

Physical Modification of Hybrid Hydrogels to Fabricate Full-Scale Construct Using 3D Bio-Printing Process

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ABSTRACT

Bioprinting for regenerative medicine has been gaining a lot of popularity in today's world. Despite being one of the rigorously studied fields, there are still several challenges yet to be solved. Geometric fidelity and mechanical complexities stand as roadblocks when it comes to the printability of the customized constructs. Exploring the rheological properties of the compositions helps us understand the physical and mechanical properties of the biomaterials which are closely tied to the printability of the filament and eventually, geometric fidelity of the constructs. To ensure the structural integrity of the constructs, viscosity enhancers such as Carboxymethyl Cellulose (CMC) and crosslinkers like CaCl_2 and CaSO_4 were used. These crosslinkers

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can be used before (pre-crosslinking) and after (post-crosslinking) the extrusion of considered compositions to investigate and compare the outcome. To do this, mixtures of Carboxymethyl Cellulose (CMC, viscosity enhancer), Alginate, and CaCl_2 and CaSO_4 (crosslinkers) were prepared at various concentrations maintaining minimum solid content ($\leq 8\%$). Each composition was subjected to a set of rheological tests like flow curve for shear thinning behavior, three points thixotropic for recovery rate, and amplitude test for gelation point. Various geometric fidelity identification tests were conducted and correlated with their physical properties. Some compositions were used to fabricate large-scale constructs (in cm-scale) to demonstrate their capability. This research is a thorough investigation of compositions when they are introduced to crosslinkers and viscosity enhancers which can be crucial for the 3D printing world.

Keywords: 3D bioprinting, rheological analysis, pre-crosslinking, hybrid hydrogel.

INTRODUCTION

The field of tissue engineering and regenerative medicine (TERM) has been growing exponentially in recent years due to 3D bio-printing and compatible biomaterials. 3D bioprinting is a revolutionary technology for fabricating functional tissue, and its growing capabilities along with the increasing availability of compatible materials have helped with its rapid expanse [1]. Spatial control and repeatability of material deposition are crucial for attaining the design of specific 3D tissue scaffolds. There are various 3D bio-printing processes such as extrusion-based [2-4], inkjet [5, 6], and laser-assisted [7, 8] bioprinting that achieves these requirements, but the extrusion-based bioprinting process has gained comparatively more attraction among them because it is the capability of printing a diverse range of materials while allowing higher cell density [9]. Highly porous 3D constructs that serve as temporary structural support for the growth of isolated cells are often fabricated using naturally growing polymeric materials and hydrogels [10]. Although hydrogels have many benefits such as high water content, biocompatibility, and biodegradability, [11-13], one of the biggest challenges with hydrogels is achieving controlled spatiality of the fabricated 3D scaffold [14]. To achieve the desired properties of a particular bio-ink and final structure, different hydrogel materials are mixed to prepare a hybrid hydrogel [15, 16]. Yield strength is one example of a mechanical property that must be correct to maintain the dimensional accuracy of the scaffold after the material is extruded from the nozzle [17]. Methods of controlling the yield strength of hydrogel materials include using viscosity modifiers [18-20], changing temperature [21-23], using an external cross-linker [24, 25], incorporating

sacrificial materials [26, 27], and controlling intrinsic rheological properties of the hydrogels [28, 29]. Researchers are using all of these methods to modify the yield strength of hydrogels, but some are more beneficial than others. Unfortunately, increasing the yield strength conflicts with another crucial aspect of tissue fabrication. The bio-ink must act as a comfortable habitat for encapsulated cells before, during, and after extrusion. While increasing the yield strength of material gives it the necessary rheological properties to maintain its post-printing shape fidelity, it also creates a material that requires more pressure to extrude, which creates a harmful environment for the encapsulated cells during extrusion [30, 31]. Increasing the solid content of a hydrogel can increase many different rheological properties that are beneficial for its strength and structural integrity, but this increase does not always guarantee better geometric fidelity [29, 32]. The higher percentage of solid content can also create barriers for the gas and nutrient flows vital for the health of the cells [33, 34]. When using sacrificial support materials, it is challenging to completely and safely remove them during the incubation period [35]. Therefore, we hypothesized that pre-crosslinking a hybrid hydrogel containing less solid content ($\leq 8\%$ w/v) [36-39] would achieve the proper rheological and mechanical characteristics needed for the construct, while simultaneously eliminating the negative effects that higher amounts of solid content have on the cell.

Various bi- and trivalent cations such as Ca^{2+} [25, 40], Ba^{2+} [41], Ga^{3+} [42], and Al^{3+} [43] have been used as physical crosslinkers. Among them, CaCl_2 and CaSO_4 are the most common [44]. Pre-crosslinking hydrogels is an approach in which the physical crosslinkers are included when mixing the hydrogel materials before the printing process [45]. Pre-

crosslinking hydrogels is an approach in which the physical crosslinkers are included when mixing the hydrogel materials before the printing process [45]. It is reported that pre-crosslinked hydrogels show a rapid shear-thinning self-healing behavior. It has also been found that pre-crosslinked gel-phase bio-ink significantly reduces cell variability (<5%) compared with its plain alginate counterpart (20-40%) [46]. In [45], alginate was pre-crosslinked with 0.55% (w/v) CaCl_2 and 0.68% (w/v) CaSO_4 respectively without any additional initiator such as glucono- δ -lactone (GDL). Pre-crosslinked hydrogels showed lower shape fidelity than post-crosslinked hydrogels with similar rheological properties. In another work, 2% alginate was pre-crosslinked with various percentages of CaCO_3 , and a building height up to 5.0 mm was reported [35]. To de measurable rheological parameters and corresponding shape fidelity of fabricated constructs. Various amounts of alginate and graphene oxide were pre-crosslinked with CaCl_2 [47]. Alginate and alginate-hydroxy apatite (HA) were pre-crosslinked with 1% CaSO_4 and pre-osteoblast cells were encapsulated to compare with chitosan and chitosan-HA [48]. The results showed that Chitosan-HA has a better bone-forming capability over alginate-HA. However, the analysis of printability and shape fidelity has not been compared and reported here. Various percentages such as 0.2% and 0.4% of CaSO_4 were used to pre-crosslink 2% alginate and 2.5% of HA with an intention to increase viscosity and consequently the shape fidelity of the constructs [49]. In another research, 0.001M CaSO_4 was utilized with 2.4% alginate to prepare a final solution of 1.2% alginate to analyze the rheological modification [50].

CaCl_2 and CaSO_4 have different behaviors regarding printability and structural integrity even though they are both physical crosslinkers that show linear viscosity in rheological tests. At a molecular level, CaCl_2 is readily soluble in water which often leads to an uncontrolled release of its Ca^{2+} ion and therefore results in heterogeneous crosslinking. The heterogeneous crosslinking creates a clumpy biomaterial that requires higher printing pressures to avoid clogging. This increase in pressure and buildup of clogs can be fatal for the cells during extrusion. It also causes scaffolds to be ununiform. Sulfate salts on the other hand exhibit more uniform crosslinking over some time due to a lower solubility. For this reason, CaSO_4 is readily printable at lower extrusion pressures and easier to handle than CaCl_2 . CaSO_4 also has better mechanical properties because it has a higher young's modulus and equilibrium modulus. As a result, compositions crosslinked with CaSO_4 exhibit better structural integrity after printing than those crosslinked with CaCl_2 .

In this paper, we used both viscosity enhancer and pre-crosslinkers (CaCl_2 and CaSO_4) to ensure better shape fidelity of hybrid hydrogels, improve mechanical properties and achieve a full-scale (in cm-scale) printed construct. We used alginate and Carboxymethyl Cellulose (CMC) maintaining $\leq 8\%$ solid content as the base hydrogel materials. As pre-crosslinkers, CaCl_2 and CaSO_4 were used. The effects of the viscosity enhancer and pre-crosslinker were characterized by a set of tests including flow analysis and geometric fidelity tests. Finally, two candidate materials were used to fabricate a large-scale freeform construct. Further, some compositions utilize lower viscosity materials in place of or alongside higher viscosity materials to provide extra crosslinking

without the unnecessary increase in viscosity. This gives the cells a more porous and friendly environment to increase proliferation and allows for lower print pressures because the crosslinking bonds can break during extrusion and reform to provide structure post-printing.

MATERIALS AND METHODS

Preparing materials

A set of hybrid hydrogels were prepared using alginate and carboxymethyl cellulose and all the compositions were pre-crosslinked with CaCl_2 and CaSO_4 respectively. As candidate material, medium (viscosity ≥ 2000 cps of 2% in water) viscous Alginate (A) (alginic acid sodium salt from brown algae) and carboxymethyl cellulose (CMC) (pH: 6.80) (Sigma-Aldrich, St. Louis, MO, USA) were used. Alginate is the most common biopolymer, composed of (1-4)-linked β -Dmannuronic (M) and α -Lguluronic acids (G). Carboxymethylcellulose (CMC) is an anionic water-soluble biopolymer that is composed of β -D-glucose and β -D-glucopyranose-2-O-(carboxymethyl)-mono-sodium salt. Both of them are connected via β -1,4-glucosidic bonds [51]. Five hybrid hydrogels composed of various weight percentages of alginate and CMC were pre-crosslinked separately with 0.5% (w/v) calcium chloride (CaCl_2 , CL) and 0.5% (w/v) calcium sulfate (CaSO_4 , CS) (Sigma-Aldrich, St. Louis, MO, USA) which resulted total ten compositions in this paper. The solutions were prepared by mixing the specified amount of alginate, CMC, and pre-crosslinkers (either CL or CS) overnight with an electric stirrer to confirm the uniform mix and crosslinks. It is reported that CaSO_4 can help cross-link gradually with lower percentage [50]. We maintained a constant 8% (w/v) solid content of alginate and

CMC as shown in Table 1. Food colors are used to differentiate the compositions as shown in Table 1.

Table 1: Various compositions prepared with different weight percentages of alginate, CMC, CaCl_2 , and CaSO_4 . process parameters used.

$A_x C_y$		Pre-crosslinked		Process parameters
x	y	0.5% CL	0.5% CS	
8	0			Nozzle dia 0.84mm, print speed 20mm/s, pressure 29-45 psi
6	2			
4	4			
2	6			
0	8			

Rheological properties

Rheological tests were done with a rotational rheometer (MCR 102, Anton Paar, Graz, Austria) having a parallel plate geometry (25.0 mm flat plate) setup. The plate-to-plate gap was maintained at 1.0 mm, and all data were recorded at room temperature (25°C) having the intention that the extrusion process will be performed at room temperature. Room temperature can facilitate the quick gelation of the deposited filament [30]. Hydrogels were prepared as explained in section 2.1 and placed on the Peltier module. A set of rheological analyses such as shear thinning behavior with flow curve, recovery rate with three-point interval thixotropic test (3ITT), and gelation points with amplitude test were conducted. For flow curve analysis, a steady rate sweep test was conducted having a variable shear strain from 0.1 to 100 s^{-1} . Three different time intervals such as 60, 5, and 120 seconds were used at three different shear strains as 1s⁻

¹, 100s⁻¹, and 1s⁻¹ respectively. Amplitude sweep test was conducted at a constant 1 Hz frequency with a variable shear strain from 0.01% to 100% to determine the storage (G') and loss modulus (G'').

To analyze the shear-thinning behavior of considered compositions, the Power-Law Equation (Equation 1) was fitted to the linear region of the shear strain rate vs viscosity curve [52], Then the shear-thinning coefficients of n and K were determined using the following equation:

$$\eta = K\dot{\gamma}^{n-1} \quad (1)$$

Where η is the viscosity $\dot{\gamma}$ is the shear rate. While the material is extruded through the nozzle, shear stress occurs throughout the material and is larger along the nozzle wall.

3D printing

An extrusion-based 3D printer, BioX (CELLINK, Boston, MA), was used to extrude hydrogel layer-upon-layer and fabricate constructs. After preparing the pre-crosslinked hybrid hydrogels, they were loaded into 3.0 ml disposable syringes and extruded pneumatically on a stationary build plane. Various printing parameters shown in Table 1 can control the deposition rate of the material. Rhino 6.0 (<https://www.rhino3d.com>), a Computer-Aided Design (CAD) software was used to design and define the vectorized toolpath of a construct. Slicer (<https://www.slicer.org>), a G-code generator software was used to generate a 3D printer compatible file including the toolpath coordinates and all process parameters to construct the construct. The partial physical cross-link of the fabricated construct after the print was confirmed by a spray of 4% (w/v) CaCl₂. The overall 3D bioprinting process is schematically shown in Figure 1. The process parameters

used in this paper are nozzle diameter 0.84mm, print speed 20mm/s, bed temperature equal to room temperature, layer height 0.4mm, and infill density 50% unless and otherwise stated. However, during the usage of six sample compositions to fabricate a freeform kidney model, various process parameters needed to use as shown in Table 2.

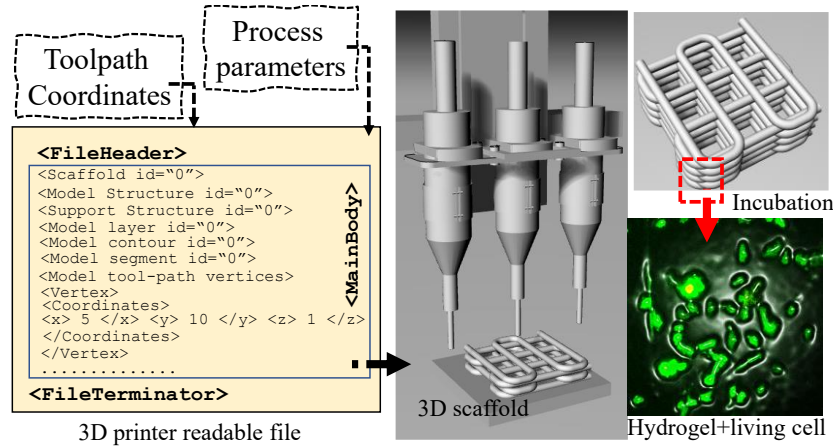


Figure 1: An overview of the 3d bioprinting process.

Table 2: Process parameters used to fabricate kidney model shown in Figure 15.

	Pre-crosslinked with CaSO_4			Pre-crosslinked with CaCl_2		
	A_2C_6	A_4C_4	A_6C_2	A_2C_6	A_4C_4	A_6C_2
Speed	30	30	25	40	40	35
Pressure	18	22	26	20	26	34
% Infill	20	20	20	20	20	20
Layer height	0.58	0.58	0.58	0.58	0.58	0.58

3D printability

The extruded filament should demonstrate a consistent surface and constant width which will subsequently create regular grids, square holes, and proper layer height. However, a hydrogel with a more liquid-like state may fuse the released filament, create

circular holes for bi-layer geometry, and eventually close the pore. Therefore, the printability (Pr) [21] of the hydrogel is defined using the following Equation:

$$P_r = \frac{\pi}{4} \frac{1}{C} = \frac{L^2}{16A_a} \quad (2)$$

Where, L and A_a are the perimeters and actual area of the pore. The under gelation (Pr < 1), ideal gelation (Pr = 1), and over gelation (Pr > 1) can be defined using the Pr value. To determine the printability, various constructs having 2.5 mm raster width (filament to filament distance) were fabricated with a zig-zag pattern using all compositions. The objective of determining the printability is to identify the shape-holding capacity of the deposited material. Pore closure or the diffusion rate was determined using the following equation:

$$Diffusion\ rate = \frac{Change\ of\ pore\ area}{Theoretical\ pore\ area} \times 100 \quad (3)$$

Pore images were captured using the CK Olympus bright field microscope (Tokyo, Japan) and analyzed using ImageJ software.

Scanning electronic microscope

The microstructures of thin films generated by various compositions were analyzed by scanning electron microscopy (Tescan Lyra 3 GMU, Brno, Czech Republic). Samples were focused on the surface and cross-sections of the films. The accelerating voltage, spot size (SS), and working distance (WD) used in this imaging are 3kV, 5.73x10⁻⁹ and 3.6 mm respectively. All samples were coated with 15nm Au/Pd using an Anatech Hummer V sputter coater (Sparks, NV, USA). Samples were mounted on ½ inch diameter pin-type stubs and mounting cubes.

Statistical analysis

All data were presented in the format of “mean $\pm$ standard deviation”. Statistical significance was evaluated at a significance level of $p = 0.05$ with a two-way ANOVA. All calculations were done with $n=3$ unless otherwise stated. Statistical software Origin Pro 2021b (Northampton, MA, USA) was used to do quantitative and graphical analysis.

RESULTS AND DISCUSSIONS

Shear-thinning behavior

Shear-thinning behavior is defined by a scenario where viscosity shows a decreasing trend with increasing the shear strain rate on hydrogels. This behavior is critical for the extrusion-based bioprinting process [20]. Characteristically, fluid flow through a nozzle experiences shear stress. The amount of shear stress experienced by the flowing fluid depends on its viscosity. Therefore, high viscous hydrogel experiences more shear stress during extrusion through a nozzle. Encapsulated living cells into those hydrogel materials can adversely be affected due to high shear stress [53]. Since shear-thinning behavior confirms the reduction of viscosity during extrusion, it can help create a protective environment for encapsulated cells in the extrusion-based 3D bioprinting technique.

Figures 2(a) and 2(b) show the log-log plot of viscosity concerning the shear strain of considered compositions pre-crosslinked with CaCl_2 and CaSO_4 respectively. For both pre-crosslinkers, almost all compositions showed a sequence of viscosity increase with increasing the percentage of alginate. Therefore, A_8C_0 pre-crosslinked with CaCl_2 showed 47%, 48%, 57%, and 66% more viscosity with respect to A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 . And A_8C_0 pre-crosslinked with CaSO_4 showed 61%, 68%, 77%, and 74% increase in viscosity

compared to A₆C₂, A₄C₄, A₂C₆, and A₀C₈, respectively. Alginate is a negatively charged linear copolymer (M and G blocks) and because of its carboxylate ion (-COO⁻), it is soluble in the water. The G-block of this material creates bonds to form gels and GM and M blocks increase the flexibility. More number of carboxylate ions (-COO⁻) facilitates the attraction with Ca²⁺ ions, creates physical bonding, and results in a higher rate of cross-linking.

All the compositions demonstrated the reduction of viscosity with increasing the shear rate. This phenomenon confirms the presence of shear-thinning behavior in those compositions. However, compositions pre-crosslinked with CaCl₂ showed more viscosity at each shear strain compared to the compositions pre-crosslinked with CaSO₄. For an example, A₈C₀, A₆C₂, A₄C₄, A₂C₆, and A₀C₈ compositions pre-crosslinked with 0.5% CaCl₂ showed 1.7%, 35.62%, 57%, 99.63%, and 30% more viscosity at shear rate 1.0 s⁻¹ compared to those compositions pre-crosslinked with CaSO₄. A close look at the molecular level of CaCl₂ will show it is readily soluble in water which often leads to an uncontrolled release of Ca²⁺ ions and therefore results in more crosslinking rate compared to compositions crosslinked with CaSO₄.

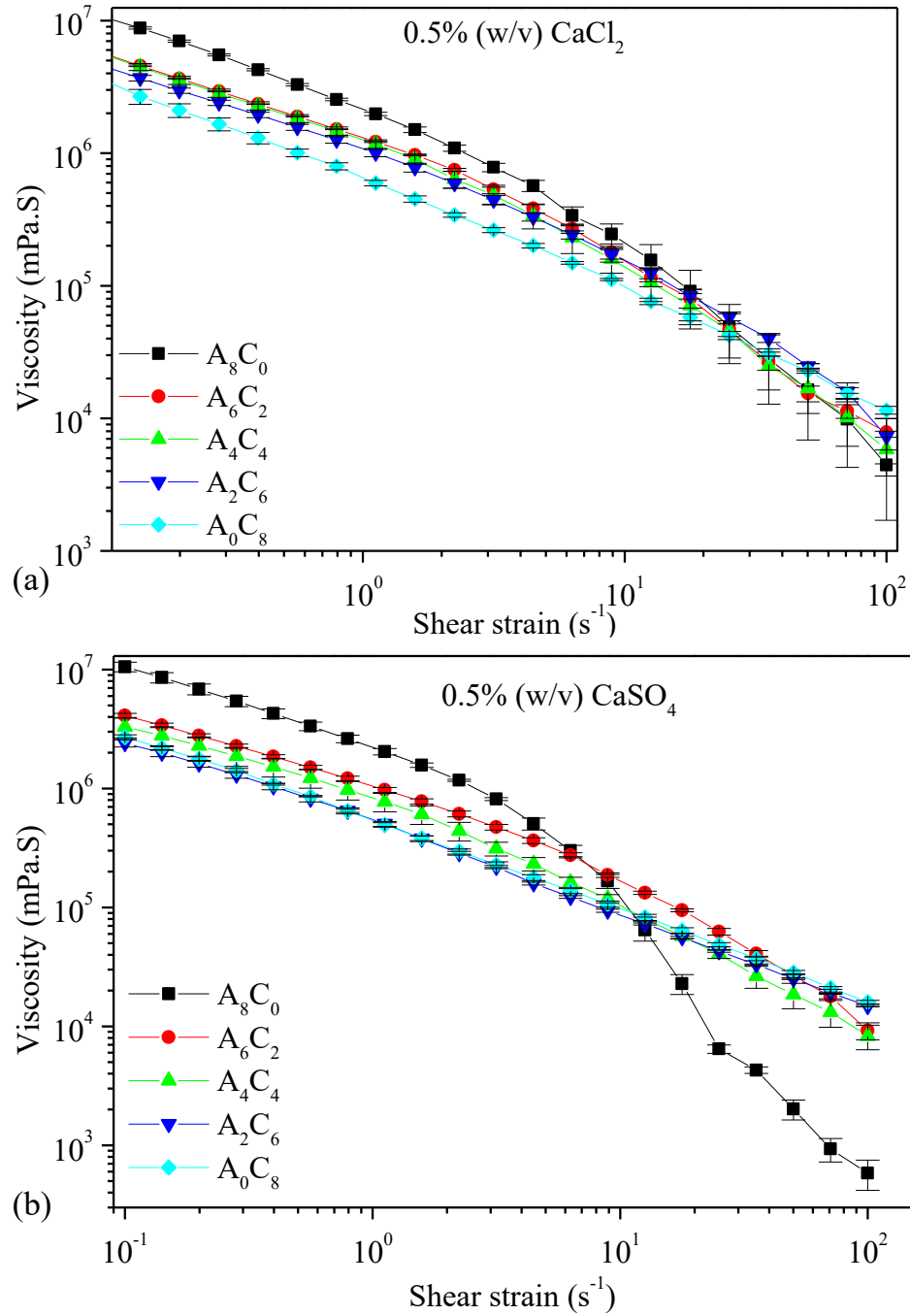


Figure 2: Viscosity concerning shear strain of various compositions pre-crosslinked with (A) $CaCl_2$ and (B) $CaSO_4$. All compositions demonstrated shear thinning behavior.

Using Equation 1, the shear-thinning coefficients, n , and K were calculated from the regressions of the linear regions of graphs in Figure 2 (a and b). Calculated values of n

and K are shown in Table 3. It is clear from n-values ($n < 1$), that all the compositions have shear thinning behavior

Table 3: Value of shear-thinning co-efficient such as shear thinning behavior identifier (n) and a constant value (K) of various compositions. Values were determined using Equation 1.

Compositions	K(mPa.S ⁿ)		n	
	CaCl ₂	CaSO ₄	CaCl ₂	CaSO ₄
A ₈ C ₀	2095363	2060350	0.16	0.08
A ₆ C ₂	1356298	1000000	0.23	0.31
A ₄ C ₄	1292658	823263	0.20	0.26
A ₂ C ₆	1098658	550326	0.27	0.28
A ₀ C ₈	689532	532165	0.22	0.28

Recovery rate

3iTT test is readily used to identify the recovery rate of hydrogel materials [15, 20]. This test is critical to determine the ability of materials to hold the filament shape right after extrusion happened. In this test, the first, second, and third intervals emulate the at-rest state of the hydrogels, decomposition under high shear i.e., hydrogel experiences high shear during extrusion, and the structural retention after hydrogel extrusion respectively as shown in Figure 3. A shear rate of 100 s^{-1} was applied on the at-rest hydrogel after the 60s which breaks down the initial network structures of the hydrogel and demonstrated an abrupt reduction of viscosity at 61s shown in Figure 3. After the hydrogel is extruded through the nozzle at a certain shear rate, it takes time to recover the internal network. In most cases, when the shear is released, a portion of the

bond remains irrecoverable. Therefore, when the shear rate was reduced to 1.0 s^{-1} from 100 s^{-1} , the amount of viscosity for all the compositions was higher than in the initial stage of the tests.

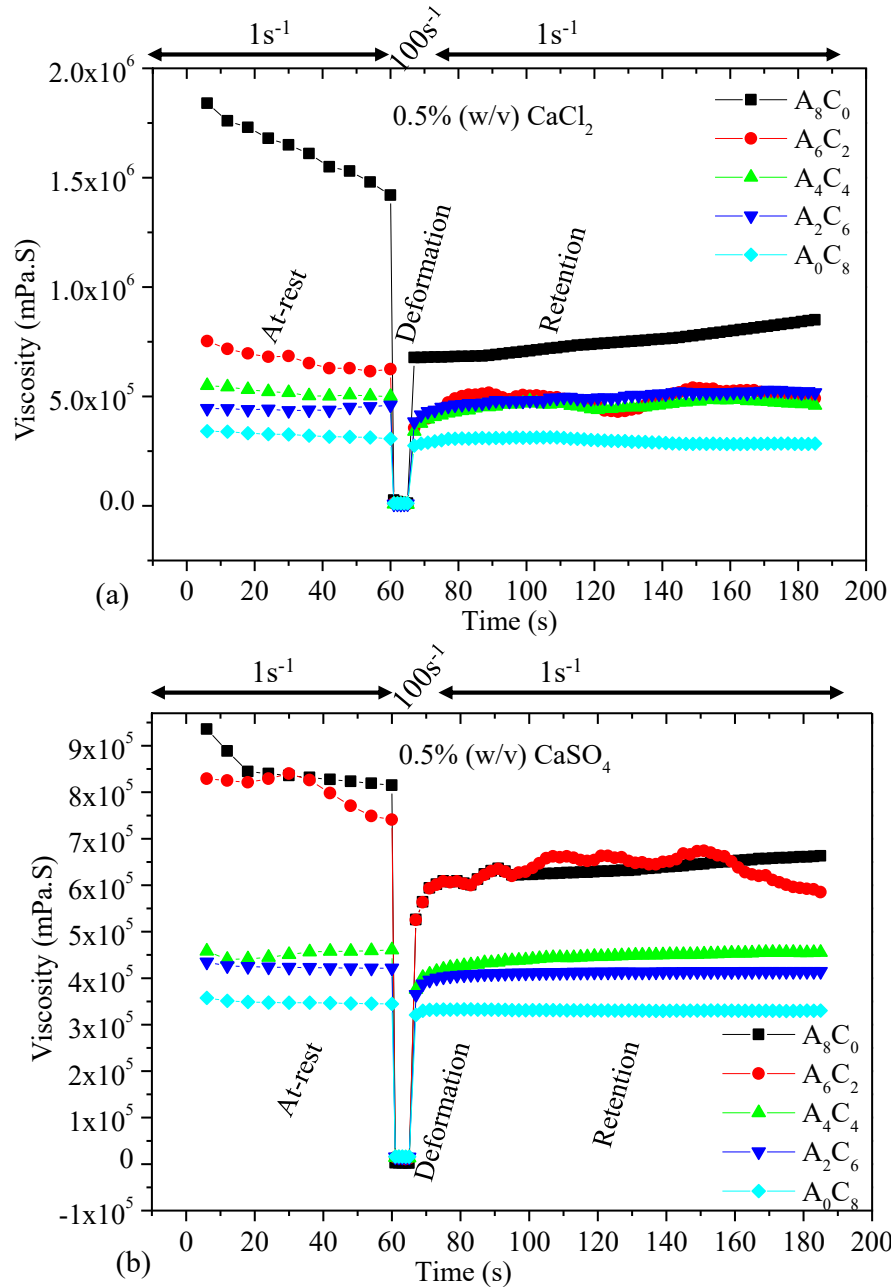


Figure 3: Viscosity with respect to time and shear strain of various compositions pre-crosslinked with (a) CaCl_2 and (b) CaSO_4 . The time between 60-65 seconds represents the extrusion scenario.

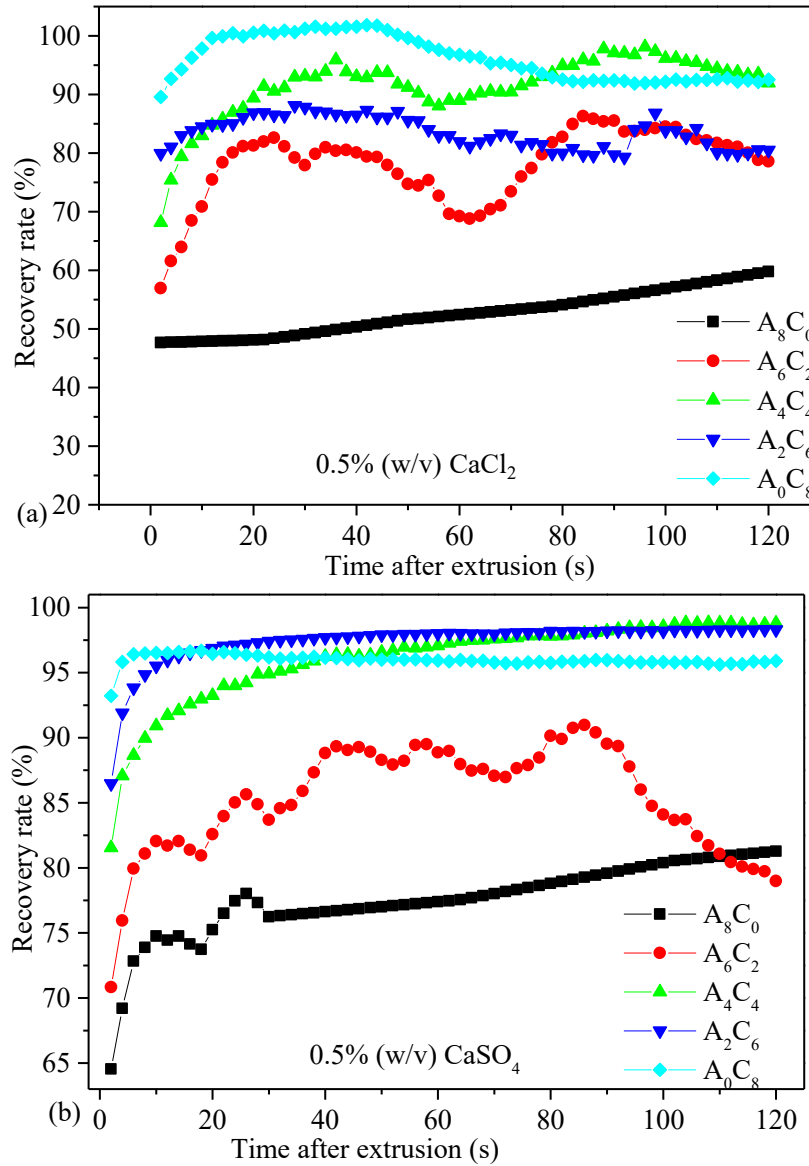


Figure 4: Recovery rate with respect to time after the extrusion happened of various compositions pre-crosslinked with (a) CaCl_2 and (b) CaSO_4 . The time between 60-65 seconds represents the extrusion scenario.

It is clear from Figure 4 that in most cases, the recovery rate increases with time right after the extrusion because deformed bonds start to retain their bonds. For an example, the recovery rates of A_8C_0 , A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 after 2.0 second of extrusion were respectively 48%, 57%, 68%, 80%, and 89% pre-crosslinked with $CaCl_2$. At the same time interval, the recovery rates of A_8C_0 , A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 pre-crosslinked with $CaSO_4$ changed to 65%, 71%, 81%, 87%, and 93% respectively. Figure 5 shows the comparison of recovery rate after 2, 60, and 90 seconds of extrusion of compositions pre-crosslinked with $CaCl_2$ and $CaSO_4$.

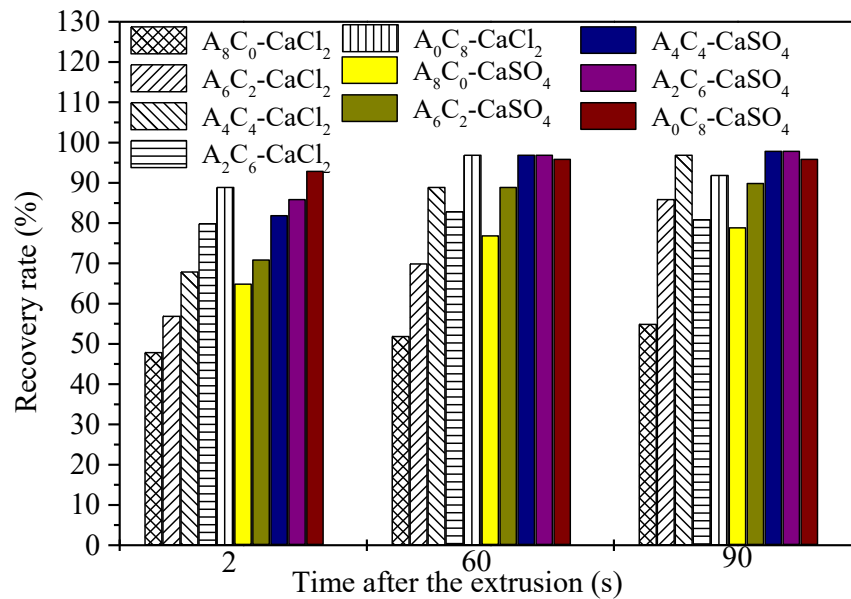


Figure 5: The comparison of the recovery rate of various compositions pre-crosslinked with $CaCl_2$ and $CaSO_4$ after 2, 60, and 90 seconds of extrusion.

Yield and flow stress

To determine the yield and flow stress, we performed an amplitude sweep test with a constant frequency of 1 Hz for all compositions as shown in Figures 6 and 7. This test defines the linear region of the material under a subsequent frequency sweep. The shear stress or deformation amplitude is varied while the frequency is kept constant during the test. Storage modulus (G' , solid-like) and loss (G'' , liquid-like) modulus were resulted from this experiment that can identify the complex modulus ($G^* = G' + iG''$). Figures 6 and 7 demonstrate that the solid-like state dominated the liquid-like state at the lower shear rate for all compositions. This phenomenon continued until a certain level where both moduli started getting reduced i.e., permanent deformation of internal bonds. This linear viscoelastic range (LVR) indicates the range at which the extrusion can happen without deforming the internal structure of the sample i.e., suspension, preserves the sedimentation without permanent deformation. The strain rate at the LVR and corresponding G' which are also known as yield stress is shown in Table 4.

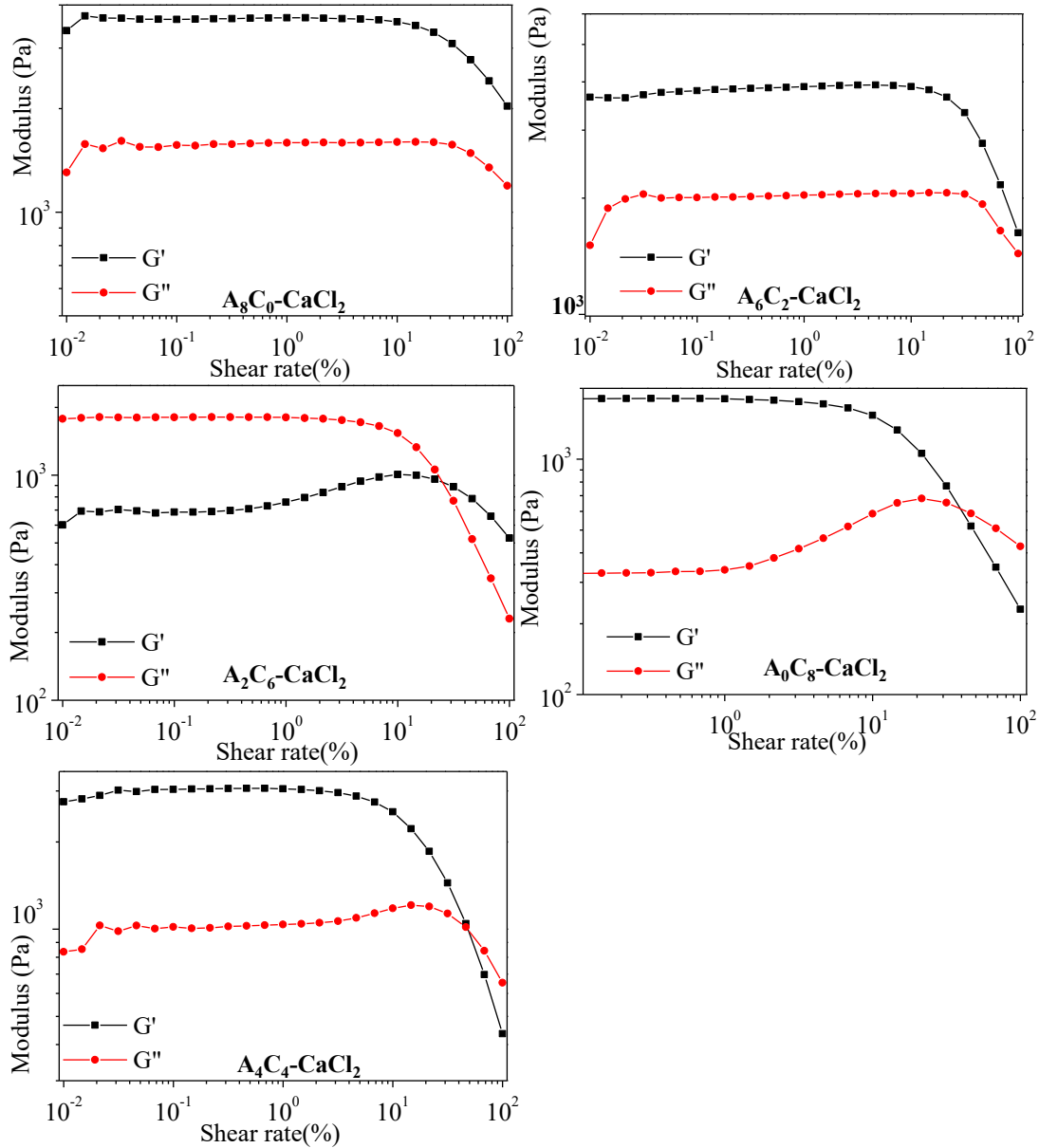


Figure 6: The storage and loss modulus, the linear viscosity range, and gel-point for A_8C_0 , A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 compositions pre-crosslinked with 0.5% CaCl_2 .

Since the storage modulus (G') dictates the loss modulus (G'') at the LVR region for all ten compositions, they showed a 'gel structure'. After exceeding the intersection points of storage and loss modulus, G'' started dominating and material started to flow. The required stress to create this flow is defined as flow stress. The relationship between

applied pressure and material flow was understood clearly by the amount of this flow stress. When the applied pressure exceeded this LVR strain rate, the material started to flow. A higher percentage of solid content pushes the LVR boundary as shown in Figures 6 and 7 during the co-existence of liquid and solid-like phases.

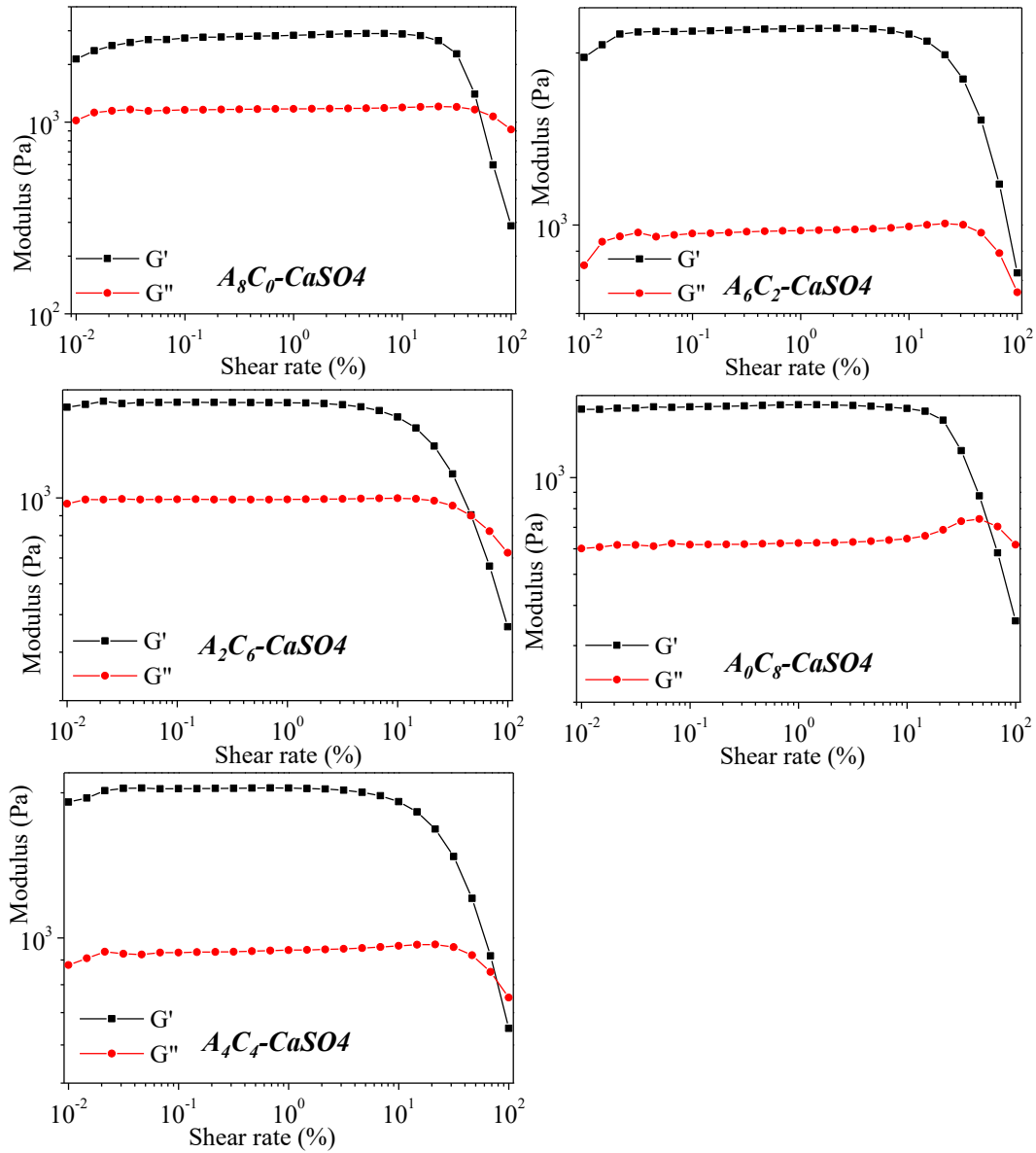


Figure 7: The storage and loss modulus, the linear viscosity range, and gel-point for A₈C₀, A₆C₂, A₄C₄, A₂C₆, and A₀C₈ compositions pre-crosslinked with 0.5% CaSO₄.

It is clear from Table 4 that all compositions pre-crosslinked with CaSO₄ showed comparatively less yield stress than pre-crosslinked with CaCl₂. Due to the molecular structure of CaCl₂, it is readily soluble in water which often leads to an uncontrolled release of Ca²⁺ ions and therefore results in more crosslinking rate compared to compositions crosslinked with CaSO₄. Alginate is a negatively charged linear copolymer (M and G blocks) that contains an abundance amount of carboxylate ions (-COO⁻), soluble in the water. The G-block of this material creates bonds to form gels and GM and M blocks increase the flexibility. More number of carboxylate ions (-COO⁻) facilitates the attraction with Ca²⁺ ions, creates physical bonding, and results in a higher rate of cross-linking. Therefore, A₈C₀, A₆C₂, A₄C₄, A₂C₆, and A₀C₈ compositions pre-crosslinked with 0.5% CaCl₂ showed 32%, 41%, 32%, 28%, and 5% more yield stress respectively compared to the same compositions pre-crosslinked with 0.5% CaSO₄. Composition without alginate i.e., A₀C₈ showed lower yield stress increment compared to others due to the unavailability of a higher number of free carboxylate ion (-COO⁻) from complex carboxymethyl cellulose.

Table 4: Yield point, corresponding yield stress, and flow-point, corresponding flow stress for A_8C_0 , A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 compositions pre-crosslinked with 0.5% $CaSO_4$ (CS) and $CaCl_2$ (CL).

Hydr ogels	Pre- crossl inkers	% strain at yield point	Yield stress (Pa)	% of strain at flow point	Flow stress (Pa)
A_8C_0	CL	10	3913	NA	NA
	CS	20	2662	55	965
A_6C_2	CL	9.6	3577	NA	NA
	CS	16	2096	105	679
A_4C_4	CL	5	2875	50	1105
	CS	6	1965	80	767
A_2C_6	CL	4.5	2408	19	1490
	CS	5	1726	50	712
A_0C_8	CL	3	1750	25	879
	CS	7	1655	60	646

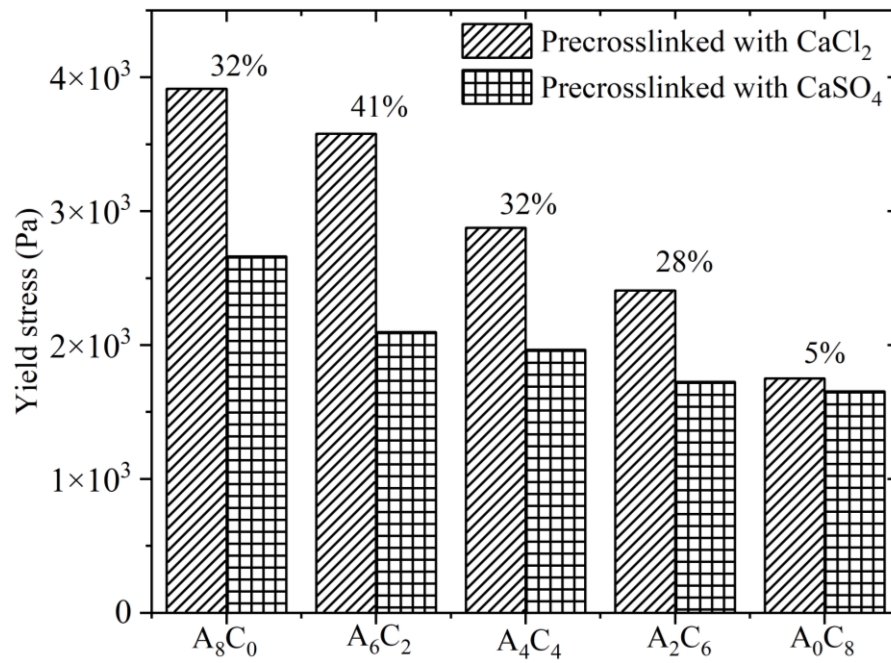


Figure 8: Comparison of yield stress for A_8C_0 , A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 compositions pre-crosslinked with 0.5% $CaSO_4$ and 0.5% $CaCl_2$.

Scanning electron microscope

The surface and cross-sectional morphology of A_6C_2 , A_4C_4 , and A_2C_6 pre-crosslinked with 0.5% $CaCl_2$ and 0.5% $CaSO_4$ were analyzed by scanning electron microscopy (SEM). Surface morphologies of all compositions turned out almost smooth whereas the cross-sections showed crystalline and fiber type morphology. Moreover, cross-sectionally almost all the compositions except A_2C_6 pre-crosslinked with $CaCl_2$ showed porous morphology with various pore sizes and roughness. Therefore, there is a high chance that the transmission of required nutrients and exchange of gases of the encapsulated cells in those compositions will be allowed. However, compositions pre-crosslinked with $CaSO_4$ showed better porosity compared to the compositions pre-crosslinked with $CaCl_2$. Morphologically, a higher percentage of CMC pre-crosslinked with $CaSO_4$ showed larger micro-porosity which aligns with our earlier investigation [54].

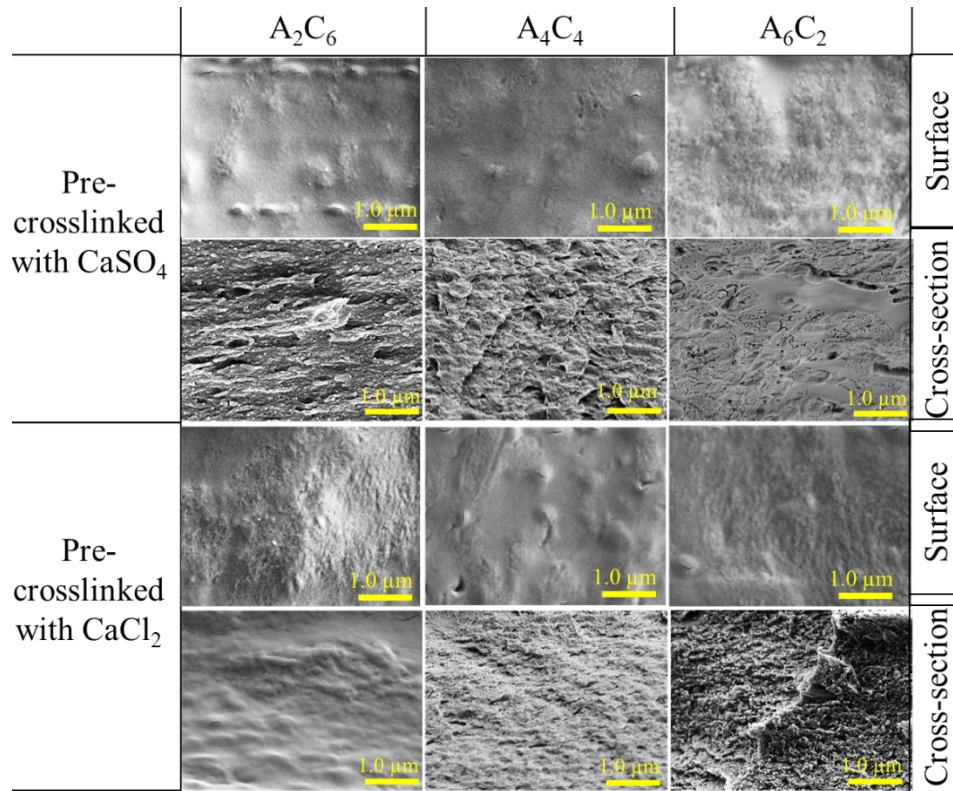


Figure 9: Microstructural investigation of various compositions using a scanning electron microscope.

3D printing

3D printing of 1D filament

Acellular filaments were deposited with the pre-crosslinked compositions of A_0C_8 , A_2C_6 , A_4C_4 , A_6C_2 , and A_8C_0 for analyzing their 3D printability as shown in Figure 10. All the filaments fabricated with $CaSO_4$ pre-crosslinked compositions were extruded using 29 psi air pressure. Since the viscosities of those compositions were decreasing with increasing the weight percent of alginate, the filament size was showing an increasing trend as shown in Figure 11 (a). Therefore, it showed a filament width change from 4.76 to 46.55% with respect to the nozzle diameter. On the other hand, in the case of compositions A_8C_0

and A_6C_2 pre-crosslinked with $CaCl_2$, we needed to increase the air pressure up to 45 psi to surpass the yield point and create a flow of those compositions. This activity resulted in uncontrolled extrusion with a random amount of material release, and eventually more filament length as shown in Figure 11(b).

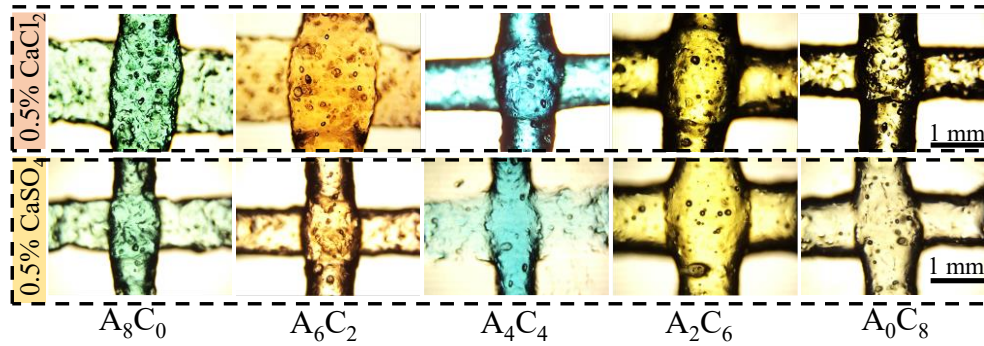


Figure 10: 3D printed filaments and inter-layer morphology created with various compositions pre-crosslinked with $CaCl_2$ and $CaSO_4$.

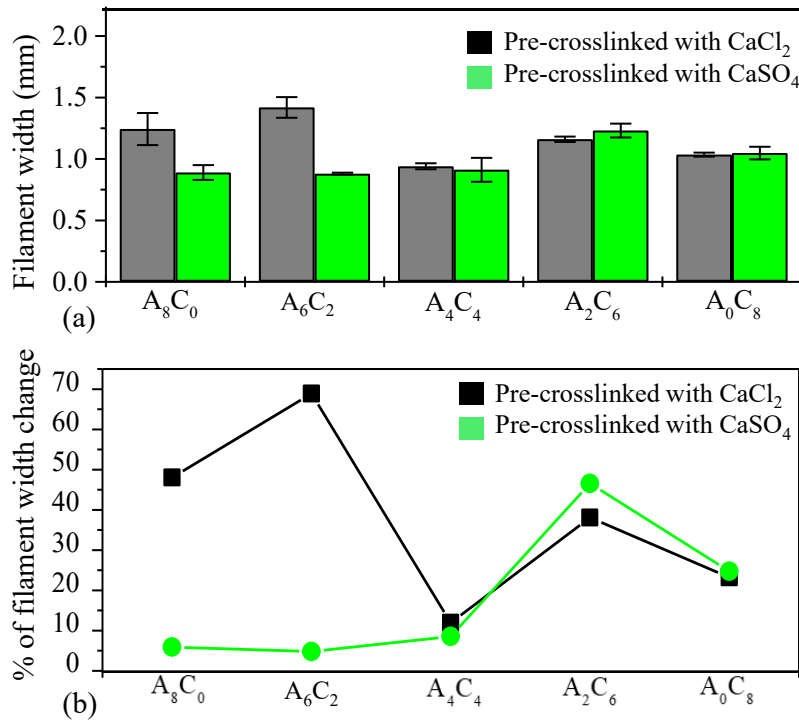


Figure 11: Filament width and % of filament width change of various compositions pre-crosslinked with CaCl_2 and CaSO_4 .

3D printing of bilayer

Acellular bi-layer constructs having a dimension of $20\text{mm} \times 20\text{ mm}$ were fabricated with the compositions of A_8C_0 , A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 pre-crosslinked with CaCl_2 and CaSO_4 for inspecting their manufacturability or printability as shown in Figure 12.

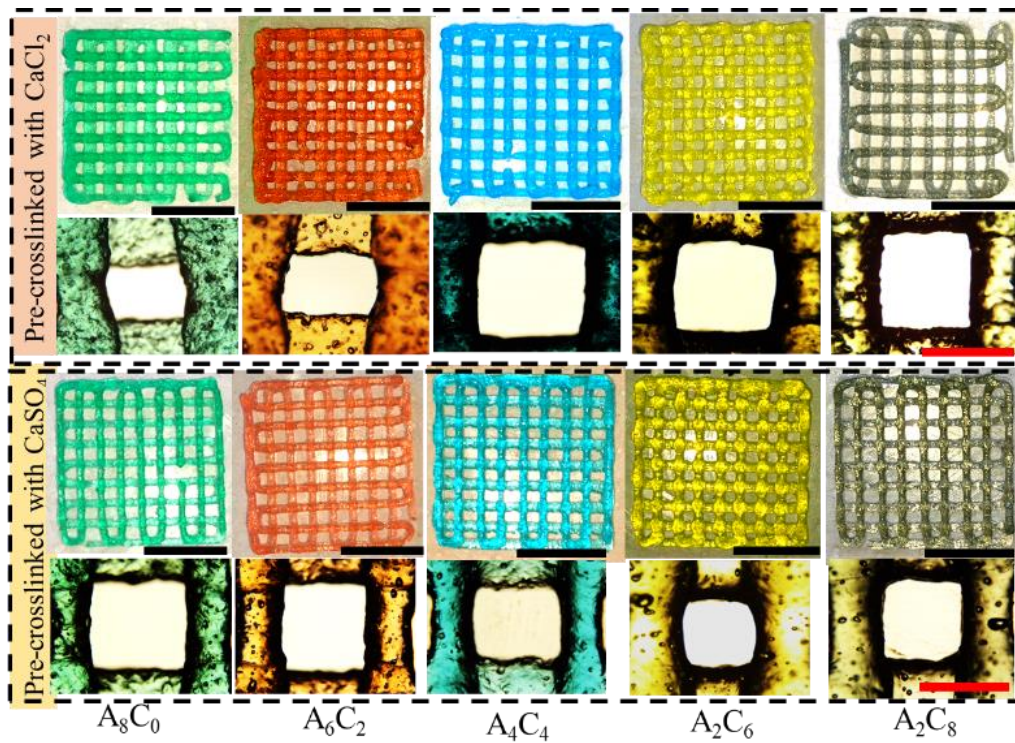


Figure 12: Bi-layer constructs fabricated with the compositions of A_8C_0 , A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 compositions pre-crosslinked with 0.5% CaSO_4 and CaCl_2 . the length of the black and red bars are 10 mm and 2 mm respectively.

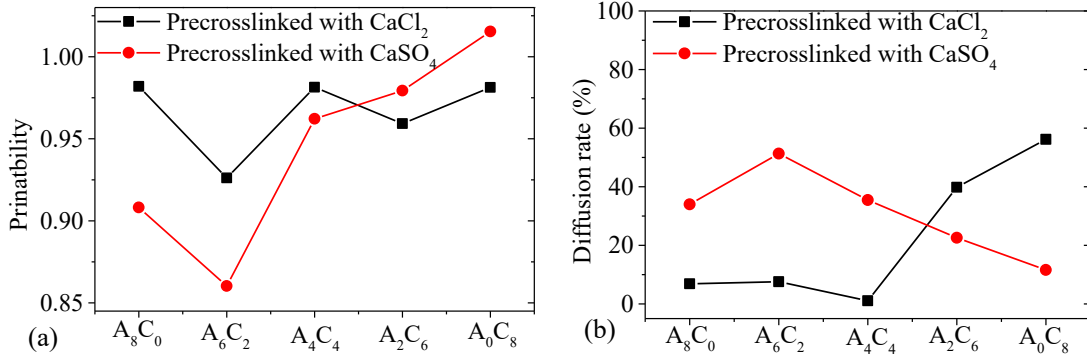


Figure 13: printability and diffusion rate of the constructs fabricated with the compositions of A_8C_0 , A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 compositions pre-crosslinked with 0.5% $CaSO_4$ and $CaCl_2$.

Even though various compositions showed the various amount of pore closure or diffusion rate, all of them showed a decent amount of printability having a range of 0.86-1.01 as shown in Figure 6(a). All the constructs fabricated with $CaSO_4$ pre-crosslinked compositions were extruded using 29 psi air pressure. Since the yield stresses of those compositions were decreasing with increasing the weight percent of alginate, the pore size of the constructs fabricated with A_8C_0 , A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 compositions was getting reduced. Eventually, the diffusion rate followed an increasing trend from 11% to 51% as shown in Figure 13(b). In the case of compositions A_8C_0 and A_6C_2 pre-crosslinked with $CaCl_2$, we needed to increase the air pressure up to 45 psi to surpass the yield point and create a flow of those compositions. This resulted in uncontrolled extrusion, more material release, and eventually more diffusion rate (56% and 39% respectively) as shown in Figure 6(b).

In our earlier works, we demonstrated the percentage of filament width change fabricated with various compositions of alginate-CMC using 250 μm and 410 μm [54, 55].

In this paper, to demonstrate the filament shape fidelity printed with a smaller nozzle diameter compared to 840 μm , filaments were deposited with a nozzle diameter of 610 μm by applying an average of 17 psi air pressure with four different compositions such as of A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 pre-crosslinked with 0.5% CaSO_4 as shown in Figure 14 (a). Analyzing the filament width with various process parameters such as nozzle diameters (250 μm and 410 μm), air pressure (10-15 psi), print distance (0.7 to 1.2 mm), and print speed (10-50 mm/s) is an ongoing work.

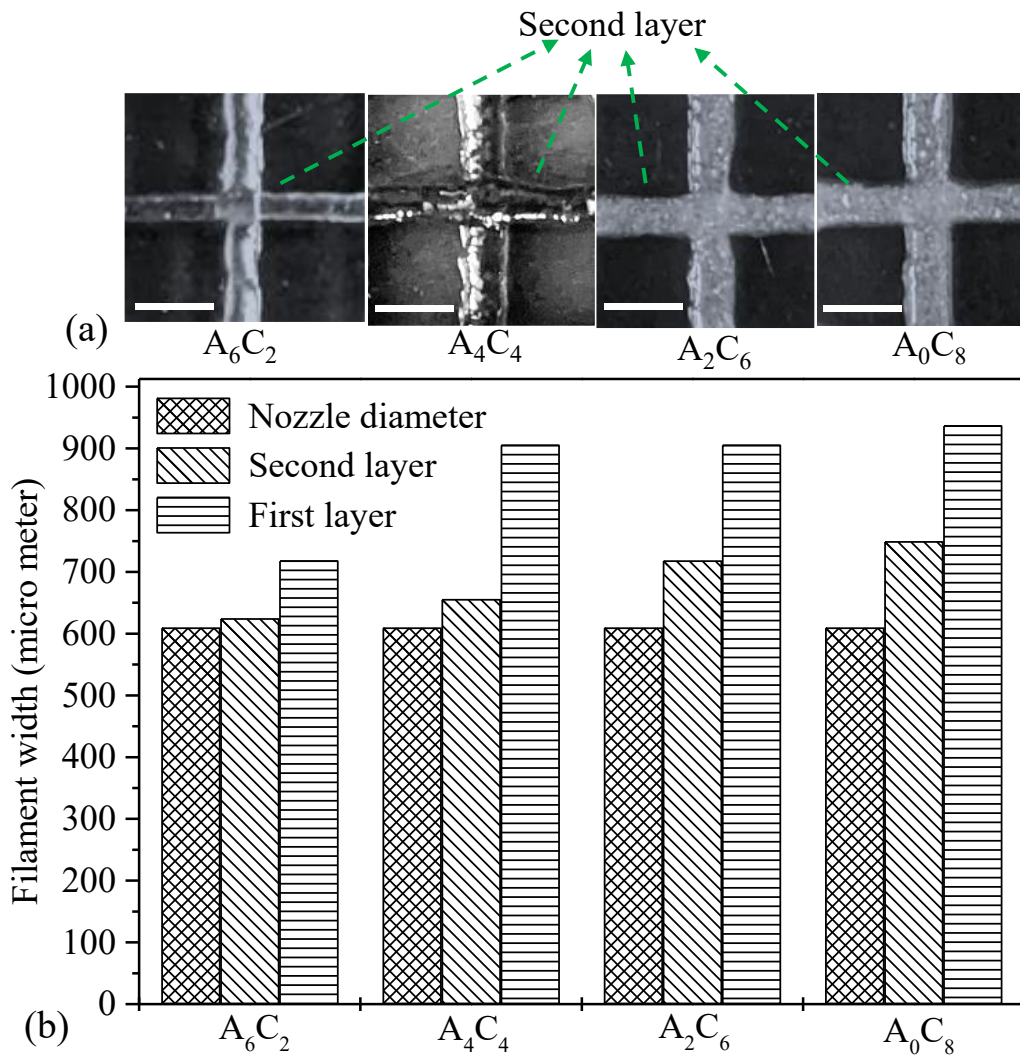


Figure 14: (a) Bi-layer constructs fabricated with the compositions of A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 compositions pre-crosslinked with 0.5% $CaSO_4$, scale bar = 1500 μm , and (b) Filament width of first and second layer. The immediately printed layer (second layer) showed a filament change of 2.5%, 7.6%, 18%, and 23% for A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 compositions respectively. Layer printed at the beginning (first layer) showed a filament change of 15%, 38%, 26%, and 25% for A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 compositions respectively. It is clear from this figure that the first layer diffused around 15%, 38%, 26%, and 25% respectively during the time of second layer deposition. Nozzle diameter was 610 μm .

Full-scale construct fabrication

To demonstrate the capability of fabricating a full-size construct, as a candidate composition, we chose A_6C_2 pre-crosslinked with $CaCl_2$ and $CaSO_4$, because of its good printability (0.96 and 0.98 respectively). A freeform liver model with a bounding box dimension and volume of $3.7 \times 1 \times 1.7$ (cm³) was used to fabricate as shown in Figure 7. No support structure was used to fabricate this construct which indicates the capability to fabricate a full-scale freeform construct without sacrificing its geometric fidelity.

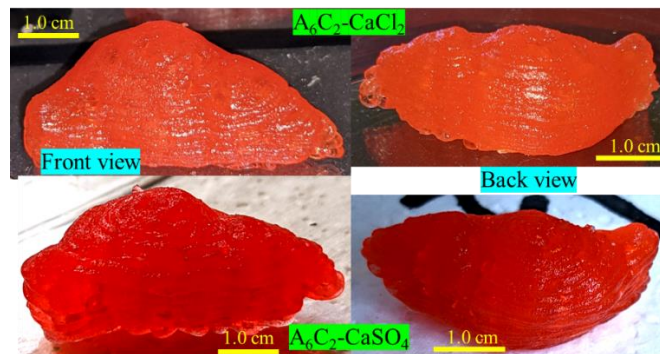


Figure 15 Full-scale scale fabrication of constructs compositions fabricated with the compositions of A_6C_2 pre-crosslinked with 0.5% $CaSO_4$ and $CaCl_2$.

We also printed a kidney model using six compositions such as A_6C_2 , A_4C_4 , and A_2C_6 pre-crosslinked with 0.5% $CaSO_4$ and $CaCl_2$, respectively as shown in Figure 15 to demonstrate the capability of those compositions to maintain the shape fidelity of freeform structure. With increasing the percentage of alginate, the structure was maintaining its internal porosity as well.

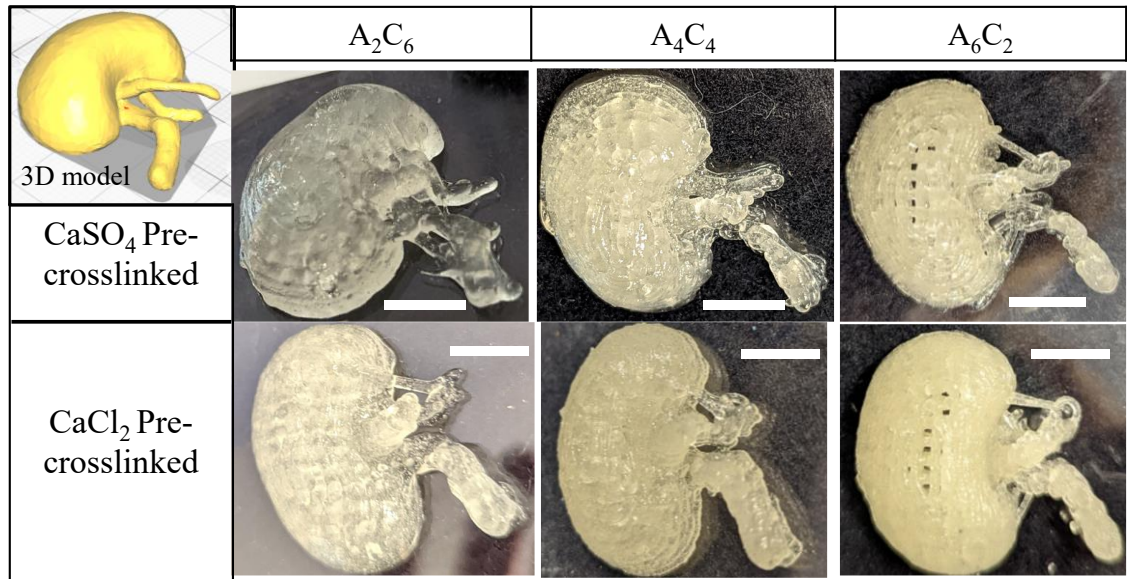


Figure 16: Full scale fabrication of constructs compositions fabricated with the compositions of A_2C_6 , A_4C_4 , and A_6C_2 pre-crosslinked with 0.5% $CaSO_4$ and 0.5% $CaCl_2$. Scale bar = 1.0 cm.

DISCUSSION AND FUTURE WORK

Five hybrid hydrogels such as A_8C_0 , A_6C_2 , A_4C_4 , A_2C_6 , and A_0C_8 were pre-crosslinked with 0.5% $CaCl_2$ and 0.5% $CaSO_4$. All pre-crosslinked hydrogels showed shear thinning behavior ($n < 1$). It is clear from the flow curve that viscosity was the percentage of alginate dependent. For the same combination of alginate-CMC, higher viscosity resulted in pre-crosslinked with $CaCl_2$. Therefore, A_8C_0 pre-crosslinked with 0.5% $CaCl_2$ showed the

highest viscosity. Amplitude tests showed that yield stress was the percentage of alginate dependent. For the same combination of alginate-CMC, higher yield and flow stresses resulted in pre-crosslinked with CaCl_2 . Therefore, A_8C_0 pre-crosslinked with 0.5% CaCl_2 showed the highest yield stress. However, we did not get any flow point and corresponding flow stress within the considered limit of shear strain (0.01 to 100%). We could identify the flow point by increasing the shear strain limit ($> 100\%$). High yield stress requires greater applied force to surpass the LVR region and make a smooth flow of material through the nozzle.

High viscous hydrogels required greater applied force to surpass yield stress and make a smooth flow of material through the nozzle. We showed in our earlier work that high applied pressure generates more shear stress and high cell death as a consequence [56]. Therefore, it is obvious that pre-crosslinked A_8C_0 will create high shear stress and highly affect the encapsulated cell whereas A_0C_8 will do minimally after extruding. However, mixing alginate with hybrid hydrogel can help quick post-crosslinking and more reliable construct geometry during the incubation period.

In our earlier work, we encapsulated various cell lines such as BxPC3 cells, Prostate Cancer stem cells, and HEK 293 cells in 4% alginate and 4% CMC where we reported 92%, 91%, and 89% of cell viability respectively after 15 incubation days [55]. We also reported the impact of various applied pressure on the overall cell viability where we observed 90% and 74% cell viability by extruding with an air pressure of 8 and 12 psi respectively [55]. A region-based cell viability analysis was performed on a cross-section of filaments fabricated by applying 8 and 12 psi air pressure where we demonstrated a higher

percentage of live cells close to the center of both filament cross-sections. Even though we did not conduct any cell work with the pre-crosslinked hydrogels in this paper, we anticipate a similar trend of cell viability in the future. In another work, we reported an increasing trend of cell proliferation encapsulated in alginate-CMC compositions. We observed 49% cell viability on day 1 and it increased up to 86% after 23 incubation days [54]. Therefore, using a higher percentage of applied pressure may result in lower cell viability close to the nozzle wall in pre-crosslinked hydrogels. However, we anticipate cell viability will be recovered with time from our previous cell work result. We analyzed an *Escherichia coli* (a common bacterial model organism) bacterial growth in 3% alginate and 3% CMC pre-crosslinked simultaneously with 0.5% CaSO_4 and 0.5% CaCl_2 using the Most Probable Numbers [57] assay where we concluded that material composition was intrinsically antimicrobial [58]. The bacterial growth test for the materials we propose in this paper is ongoing to identify if these are intrinsically antimicrobial.

Analysis of 3D printed filament, bilayer intersection, and bilayer revealed a good printability, shape-holding capability, and various amounts of filament width change for all pre-crosslinked compositions. However, A_8C_0 and A_6C_2 pre-crosslinked with CaCl_2 required a substantial amount of applied pressure. Hence, they showed a discontinuous extrusion and a higher rate of filament spread at the bilayer intersection. Same compositions pre-crosslinked with CaSO_4 required 35% less applied pressure and showed a defined filament structure throughout the printing. Therefore, compositions pre-crosslinked with CaSO_4 can be considered to fabricate a full construct ensuring good

geometric fidelity and cell viability. Analyzing the geometric fidelity of the 3D printed structures fabricated with all the compositions mentioned here is our ongoing research.

CONCLUSION

This research represents a rheological investigation of various compositions prepared with medium viscous alginate and CMC pre-crosslinked with CaCl_2 and CaSO_4 maintaining a solid content of 8%. The effect of various pre-crosslinkers and viscosity enhancers were identified in terms of viscosity, recovery rate, and yield stress. The relationship between the printability and rheological properties of the compositions was identified. From the applied pressure standpoint, to fabricate a full construct ensuring good geometric fidelity and cell viability, compositions pre-crosslinked with CaSO_4 can be good candidates. The illustrated rheological tests and corresponding printability of those compositions can direct the 3D bio-fabrication of the tailored anisotropic constructs, which will assist in future efforts to fabricate functional tissues.

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