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Rheological Investigation of Pre-Crosslinked Hybrid Hydrogels for 3D Bio-printing Processes.

Slesha Tuladhar, Cartwright Nelson, and Ahasan Habib
Sustainable Product Design and Architecture Department, Keene State College, Keene, NH 03435, USA.

Abstract

Despite being a very popular topic and researched by several scientists, the entire 3D bioprinting process is still subjected to several challenges like geometric fidelity, mechanical complexities, cell viability, and proliferation. Rheological investigations along with the proper design of experiments help to explore the physical and mechanical properties of biomaterials and 3D printed scaffolds that are directly associated with their geometric fidelity. To ensure post-printed structural integrity, viscosity thickeners and crosslinkers were used in this research. Mixtures of Carboxymethyl Cellulose (CMC, viscosity enhancer), Alginate, and CaCl2 and CaSO4 (crosslinkers) were prepared at various concentrations maintaining minimum solid content. For each composition, a set of rheological tests was performed in form of flow, thixotropic, amplitude, and frequency tests. This research presents an overview of controlling the rheological properties of various bio-inks that are viscosity enhancer and pre-crosslinkers dependent, which opens doors to looking at 3D bioprinting in a very different way.

Keywords
3D bioprinting, rheology, pre-crosslink, printability, and hybrid hydrogels

1. Introduction
Three dimensionally printed scaffolds with biomaterials have significant effects on the growth of encapsulated or seeded living cells and eventually tissue regeneration. A digital model along with a computer-controlled 3D printer is used to extrude bio-ink (bio-materials and living cells mix) in a layer-upon-layer fashion [1, 2]. Due to users' ability to control, bio-ink can be deposited in a defined location to achieve customized geometry and porosity of the fabricated scaffolds. Among various available 3D bioprinting techniques, extrusion-based bio-printing allows the usage of a diverse range of materials and compositions including heterogeneous bio-ink [3]. The unique bio-compatibility and capacity to arrange 3D environment with a high water content make hydrogels demanding candidate for bio-printing [4]. Hydrogels are subjected to various mechanical challenges during the 3D bio-printing process. Specifically, the flowability and retention of the deposited filament structure after printing while extruding through a small nozzle are important to have a successful 3D bio-printing process [5]. To ensure the filament geometry and eventually the overall scaffold geometries, rheological properties of hydrogels play an important role. However, focusing on the filament geometry and increasing the viscosity accordingly can negatively impact encapsulated cells during extrusion when a hydrogel needs to create a suitable habitat [6]. Sometimes viscosity is interchangeably used as the rheological properties of hydrogel materials, however, it solely cannot represent the complex rheological behavior of hydrogel materials [7, 8]. In addition to viscosity, two dynamic moduli such as storage modulus (G') and loss modulus (G") are critical [8]. Storage modulus (G') and loss modulus (G") indicate the solid-like and liquid-like characteristics of the hydrogel material, respectively.

Rheological properties can be controlled by mixing multiple hydrogels [9, 10], viscosity modifier [11-13], temperature [14-16], external cross-linker [17, 18]. Among them, using external cross-linker during preparation of bio-ink i.e., pre-crosslinking gets interest to the research community. Recently, we demonstrated the effect of pre-crosslinkers such as $CaCl_2$ and $CaSO_4$ on the rheology of our already-proposed hybrid hydrogels such as Alginate and Carboxymethyl Cellulose (CMC) limiting the solid content 6% (w/v) [19]. To distinct the effect on higher solid content such as 8% (w/v), we will pre-crosslink the same compositions of alginate and CMC with the same percentage (0.5%) of $CaCl_2$ and $CaSO_4$ separately in this paper. A set of rheological data was taken, and the results were compared between the compositions pre-crosslinked with $CaCl_2$ were $CaSO_4$. 3D filaments were fabricated with all pre-crosslinked compositions and compared in terms of printability. A set of rheological tests enlisted in Table 1 (b) will be conducted to determine the rheological properties.

Table 1: (a) Various Composition prepared with different weight percentages of alginate and CMC and (b) An overview of all rheological tests.

Compositions	A %(w/v)	C %(w/v)	Pre-crosslinkers	
			CL, 0.5%	CS, 0.5%
A_6C_2	6	2		
A_4C_4	4	4		
A_2C_6	2	6		

(a)

Rheological tests	Variables	Outcome
Steady rate sweep	Shear rate (s^{-1}): 0.1 to 100	Flow curve, viscosity, shear stress, shear-thinning behavior
Amplitude sweep	Shear strain (%): 0.1 to 100	Storage modulus (G') and loss modulus (G"), loss tangent ($\tan\delta$)
3iTT	Time (s)/Shear rate (s^{-1}): 0-60/1, 61-65/100, 66-185/1	Recovery rate of the hydrogel

(b)

2. Hybrid hydrogel preparation

Alginate (alginic acid sodium salt from brown algae) and medium viscous carboxymethyl cellulose (CMC) (pH: 6.80) (Sigma-Aldrich, St. Louis, MO, USA) were used as biomaterials to prepare the bio-ink. Alginate is a common biopolymer, composed of (1-4)-linked β-Dmannuronic (M) and α-Lguluronic acids (G). Alginate is a negatively charged linear copolymer (M and G blocks) which is soluble in the water and supports cell growth and exhibits high biocompatibility. The G-block of this material creates bonds to form gels and GM and M blocks increase the flexibility. Carboxymethylcellulose (CMC) is another naturally or chemically derived anionic water-soluble biopolymer. CMC is a copolymer of β-D-glucose and β-D-glucopyranose-2-O-(carboxymethyl)-mono-sodium salt which are connected via β-1,4-glucosidic bonds [20]. This material is widely used as thickener [21] which is also non-toxic and non-allergenic in nature. Each glucose monomer has three hydroxyl groups which can be substituted by a carboxyl group. More substitution of the hydroxyl group by a carboxyl group makes the cellulose more soluble, thicken and stable [20]. Pre-crosslinking was done using calcium chloride ($CaCl_2$) and calcium sulfate ($CaSO_4$) (Sigma-Aldrich, St. Louis, MO, USA). Three hybrid hydrogels composed with various weight percentages of alginate and CMC were pre-crosslinked with $CaCl_2$ and $CaSO_4$ separately, which resulted in a total of six compositions maintaining the total solid content 8% (w/v) in this paper as shown in Table 1(a).

3. Rheological tests

Rheological measurements were performed using a rotational rheometer (MCR 102, Anton Paar, Graz, Austria) with parallel plate geometry (25.0 mm flat plate). All measurements were recorded with a 1.0 mm plate-plate gap width at room temperature (25°C). We conducted the rheological measurement at room temperature because our extrusion process was performed at room temperature which also facilitated the quick gelation of the deposited filament [6]. A set of rheological tests such as steady rate sweep test, amplitude test, and three-point interval thixotropic test were done. An overview of those tests is shown Table 1(b).

3.1. Shear thinning behavior

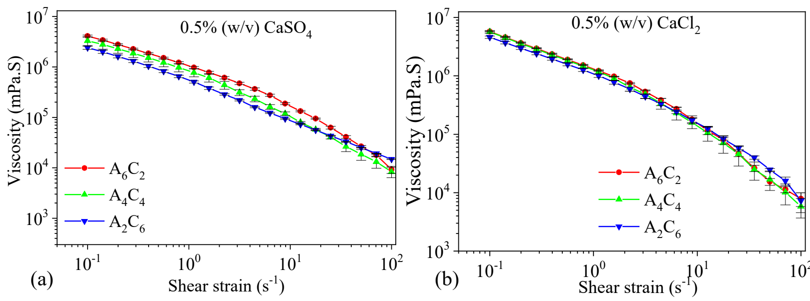
Shear-thinning behavior is characterized by an event where viscosity shows a decreasing trend with increasing the shear strain rate on hydrogels. This behavior is crucial for the extrusion-based bioprinting process [13]. Hydrogels flowing through a nozzle experiences shear stress. The amount of shear stress experienced by the flowing fluid depends on its viscosity. Therefore, hydrogel with high viscosity experiences higher shear stress during extrusion through a nozzle. Eventually, this shear stress can adversely affect the encapsulated living cells into those hydrogels [22]. Reduction of viscosity during material release due to shear-thinning behavior can help create a protective environment for encapsulated cells in the extrusion-based 3D bioprinting technique.

Figures 1(a) and 1(b) show the log-log plot of viscosity with respect to 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 i.e., $A_6C_2 > A_4C_4 > A_2C_6$. Composition A_6C_2 pre-crosslinked with $CaCl_2$ showed 1% and 25% higher viscosity with respect to A_4C_4 and A_2C_6 at 0.1 s^{-1} shear rate. And the same composition pre-crosslinked with $CaSO_4$ showed 19.26% and 41.37% higher viscosity with respect to A_4C_4 and A_2C_6 at 0.1 s^{-1} shear rate. 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 Ca^{2+} ions, creates physical bonding, and results in a higher rate of cross-linking.

Same compositions pre-crosslinked with $CaCl_2$ showed higher viscosity than pre-crosslinked with $CaSO_4$. Comparing Figures 1(a) and 1(b), it is clear that compositions A_6C_2, A_4C_4, and A_2C_6 pre-crosslinked with $CaCl_2$ showed 28%, 41%, and 48% higher viscosity at 0.1 s^{-1} shear rate than those same compositions pre-crosslinked with $CaSO_4$ respectively. A close look towards the molecular level of $CaCl_2$ will show it is readily soluble in water which often leads to an uncontrolled release of Ca^{2+} ions and therefore results in more crosslinking rate compared to compositions crosslinked with $CaSO_4$. The shear thinning co-efficient of n and K were determined using the following equation:

$$\eta = K\dot{\gamma}^{n-1} \tag{1}$$

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. Using Equation 1, the shear-thinning coefficients, n, and K were calculated from the regressions of the linear regions of graphs of Figure 1 (a and b). Calculated values of n and K are shown in Table 1. It is clear from n-values (n<1), all the compositions have shear thinning behavior.



Compositions	K(mPa.S^n)		n	
	$CaCl_2$	$CaSO_4$	$CaCl_2$	$CaSO_4$
A_6C_2	1356298	1000000	0.23	0.31
A_4C_4	1292658	823263	0.20	0.26
A_2C_6	1098658	550326	0.27	0.28

(c)

Figure 1: Shear thinning behavior (viscosity vs shear rate, sample n=3) of the compositions pre-crosslinked with (a) 0.5% $CaSO_4$, (b) 0.5% $CaCl_2$, and (c) the values of various shear thinning co-efficient.

3.2. Hydrogel recovery rate

To determine the recovery rate after extrusion, a three point-interval-thixotropy-test was conducted on all the compositions which is directly related to the shape fidelity of the filament. The first interval of the trial imitates the at-rest state of sample, the second interval resembles the hydrogel decomposition under high shear, and third interval reflects the structure retention after hydrogel extrusion.

A shear rate of 1.0 s^{-1} was applied for 60 seconds at first. Then, the shear rate was increased to 100 s^{-1} for 5 seconds. Finally, shear rate was reduced to 1.0 s^{-1} and held for 180 seconds. During extrusion, the internal weak physical bonds got destroyed and compositions showed a sharp drop of viscosity for all six compositions. However, when the extrusion pressure was released, bonds started to recover and retain the viscosity as shown in Figure 3(a, b). Figure 3(a) shows that the recovery rate of A_2C_6 pre-crosslinked with $CaSO_4$ 58% and 86% after 1 and 110 seconds of releasing the extrusion pressure. Compositions A_4C_4, and A_2C_6 pre-crosslinked with $CaSO_4$ showed 68% and 80% recovery rate respectively after 1 second of releasing the extrusion pressure while they showed 91% and 94% recovery rate respectively after 110 seconds of releasing the extrusion pressure. Same trend was observed for all three compositions pre-crosslinked with $CaCl_2$. Therefore, it is a good indication that the deposited filament will hold the shape to maintain the geometric fidelity. Even though A_2C_6 pre-crosslinked with $CaSO_4$ and $CaCl_2$ showed very promising recovery rate, there is a high chance this combination will demonstrate over-deposition and eventually poor shape fidelity due to lower initial viscosity than other considered compositions. In terms of recovery rate and initial viscosity, all three compositions can be good candidates for 3D bio-printing.

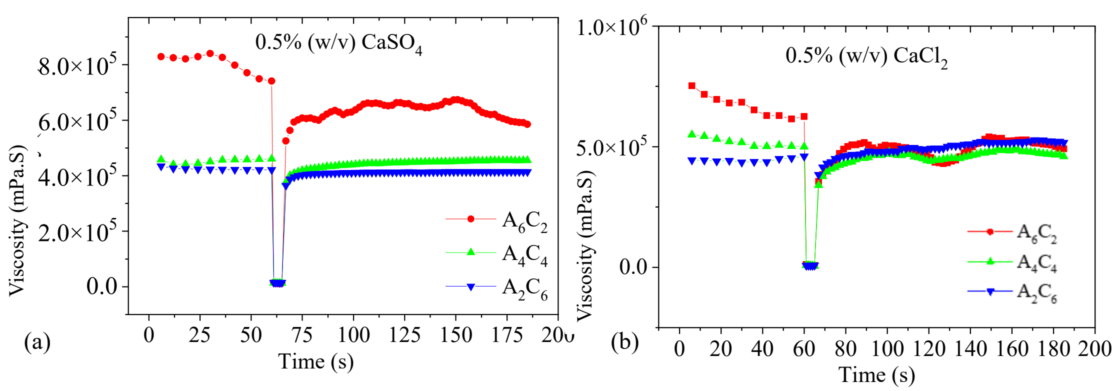


Figure 2: (a) Three-point thixotropic test and (b) Recovery rate of various compositions at 60s and 120s.

4. 3D scaffold fabrication

BioX (CELLINK, Boston, MA), an extrusion-based 3D bio-printer was used to fabricate the 3D filaments. Hydrogels were prepared following section 2, loaded into a 3.0 ml disposable nozzle, and extruded pneumatically on a stationary build plane. The printing parameters we used in fabricating the scaffolds were a nozzle diameter of 840 µm, print speed of 20 mm/s, air pressure varying from 29-45 psi. A visual basic-based Computer-Aided Design (CAD) software, Rhino 6.0 (https://www.rhino3d.com), was used to design and define the vectorized toolpath of a scaffold. Slicer (https://www.slicer.org), a G-code generator software was used to generate a Bio-X compatible file including the toolpath coordinates and all process parameters to build the scaffold. We followed a layer-upon-layer fashion to release the materials. The images of fabricated filaments were captured using the CK Olympus bright field microscope (Tokyo, Japan). The width of the filament is determined using ImageJ software.

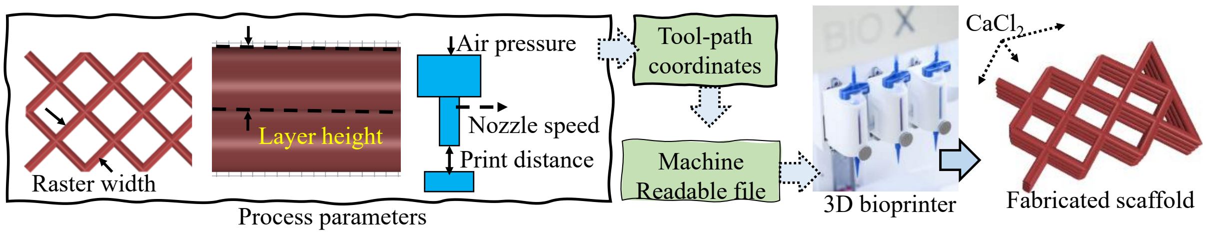


Figure 3: Preparing machine-readable file and execution of 3D fabrication.

Filaments without encapsulating any cell i.e., acellular were deposited with the compositions of A_6C_2, A_4C_4, and A_2C_6, for printability and shape fidelity investigation. All the filaments fabricated with CaSO4 pre-crosslinked compositions were extruded using 29 psi air pressure. Fabricated filaments with their width are shown Figure 5. This figure indicates that with increasing the solid content of alginate into the composition, the diffusion of the filament decreased, i.e. properly holding the geometry of the filament, which eventually will improve the overall shape fidelity of the fabricated scaffold. Filament fabricated with $CaSO_4$ pre-crosslinker showed relatively lower deviation with respect to the nozzle diameter compared to the filament fabricated with $CaCl_2$ pre-crosslinker. Therefore, filament fabricated with $CaSO_4$ pre-crosslinker showed a filament width change from 10 (A_6C_4) to 46.55% (A_4C_4) with respect to the nozzle diameter where filament fabricated with $CaCl_2$ pre-crosslinker showed a filament width change from 17% (A_4C_4) to 72% (A_2C_6). In case of compositions 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 5(b).

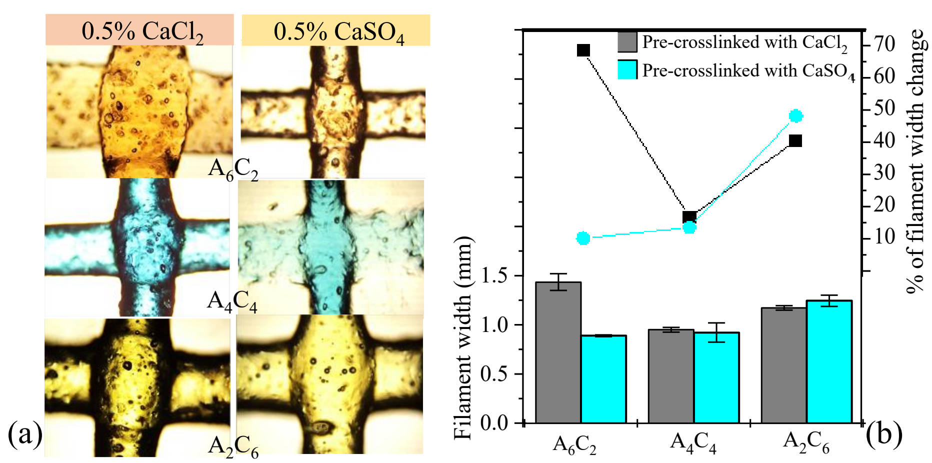


Figure 4: (a) Scaffolds and filaments fabricated with various compositions and (b) Comparison of filament width pre-crosslinked with $CaSO_4$ and $CaCl_2$.

5. Discussion

Various efforts have been reported to prepare hybrid hydrogels to achieve the proper rheological properties to help print the scaffolds to maintain the shape fidelity [23]. In our earlier work, we used medium viscous CMC into alginate to analyze printability, shape fidelity and cell viability [24]. We also reported the effect of low viscous CMC on bio-printing process [25]. In this paper, a hybrid hydrogel was prepared mixing carboxymethyl cellulose (medium molecular weight) at different weight percentages (2%, 4%, 6%) with various percentages of alginate (6%, 4%, 2%) with an overall 8% (w/v) total solid content. All three compositions were pre-crosslinked with $CaSO_4$ and $CaCl_2$ separately and therefore, we experimented with total six compositions. It was clear from the rheological standpoint that compositions having higher percentage of alginate resulted higher viscosity pre-crosslinked with either $CaSO_4$ or $CaCl_2$. Each composition pre-crosslinked with $CaCl_2$ showed higher viscosity that pre-crosslinked with $CaSO_4$. For an example, the K-value (viscosity value at 1.0 s^{-1} shear rate) of composition A_6C_2 pre-crosslinked with $CaSO_4$ was within the recommended limit of viscosity (300-100000 cps) where it surpassed the limit pre-crosslinked with $CaCl_2$. Hence, A_6C_2 pre-crosslinked with $CaSO_4$ has better chance to ensure a good shape fidelity of the 3D printed filament maintaining a good cell viability where the same composition pre-crosslinked with $CaCl_2$ can harm the encapsulated cell resulting cell death during extrusion. The K-value for other two compositions such as A_4C_4 and A_2C_6 pre-crosslinked with $CaSO_4$ were also within the recommended limit (823263 mPa.S. and 550326 mPa.S. respectively) where the same compositions surpassed (>100000 mPa.S.) the recommended viscosity limit pre-crosslinked with $CaCl_2$. Therefore, it can be concluded that all three compositions pre-crosslinked with $CaSO_4$ can be a potential bio-ink for extrusion-based bio-printing process where the same compositions pre-crosslinked with $CaCl_2$ can be used to fabricate large acellular scaffolds and seed the cell later.

High viscous hydrogels require 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 [28]. Analysis of 3D printed filament and bilayer intersection revealed a good printability, shape-holding capability, and various amounts of filament width change for all pre-crosslinked compositions. However, A_6C_2 pre-crosslinked with $CaCl_2$ required a substantial amount of applied pressure. Hence, it 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 can be considered to fabricate a full scaffold 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.

6. Conclusion

This research identified a rheological analysis of various compositions prepared with alginate and medium viscous CMC pre-crosslinked with maintaining a solid content of 8%. The effects of alginate and $CaCl_2$ and $CaSO_4$ as a pre-crosslinker on the hybrid hydrogels were determined in terms of viscosity and recovery rate. The relationship between the printability and rheological properties of the compositions were demonstrated. The illustrated rheological tests and corresponding printability of those compositions can direct the 3D bio-fabrication of the tailored anisotropic scaffolds, which will assist in future efforts to fabricate functional tissues.

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