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Optimization of vascularization-inducing hydrogel bioinks for 3D printing

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Disclaimer

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ABSTRACT

This study seeks to validate the reproducibility of previous bioprinting work at Lawrence Livermore National Laboratory (LLNL) on a new Aerotech motion controller system and to modify an existing bioink, fibrin, by adding varying percent volumes of hyaluronic acid (HA). Endothelial and fibroblast cells bioprinted in fibrin gels using the Aerotech system were confirmed to be more than 77 percent viable after one day, and all bioprinted samples retained sterility after one week of culture. To characterize cell behavior in fibrin with HA addition, static co-culture gels with varying percent volumes of HA were cultured *in vitro* for one week. Resulting confocal microscope images showed increased cell network formation in all concentrations of HA compared to the control (no HA), and rheological tests mimicking static gel compositions displayed positive correlations between gelation time, gel stiffness (G'), and hyaluronic acid concentration. Although the current data is insufficient to quantitatively associate HA concentration with the level of cell vascularization, future work will aim to develop a targeted HA concentration in fibrin for maximum cell network formation, to optimize the printing process parameters for this new bioink composition, and to analyze cell viability in bioprinted fibrin-HA structures.

INTRODUCTION AND BACKGROUND

What is 3D bioprinting?

Three-dimensional printing (3DP) is the bottom-up approach to manufacturing that builds structures layer by layer, allowing for significant user customizability and geometric complexity. What makes 3D bioprinting**,** a subcategory of 3DP, unique is the material used for printing. Rather than using plastics or metals, as is common in many 3DP processes, bioprinting utilizes bioinks – fluidic materials with biological constituents, such as cells – to print 3D structures in a layer-by-layer fashion. It is important to note, however, that although bioprinted structures may be geometrically complex, bioinks merely serve as scaffolds onto which co-printed cells are able to attach and proliferate. The vasculature that forms after printing is entirely cell-specific and uncontrolled; that is, bioinks provide a suitable *environment* for cells to build their own vasculature, but the printing process and engineered structures themselves do not in any way *manufacture* the cell vascular networks. It is hoped that, by allowing cells the undisturbed freedom to grow and vascularize, the bioprinted structures as a whole will more closely mimic and model the natural behavior of tissue constructs in the human body.

Bioinks are first and foremost biocompatible materials, as they must be co-printed with cells and ensure cell viability and growth after printing. The common bioinks used for 3D bioprinting – namely, collagen, gelatin, alginate, hyaluronic acid, and fibrin – are hydrogels, or gels in which the liquid component is water. These hydrogels are typically extruded in liquid form and polymerized through chemical or ionic cross-linking after printing, which minimizes clogging in the dispensing tip and fluidic lines.[3] An important parameter to consider in printing hydrogels is the storage modulus (G'), which is the elastic component of the viscoelastic modulus (G*) that essentially measures the stiffness of a gel. A gel with a G' value that is too high will hinder the growth of surrounding cells, as the stiff environment makes it difficult for cells to branch out and proliferate. A G' value that is higher than the acceptable

threshold, experimentally found to be in the vicinity of 200 Pascals[1], can also limit the efficiency of nutrient absorption, as mass transport becomes increasingly difficult with stiffer gels. On the other hand, a gel with a G' value that is too small will lack mechanical integrity and break down prematurely. The optimal range for the storage modulus has been found to be from 100 to 200 Pa[1].

The bioinks used in this study are hydrogels that are mixed with and co-printed with endothelial (umbilical cord) and fibroblast (lung) cells. The hydrogel components of these bioinks include alginate, a polysaccharide derived from brown algae, hyaluronic acid (HA), a polymer associated with tissue repair, and fibrin, a natural blood-clotting agent formed from the protein-enzyme interaction of fibrinogen and thrombin in the event of tissue damage. Because of the mechanically stable nature of fibrin, after bioprinting, the gel matrix acts as a temporary scaffold onto which cells are able to attach, grow, and form unique vascular networks. Alginate, with its rather viscous viscoelastic properties, forms a structurally stable gel when bioprinted alone, although ionic cross-linking through immersion in calcium chloride further stiffens and increases its mechanical integrity. Hyaluronic acid, however, does *not* form a structurally stable standalone gel, but because of its role in tissue regeneration, its effect on cell vascularization is of prime interest. Thus, this study attempts to combine hyaluronic acid with fibrin to develop a novel bioink that is both mechanically robust and conducive to cell vascularization.

3D bioprinting challenges

There are several challenges associated with bioprinting that affect the quality of printing. For one, because bioinks polymerize over time at room temperature, clogging at the dispensing tip and within fluidic lines is a roadblock that sets a time limit for the bioink loading and printing processes. Accidentally introducing air bubbles into peristaltic tubing lines can cause sputters and gaps in pre-coded print patterns, whereas back pressure within the fluidic lines can cause unintentional splashes onto the printing substrate, known as satellite droplets. The biggest challenge, however, in bioprinting with cells is ensuring cell viability. The viscous nature of hydrogel bioinks adds the caveat of fluidic shearing at the extrusion tips – a stressful experience for cells, even with the layer of protection provided by the surrounding gel matrix.

Despite these challenges, however, reliable bioprinting can be and has been achieved with an optimized bioprinting procedure and adaptable process control parameters, which are discussed in detail in Appendix III.

BIOINK CANDIDATES

Fibrin-hyaluronic acid

Fibrinogen (FG) from bovine plasma (Sigma-Aldrich) was weighed out in powder form using an analytical balance (Mettler Toledo) and dissolved in DPBS (Dulbecco's phosphate-buffered saline, Life Technologies) to final concentrations of 20 mg/mL and 25 mg/mL. Hyaluronic acid (HA, Sigma-Aldrich) was also weighed out using the analytical balance to a final concentration of 5 mg/mL. Thrombin (TH) from bovine plasma (Sigma-Aldrich) was made into a stock 50 U/mL concentration and diluted to 5 U/mL for 3D bioprinting using 40 mM calcium chloride ($CaCl_2$, Sigma-Aldrich). FG and HA solutions were

placed in a bead bath set at 34 °C to speed the dissolution process, whereas thrombin was kept incubated at below 4 °C to preserve the enzymatic potency. Fibrinogen-HA solution and thrombin were mixed to a 25:2 ratio prior to static gel rheological tests and to a 25:1 ratio prior to 3D bioprinting.

Fibrin (control)

Fibrinogen and thrombin (25:2 ratio rheology, 25:1 bioprinting) without the addition of hyaluronic acid was also tested as a control variable. The same final concentrations of each solution – 20 mg/mL and 25 mg/mL for fibrinogen, 50 U/mL and 5 U/mL for thrombin – were made to allow a direct comparison between fibrin-HA and fibrin bioinks.

Alginate

Sodium alginate (Sigma-Aldrich) was weighed out and dissolved in an alginate buffer consisting of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 118 mM sodium chloride (NaCl), 5.6 mM potassium chloride (KCl), and 2.5 mM magnesium chloride ($MgCl_2$) to a final concentration of 5% alginate gel. Calcium Chloride ($CaCl_2$, Sigma-Aldrich) dissolved in deionized (DI) water to a final concentration of 40 mM was added to ionically cross-link the alginate gel after bioprinting.

RHEOLOGY

Why is rheology important?

To characterize the mechanical properties of a bioink, it is important to first understand the rheology, or flow behavior, of the fluidic material. The rheological tests conducted in this study, isothermal time sweeps, are oscillatory tests that measure the cross-linking and gelation of bioinks over time at a constant temperature. These tests capture the time of gelation and steady-state stiffness of the gels, both of which give valuable information about (a) how reliably the gels can be printed and (b) how easily the cells within the gel matrix are able to proliferate. In a positive feedback loop, this information allows for even further bioink optimization, as concentration and volumetric changes can be made to enhance properties that are more desirable for bioprinting. In reference to previous work[1], the parameters used in these oscillatory tests are 1 Hz at 5% strain.

Protocol for testing bioink rheology

In order to obtain consistent and reproducible rheological data, it is important to establish and adhere to a standard protocol. All experiments were done with a modular compact rheometer (Anton Paar, Ashland, VA) using a 24.974 mm aluminum parallel plate measuring geometry (Anton Paar). To prevent dehydration of the hydrogels during rheological sweeps, a solvent trap was made by filling a circular ring (Anton Paar) with a rope-twisted sterile wipe soaked in distilled water. The following steps were followed for each tested sample.

Rheometer and RheoCompass preparation

1. Turn on the rheometer, and wait for a triumphant beeping noise before opening Anton Paar RheoCompass software.
2. Under the *Measuring Set* tab on the top panel, select *Configuration Peltier.*
3. Click on *Control Panel* at the top right corner of the software window, and select *Initialize.* (Note: If the measuring geometry was accidentally left in the rheometer from previous use, the software will prompt the user to remove the measuring system first.)
4. Insert the measuring geometry (parallel plate).
5. Insert the solvent trap and re-soak the twisted wipe with DI water, if needed.
6. Set the temperature (bottom left corner of *Control Panel*) to 25 °C.
7. Click on the *Set Zero-Gap* button (should appear in the *Control Panel* window by this point), which homes the measuring system. A default of 1 mm is used for the parallel plate configuration.
8. Select the middle arrow to move the system to waiting position. A value of 20-30 mm works well for loading a 500 µL sample.
9. Under *My Apps* at the bottom left corner of the software window, select the desired test and preset any parameters (e.g. frequency bounds, percent strain, duration). This will automatically create a new project in RheoCompass.

Sample preparation: fibrin-HA gels

1. Sterile filter fibrinogen, hyaluronic acid, and thrombin solutions.
2. Use 1.5 mL Eppendorf tubes and a 1 mL micropipette to contain and mix fibrinogen and hyaluronic acid at the required concentrations and volumes.
3. Prepare separate 0.6 mL Eppendorf tubes by adding thrombin at the required concentration and volume (recall a 25:2 ratio of FG-HA to TH for static gels).
4. When the rheometer is configured and ready to begin testing a sample, use a micropipette to draw 500 µL of FG-HA solution. Dispense carefully into the thrombin-loaded tube and mix gently.
5. Aspirate 500 µL of FG-HA-TH solution and immediately dispense onto the rheometer measuring surface, being careful to center the fluid and avoid bubbles.
6. Select the bottom arrow on the *Control Panel* window to move the measuring set to the trimming position.
7. Wait for a prompt to trim the sample, and use a small, dry sterile napkin to wipe away excel fluid. Click *Continue* when finished.

Carrying out a test

1. Under your selected project and test, click *Start Test* and quickly input the sample name.
2. Click *Start* and view your data points in real time under the *Diagrams* tab.

The standard timespan of an oscillatory test for a gel-like sample is 30 minutes. Note that in the duration of a test, the rheometer will pick up oscillations arising from the opening and closing of table

drawers and doors nearby. Be sure to post warning signs and take care not to disturb the rheometer system during a test, or else there may be a spike in the data that will affect all subsequent measurements. If entering or exiting the lab is necessary during this half-hour period, be sure to open and close doors very gently.

Fibrin-hyaluronic acid bioink optimization

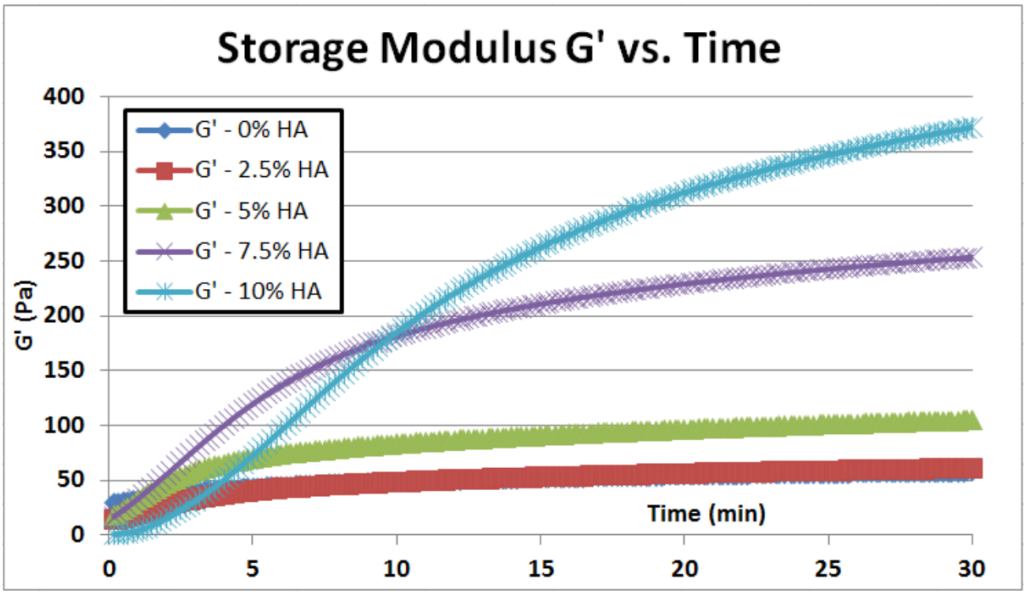

Figure 1: Isothermal time sweep of fibrin gels with varying percent volume of hyaluronic acid.

It is apparent from the rheological data in Figure 1 that the gels with the most optimal steady-state stiffness (falling in the 100-200 Pa range[1], as determined from previous work) are the fibrin gels with 5 percent and 7.5 percent volume of added hyaluronic acid. This seems reasonable, as literature deems there to be a saturation point above which higher hyaluronic acid concentration over-stiffens the fibrin gel matrix, thereby deterring cell growth and proliferation[2]. These percent volumes of hyaluronic acid correspond to the gels with the brightest confocal microscope images and the greatest depth of cell vascularization and proliferation on a three-dimensional level. The confocal microscope images can be seen below in Figure 2.

BIOCOMPATIBILITY

In order to have practical applicability in tissue engineering and bioengineering, bioinks must allow cell vascularization and proliferation – that is, they must be biocompatible and suitable for cells to form their own networks. From a mechanical standpoint, this biocompatibility is quantified through rheological tests that measure the steady-state stiffness of the gels, which correlates to the ease at which cells within these gels are able to branch out and absorb nutrients from the surrounding media. The rheological tests shown above show how stiffness moduli vary with time, but to associate these

numerical stiffness values with cell viability and vascularization, static gel cell cultures and 3D printed structures were kept alive *in vitro* for one week, fixed, and then stained with fluorescent staining or a live/dead cytotoxicity assay to allow confocal imaging of structures and networks.

Methods

Static co-culture fibrin gels with hyaluronic acid

Materials

Static fibrinogen-hyaluronic gels were made in a 48-well plate using 20 mg/mL fibrinogen diluted to 10 mg/mL per well, 50 U/mL thrombin (lyophilized powder, Sigma-Aldrich), and varying percent volumes of 5 mg/mL hyaluronic acid (0% - 10%, not accounting for thrombin volume). Fibrinogen was prepared fresh on the day of the experiment by weighing out solid powder and dissolving completely in 1X DPBS (no ions). Thrombin and hyaluronic acid were also weighed out in powder form and dissolved in deionized water and DPBS, respectively, to obtain a final concentration of 50 U/mL and 5 mg/mL. All solutions were sterile-filtered using a 35 µm syringe filter (EMD Millipore, Darmstadt, Germany) prior to the addition of cells.

Material Added	0% HA	2.5% HA	5% HA	7.5% HA	10% HA
HA*	0	6.25	12.5	18.75	25
DPBS*	125	118.75	112.5	106.25	100
FG*	125	125	125	125	125
TH*	20	20	20	20	20

Table 1: Material composition per well in a 48-well plate. * Units: µL.

Cell passaging

Before starting any static gel experiments, Normal Human Lung Fibroblasts (NHLFs, Lonza, Switzerland) and Human Umbilical Vein Endothelial Cells (HUVECs, Lonza, Switzerland) were cultured in flasks for 4-6 days until confluent. When the cells were ready to passage, a co-culture passaging method was used:

1. Remove media and rinse with 10 mL of HBSS(-) (Hank's Balanced Salt Solution without ions, Life Technologies).
2. Remove HBSS(-) and add 3 mL (HUVECs) or 6 mL (NHLFs) of 0.025% trypsin (1:1 dilution of stock trypsin in HBSS(-)). Incubate at 37 °C for 1 minute.
3. Remove from incubator and tap against a hard surface to fully detach cells. Check flask under microscope to ensure no cells are attached.
4. Add 13 mL of EGM-2 (HUVECs) or 10 mL of FGM-2 (NHLFs) to dilute the trypsin reaction.
5. Add solution (media, trypsin, and cells) into one 50 mL conical tube.
6. Repeat steps 1-4 for other flask and cell type. Aliquot into a separate 50 mL conical tube.
7. Centrifuge two conical tubes together at 200G for 5 minutes. Check that both cells have formed pellets at the bottom of the tubes.

8. Remove media-trypsin suspension from one tube and add known amount of media (e.g. 3 mL). Mix well with a micropipette until the pellet has completely broken and dissolved.
9. Count cells using a hemocytometer. Record the live cell count (cells/mL).
10. Repeat steps 8-9 for other cell type.

For co-culture gels, a concentration of 2 million NHLFs/mL of gel and 1 million HUVECs/mL of gel is needed. Knowing the live cell count from the hemocytometer and the volume of each gel in the 48-well plate (250 µL), the corresponding volumes of cell-media solution were taken from the NHLF and HUVEC 50 mL conical tubes and aliquoted into a 15 mL conical tube. If needed, EGM-2 of arbitrary amount was added to form a larger volume of suspension before centrifuging again at 200G for 5 minutes. The media suspension was then removed, and the pellet was re-suspended in and mixed with 500 µL of DPBS (5 x 100 µL of DPBS, the lowest concentration of DPBS used in a single well).

Forming and culturing gels

First, 20 µL of 50 U/mL thrombin was added to five wells on the 48-well plate. After dissolving the DPBS-suspended pellet fully by mixing with a micropipette, 100 µL of DPBS was aliquoted into five separate 1 mL Eppendorf tubes (autoclaved), labeled 0%, 2.5%, 5%, 7.5%, and 10%, respectively. Then, the respective volumes of fibrinogen, additional DPBS, and hyaluronic acid were added to the Eppendorf tubes according to Table 1, mixing gently and taking care to avoid bubbles. If bubbles were accidentally introduced, closing the tube cap and tapping the bottom onto a hard surface as well as gently flicking the sides of the tube got rid of the bubbles.

Next, the contents of the Eppendorf tubes were added to the thrombin-loaded wells and aspirated and dispensed 2-3 times with a pipette to mix well. Again, care was taken not to introduce air bubbles into the gels, as bubbles deter the cells from attaching to the gel. After all the gels were polymerized (if needed, incubate at 37 °C for 30 minutes), 500 µL of EGM-2 with aprotinin at 100 KIU/mL-media was added to each well, and the 48-well plate was incubated at 37 °C.

The gels were then cultured *in vitro* for one week and fed an aprotinin-infused EGM-2 media every two days. After one week (seven days), the gels were fixed and fluorescently stained to view the cell network formation.

Fluorescent staining

In order to visually assess and analyze the level of cell vascularization and proliferation within the static gels after one week, the cells were first fixed with formalin (Sigma-Aldrich) and then washed with PBS thrice to remove the residual fixative. Then, a fluorescent staining protocol was used to mark the presence of CD31, an endothelial cell protein, and the fibroblast cells:

1. Permeabilize cells with PBS + 0.5% TWEEN for 15 minutes at room temperature.
2. Wash gels thrice in PBST for 5 minutes duration each time.
3. Block samples with blocking solution (2% BSA in PBS + 0.1% TWEEN, otherwise called PBST) for 1 hour at room temperature.
4. Add primary antibody, CD31 (mouse anti-human, Dako, Carpinteria, CA), at 1:300 dilution in blocking solution. Incubate overnight at 4 °C.

5. Wash gels thrice in PBST for 5 minutes duration each time.
6. Add secondary antibody, Alexa Fluor (488 goat anti-mouse, Life Technologies), at 1:500 dilution in blocking solution. Incubate at room temperature for at least 2 hours.
7. Wash gels in PBST four times for 20 minutes duration each time.
8. Stain with 1 ug/mL Hoescht stain (Thermo Scientific) for 30 minutes.
9. Wash with PBST once. Leave in PBS and wrap with Parafilm for storage.

After Step 9 of the fluorescent staining process, the gels may be imaged using a fluorescence microscope or a confocal microscope, depending on the resolution needed and the time allotted to imaging. Fluorescence microscope images are less clear but faster to capture, whereas resolution and imaging speed are higher and slower with confocal microscopy, respectively. In this study, confocal microscopy was used to more clearly capture the 3D structures and vascular network formation.

Confocal microscopy

For gels that were stained fluorescently, a confocal microscope with two channels – GFP (green) and DAPI (blue) – was used to capture CD31 and Hoescht fluorescence, respectively. The software used to capture and render images was Zen, gray edition. To capture an image, a sample was first loaded and brought into focus using the microscope viewing lens. Then, in the *Acquisition* tab, *Default Green Blue Fast Line* was selected as the experiment type and the GFP and DAPI channels were tentatively set to 650 µm. Extra care was taken not to overexpose, or bleach, the samples by periodically checking the read indicator during scans and adjusting the channel gain. In the *Z-stack* tab, the first and last layer of the sample were set by adjusting the focusing knob forward and backward until structures were barely visible. After hitting *smallest* under the same tab, selecting *Start Experiment* began the 3D confocal imaging. The images were then saved as .czi files on a portable USB.

Live/dead cytotoxicity

For 3D bioprinted structures, the above fluorescent staining procedure could also be used to view the formation of vasculature networks. However, because cell viability and sterility were the two main concerns with bioprinting using the new Aerotech system, on the second day of bioprinting, after checking and confirming that the samples were contamination-free, the printed structure was fixed in formalin for 30 minutes, washed with PBS thrice to remove residual fixative, and immersed in a cytotoxicity assay (CytoScan WST-1 Cell Cytotoxicity Assay, GBiosciences) for 30 minutes. Then, a confocal microscope with green (m-GFP) and red (m-Cherry) fluorescent channels was used to see the live and dead cell structures and assess cell viability after bioprinting.

Results

Confocal images of static 3D gel cultures are shown in Figure 2. The gels are labeled with the percent volume of 5 mg/mL hyaluronic acid in the fibrin gels, and the differing levels of cell vascularization and growth can be seen clearly. The green fluorescent stain marks the CD31 protein in the endothelial cells, whereas the blue Hoescht stain highlights the presence of fibroblasts.

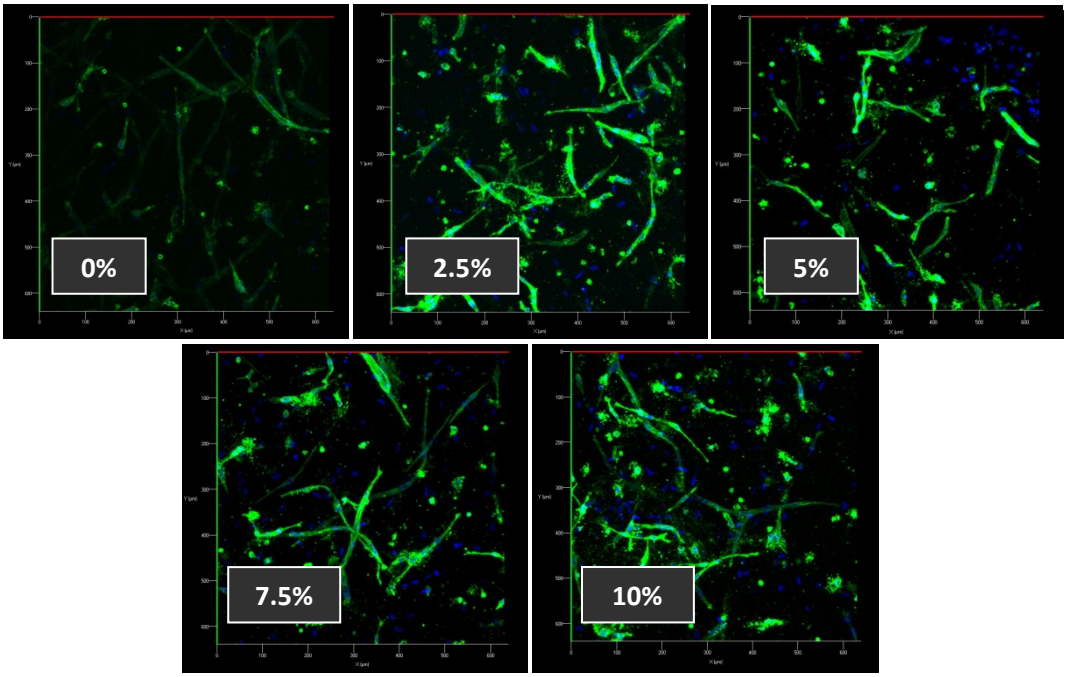

Figure 2: Confocal images of 3D fibrin gels with varying v% hyaluronic acid (HA) using a HUVEC-A line primed for angiogenesis. *Scale bars in 100 µm.*

Although it can be determined from the images in Figure 2 that adding hyaluronic acid to fibrin gels induces greater network formation, there is currently a lack of information to precisely quantify the relationship between percent volume of hyaluronic acid and cell vascularization. That is, the endothelial and fibroblast cells seem to thrive and vascularize in each fibrin gel with hyaluronic acid, regardless of the percent volume of HA added. One potential reason for this phenomenon is that the endothelial cells used in these static gels, HUVEC-A, is a new cell line that is purportedly primed for angiogenesis, or vessel formation. To test this hypothesis, however, more experimental data with the introduction of a control (non-primed HUVEC cells) is needed. With the current data, the only conclusive statement that can be made – that the HUVEC and NHLF cells thrive in fibrin gels with hyaluronic acid – is qualitative.

Figure 3 shows the resulting confocal image of a bioprinted structure after fixing with formalin and immersing in a cytotoxicity assay. ImageJ analysis of the green (live) cells found that 77.3% of the cells remained viable one day after bioprinting – an incredibly promising result, given that this was the very first time printing with cells using the new Aerotech motion controller system and the displacement pumps.

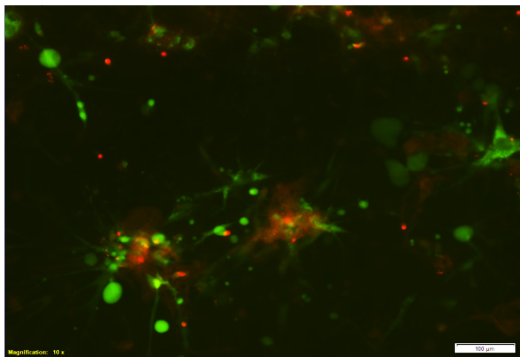

Figure 3: Cytotoxicity assay of a 3D bioprinted structure after one day.

3D BIOPRINTING

Aerotech motion controller system

This study uses an extrusion-based bioprinting (EBB) system with displacement pumps that drive fluid flow. Figure 4 shows the printing setup of the Aerotech motion controller, the two displacement-driven pumps, the print stage, and the fluidic lines. The entire system is contained within a BSL-2 cleared biobubble to ensure safe handling of biohazardous materials.

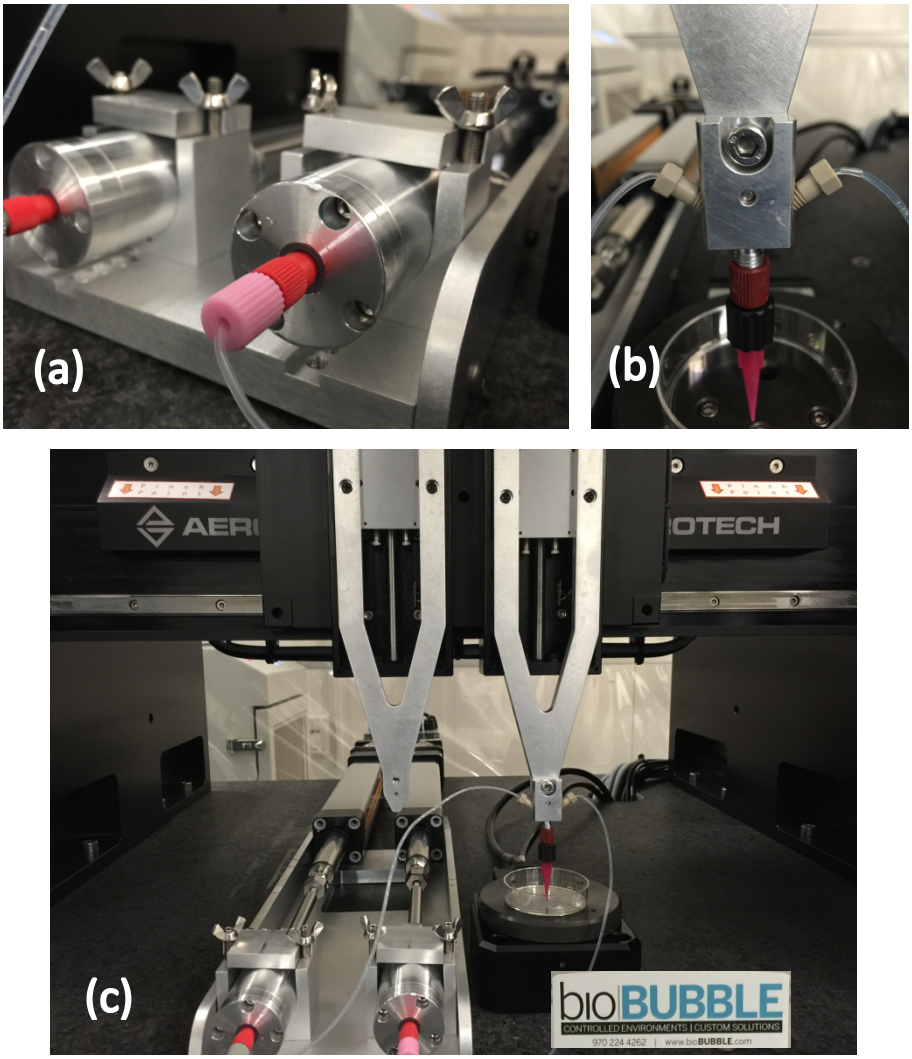

Figure 4: (a) Displacement pumps, (b) static fluid mixer, (c) overview of printing setup.

In contrast to pneumatic pumps, which are commonly used in EBB systems and problematic due to the variability of bioink viscosity and rheological behavior during an isobaric printing process, displacement pumps are driven not by pressure but by distance. That is, as a bioink polymerizes and becomes more viscous and difficult to extrude, the displacement pump in turn pushes the fluid with a greater force to achieve the same preprogrammed distance. As a result, structures that are bioprinted

using displacement pumps are much more controlled and reliable, allowing for the freedom to design complex 3D structures sans equipment-related precision limitations. That is not to say, however, that there is not a different set of challenges associated with this bioprinting setup. A detailed, optimized 3D bioprinting protocol for the Aerotech motion controller and displacement pumps is included in Appendix III.

Bioprinted samples

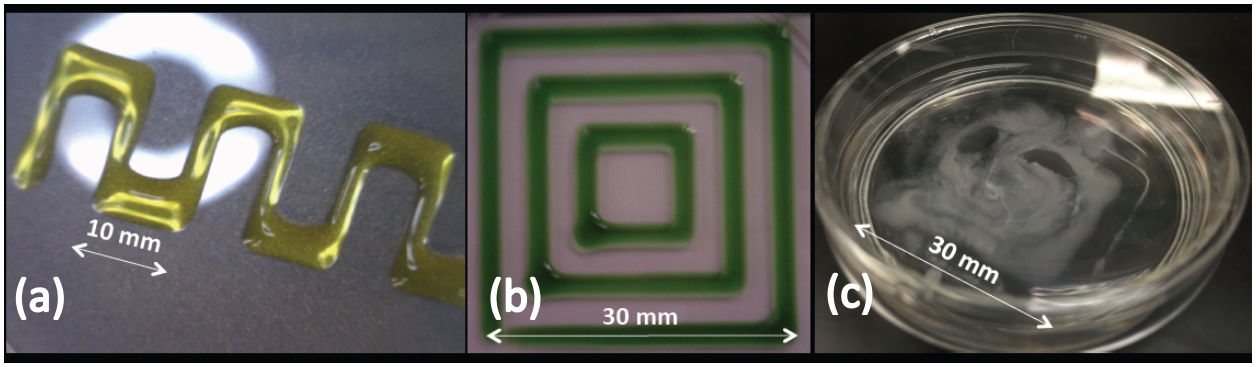

Figure 5: (a) 5% alginate, waveform pattern, (b) 5% alginate, concentric squares pattern, (c) 25:2 fibrin with HUVEC-NHLF co-culture cells, concentric squares pattern.

The first two images above (Figure 5a, b) are alginate gels that have been printed without cells in a culture plate as a proof of concept. Both patterns – the waveform and concentric squares – were programmed in AeroBasic .pgm code, which, through an interfacing software program called A3200 Motion Composer, directly controls the Aerotech axes positions and feed rate. As is apparent in the images, the precision of the cell-free printed structures is quite high, especially compared to structures that have been printed in the past using a MakerBot Replicator2 bioprinter.

The rightmost image (Figure 5c) is a fibrin gel co-printed with endothelial and fibroblast cells into a concentric square pattern and suspended in EGM-2 infused with aprotinin (to help slow the breakdown of the gel). Clearly, the structural integrity of this gel was undesirable, as shortly after printing and the introduction of a media suspension, the gel congealed into a blotched structure. However, as stated above in the discussion regarding biocompatibility, the percent of live cells in this sample – over 77 percent – is promising. With further material and process parameter optimization, it is projected that future bioprinted structures will be both mechanically robust and well-suited for cell proliferation and vascularization.

CONCLUSIONS

The reliability and controllability of a new bioprinting system consisting of an Aerotech motion controller with micron-scale precision and displacement-driven pumps was demonstrated both with and without the addition of cells. To develop and characterize a new bioink, hyaluronic acid at varying volumetric concentrations was added to fibrin gels and tested for biological compatibility with cells in a

static gel and for mechanical strength via rheological tests. Although the structural integrity of fibrin gels co-printed with cells, attributed to the material composition of the gels, is still to be optimized, all bioprinted samples retained complete sterility after one week, and bioprinted cells remained, at large, viable after one day of culture in media. In addition, this study successfully developed a new, reliable bioprinting procedure for printing hydrogel-based bioinks, and detailed loading and printing procedures, included in Appendix III, can be used as guidance in future work.

ACKNOWLEDGMENTS

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APPENDICES

I: Modifying Motion Composer .pgm code for 3D bioprinting

All of the printed samples are coded in A3200 Motion Composer (Ver. 5.02.000, Aerotech, Inc.), a coding platform that is specific to the Aerotech motion controller system. The saved programs are AeroBasic program (.pgm) files that are syntactically identical to G-code, with some minor configurations to account for the printing setup. The following steps walk through different commands that are used to control the displacement pumps and axes during a simple printing process.

Step 1: Pre-programming setup

Before beginning a program, it is important to specify the units and define any variables. The following is a standard setup code with comments (all text following semicolons in a line of code) that explain the specific purpose and action of the preceding command. Note that these commands match

those in the G-code database, and that although not a requirement, it is good practice to include comments throughout a code for clear logic flow and readability.

```
; __ OVERVIEW __
; This program will print out a square using one fluidic material.
; __ VARIABLE REFERENCE KEY __
        ; PDISP1:        right displacement pump
        ; PDISP2:        left displacement pump
        ; G1:    a command to move the axis
        ; X:     x-axis control
        ; Y:     y-axis control
        ; ZL:    left Aerotech stage mount control
        ; ZR:    right Aerotech stage mount control
; __ START SETUP CODE __
        G71;    establishes metric units
        G76;    sets time base to seconds
        G91;    sets to relative positioning
```

Note that the lines before "START SETUP CODE" simply explain the program and define the commands that are used. Also note that PDISP1 and PDISP2 are control commands that directly call the right and left displacement pumps; there is no need to define the variables separately.

Step 2: Begin writing code

The actual program will vary depending on the desired print configuration. The following code is a continuation of the above setup program and walks through the steps of printing out a simple square pattern using one displacement pump. Note that more complex geometries can also be designed using these same coding principles.

```
; __ PRINTING A SQUARE __
FREERUN PDISP1 0.02;            directs the right displacement pump to move in the positive
                                ; direction at a flowrate of 0.02 mm/s
DWELL 3;                        directs the Aerotech motion controller to wait for 3 seconds
                                ; before moving, allowing the fluid adequate time to reach the
                                ; dispensing tip.
G1 X20.000000 Y0.000000 F3.75;          prints a 20 mm horizontal line, rightward, at 3.75 mm/s
G1 X0.00000 Y20.000000 F3.75;           prints a 20 mm vertical line, upward
G1 X-20.000000 Y0.000000 F3.75;         prints a 20 mm horizontal line, leftward
G1 X0.0000000 Y-20.000000 F3.75;        prints a 20 mm vertical line, downward (closes square)

FREERUN PDISP1 STOP;            stops the displacement pump
G1 ZL-20.000000;                raises the dispensing tip up by 20 mm to allow easy sample
                                ; change/removal
```

II: CNC Operator Interface

The following is a screen capture of the A3200 CNC Operator Interface software platform that controls the x-, y-, and z-axes of the Aerotech motion controller, as well as the theta direction of the rotary stage.

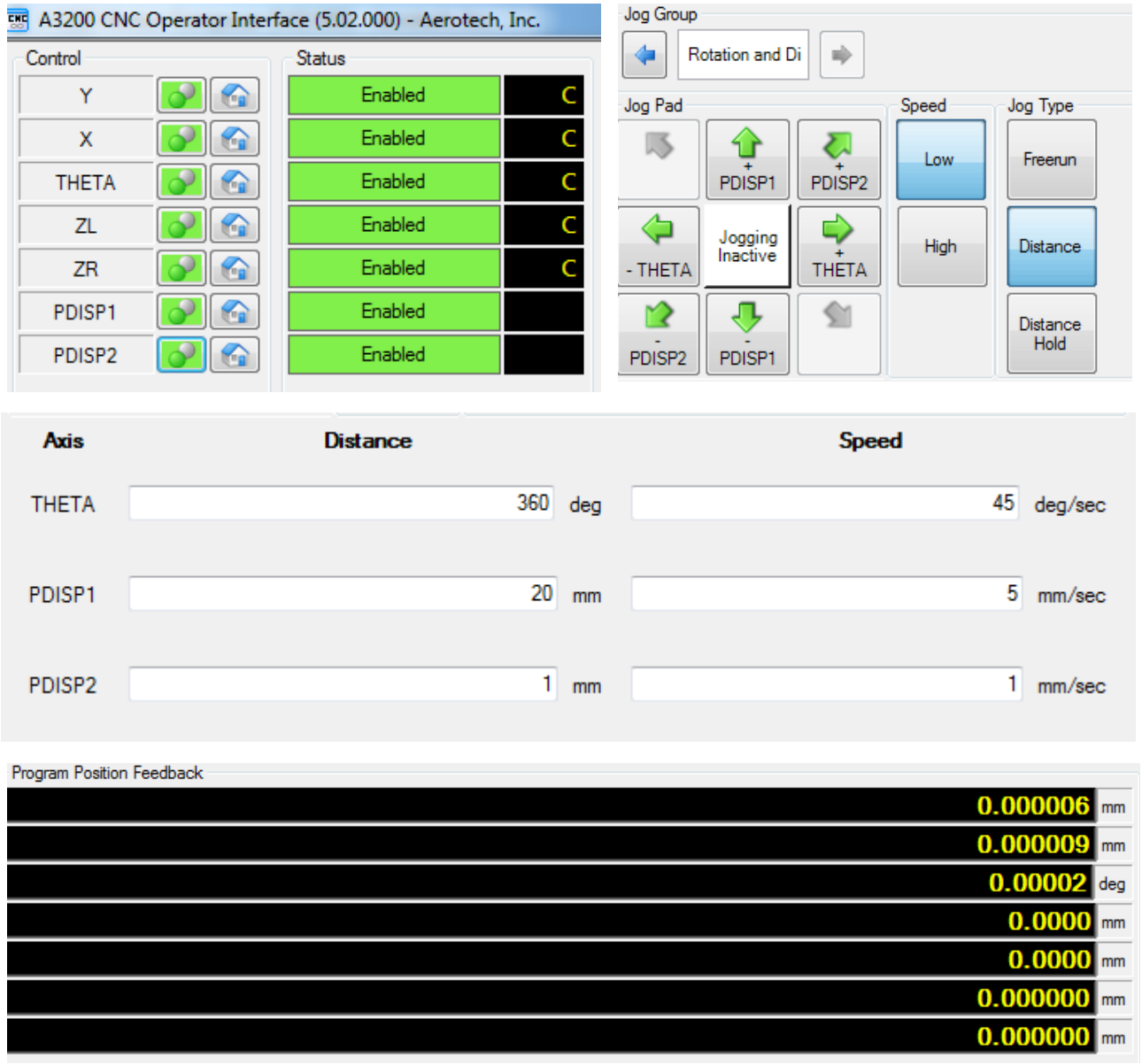

Figure 6: CNC Operator Interface controls.

III: 3D bioprinting protocol

To ensure sterile, reproducible 3D bioprinted structures and to avoid errors in printing, which can be caused by introduction of air bubbles, backpressure resulting in satellite droplets, and uneven mixing, the following, optimized bioprinting protocol was developed:

Step 1: Decontamination

Decontaminate the biobubble with 70% ethanol, wiping (sterile wipe) all surfaces and equipment, including displacement pump parts, the rotary stage, and the Aerotech stage mounts. Autoclave all heat-resistant peristaltic tubing and fluidic parts at 250 °F until the machine stops (beeping noise, approximately 30 minutes). Wait until autoclaved parts cool to room temperature before introducing any cell-laden bioinks. For parts that cannot be autoclaved (3 cc syringes, syringe caps, piston shaft caps, dispensing tips), soak in an ethanol bath for at least 20 minutes and allow time for the alcohol to evaporate. Spray gloved hands with ethanol before handling any materials within the biobubble.

Step 2: Loading a bioink

Use a syringe cap to close off the bottom of a 3 cc syringe (Nordson EFD) to prevent fluid from leaking outward. Using a micropipette, dispense the required volume of bioink and aspirate and dispense the bioink to evenly mix all fluid constituents. Manually prime the piston shaft (with cap screwed in) to the optimal printing position, such that the end of the cap directly contacts the bioink. If there is built-in pressure preventing the shaft from advancing forward in the syringe, flip the syringe (and shaft) upside down, unscrew the syringe cap, and manually push the syringe forward. (Note that doing this also helps eliminate bubbles, which reduces the chance of introducing air gaps into the fluidic lines). With the syringe cap removed, place the syringe and shaft into the piston cylinder and attach the fluidic line that connects to the dispensing tip. Secure the configuration with appropriate wing nuts and clamping rings, and attach the opposite end of the piston shaft (with a hexagonal nut) into the threaded end of the pump piston. If the piston is too close or too far to attach the shaft, manually adjust the appropriate PDISP position in the CNC Operator Interface.

Step 3: Bioprinting

Prime the bioink by selecting *Distance* under *Jog Type* in the A3200 CNC Operator Interface platform and advancing the respective displacement pump forward 5 mm at a time until the fluid in the syringe flows through the peristaltic tubing lines and almost reaches the dispensing system. Then, reduce the distance to 1 mm or less and advance until the fluid lies flush with the end of the dispensing tip. This may be difficult to achieve – in which case, use a sterile wipe to absorb fluid spillage. Switch to the A3200 Motion Composer program and open the desired AeroBasic .pgm file. Click *Run* (green arrow resembling a *play* button) in the top left panel. The Aerotech and displacement pump systems should now begin bioprinting a structure according to the preprogrammed code.

IV: Summary of ideas for future work

Custom manufacturing parts for the Aerotech system

In an effort to customize and add flexibility to the Aerotech motion controller system, two proof of concept custom syringe clamps were designed in SolidWorks and prototyped in ABS plastic. The left syringe clamp would ideally screw into the Aerotech stage mount and allow a disposable syringe to snap into place. The second design, a rack, would allow several different syringes to be mounted at once, thereby enabling multi-material bioprinting, utilizing the two displacement pumps and two pneumatic pumps available for use, and eliminating the need for changing syringes or tips in between print layers. Although the dimensions and scaling of these particular designs would need to be adjusted for proper fitting to the motion controller prior to manufacturing in a more durable material, such as a metal, customized parts beyond those shown below – such heat-resistant and autoclave-able parts for the displacement pumps and dispensing system – would be valuable for future work.

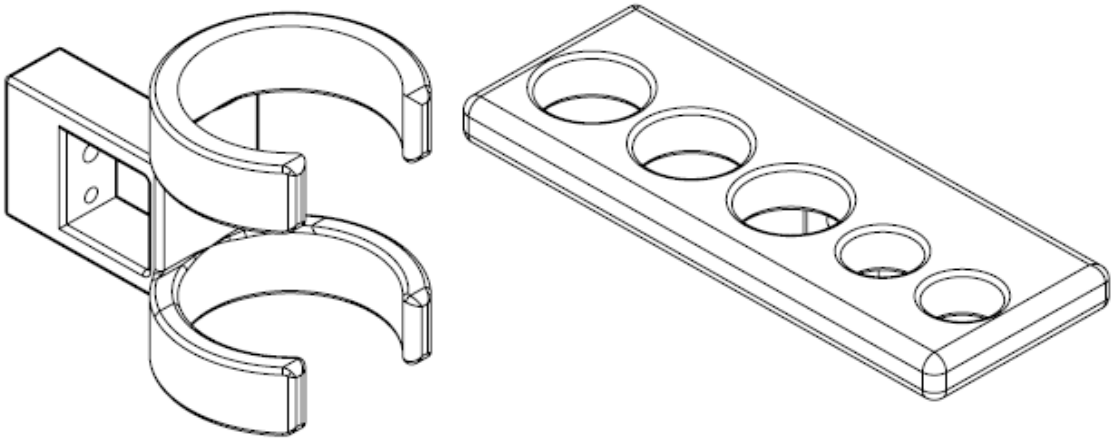

Figure 7: (Left) Single syringe clamp design, (right) multi-syringe rack design.

Refining the fibrin-hyaluronic acid bioink

In an effort to further optimize the fibrin-hyaluronic acid gels for biocompatibility and to pinpoint and quantify the relationship between cell vascularization and HA concentration, a new set of static co-culture gels was designed using 1 mg/mL, 3 mg/mL, and 5 mg/mL hyaluronic acid with the same per-well material compositions as previously shown in Table 1.

Due to a shortage of fibroblast (NHLF) cells available at the time of making gels, only 0% (control), 5%, and 7.5% volume of HA gels were made using a 1:1 ratio between endothelial cells (HUVECs) and NHLFs. The two non-control percent volumes, 5% and 7.5%, were chosen based on confocal image and rheological data from previous gel sets, which showed high levels of vascularization and near-optimum storage moduli at those percent volumes of hyaluronic acid. Preliminary results from viewing the gels under a fluorescence microscope seem to indicate that a hyaluronic acid concentration of 1 mg/mL results in the best vascular network formation, with the highest interconnectivity and vasculature density occurring at a HA concentration of 1 mg/mL at 5 percent volume.

Since the hyaluronic acid (powder, Sigma-Aldrich) used in this study reportedly has a maximum solubility of 5 mg/mL, the denser vascular network formation observed in lower concentrations of hyaluronic acid might be a sign that the optimum HA concentration, when combined with fibrin, is near or below 1 mg/mL. Analysis of these static gels under a confocal microscope, as well as further study of fibrin-hyaluronic acid gels at sub-1 mg/mL concentrations of HA (using the standard 2:1 NHLF to HUVEC cell ratio) would be needed to make any quantitative statements. In a continuation of this study, future work could focus on optimizing the Aerotech system to bioprint fibrin-hyaluronic acid bioinks and to assess the viability of cells within the printed co-gel matrix.