Granular Gel Support-Enabled Extrusion of Three-Dimensional Alginate and Cellular Structures

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Abstract: Freeform fabrication of soft structures has been of great interest in recent years. In particular, it is viewed as a critical step toward the grand vision of organ printing - the on-demand design and fabrication of three-dimensional (3D) human organ constructs for implantation and regenerative medicine. The objective of this study is to develop a novel granular gel support material-enabled, two-step gelation-based “printing-then-gelation” approach to fabricate 3D alginate structures using filament extrusion. Specifically, a granular Carbopol microgel bath holds the ungelled alginate structure being extruded, avoiding the instantaneous gelation of each printed layer as well as resultant surface tension-induced nozzle clogging. Since Carbopol microgels react with multivalent cations, which are needed for alginate crosslinking, gelatin is introduced as a sacrificial material to make an alginate and gelatin bioink for extrusion, which gels thermally (step-one gelation) to initially stabilize the printed structure for removal from Carbopol. Then gelatin is melted and diffused away while alginate is ionically crosslinked in a 37°C calcium chloride bath (step-two gelation), resulting in an alginate structure. The proposed “printing-then-gelation” approach works for alginate structure fabrication, and it is also applicable for the printing of cellular constructs and other similar homogeneous soft structures using a two-step or even multi-step approach. The main conclusions are: 1) the 0.8% (w/v) Carbopol bath with a neutral pH value may be most suitable for soft structure printing; 2) it is most effective to use a 0.9% (w/v) NaCl solution to facilitate the removal of residual Carbopol; and 3) alginate structures fabricated using the proposed approach demonstrate better mechanical properties than those fabricated using the conventional “gelation-while-printing” approach.

Keywords: alginate, extrusion, gelatin, granular gel, three-dimensional bioprinting

1. Introduction
Freeform fabrication of soft structures has been of great interest in recent years. In particular, it is viewed as a critical step toward the grand vision of organ printing – the on-demand design and fabrication of three-dimensional (3D) human organ constructs for implantation and regenerative medicine [1-6]. The development of freeform soft structure fabrication has been stimulated by various advances in materials and manufacturing technologies. Of various advanced materials used for soft structure fabrication for biomedical applications, alginate is highly valued as a model material for its wide applicability as a versatile biomaterial [7, 8]. Specifically, alginate can be chemically or physically modified to have disparate material properties including mechanical stiffness, swelling, degradation, cell attachment, and binding or release of bioactive molecules. Therefore, it has been used as scaffold for tissue
engineering, delivery vehicle for drugs, and model extracellular matrix material for biological studies. Fabrication of alginate structures has been of great interest in the field of biofabrication [7, 9-15].

The objective of this study is to develop a novel granular gel support material-enabled, two-step gelation-based approach to fabricate 3D alginate structures using filament extrusion. Specifically, a granular Carbopol microgel bath holds the ungelled alginate structure during extrusion, avoiding surface tension-induced nozzle clogging. Since Carbopol microgels react with multivalent cations, which are needed for alginate crosslinking, gelatin is introduced as a sacrificial material to make an alginate and gelatin bioink for extrusion. The gelatin in the blended bioink gels thermally (step-one gelation) to initially stabilize the printed structure for removal from Carbopol. Then gelatin is melted and diffused away while alginate is ionically crosslinked in a 37°C calcium chloride (CaCl₂) bath (step-two gelation), resulting in an alginate structure.

2. Background and Proposed “Printing-then-Gelation” Approach

While 3D complex alginate structures have been successfully drop-on-demand (DOD) inkjet [15] and laser [16, 17] printed, unfortunately, thus far there are no reported 3D complex alginate structures using any filament-based extrusion fabrication approaches. During typical inkjet and laser printing, sodium alginate solution is ejected into a calcium chloride (CaCl₂) solution, which serves as a dual-purpose crosslinking and support material for alginate structure fabrication. Usually, their standoff distance, the distance between a dispensing orifice/location and a receiving substrate, is a few millimeters or higher for jets to form and subsequently break up. Typically, during inkjet and laser printing, each printed droplet gels immediately after deposition in order to maintain the mechanical integrity of the printed shape in a “gelation-while-printing” approach [15-19]. However, the dispensing orifice during extrusion must remain in close proximity to a receiving substrate for optimum process control. If a sodium alginate filament is directly extruded into a calcium chloride solution to be gelled and build up an alginate structure, the surface tension of the calcium chloride solution easily draws this ionic crosslinking solution toward the orifice via the filament and then clogs it. As a result, the printing and gelation speeds must be precisely matched to enable 3D printing by conventional filament extrusion.

Considering the easy implementation and fabrication efficiency advantages of filament extrusion, it is of great interest to implement extrusion for alginate structure fabrication in addition to DOD inkjet and laser printing. For extrusion to be a robust 3D alginate structure printing technique, the well-accepted “gelation-while-printing” approach should be revolutionized to avoid the surface tension-induced nozzle clogging due to the proximity of the extrusion nozzle. Herein we propose an alternative “printing-then-gelation” approach, in which a liquid alginate structure is first printed inside a fluid support material and then gelled after printing. This requires innovative support materials to effectively stabilize the liquid alginate structure during fabrication until it is ready for gelation. Ideally, such materials should easily flow when an external force higher than their yield stress is applied, but maintain their form and cease to flow when they are subjected to a relatively weak force, holding their shape under low stresses. In addition, such support materials should be able to fill any crevasses introduced by nozzle translation inside the bulk reservoir, eliminating the need for a fluid filler [20] to fill crevasses. Fortunately, granular gels such as Carbopol made from reproducible soft micro-scale particles (~ 5 µm) [21] smoothly transition between the fluid and solid states, making them ideal support materials for alginate extrusion.
Widely used in personal care products, Carbopol is a family of commercial thickening agents composed of poly(acrylic acid), \((\text{CH}_2\text{CH(\text{CO}_2\text{H})}_n\). It is strongly hydrophilic and swells by a factor of \(\sim 1000\) in water \([22]\). Each particle inside Carbopol is a separate sponge-like gel with internal crosslinks formed by polyalkenyl polyethers to prevent complete dissolution; some chains extend from the core, and their entanglement strengthens inter-particle interactions. Because of Carbopol’s optical clarity, availability, and tunable yield stress behavior, it has also been widely used to study the behavior of yield stress fluids \([23]\). For its unique jamming/unjamming transition property, Carbopol is selected in this study to explore its use for alginate structure fabrication. During extrusion, Carbopol fluidizes at the nozzle tip as it travels then rapidly solidifies, keeping extruded filaments in place until an entire structure is completed.

Since the driving force for Carbopol swelling is electrostatic, the addition of ionic species to Carbopol dispersions causes significant de-swelling and reduction in yield stress \([24]\). Moreover, divalent and trivalent cations, which are typically used for the crosslinking of alginate solutions, are reactive with Carbopol and form a precipitate, so extruded alginate structures cannot be crosslinked within Carbopol. This issue calls for a new fabrication approach to make alginate structures using Carbopol-based extrusion.

As introduced, we use a two-step gelation approach based on the reversible thermal gelation of gelatin and ionic gelation of sodium alginate as illustrated in Figure 1 to overcome this challenge. Gelatin is derived from collagen, a stiff helical protein with the repeating amino acid sequence glycine-\(X_1-X_2\) in which \(X_1\) and \(X_2\) are often proline and hydroxyproline. Three individual molecules combine into a triple helix; helices aggregate into larger fibers. Gelatin is produced by heat and chemical treatments which denature (uncoil) and fragment the protein chains, resulting in shorter chains with the same repeating sequence; although chemically similar, the fragments are no longer capable of self-assembling into well-organized fibers like native collagen due to entropic considerations. In native collagen, hydrophobic interactions and hydrogen bonding are sufficient to overcome the entropic barrier to self-assemble, but smaller molecules in gelatin have more available configurations and therefore the entropic barrier is too high for long range order. However, upon cooling, some segments in gelatin return to the triple helical conformation, forming junctions between gelatin molecules, which result in a bulk thermal hydrogel. Herein we extrude gelatin at elevated temperature (37°C), gel it at room temperature, and finally remove it at 37°C.

For the proposed Carbopol-enabled two-step gelation approach, we first prepare the bioink using sodium alginate (NaAlg) and gelatin at an elevated temperature and extrude it to fabricate a branched structure layer-by-layer inside a room temperature Carbopol granular gel bath (Figure 1a). Due to the temperature drop inside the Carbopol bath, the chains of gelatin undergo a conformational disorder-order transition and tend to recover the collagen triple-helix structure, becoming entangled and forming a stable gel. This step-one gelatin gelation is a slow thermal process. Fortunately, Carbopol maintains the shape of the printed structure until the gelatin is completely gelled at room temperature (Figure 1b). Once the gelatin is gelled, the printed alginate/gelatin structure is mechanically strong enough for us to take it out and place it in a sodium chloride (NaCl) bath to de-swell and collapse the residual granular microgels of Carbopol, which may be still adsorbed to the printed structure (Figure 1c). Finally, the clean Carbopol-free structure is immersed in an ionic crosslinking solution at 37°C. Sodium alginate consists of a family of unbranched binary copolymers of 1,4 linked \(\beta\)-D-mannuronic acid (M units) and \(\alpha\)-L-guluronic acid (G units). Depending on the source and processing method, these subunits are arranged in three types of blocks along the length of
alginate chains: M blocks, G blocks, and strictly alternating MG blocks. It undergoes gelation when it interacts with divalent ions such as Ca\(^{2+}\) or trivalent ions such as Al\(^{3+}\). The gelation occurs as such cations form interchain ionic bonds between G blocks, giving rise to a stable 3D network of calcium alginate. Meanwhile, gelatin melts and diffuses away at 37°C (Figure 1d), resulting in the final alginate structure (Figure 1e).

3. Methods
3.1. Materials Preparation
Yield-stress suspension preparation: Carbopol (ETD 2020, Lubrizol, Wickliffe, Ohio) was used as the support material during alginate extrusion. Stock Carbopol (1.0% (w/v)) was prepared by dispersing the appropriate amount of dry Carbopol powder in deionized (DI) water with continuous mixing, which was continued for a minimum of 20 minutes to ensure thorough hydration of Carbopol particles. Then, aqueous 50% sodium hydroxide (NaOH, Sigma-Aldrich, St. Louis, MO) was added to adjust the pH value to 6.0 ± 0.1, 6.5 ± 0.1, 7.0 ± 0.1, 7.5 ± 0.1, and 8.0 ± 0.1. Lower concentration Carbopol solutions (0.2%, 0.4%, 0.6%, and 0.8% (w/v)) were prepared by combining the appropriate volume of DI water with neutralized suspensions and mixing thoroughly. Carbopol suspensions were measured by weight to avoid viscosity and bubble errors; the 1.0 g/mL density of Carbopol suspensions was verified by centrifugation of a known weight in a graduated vial. Neutralized suspensions were stored at room temperature in the dark in sealed containers to prevent degradation and evaporation.

Bioink preparation: Sodium alginate (Sigma-Aldrich, St. Louis, MO) and gelatin (Type A, 300 bloom, from porcine skin, MP Biomedicals, Solon, OH) were used together as the major constituents of the bioink. After evaluating the extrudability, temperature sensitivity, and strength of printed structures, 3% (w/v) and 10% (w/v) were determined as the final concentrations of sodium alginate and gelatin, respectively. The bioink composed of alginate and gelatin was prepared by dispensing the required amount of each powder in DI water with continuous stirring at 37°C until completely dissolved.

Carbopol cleaning solution preparation: Aqueous sodium chloride (NaCl, Sigma-Aldrich, St. Louis, MO) was used to rinse Carbopol from printed structures. The NaCl solution (0.9% (w/v)) was prepared by dispensing the required amount of NaCl in DI water with continuous stirring until completely dissolved, and stored at 4°C. Dulbecco’s Modified Eagles Medium (DMEM; Sigma-Aldrich, St. Louis, MO) and 10% (w/v) sucrose were also evaluated as rinsing solutions.

Crosslinking solution preparation: Calcium chloride (CaCl\(_2\), Sigma-Aldrich, St. Louis, MO) was used to gel sodium alginate after Carbopol was rinsed away from printed structures. CaCl\(_2\) solution (2% (w/v)) was prepared by dispensing the required amount of CaCl\(_2\) in DI water with continuous stirring until completely dissolved and warmed to 37°C immediately before use in order to melt gelatin inside the printed structures during ionic crosslinking.

3.2. Carbopol Rheological Properties Measurement
Rheological properties of Carbopol baths were measured using a rheometer (ARES LS1, TA, New Castle, DE) at room temperature. The cone-plate rheometer had a plate diameter of 50 mm, a cone-to-plate gap distance of 46 μm, and a cone angle of 2.64°. Viscosity and shear stress were measured by varying the shear rate from 0.01 s\(^{-1}\) to 100 s\(^{-1}\) using a rotary testing setup. Storage modulus (G’) and loss modulus (G”) were assessed by varying the strain from 0.01% to 100% at an oscillation frequency of 10.0 rad/s using an oscillatory testing setup.
3.3. 3D Bioprinting System and Straight Tube Printing and Characterization

The extrusion system was implemented on a micro-dispensing pump machine (nScrypt-3D-450, nScrypt, Orlando, FL). The motion stages of the system were numerically controlled to coordinate the lateral $XY$ and vertical $Z$ movement of the printhead for layer-by-layer deposition. The $37 \degree C$ printing temperature was controlled using a heating tape (SRT051-020, Omega, Stamford, CT) with temperature control accuracy of $\pm 0.1 \degree C$, a temperature controller (CSi32J-C24, Omega, Stamford, CT), and a thermocouple probe (JM02SS-040U-6, Omega, Stamford, CT).

Straight tubes were extruded into Carbopol bath via a 25 gauge nozzle with an inner diameter of 250 $\mu m$. Printing conditions were pre-determined based on preliminary testing for a stable extrusion process. Specifically, the printing path speed was 1 mm/s, the printing pneumatic pressure was 15 psi, and the layer height was 200 $\mu m$. The dimensions of printed structures were measured using microscopy (EVOS XL Core, Grand Island, NY).

3.4. Carbopol Cleanup and Gelatin Removal Test

Three different biologically relevant solutions, DMEM, 0.9% (w/v) sodium chloride (NaCl) in DI water, and DI water supplemented with 10% (w/v) sucrose, were used to rinse away Carbopol particles. To investigate the rinsing efficiency using these three solutions, 10 g of 0.8% (w/v) Carbopol suspension with a pH value of 7.0 was injected into cellulose dialysis tubing (Sigma-Aldrich, St. Louis, MO); then it was immersed in a DMEM, 0.9% (w/v) NaCl, or 10% (w/v) sucrose bath (400 mL). The change of rheological properties of the Carbopol suspensions in each solution was measured after 20, 40, 60, 80, 100, and 120 minutes (one for each solution at each time point).

After the residual Carbopol was rinsed away, the thermally gelled straight tubes were immersed in a CaCl$_2$ bath at $37 \degree C$ for 0, 15, and 60 minutes, respectively, in order to crosslink alginate while removing gelatin. Then the tubes were taken out of the CaCl$_2$ bath and liquefied using 1 mL 0.055 mol/L sodium citrate solution (VWR, West Chester, PA) at $37 \degree C$. Finally, 10 $\mu$L liquefied solution was mixed with 5 $\mu$L 0.4% trypan blue stain (Sigma-Aldrich, St. Louis, MO) to observe the presence of residual gelatin using optical microscopy (EVOS XL Core, Grand Island, NY).

3.5. Tensile Test Samples Preparation and Testing

Sample for tensile testing were fabricated by three different methods using the same alginate-gelatin bioink: printing-then-gelation (proposed), gelation-while-printing (conventional), and casting. The proposed “printing-then-gelation” approach is described in the manuscript. When preparing the “gelation-while-printing” samples, an ice plate was used to maintain the Carbopol bath at low temperature. The extruded bioink filament was thermally gelled before an adjacent filament was deposited. All other printing conditions and fabrication procedures were the same as those during the proposed “printing-then-gelation” approach. The cast samples were made by pouring the bioink into a polydimethylsiloxane (PDMS) mold and keeping it at room temperature for 1 hour for the thermal gelation of gelatin. Then the cast samples were taken out of the PDMS mold and immersed in a 2% (w/v) CaCl$_2$ solution at $37 \degree C$ for 24 hours.

The Young’s modulus and fracture strength of fabricated samples were determined using tensile testing. Three samples made by each fabrication process were tested using an eXpert 4000 micro test system (Admet, Norwood, MA, USA) at room temperature. Samples were
tested to failure at a constant cross-head speed of 0.02 mm/s. The stress-strain curve was determined based on the load and displacement data and the sample geometry.

4. Fabrication Results and Discussion

4.1. Effect of Carbopol Preparation on Printing Quality

The effect of Carbopol bath preparation on the printing quality is first characterized for better alginate structure fabrication. The yield stress and storage and loss moduli of Carbopol baths are measured and presented as functions of concentration (0.2%, 0.4%, 0.6%, 0.8% and 1.0% (w/v)) and pH value (6.0, 6.5, 7.0, 7.5 and 8.0) (Figure 2a, 2b, and 2c). The error bars indicate plus and minus one-sigma standard deviations (±1σ) in this study. Because the Carbopol polymer is acidic, dispersions in pure water have a pH value of around 3, and the individual particles each occupy a relatively small volume. In addition, Carbopol undergoes a sol-gel transformation in aqueous solution as the pH value of its bath is about or above 5.5, so only Carbopol baths with a pH value equal to or higher than 6.0 are studied. Generally speaking, as the dispersion is neutralized by the addition of base, carboxylic acid groups along the Carbopol polymer chains are deprotonated, leaving anionic carboxylate groups in the polymer; the electrostatic repulsion between these sites causes extensive swelling.

Herein the printing quality is investigated based on the wall thickness of printed straight alginate/gelatin tubes, which are designed to have a mean diameter (defined by the dispensing nozzle center) of 4 mm and a height of 4 mm. The bioink, made of 3% (w/v) alginate and 10% (w/v) gelatin, is deposited in Carbopol baths with different concentrations and pH values. The presented wall thickness information (Figure 2d and 2e) is based on the average of three independent measurements before thermal gelation. It is found that the wall thickness decreases significantly when the Carbopol concentration increases from 0.2% to 0.8% and then stabilizes (Figure 2d). In addition, burrs are observed at the tube outer surface during printing inside low concentration baths. These observations are attributed to the mechanical and rheological properties of Carbopol baths. At low concentrations, Carbopol baths have a lower yield stress and lower storage and loss moduli as seen from Figure 2a, 2b, and 2c. Moreover, with the increase of concentration, the volume fraction of the solid phase is found to increase at the expense of that of the liquid phase [25], resulting in a stiffer fluid. As such, Carbopol baths with a high concentration are more desirable to hold the shape of printed structures with a better feature resolution such as a smaller wall thickness. However, it is further noted that it becomes difficult to clean structures printed inside a higher concentration Carbopol bath. To balance these two effects, the 0.8% (w/v) Carbopol bath is selected as the support fluid in this study. At a given concentration such as 0.8% (w/v), the wall thickness increases gradually with the increase of pH value (Figure 2e). This is attributed to the decreasing yield stress (Figure 2a) as well as the reduction of both storage and loss moduli (Figure 2b and 2c), resulting in less resistance to the swelling of printed structures. For future bioprinting and tissue engineering applications, a Carbopol bath with a neutral pH value is favored even though a lower pH value bath may enable better printing resolution.

4.2. Dimension variation of printed alginate/gelatin tubes

After thermal gelation at room temperature for 15 minutes, the dimension variation of printed alginate/gelatin tubes is investigated during storage inside a Carbopol bath at 4ºC (Figure 3a). The wall thickness is measured every 12 hours for 3 days. During the three-day observation, the wall thickness increases initially during the first 12 hours and then shrinks gradually inside Carbopol baths with different concentrations and pH values (Figure 3b and 3c). The observed general trend is the result of two main competing effects: water diffuses into printed tubes under an osmotic pressure, which can be described using Darcy’s law, and alginate
polyanions gradually diffuse out of the tubes to the surrounding Carbopol bath, which can be captured using Fick’s second law. For our system, the characteristic timescales for both processes are estimated as follows.

4.2.1. Water permeation into printed tubes
According to the Morse equation, the osmotic pressure can be determined as: \( \Pi = RTC \), where \( R \) is the gas constant, \( T \) is the thermodynamic temperature, and \( C \) is the molar concentration. Thus, \( \Pi \) is determined as 13,000 Pa for a typical molar concentration value of \( 5.6 \times 10^{-3} \) mol/L based on the molecular weight and concentration of gelatin.

According to Darcy’s law, the permeation flow rate \( Q = \frac{-KAP}{\mu L} \), where \( K \) is the permeability \((1.4 \times 10^{-18} \ m^2) \) [26], \( A \) is the cross-sectional area \((3.6 \times 10^{-6} \ m^2)\), \( \mu \) is the viscosity of water \((10^{-3} \ Pa \cdot s)\), and \( L \) is the distance from the surface to the center of tube wall \((5 \times 10^{-4} \ m)\). Then the diffusion time of water permeating into a printed tube is approximated based on the tube volumetric change and flow rate as follows: \( t = \frac{\Delta V}{Q} \), where \( \Delta V \) is the tube volumetric change between two observations \((5.4 \times 10^{-9} \ m^3\), right after fabrication and after 12 hours). Assuming \( \Pi = 13,000 \) Pa and \( K = 1.4 \times 10^{-18} \ m^2 \) (0.0014 mD), \( t = \frac{5.4 \times 10^{-9}}{93,600 \times 0.014 \times 10^{-16}} = 41,209 \text{s} = 11.4 \text{ hours} \).

4.2.2. Alginate diffusion out of printed tubes
According to Fick’s second law of diffusion: \( \frac{\partial C}{\partial t} = D_e \frac{\partial^2 C}{\partial x^2} \), where \( C = C_{(x,t)} \) is the alginate concentration at length \( x \) from the surface at time \( t \), and \( D_e \) is the effective diffusion coefficient, the unidirectional diffusion can be expressed as: \( C_{(x,t)} = C_0 \left[ 1 - \text{erf} \left( \frac{x}{2\sqrt{D_t}t} \right) \right] \), where \( \text{erf}(\cdot) \) is the error function. Under the conditions of \( x = 4 \times 10^{-4} \ m\), \( C_{(x,t)} = 3\% \times \frac{0.08}{3} = 0.08\% \), \( C_0 = 3\% \), and \( D_e = 10^{-13} \ m^2/s \) [27], the diffusion time is estimated as \( t = 16,000 \text{s} = 45.4 \text{ hours} \).

Based on the above estimates, water diffusion is the dominant fast (11.4 hours) process which drives initial swelling, while the slower (45.5 hours) process of polyanion diffusion results in slow de-swelling, which explains the observations shown in Figure 3b and 3c. After 12 hours, the water diffusion effect vanishes while the polyanion diffusion effect sustains, resulting in a shrinking structure.

Specifically, Figure 3b shows that the tube wall is always thicker during three-day observation when using lower concentration Carbopol baths. At low concentrations, Carbopol particles swell to their maximum capacity, which is limited only by the internal crosslink density, and may not absorb all water in the bath. Therefore, a significant portion of water is free to diffuse into printed tubes and makes them swell. This trend weakens with higher Carbopol concentrations as the proportion of free or weakly bound water approaches zero, which is consistent with previous microrheology data [25]. That is, as the concentration
increases, the swelling of each Carbopol particle is limited by the amount of water present, rather than by its crosslinks, which prevent its complete dissolution; since all water is tightly bound by highly concentrated Carbopol particles, very minimal initial swelling of printed tubes is observed. The tube wall shrinks over time since surrounding Carbopol particles draw water out of printed tubes, indicating that the osmotic pressure in the Carbopol bath is actually higher than that inside printed tubes.

Figure 3c shows the effect of pH value on the wall thickness over time. At the two lowest pH values (6.0 and 6.5), Carbopol particles are more swollen, binding water tightly and resulting in a higher yield stress than at neutral or higher pH values. As a result, the wall thickness is smaller and decreases more strongly at the pH value of 6.0 and 6.5 than at higher pH values since the Carbopol bath may not have available free water to allow swelling, and instead draws water out of printed structures over time. In addition, gelatin tends to be more soluble at low pH values and may diffuse away from printed structures along with alginate, which would explain the greater magnitude of wall shrinkage: 14.1% shrinkage at pH = 6.0 versus 8.7% shrinkage at pH = 8.0 after three days. At and above neutral pH values, however, the yield stress drops, and the swelling effect stabilizes to nearly identical behavior at pH values from 7.0 to 8.0. This indicates that the balance of osmotic pressure between the Carbopol bath and printed structures is relatively insensitive to the pH range from 7.0 to 8.0. It is likely because Carbopol particles are already fully swollen and have only very slight differences in ionic character over this range. The dimensions of printed structures remain more stable because the gelatin is less soluble in this pH range; hence, there is a relatively small change in the wall thickness. For the remainder of this study, printed structures are only thermally gelled for 15 minutes to minimize dimension variation.

4.3. Post-Fabrication Carbopol Removal
Before the ionic crosslinking of alginate within printed structures using a calcium chloride solution, residual Carbopol particles should be removed to avoid Carbopol precipitation during ionic crosslinking. Three different biologically relevant solutions, Dulbecco’s Modified Eagles Medium (DMEM), 0.9% (w/v) sodium chloride (NaCl) in DI water, and 10% (w/v) sucrose in DI water are tested to facilitate the removal of Carbopol particles. As shown in Figure 4a and 4b, we find that both the yield stress and zero-shear-rate viscosity of the 0.8% (w/v) neutral pH Carbopol suspension decrease significantly in the DMEM and NaCl solutions after immersing the Carbopol bath in all the three solutions for the same soaking time. The rate of strength and viscosity decrease inside the NaCl solution is even faster than that in the DMEM solution. In contrast, the yield stress and zero-shear-rate viscosity of the 0.8% (w/v) Carbopol bath in the sucrose solution only decrease slightly after immersion for two hours. Carbopol can be viewed as a suspension of swollen microgels, and its swelling is a result of electrostatic interactions and therefore sensitive to the ionic character of suspending media. Since sucrose is a non-ionic solute, it has little impact on the rheological properties of Carbopol suspensions. Physiological saline solutions such as the NaCl solution, however, have a strong influence on the rheological properties of Carbopol suspensions. Electrostatic repulsions within Carbopol microgels in the starting suspension cause Carbopol microgels to swell. Additional ions from added NaCl effectively shield the electrostatic repulsions, causing Carbopol microgels to shrink, and the suspension becomes more fluidic, as indicated by the decrease in yield stress and viscosity. Because the DMEM solution contains non-ionic components as well as salts to achieve the same osmotic strength as physiological saline, the physiological saline solution has a slightly higher ionic strength than DMEM. Therefore, it is most effective to use the 0.9% (w/v) NaCl solution to induce the
fluid-like behavior of Carbopol and rinse residual Carbopol away before the ionic crosslinking of printed structures.

4.4. Gelatin Removal and Alginate Gelation
Printed structures are further crosslinked by being immersed in a 2% (w/v) calcium solution at 37°C for 15 minutes, resulting in alginate-based tubular structures. Gelatin melts and is largely removed in the 37°C bath as tested using a trypan blue dye at 0, 15, and 60 minutes without any mechanical agitation (Figure 5a, 5b, and 5c). After 15 minutes, most gelatin is effectively removed. Since gelatin is biodegradable and beneficial to keep inside most biological tissue structures, we only immerse printed structures in the calcium chloride solution for 15 minutes for ionic crosslinking. The immersing time can be longer depending on the tolerance of gelatin and the geometrical complexity of printed structures.

We further investigate the mechanical properties of alginate samples with the dimensions shown in Figure 6a prepared using different fabrication approaches. As tested, the alginate structures fabricated using the proposed “printing-then-gelation” approach have an intermediate Young’s modulus value of 54.6 kPa, which is much higher than those extruded using a conventional “gelation-while-printing” approach (31.4 kPa), but slightly lower than those prepared using casting (65.1 kPa) (Figure 6b). Similarly, their fracture strength follows the same trend as: 127.1, 116.5, and 85.6 kPa for samples fabricated using the casting, “printing-then-gelation”, and “gelation-while-printing” approaches, respectively (Figure 6c), demonstrating the good mechanical properties of alginate structures fabricated using the “printing-then-gelation” approach.

4.5. Representative Fabrication Result
To further demonstrate the applicability of the proposed two-step gelation fabrication approach, Y-shaped tubular structures, as a simplified basic unit of vascular constructs [15, 28], are designed and fabricated using a bioink consisting of 3% (w/v) alginate and 10% (w/v) gelatin under the same printing conditions as for straight tube printing. As aforementioned, the 0.8% (w/v) Carbopol bath was adjusted to neutral pH for fabrication of biologically relevant structures. The base portion of the Y-shaped structures has a mean diameter of 4 mm and a height of 4 mm, and the bifurcated portions have an inclination of 45 degrees, a mean diameter projected along the horizontal plane of 4 mm, and a height of 4 mm. The total height of the Y-shaped structures is around 8 mm, and the mean wall thickness is around 1 mm. The printing trajectories for the base, junction, and bifurcated portions as well as the printing procedure are illustrated in Figure 7a. During the printing of the bifurcated portion, the connecting dashed line of the printing trajectory indicates that the pneumatic pump is turned off when the dispensing nozzle moves from one branch to the other. After immersion in the NaCl bath for 15 minutes, printed tubes are tested for Carbopol removal inside their lumen by flowing a DMEM solution supplemented with red dye for better visibility through the alginate/gelatin structure. As seen from Figure 7b, Carbopol microgels have been effectively removed from the lumen. Figure 7c shows a printed Y-shaped alginate structure from different angles, demonstrating the effectiveness of printing 3D alginate structures using the proposed two-step gelation approach.

5. Application to Cell Printing
The proposed “printing-then-gelation” approach is further applied for the printing of cell-laden straight and Y-shaped tubular constructs using NIH 3T3 murine fibroblast-based bioink containing alginate and gelatin. Cell suspension was prepared per the protocol described in previous studies [15, 17] and mixed with an alginate and gelatin blend solution. The final
bioink contained 3% (w/v) alginate, 10% (w/v) gelatin, and $5 \times 10^6$ cells/mL cells and printed with the following printing conditions: the printing path speed was 1 mm/s, the printing pressure was 15 psi, and the layer height was 200 μm.

Figure 8(a) illustrates a printed cellular tube with top and front views. Cell viability in printed straight tubes was tested with unprinted bioink as the control group. In addition to cellular tubes tested immediately after printing, some printed cellular constructs were incubated for 1, 2, and 3 days at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS; HyClone, Thermo Fisher Scientific, Waltham, MA) base medium supplemented with a 1% antibiotic/antimycotic solution (Sigma-Aldrich, St. Louis, MO). For viability testing, the printed and/or incubated tubes were liquefied using aqueous 0.055M sodium citrate (VWR, West Chester, PA); 30 μL of the liquefied suspension was mixed with fluorescein diacetate (FDA, Sigma, St. Louis, MO) to a concentration of 10 μg/mL and incubated at room temperature for 5 minutes. Stained green live cells were counted under a fluorescent microscope (470 nm excitation, 525 nm emission, EVOS FL, Grand Island, NY); total cells were counted using the transmitted light channel. The control group was also incubated and tested following the same protocol.

Cell viability data is shown in Figure 8b with plus/minus one standard deviation (±1σ) error bars. It can be seen that most cells can adequately survive the proposed fabrication process with a cell viability higher than 80% under representative printing conditions, proving the potential of the proposed “printing-then-gelation” approach as a promising biofabrication technique. The cell death is attributed to pressure-induced damage [29] during extrusion as well as alginate gelation-induced injury [30]. The post-printing cell viability slightly increases from 82.5% to 86.1%, which is attributed to reversible cell injury during printing [11, 31] and possible proliferation.

6. Conclusions and Future Work
This study introduces a novel granular gel support material-enabled, two-step gelation-based approach to fabricate 3D alginate structures using filament extrusion, which is a “printing-then-gelation” approach. By using a granular Carbopol microgel bath to hold the ungelled alginate structure being extruded, instantaneous gelation of each printed layer is avoided, eliminating the potential interfacial heterogeneity or discontinuity between two sequentially deposited layers. As demonstrated, the proposed “printing-then-gelation” approach works for alginate structure fabrication, and it is also applicable for the printing of cellular constructs and other similar homogeneous soft structures using a two-step or even multi-step approach. The main conclusions are as follows: 1) the 0.8% (w/v) Carbopol bath with a neutral pH value may be most suitable for soft structure printing; 2) it is most effective to use a 0.9% (w/v) NaCl solution to facilitate the removal of residual Carbopol; and 3) alginate structures fabricated using the proposed approach demonstrate better mechanical properties than those fabricated using the “gelation-while-printing” approach.

For most soft structure fabrication applications, the printing precision is not of great concern. If there is a high-precision requirement for printed structures, possible structural deformation and swelling during and after printing should be carefully compensated. Furthermore, future work may include: 1) determination of achievable shape and feature size using the proposed printing approach, 2) development of more versatile yield stress support fluids to expand the application of the “printing-then-gelation” approach, and 3) printing of multicellular constructs and evaluation of their post-printing performance including hydrogel degradation,
tissue fusion, and relevant biological and mechanical characterization under *in vitro* and *in vivo* conditions, in particular, for vascular tissue substitution.

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