosslinker concentrations with 1% and 2% alginate at body temperature (37 ˚C) and room temperature (22 ˚C) (n = 3).

Gelation time was determined for each crosslinker with 1% and 2% alginate, at both room temperature (22 ˚C) and body temperature (37 ˚C), as shown in Figure 2. Significant differences were seen in samples containing the 60:180, 80:160, and 120:120 crosslinkers, in which 1% alginate gels took longer than 2% alginate gels to reach full gelation at room temperature. Interestingly, there was no trend between the 1% and 2% alginates in gelation time. However, most samples appear to have reached gelation time slower at room temperature. We hypothesize that increased energy of the body temperature solutions allowed calcium ions to homogenize into layers of alginate polymers quicker, which consequently led to shorter gelation times. The data did not support existing literature gelation times and our understanding of crosslinking because elevated concentrations of GDL should have promoted the dissociation of calcium throughout the alginate (lowering gelation time), but here elevated ratio of GDL slowed gelation. Differences from expected results may come from the method of crosslinker addition, tube type, inversion rate, or other factors. The 120:120 crosslinker should have had the slowest gelation time according to Kalaf et al. (2016), who suggested that equimolar concentrations of

$CaCO_3$:GDL take the longest to fully gelate. Error in these data may come from the visual determination of one homogenous gel, which can easily vary from researcher to researcher.

3.2 - Strain Testing

Compression and strain analyses were used to gauge the ability of injectable hydrogels to biomechanically benefit patients experiencing IVD. Three tests were performed for each hydrogel, which is represented well in Figure S2-K in Appendix 6.2.1 (page 21), illustrating three different trials for the 120:120 2% composition and their representative models of best fit. This particular composition cracked while in the instron, shown by the plateaus and/or decreased force at roughly 80% change in height. One trial of the 60:180 1% composition, all trials of the 120:120 1% composition, all trials of the $CaCl_2$ 1% composition, two trials of the 60:100 1% composition, and one trial of the 60:120 1% composition also cracked and therefore went to failure. These cracks make the strain curve less uniform and therefore the curve of best fit is less accurate. The R^2 values for the three models of the 120:120 2% composition were 0.9781, 0.9884, and 0.9760. However, as shown by Figure S2-G in Appendix 6.2.1, the models are extremely accurate for the compositions that did not crack. Figure S2-G shows three different trials for the 60:100 2% composition. This composition always slipped out of the instron prior to going to failure, and as the curve is incredibly smooth, it encourages an accurate model. The R^2 values for the three models of this composition were 0.9980, 0.9987, and 0.9985. Additional graphs for other compositions are also in Appendix 1. Figure 3 below illustrates the average model strain curve for each composition. Curves that require more force to reach a 100% change in height are significantly more tough and brittle than those who reach 100% change in height at a lower amount of force.

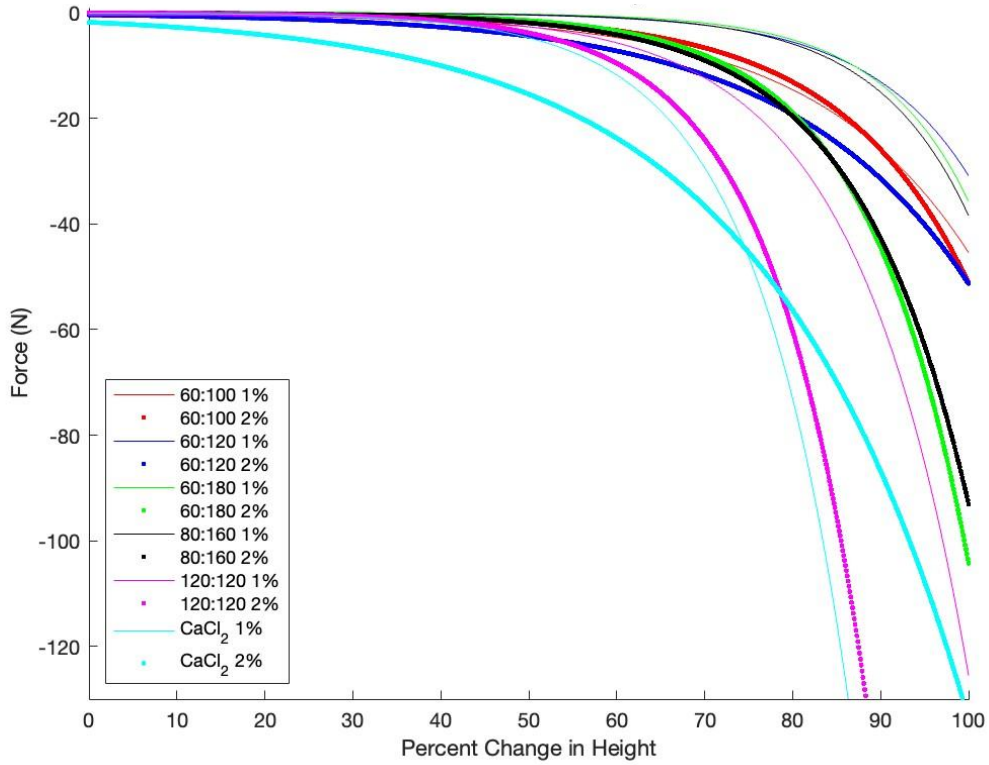

Figure 3. Modeled instron compression experiments of gels made from crosslinkers with 1% and 2% alginate (averaged line of best fit, n = 3).

For the purpose of creating a replacement or supplementary IVD, there is a trade off between the amount of strain a gel can handle prior to failure and the comfort of the patient. A primary purpose of IVDs is to cushion the spine and prevent the bones from rubbing against each other. Having a gel that can sustain more strain may not provide enough cushion and would create more pain. However, each gel must be able to withstand 70 N without failure as that is the amount of force placed on an IVD when a person is standing.[8] Therefore, the ideal composition would be one that reaches full compression (100% change in volume) slightly after 70 N, as it would have the most cushion while still being able to withstand daily activities. From our data, the 80:160 2% and 60:180 2% compositions are ideal for an IVD replacement or supplement. The equations for the models of the 80:160 2% and 60:180 2% compositions are

$$y = -0.03863 * e^{0.07787x} \text{ and } y = -0.02056 * e^{0.08531x}$$

respectively, where *y* is the force applied and *x* is the percent change in height. Additional R^2 values and model equations are in Appendix 6.2.1 (page 21).

3.3 - Swell Testing

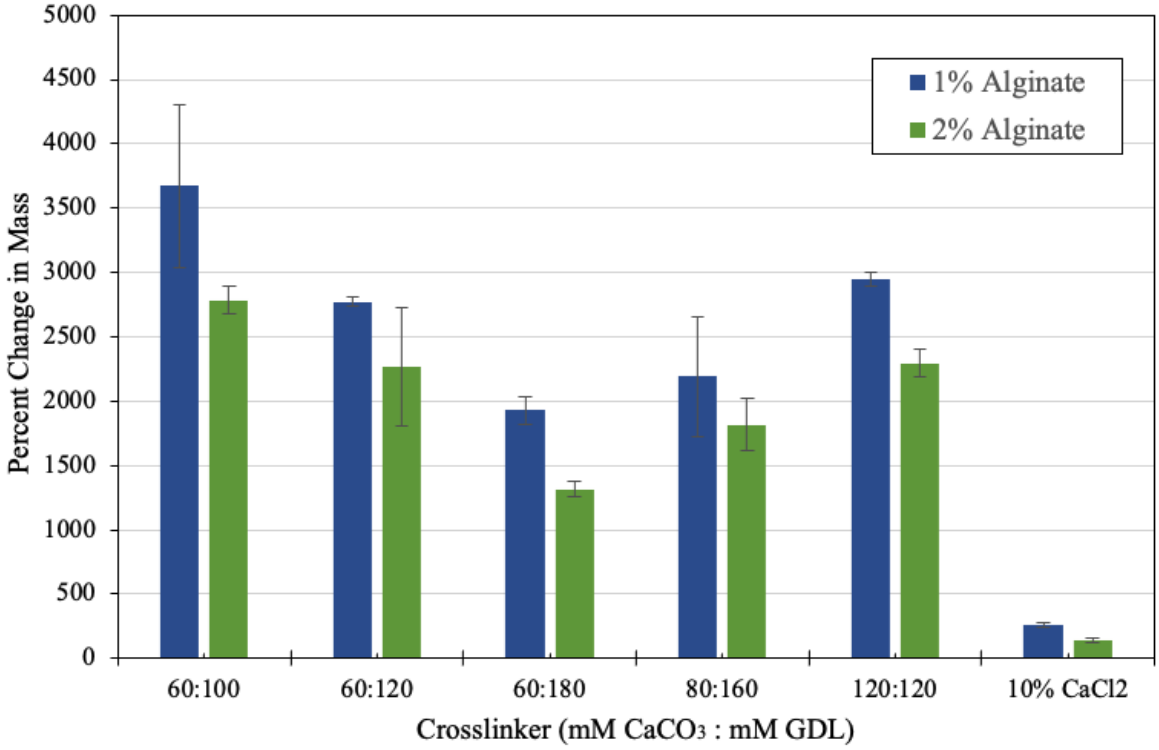

Figure 4. Plots showing the final average percent change for each hydrogel (mean ± SD, n = 3).

The swell testing data, shown above in Figure 4, illustrates differences in both crosslinker composition and ratio, as well as alginate percentage. A key finding, consistent with trends in hydrogel research[6-7], is that faster the gelation times corresponded with smaller percent change in mass. This is best demonstrated by the 10% $CaCl_2$ solution control, which had an extremely fast gelling alginate and, consequently, an extremely low percent change. This can also be seen in other samples as the data trends downwards (for both percentages of alginate) from 60:100 to 60:120 and finally to 60:180. In all three of these solutions, the concentration of $CaCO_3$ present was identical but the presence of more GDL resulted in more Ca^{2+} cations available for

crosslinking, resulting in decreased gelation time. Furthermore, 60:120 and 80:160 both have the same ratio of $CaCO_3$ to GDL, but 80:160 has a smaller average percent change for a similar reason: it has more available Ca^{2+} cations and thus gels faster. 120:120 presents an interesting challenge to this conclusion, however, as one would assume it to have the most available Ca^{2+} cations as it has the highest concentration of $CaCO_3$ to be dissociated. Nevertheless, it also represents the second highest average percent change in mass. Sources of error include excess volume (not part of the gelled sample) and disturbances during periodic measurements, as mass was measured multiple times between initial and final values.

To make a recommendation as to which solution would serve best for a replacement or supplementary IVD, there is no clear answer based solely on swell testing. While swell testing can inform researchers about the capabilities of each sample regarding retention (which is important if we want to deliver drugs or cells to the IVD), it cannot be used to conclude optimal crosslinker ratio or alginate percentage for patient benefits. There is a delicate trade-off between other tests and the amount of added volume hydrogels must retain.

3.4 - Pore Analysis

The rate at which the cross linkers gel also impacts the size of the pores. Figure 5 demonstrates that, on average, the 1% gels had larger pore sizes. According to previous studies, the 60:100 crosslinker solutions should have the smallest pore size and the 10% $CaCl_2$ should have the largest pore size[6]. The 10% $CaCl_2$ 1% gel had the largest pore area which is consistent with our hypothesis as it gels the most rapidly, leaving very little time for pores to develop. The average area size of the 10% $CaCl_2$ 2% gel was 0 mm, which was due to there being no pores in the gel, not because the pores were microscopic. The 60:100, 60:120, 60:180, and 120:120 2%

gels and the 60:100 and 80:160 1% gel followed trends of increasing pore sizes. However, the 60:120, 60:180, 120:120 1% gels and the 80:160 2% gel did not follow the increasing trend. This may be because pore volume is not being taken into account, which is an important consideration of actual pore size. When taken into account, pore volume would be another factor that corroborates the idea that variations in cross linker concentrations can indicate what kind and how many pores that would be observed in the gel. In addition to disturbances from lyophilization and gold sputtering, 1- and 2-dimension measurements of the 3-dimensional pore is a source of error in these analyses.

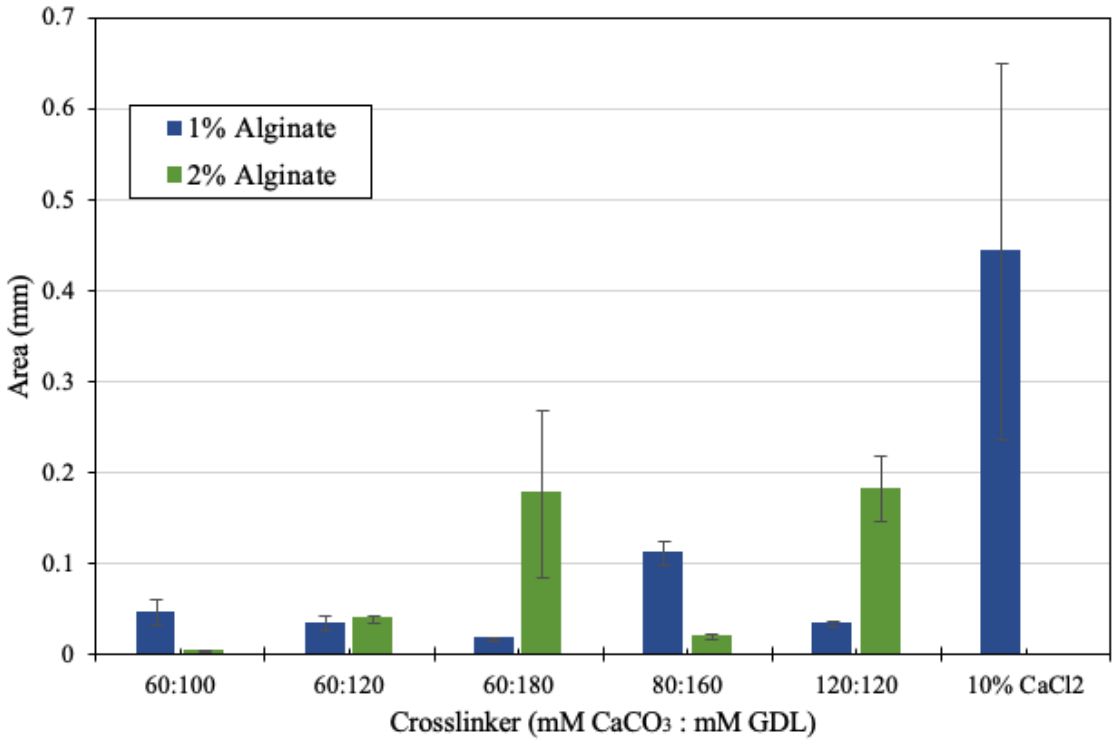

Figure 5. Average pore area of various cross linkers as well as standard error, determined using SEM imaging for 1% and 2% gels (mean ± SE, n = 30).

The standard deviation of mean pore area of the various cross linkers also shows a lot of variety. The 1% CaCl2 gel had the largest deviation in the average pore size, which indicates the presence of both large and small pores. The 60:100, 60:120, and 80:160 1% and 2% gels and the 60:180 and 120:120 1% gels have relatively small standard deviations, which point to more

consistent pore sizes. However, the 60:180 and 120:120 2% gels had relatively large standard deviations. These large standard deviations may represent inconsistent pore sizes. As well, these errors may be attributed to the sample size being only a portion of the pores present in the cross-sections as well as pores collapsing if too large. If there were more pores sampled, the standard deviations should decrease.

Hydrogel pore size is an important factor in cell adhesion, metabolism, migration, and proliferation. Smaller pores are favorable for cell attachment and intracellular signaling while larger pores are favorable for effective nutrient supply and oxygen diffusion.[9] Typical alginate gels have a pore size of around 5 nm as this nanoporous size allows for rapid diffusion of small molecules through the gel with 0.1 mm being the minimum standard for pore size.[10] Additionally, according to Kalaf et al. (2017), when determining which concentration proportion was ideal for the gel, pore sizes of roughly 0.165 mm were sufficient for cellular infiltration with pore diameters much larger for cellular migration and hydrogel integration.[11,*] According to the data, the 60:180 and 120:120 2% gels are the two gels that match the ideal pore size. Pore sizes are indicative of how much volume the gel can hold, so pores that are too small would not be beneficial in the context of hydrogels. As well, large pores that collapse are also not ideal hydrogels as collapsing pores indicates a lack of structural integrity. These pores are necessary for facilitating infiltration and cartilage growth. Another consideration when in the context of injectable hydrogel alternatives is the possibility of ice crystal formation if the gels were stored in the freezer, which could introduce pores in an alternative manner. Uniformity in pore sizes, which we did not achieve, is also important to consider as a desirable hydrogel has more uniform pore sizes. Ultimately, the pore size of the gel was dependent on the concentration ratio of crosslinkers.

* Though Thorvaldsson et al. measured pore area, and area usually has units of ‘units^2’, their analysis referred to area as “pore size", measured it in ‘μm’ rather than ‘μm^2’. Therefore, in our analysis, pore area is in units of ‘mm’ rather than ‘mm^2’. (Confirmed by Professor Hixon, most hydrogel literature uses such nomenclature.)

4. Conclusion

Alginate is a polymer that can be fine tuned to exhibit properties that mimic that of native NP tissue. This study aimed to provide further physical and mechanical characterization of alginate, exploring factors such as gelation time and crosslinking molar ratios on pore size and strain properties. These qualities can affect short and long term degradation, structural stability, and hydration. Understanding these properties and how to achieve them can assist in creating an injectable alginate that seeks to restore intervertebral disc function, allow cellularization, and regenerate disc tissue. This study found that the 2% gel with 60:180 (mM $CaCO_3$: mM GDL) crosslinker concentrations was the most suitable alginate hydrogel to be used as an injectable IVD.

Future studies should perform more biologically-relevant tests on degradation time, rheology, and cell compatibility. Another physical characterization test of the hydrogel that should be performed is pore volume analysis. In combination with pore size, this could lead to a better understanding of the relationship between pores and gelling rates. In addition, changing the preparation of the gels can also give more sample variety. As well, examining more crosslinking solutions can help understand what concentrations would be favorable.

5. Bibliography

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6. Appendix

6.1 - Experimental Procedures

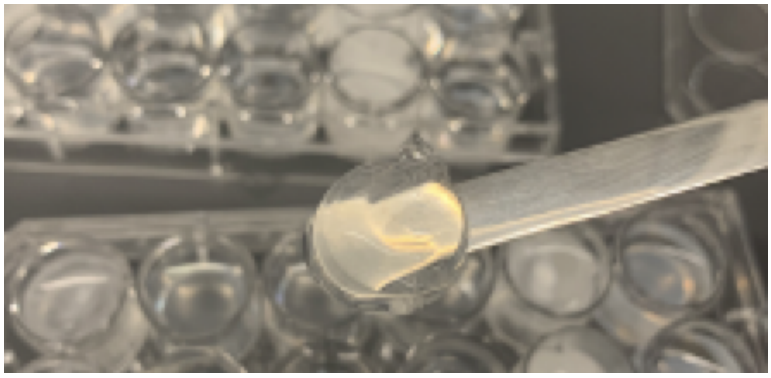
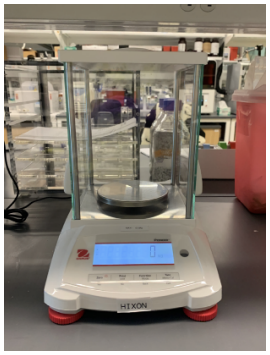


Figure S1. Completely crosslinked gel following Procedure 4 of Table 1.

Table S1. Equipment description table for all experimental procedures.

Instrument	**Photo**	**Manufacturer/Model /Serial Number**	**Notes**
Scanning Electron Microscope (SEM)		Tescan Vega 3 (With Dr. Cullen - no S/N)	Used to image the pore sizes of the alginate hydrogel
Gold Sputtering System		Anatech LTD Hummer 6.2 Sputtering System Dartmouth Asset #: 250642	Used to gold-coat lyophilized gels prior to SEM imaging

Instron Machine		Mechanical Test Frame-Small Instron Model: 4442 System ID #: 4442P1017	Use to measure how hydrogel responds to applied forces by going to failure or maximum compression
Lyophilizer		Labconco Model: FreeZone 2.5 L S/N: 220531842E	Used to the remove water from the alginate before putting it under the SEM
Scale		Pioneer Precision Model: PX523/E S/N: C151200871	Used to measure mass of reagents for creation of stock solutions

6.2.1 - Results & Discussion: Instron Data

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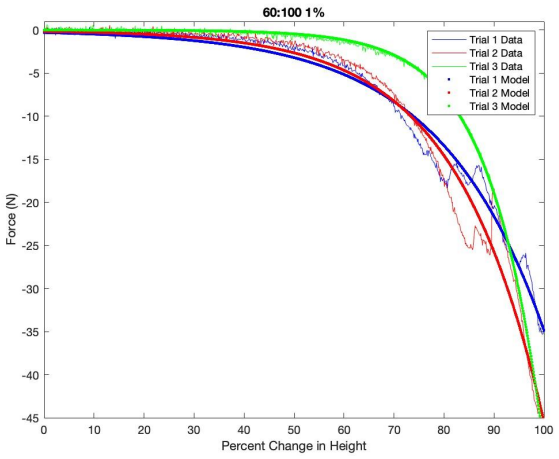

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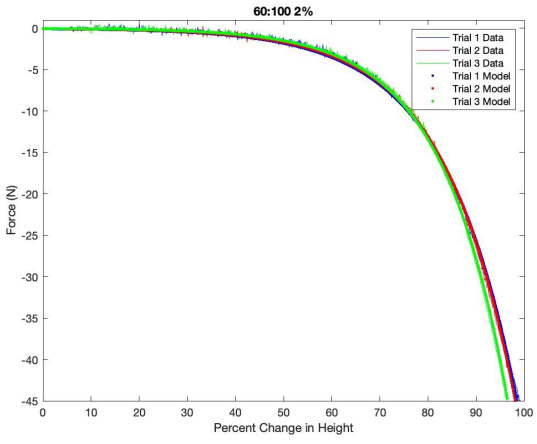
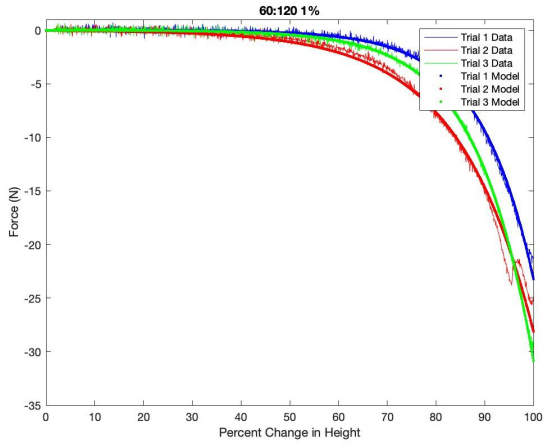
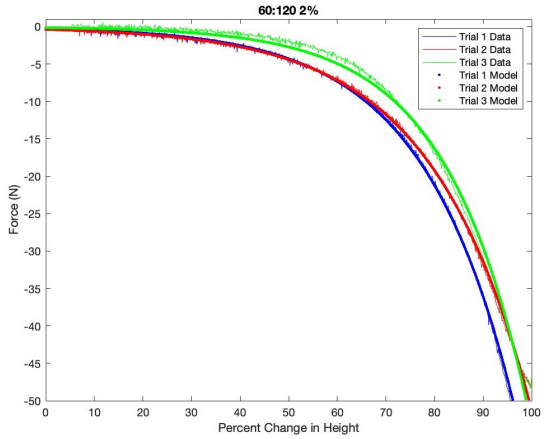


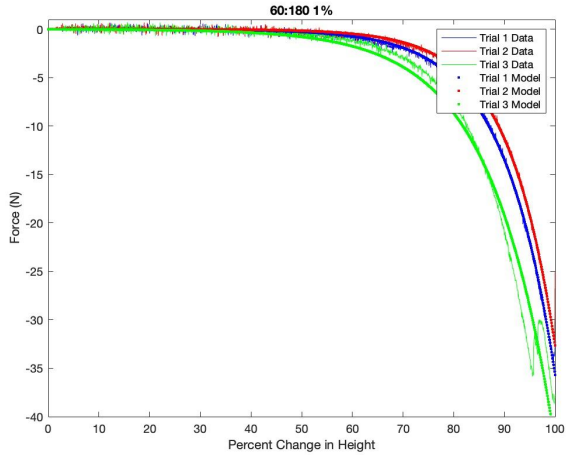
B


60:120 1%
Trial 1 Data
Trial 2 Data
Trial 3 Data
Trial 1 Model
Trial 2 Model
Trial 3 Model
Force (N)
Percent Change in Height

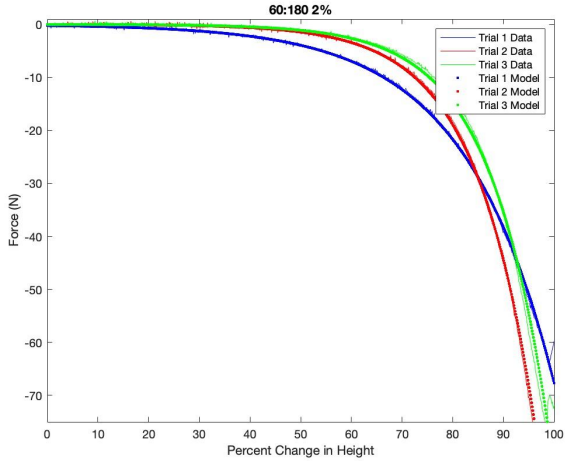
H


60:120 2%
Trial 1 Data
Trial 2 Data
Trial 3 Data
Trial 1 Model
Trial 2 Model
Trial 3 Model
Force (N)
Percent Change in Height

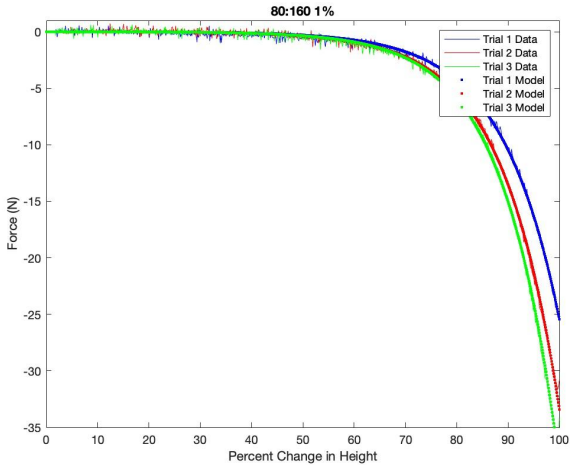
C


60:180 1%
Trial 1 Data
Trial 2 Data
Trial 3 Data
Trial 1 Model
Trial 2 Model
Trial 3 Model
Force (N)
Percent Change in Height

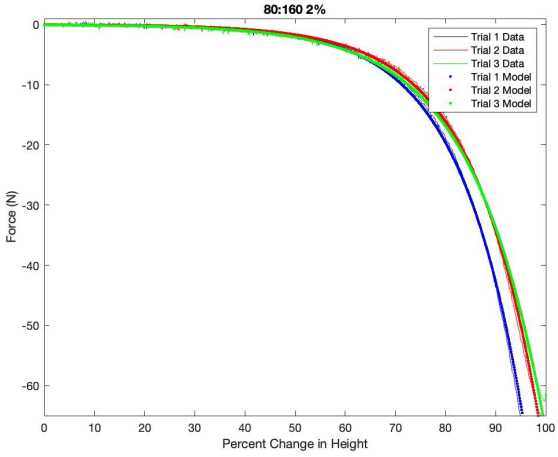
I


60:180 2%
Trial 1 Data
Trial 2 Data
Trial 3 Data
Trial 1 Model
Trial 2 Model
Trial 3 Model
Force (N)
Percent Change in Height

D


80:160 1%
Trial 1 Data
Trial 2 Data
Trial 3 Data
Trial 1 Model
Trial 2 Model
Trial 3 Model
Force (N)
Percent Change in Height

J


80:160 2%
Trial 1 Data
Trial 2 Data
Trial 3 Data
Trial 1 Model
Trial 2 Model
Trial 3 Model
Force (N)
Percent Change in Height

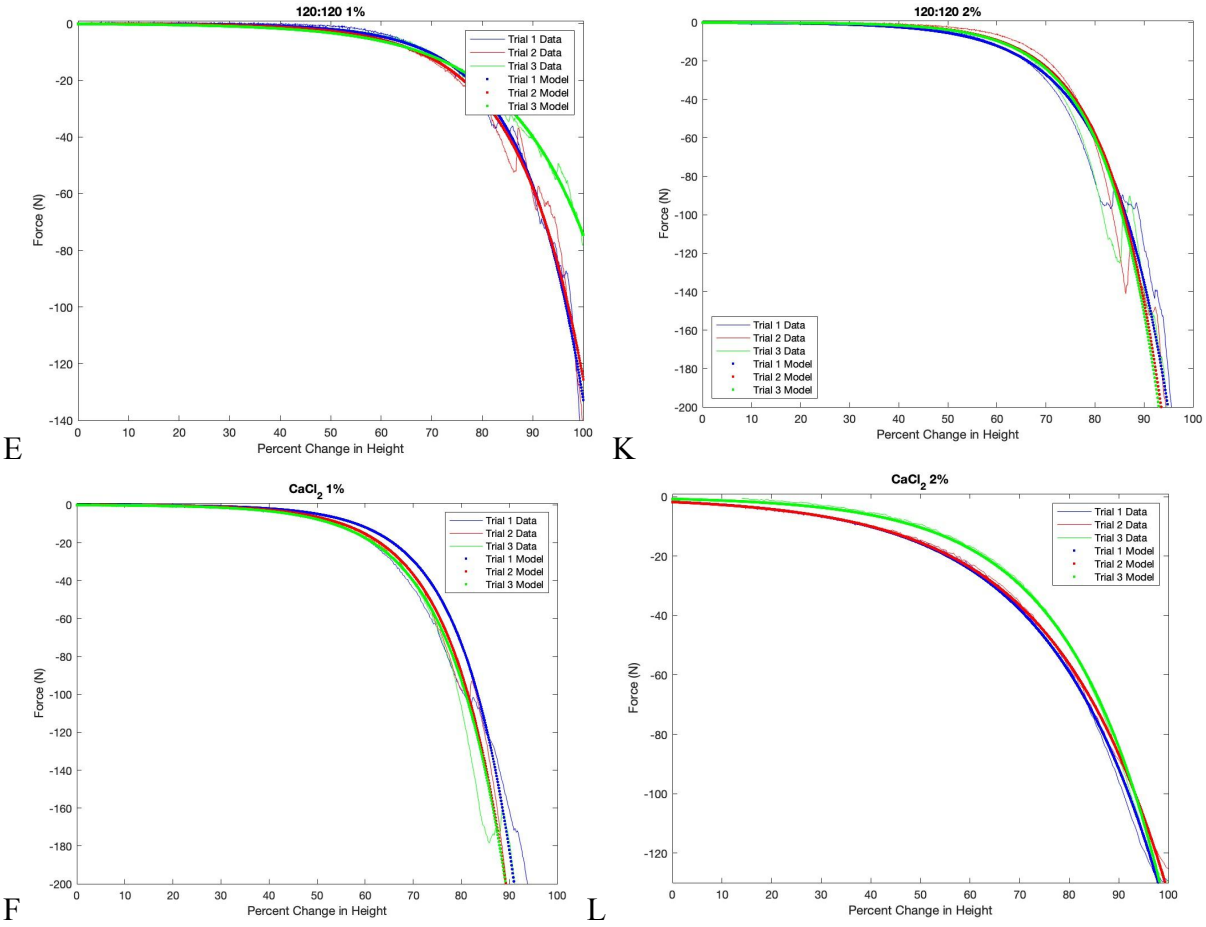


Figure S2. Compiled and modeled Instron compression experiments of gels made from crosslinkers with 1% (A-F) and 2% (G-L) alginate.

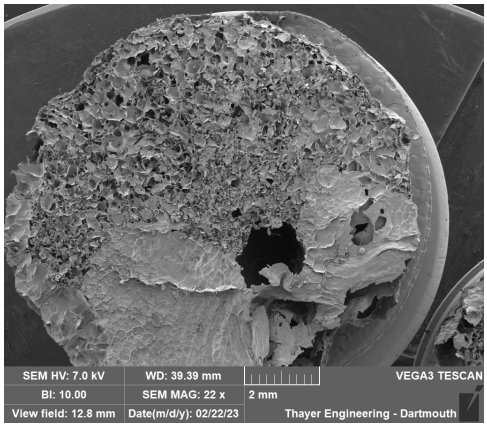
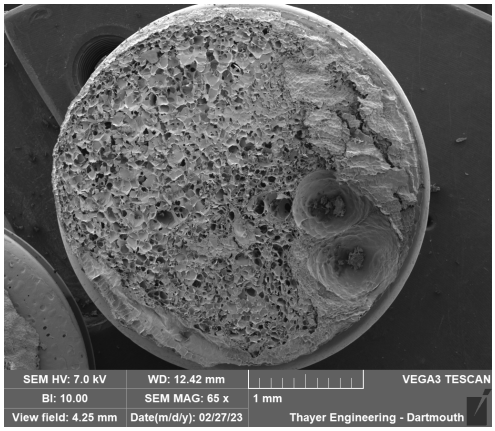
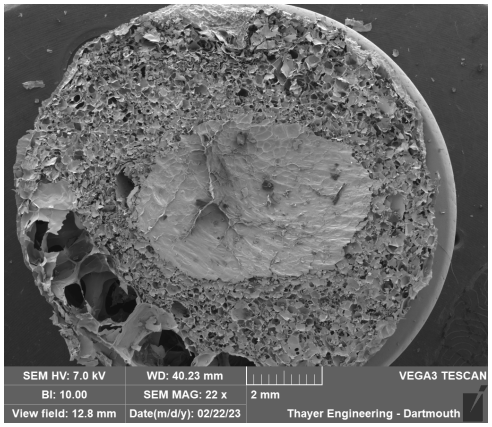
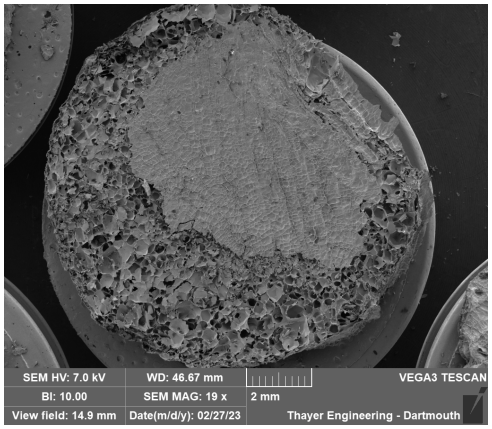
Table S2. Curve analyses used for the models of best fit illustrated in Figure S2.

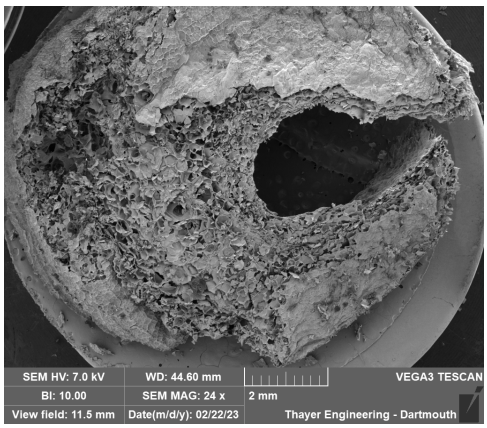
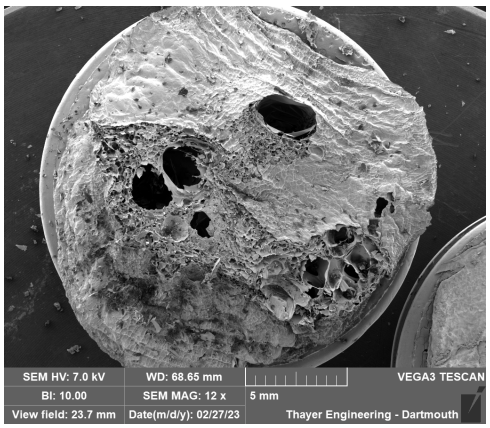
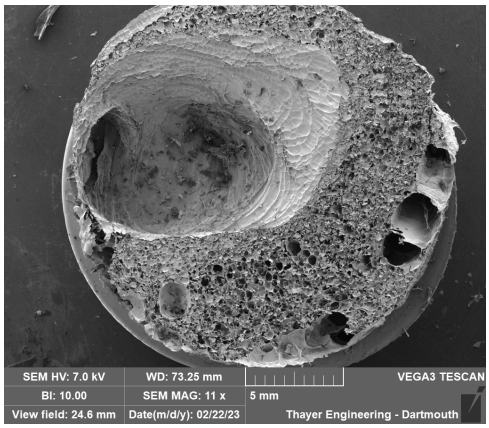
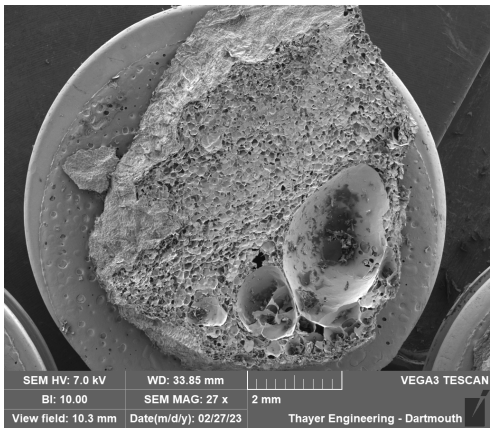
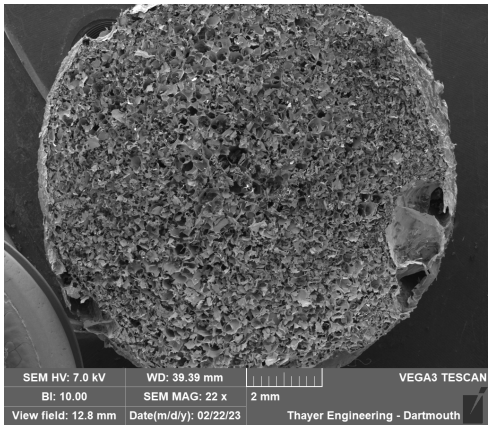
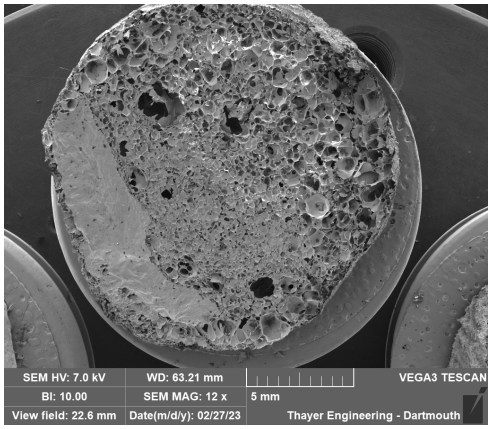
Curve Analysis for Instron Models					
Composition	R^2 Trial 1	R^2 Trial 2	R^2 Trial 3	Average R^2	Model Equation
60:100 1%	0.9790	0.9714	0.9992	0.9832	y = -0.1539(e^(0.05688x))
60:100 2%	0.9980	0.9987	0.9985	0.9984	y = -0.05591(e^0.0682x))
60:120 1%	0.9964	0.9915	0.9987	0.9955	y = -0.005676(e^(0.08601x))
60:120 2%	0.9989	0.9988	0.9954	0.9977	y = -0.3799(e^(0.04905x))
60:180 1%	0.9986	0.9977	0.9818	0.9927	y = -0.002105(e^(0.09738x))
60:180 2%	0.9987	0.9985	0.9937	0.9970	y = -0.02056(e^(0.8531x))
80:160 1%	0.9977	0.9984	0.9983	0.9982	y = -0.00324(e^(0.0938x))
80:160 2%	0.9984	0.9978	0.9984	0.9982	y = -0.03863(e^(0.07787x))
120:120 1%	0.9898	0.9864	0.9912	0.9891	y = -0.05453(e^(0.7742x))
120:120 2%	0.9781	0.9884	0.9760	0.9808	y = -0.0381(e^(0.09211x))

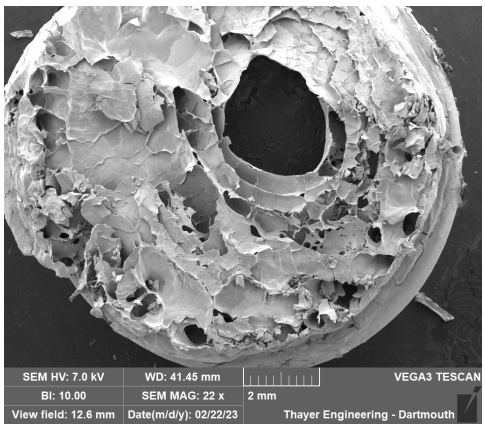
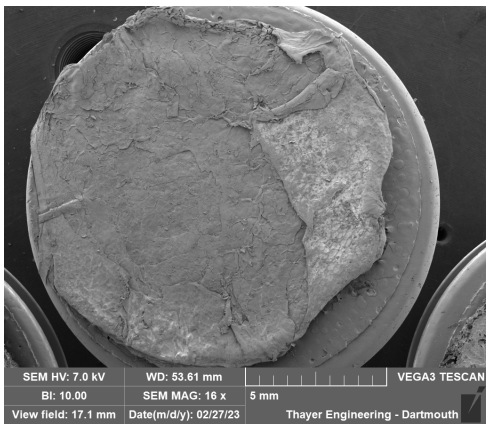
CaCl2 1%	0.9713	0.9978	0.9895	0.9862	y = -0.04922(e^(0.09129x))
CaCl2 2%	0.9969	0.9983	0.9972	0.9974	y = -1.775(e^(0.04322x))

6.2.2 - Results & Discussion: SEM Images

Table S3. SEM Images of all gels. Note that the given magnifications are not representative of the displayed images.

Crosslinker	**Image**	
	1% Gel	**2% Gel**
60:100		
60:120		

60:180		
80:160		
120:120		

10% CaCl2	 SEM HV: 7.0 kV WD: 41.45 mm VEGA3 TESCAN BI: 10.00 SEM MAG: 22 x 2 mm View field: 12.6 mm Date(m/d/y): 02/22/23 Thayer Engineering - Dartmouth	 SEM HV: 7.0 kV WD: 53.61 mm VEGA3 TESCAN BI: 10.00 SEM MAG: 16 x 5 mm View field: 17.1 mm Date(m/d/y): 02/27/23 Thayer Engineering - Dartmouth