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Abstract

Three-dimensional bioprinting is a tissue engineering and regenerative medicine technique that utilizes biomaterials to print clinically relevant scaffolds. These scaffolds are used for tissue repair and to treat disease, such as end-organ failure. The main problem with 3D bioprinting is its inability to print soft biomaterials, which collapse due to gravitational forces. In addition, the materials that can be used do not mimic native tissue's mechanical properties and texture. To combat this, Dr Feinberg's laboratory at Carnegie Mellon University developed the freeform reversible embedding of suspended hydrogels (FRESH) method. The FRESH method allows for soft biomaterials to be supported in a gelatin bath during printing and then crosslinked for stability.

The goal of this study was to adapt and apply the FRESH method to print 3D alginate structures on an Allevi 1 bioprinter. Alginate is a natural, biocompatible polymer that has a similar structure to living tissue extracellular matrix. It currently is used in multiple biomedical applications including tissue engineering, wound healing, and drug delivery. Alginate, however, is a soft biomaterial and needs to be crosslinked with divalent cations to form stable structures. With the FRESH method, we successfully printed lattice and cylindrical structures using alginate. We demonstrated that the FRESH method on Allevi 1 is viable and robust and can be used as part of a teaching laboratory in the Department of Engineering to introduce students to bioprinting. A future direction we are pursuing is to incorporate bioprinting into research using other biomaterials, such as collagen.

Introduction

All tissues contain an Extracellular Matrix (ECM) which acts as a scaffold to support cells biochemically and biomechanically (1). Due to its importance, tissue engineers utilize biocompatible

natural or synthetic components called biomaterials that model native tissue ECM to augment or replace function and to facilitate healing for tissue repair (2).

One application for biomaterials is in three-dimensional (3D) bioprinting. This tissue engineering and regenerative medicine technique utilizes a bioink often containing cells, biomaterials, and/or crosslinkers to print clinically relevant, patient specific scaffolds to be used for tissue repair and to treat disease, such as end-organ failure (3, 4). One issue with 3D bioprinting for tissue application, however, is the difficulty in printing soft biomaterials, which collapse due to gravitational forces (4). This is an issue because soft biomaterials more closely mimic native tissues mechanical properties and extracellular matrix (4).

One soft biomaterial of interest for bioprinting is alginate. Alginate is a natural, biocompatible polymer that has a similar structure to living tissue extracellular matrix (5). It currently is used in multiple biomedical applications including tissue engineering, wound healing, and drug delivery (5). It, however, needs to be crosslinked with divalent cations to form stable hydrogels, and thus is difficult to bioprint directly (5).

To combat this problem, Dr Feinberg's laboratory at Carnegie Mellon University developed the freeform reversible embedding of suspended hydrogels (FRESH) method. The FRESH method allows for soft biomaterials to be supported in a gelatin bath during printing and then crosslinked for stability (4). We aim to adapt and apply this FRESH method to print 3D alginate structures on an Allevi 1 bioprinter.

Methods

The Allevi FRESH method protocol was adapted and utilized for this study

Gelatin Slurry Preparation: The gelatin slurry was composed of 4% gelatin and 0.16% calcium chloride ($CaCl_2$). Gelatin was dissolved in 250 mL first at 55° C on a stir plate. Then the solution was cooled to 45° C to dissolve the $CaCl_2$ (Figure 1A). Once dissolved the solution was placed in 20° C overnight to gel. During this time, 1 L of 0.16% $CaCl_2$ was made at a temperature of 45° C on a stir plate then placed in 20° C overnight.

After 24 hours, 250 mL of 0.16% $CaCl_2$ was added to the gelled solution and placed in 4° C for 1hr (Figure 1B). This step created ice crystals to help prevent the solution from overheating during the blending process. Once ice crystals formed, the gelatin slurry was blended using "pulse" for 1 minute (Figure 1C). The slurry's consistency was then evaluated using a pipette and aliquoted into 50mL tubes for centrifuge (Figure 1C). The 50mL tubes were centrifuged 3 times, each time the supernatant was extracted, and the gelatin was resuspended in 0.16% $CaCl_2$ (Figure 1D). This step removed any soluble gelatin in the slurry. After the last centrifuge, the gelatin was not resuspended, but dislodged and placed in a petri dish for printing (Figure 1E). Two Kimwipes were then placed on top of the petri dish to soak up remaining liquid.

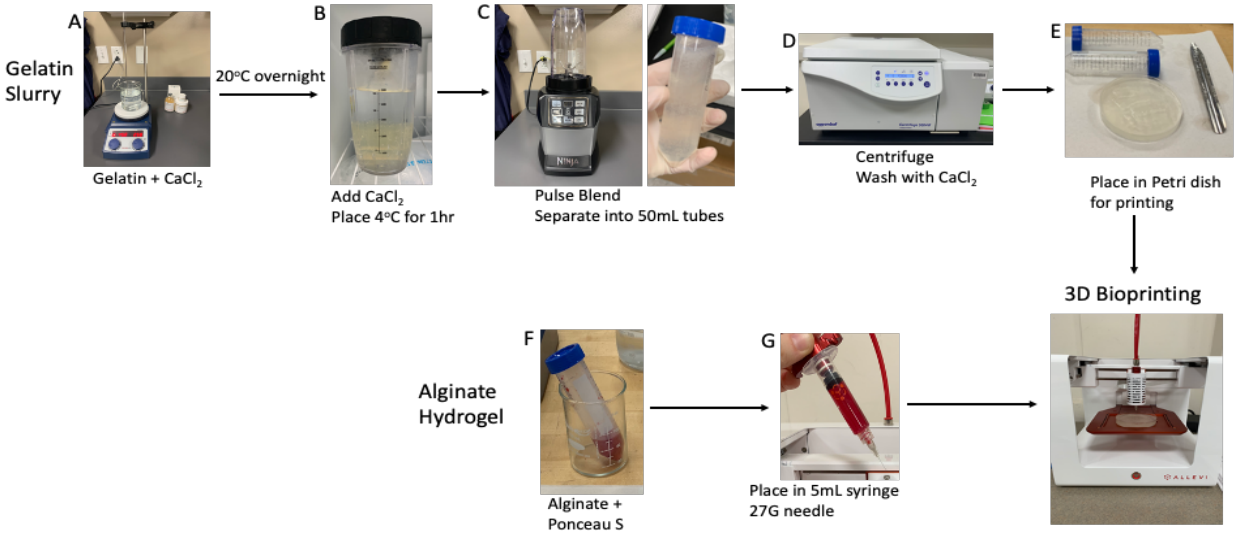

Figure 1: An overview of the preparation for the support bath, gelatin slurry (A, B, C, D, E). Gelatin and $CaCl_2$ are combined and stored overnight at 20° C to gel (A). Addition $CaCl_2$ is added, and the solution is placed in 4° C for 1hr (B). The solution is then blended into a slurry and into 50mL tubes (C). The slurry is next centrifuged 3 times and placed in a petri dish for printing (D, E). An overview of the preparation of the alginate biomaterial hydrogel (F, G). A combination of alginate and a stain are mixed at 60° C (F). Once cooled the mixture is placed in a syringe with a 1 in 27G needle for printing (G).

Alginate Preparation: 2% Alginate combined with a red Ponceaus S dye or black India Ink was dissolved and vortexed at 60° C (Figure 1F). Once cooled, the hydrogel was placed in a syringe with a 1inch 27G needle for printing (Figure 1G).

3D Structures: 3D cylindrical, lattice, and BME shaped structures were designed using Autodesk Fusion 360. Then they were exported as .stl files and uploaded to the Allevi software for printing.

Print Set Up: The Allevi 1 Bioprinter and an air compressor was used for printing. The printer was first auto-calibrated to set the X and Y directions and manually calibrated to offset the Z direction by 1mm, so the print was suspended in the slurry while printing. After calibration, the .stl file was uploaded to the Allevi software. Layer height for the print was set to 0.15mm, speed 6mm/s, and zigzag infill 1mm. Printer parameters were next set to 25 psi and 25^{o} C for pressure and temperature, respectively. After the alginate was placed in the extruder and the petri dish with slurry was placed on the print plate, the printing process was initialized.

Crosslinking: During printing, 250mL of 1% $CaCl_2$ was made at a temperature of 45^{o} C on a stir plate. After printing, the petri dish was placed in a glass container and the 1% $CaCl_2$ was poured into the container to crosslink the alginate and dissolve the slurry. The temperature of the solution was at 45^{o} C to assure the slurry did not dissolve before the alginate stabilized. The solution was also poured to the side of the petri dish, so the print was not disturbed before crosslinking. The alginate crosslinked in this solution for about 2 hours.

Results and Discussion

Bioprinting with no Support Bath: Printing into a support bath adds an additional complexity to the bioprinting process and so we initiated our studies by assessing the print quality of alginate and gelatin alone. 3D cylindrical shaped structures were designed using Autodesk fusion 360 and printed using 10% gelatin and 2% alginate to determine if intact, hollow cylinders could be obtained. As shown in figure 2, the 10% gelatin structure displays an intact hollow structure (A). On the other hand, 2% alginate, a softer biomaterial, formed beads instead of forming a cylindrical structure (Figure 2B). This demonstrates the need for a support material to print soft biomaterials during the printing process.

Bioprinting with the FRESH method: 3D cylindrical, lattice, and BME shaped structures were designed using Autodesk Fusion 360 then printed using the FRESH method and an Allevi 1 Bioprinter. Figure 2 displays the printing of the BME, cylindrical, and lattice structures into a gelatin support bath (C, D, E). The beading did not occur, as it did without the support bath indicating that the FRESH method was beneficial. Following printing, the plate was placed in a heated calcium chloride bath to simultaneously cross-link the alginate and dissolve the FRESH support material (Figure 2 F, G, H). These demonstrate that 1% $CaCl_2$ at 45° C can be used to crosslink and stabilize the alginate structures while dissolving the gelatin support bath.

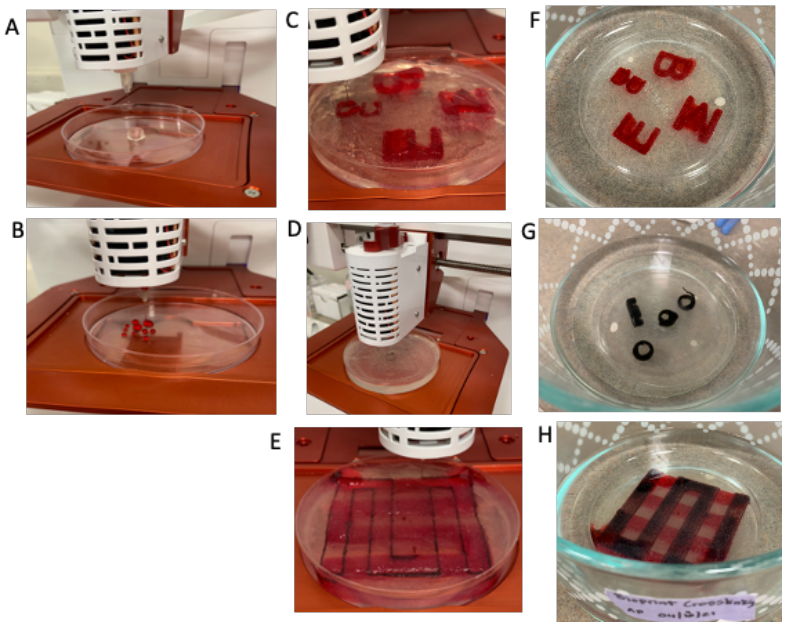

Figure 2: Bioprinting with no support bath (A, B). 10% gelatin was bioprinted into a cylindrical structure using an Allevi 1 bioprinter (A). 2% Alginate, a soft biomaterial, was bioprinted into a cylindrical structure (B). Bioprinting alginate BME, cylindrical and lattice structures using the FRESH method (C, D, E). These structures were then crosslinked using $CaCl_2$ (F, G, H).

Pressure Investigation: Although our layer height was set to 0.15mm, we were only able to print about 9 layers before the print height exceeded the support bath height of 15mm. This problem is shown in figure 3 with the Letter E print where a layer of alginate hydrogel on top of the slurry is evident (A). This could indicate the pressure was too high causing over extrusion of the biomaterial. Thus, we next investigated the pressure print parameter. We did this by printing 3 layers of a rectangular alginate structure at varying

pressures (25 psi, 20 psi, 15 psi, 10 psi, and 5 psi). All other print parameters remained the same (zigzag 1mm infill, 25° C temperature, 6mm/s speed, 0.15mm layer height). By lowering the pressure, the bioprinting produced a more defined and precise structure (Figure 3B). We next printed DU letter shaped structures using a pressure of 10 psi. This structure was able to print more layers and did not contain the extra layer of alginate on top of the slurry (Figure 3C).

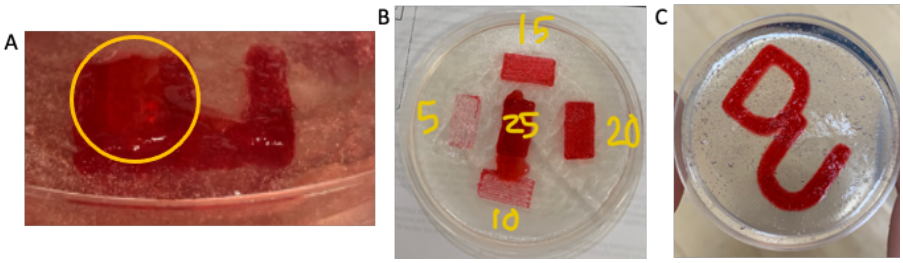

Figure 3: Letter E structure printed at a pressure of 25 psi with a layer of Alginate on top of the slurry (A). 5 rectangular structures were printed at varying pressures in psi with the indicated pressure in yellow writing (B). A pressure of 10 psi was utilized to print a DU structure to demonstrate the ability to print more than 9 layers (C).

Conclusion

Through this study, we demonstrated that soft biomaterials were difficult to bioprint without a gelatin support bath, and that the FRESH method on Allevi 1 was a viable way to print soft biomaterials. We conclude that bioprinting using the FRESH method can be adapted and used as a teaching technique within the Department of Engineering to introduce students to bioprinting due to its promising advantages in the tissue engineering and regenerative medicine field.

Future directions focus on exploring key ideas that may impact scaffold composition and bioprinting technique to expand application possibility. One future direction includes printing a more diverse variety of structures to demonstrate the techniques versatility. Another direction is to incorporate bioprinting into research using other biomaterials, such as collagen, and to investigate how printed scaffolds interact with mammalian cells. Lastly, we plan to determine how composition and printing parameters impact key tissue engineering parameters like mechanical strength and degradation rate.

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