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RHEOLOGICAL ANALYSIS OF A CELLULOSE NANOFIBRIL COMPOSITE HYDROGEL BIOINK FOR BIOPRINTING APPLICATIONS

Roger Carrick[1*], Alex Czekanski[1], Elftherios Sachlos[1]
[1]Mechanical Engineering, York University, Toronto, Canada
*carrick@yorku.ca

Abstract—Biocompatible hydrogels, or bioinks, are an essential component of 3D bioprinting technology. Bioinks must balance being able to support cells, biocompatibility, controlled degradation rates, printability as well as a host of mechanical properties. One step towards the development of a bioink is the understanding of its rheological properties. In this work we examine the properties of a gelatin-alginate-cellulose nanofibril (CNF) composite hydrogel, as well as the individual components. We found that the proposed bioink had a relatively weak structure due to the purely physical crosslinking mechanisms employed. The hydrogel did have excellent shear-thinning properties as well as a highly tunable viscosity making it a good candidate for bioprinting applications.

Keywords-rheology; bioink; 3D bioprinting

INTRODUCTION

3D bioprinting, the application of 3D printing techniques to produce living tissues, has exploded in popularity over the past decade. 3D bioprinting offers unprecedented control and repeatability in the fabrication of complex tissue structures, far surpassing the capabilities of traditional biofabrication techniques [1]. One of the key components of a bioprinting system is a cell encapsulating bioink that not only supports and transports cells, but also behaves as a temporary extracellular matrix (ECM), giving structure to the tissue as it matures. A successful bioink must be non-cytotoxic, biodegradable or bioresorbable and be able to be cross-linked. For extrusion based 3D bioprinting processes, the bioink should be shear-thinning to minimize the stress on cells during extrusion and have enough structural integrity to accurately reproduce complex geometries without collapsing[1].

Gelatin is the denatured form of collagen produced through hydrolysis. Gelatin shares many of the same properties as collagen, however it has a higher solubility in water and lower antigenicity due to being denatured [2]. The type of collagen and processing method used to produce the gelatin can lead to different properties and molecular weights [3]. Gelatin is commonly used in tissue engineering and undergoes physical crosslinking at lower temperatures.

Sodium Alginate is by far the most common natural polymer used in tissue engineering applications. Alginate one of the main structural components of marine brown algae (up to 40% by weight) and is also found in some soil bacteria. As such it is easily produced and well-studied. Alginate is commonly used to produce hydrogels through crosslinking with cations (commonly Ca^{2+}) in solution. As this method is relatively mild, it sees extensive use when looking to encapsulate cells within a hydrogel. There are many alginate hydrogel bead based products on the market for wound dressings [2]. Combining alginate and gelatin hydrogels in varying compositions is common in order to tailor the gel and degradation properties of the hydrogel [4].

Cellulose is the most common naturally occurring form of glucose polymer and can be sourced from plants (such as wood pulp or cotton) or from bacterial sources. Cellulose can form a hydrogel through entanglement of cellulose chains as well as hydrogen bonding interactions between polymer chains [5]. Additionally, Cellulose Nanofibrils have a high aspect ratio and Young's modulus (~145GPa) which makes them an excellent candidate as a reinforming material [6].

Understanding the rheological properties of hydrogels is an important step in the development of a successful bioink. In this paper we will examine the properties of a gelatin-alginate-cellulose nanofibril composite bioink as part of a broader study into the development of a 3D bioprinting process.

METHODS AND MATERIALS

A. Materials

Gelatin from porcine skin (Type A) with a gel strength of 300 and Alginic acid sodium salt (Sodium Alginate) from brown algae (medium viscosity) was purchased from Sigma-Aldrich Ltd., Canada. Phosphate Buffered Saline (PBS) 10X Liquid Concentrate was purchased from BioShop Canada Inc. and diluted to 1X using deionized water. Unmodified Cellulose Nanofibrils (CNF) (Width 10-13nm, Length 1000-3000nm) were purchased in a freeze-dried state from Cellulose Lab (Canada).

B. *Hydrogel Bioink Preparation*

Gelatin hydrogels were prepared at concentrations of 2, 4, 6, 8 and 10% (w/w). Gelatin Powder was added to PBS at 60°C and mechanically stirred for 30 minutes. Alginate was prepared at concentrations of 2, 4 and 6% (w/w) in PBS, and similarly mechanically stirred for 30 minutes at 60°C. The CNF hydrogel was prepared at concentrations of 1,2 and 3% (w/w) in PBS. The freeze-dried CNF were first added to PBS and mechanically stirred for 10 minutes. They were then homogenized using an ultrasonic homogenizer (Qsonica Q700) at 50% amplitude for 2 minutes. Composite hydrogels were prepared using the same procedures sequentially, with CNF added first, followed by Gelatin and finally Alginate. Hydrogels were stored at 4°C and prior to testing were raised to a temperature of 40°C through immersion in a water bath.

C. *Rheological Measurement*

All rheological measurements were conducted using a TA Discovery HR-3 Hybrid Rheometer equipped with 40mm parallel plates, and a gap of 1mm. Temperature was controlled using a Peltier lower plate and all tests were conducted at 25°C. The viscoelastic properties were measured using a strain sweep between 0.1% and 100% at a rate of 10rad/s. A frequency sweep was conducted 0.1 and 100rad/s at a strain of 2% to determine the gel strength. Finally, the viscosity was measured through a steady state flow test conducted at frequencies between 0.01 and 100Hz. Steady state sensing was used to determine the steady state flow characteristics with an equilibrium time of 30s and a tolerance of 5%. For all tests, the temperature of the plates started at 40°C and then was cooled to 25°C and held at that temperature for 30s to condition the samples.

RESULTS

A. *Storage and Loss Modulus*

The results of the strain sweep seen in Figure 1 show that, of the individual components, only alginate shows any strong viscoelastic properties. Gelatin displayed almost zero storage and loss modulus for any value below 10%. CNF also showed low values of G', and G'' but in comparison to gelatin and alginate, it showed higher values of loss modulus than storage modulus. Alginate showed the highest values of storage and loss, with the values increasing linearly with increasing concentration.

In the composite hydrogel formulations, the gelatin-alginate hydrogels showed viscoelastic properties greater than the simple combination of the components. Again, there appears to be a linear relationship between both the addition of gelatin and alginate. With the addition of just 2% CNF, the storage and loss modulus were almost doubled.

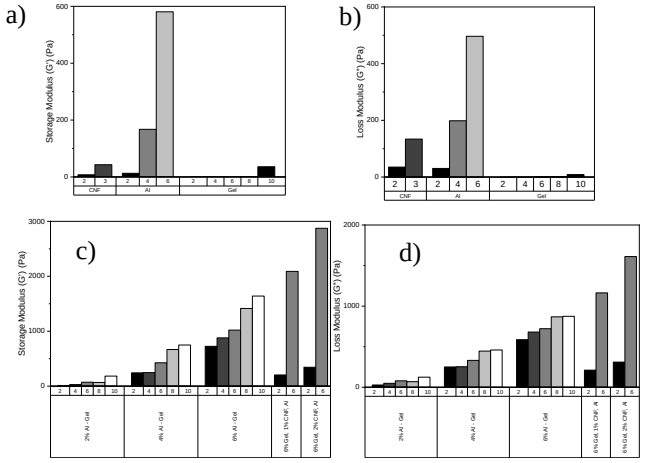

Figure 1: a) Storage (G') and b) Loss (G'') modulus for base materials (CNF, Alginate and Gelatin). C) Storage and d) Loss modulus for composite bioinks.

B. *Frequency Dependence*

Figure 2 shows the frequency dependence of the viscoelastic parameters for select single component and composite hydrogels. All hydrogels showed increasing modulus with increased frequency, with hydrogels containing higher quantities of alginate showing the greatest frequency dependence.

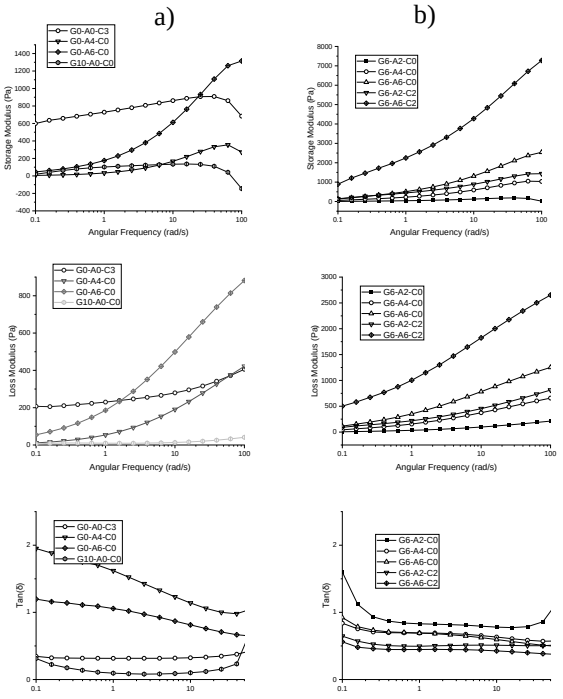

Figure 2: Storage modulus, loss modulus and tan(δ) for a) select single component hydrogels and b) select composite hydrogels

C. *Viscosity*

The viscosity measurements for the single component (Figure 3)hydrogels showed that gelatin alone had very low viscosity. Alginate and CNF hydrogels had significantly higher viscosity at low shear rates, and both showed strong shear-thinning properties at higher shear rates. In the

composite hydrogel systems, compositions with higher alginate content showed the highest viscosity and shear-thinning properties. The addition of CNF significantly amplified the viscosity while maintaining the shear-thinning behavior.

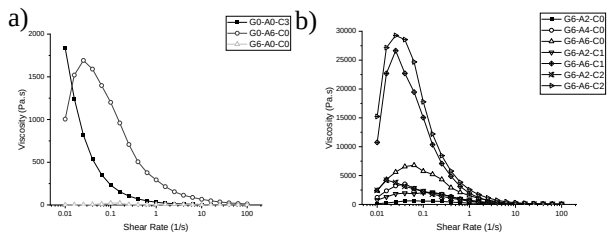

Figure 3: Viscosity vs Shear Rate for a) single component hydrogels and b) composite hydrogels

DISCUSSION

In this study we examined the rheological property of a candidate hydrogel for 3D bioprinting applications. We have shown the properties of the individual base hydrogel components, gelatin, alginate and CNF, as well as the properties of various compositions of composite hydrogels.

A. Storage and Loss Modulus

The storage modulus (G') gives us information about the structure, or elasticity of the hydrogel, with higher values representing a more structure. The loss modulus (G'') represents the ability of the material to absorb or dissipate energy. Materials that are G' dominant (G'>G'') behave more like a gel, while materials that are G'' dominant (G''>G') behave more like a fluid [7]. Gelatin had little structure or damping ability on its own given the current working temperature of 25°C. This would likely change at lower temperatures below the sol-gel transition temperature of gelatin. Alginate showed G' dominant behavior at higher concentrations (6% (w/w)) while lower concentrations showed G'' dominant behavior, or more fluid like behaviour. Finally, both concentrations of CNF were strongly G'' dominant.

In the composite hydrogels, the combination of gelatin and alginate led to all concentrations showing gel behaviour (G' dominant). The strength of the gel increased with increasing concentrations of gelatin and alginate. This suggests an interaction between gelatin and alginate polymer chains. In [8], they showed that hydrogen bonds formed between and within the carbonyl, amino and hydroxyl groups, as well as other electrostatic interactions. Similarly, the addition of CNF further increased the G' of the combinations and increased the difference between storage and loss modulus.

B. Frequency Dependence

Frequency dependence is an important rheological property of a hydrogel to understand is it gives insight into the strength of a gel. A gel with low frequency dependence has a robust 3-dimensional network. Of the individual components, gelatin showed little frequency response, but gelatin also had almost zero storage or loss modulus. Alginate, particularly at higher concentrations, showed strong frequency dependence. This suggests a weak 3D network. This is to be expected as un-crosslinked alginate will only have weak hydrogen or electrostatic interactions between polymer chains. CNF did not show as much frequency dependence suggesting a stronger 3D network, particularly at lower frequencies.

The composite hydrogels followed similar trends as the base components. Examining the ratio of G'/G'' (tan(δ)), it can be seen that for alginate only hydrogels, the loss modulus increases more rapidly than the storage modulus, while in all cases of the composite hydrogels, tan(δ) does not change with frequency. This suggests that the gelatin has a stabilizing effect on the gel. While it is not a strong 3D network, it still reduces the flow properties at higher frequencies.

Materials with a tan(δ) below 1 are more elastic (G' dominant), and the composites with higher alginate and CNF concentrations all had tan(δ)<1. Previous studies have linked a tan(δ) in the range of 0.25-0.45 as having optimal properties for printability in 3D bioprinting applications [9]. The composites containing CNF fell within that printability window suggesting that they may work well for the intended application.

C. Viscosity

Viscosity is an important parameter to understand for a potential bioink. The bioink should resist flow under stationary conditions but should flow easily under high shear rates such as those seen in a needle during extrusion. This not only reduces the pressure needed to extrude the material but also reduces the shear stress on the cells contained within the bioink. High levels of shear stress during extrusion have been linked to lower survival rates of cells [10,11]. Therefore, a bioink with shear-thinning behaviour is desired.

The tests show that alginate has a strong shear-thinning behaviour with the viscosity reducing to almost zero beyond 10Hz. CNF showed an even stronger shear thinning behaviour with the viscosity dropping to near zero at only 1Hz.

The composite hydrogels again all showed similar behaviour as their separate components. A notable point is that the viscosity of the composites containing Gelatin, Alginate and CNF had almost 6X the viscosity as the composites with the same composition without CNF while maintaining the strong shear-thinning behavior. This suggests that CNF are very potent as viscosity modifiers and that by tuning the amount of CNF we can achieve the viscosities that will best support printability.

CONCLUSIONS AND FUTURE WORK

In this work, a set of rheological experiments were conducted on a composite gelatin-alginate-CNF hydrogel as well as the individual components. The results show that there are interactions between the components can give properties greater than the sum of their components. The composite hydrogel had a relatively weak elastic structure due to the only crosslinking being due to weak physical interactions such as hydrogen bonding and electrostatic bonds. The material showed strong shear-thinning behavior and a viscosity that

could be easily tuned by modifying the composition, particularly the CNF content.

Future work will include examination of the mechanical properties of the hydrogel following ionic crosslinking of the alginate and the reinforcing properties of CNF, exploration of the properties of the hydrogel at lower temperatures, as well as a printability study to determine the ability of the hydrogel to support complex architectures.

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