Biomaterials Science

A 3D-bioprinted GelMA hydrogel scaffold with tunable stiffness as a tissue model of the epithelial mesenchymal trophic unit

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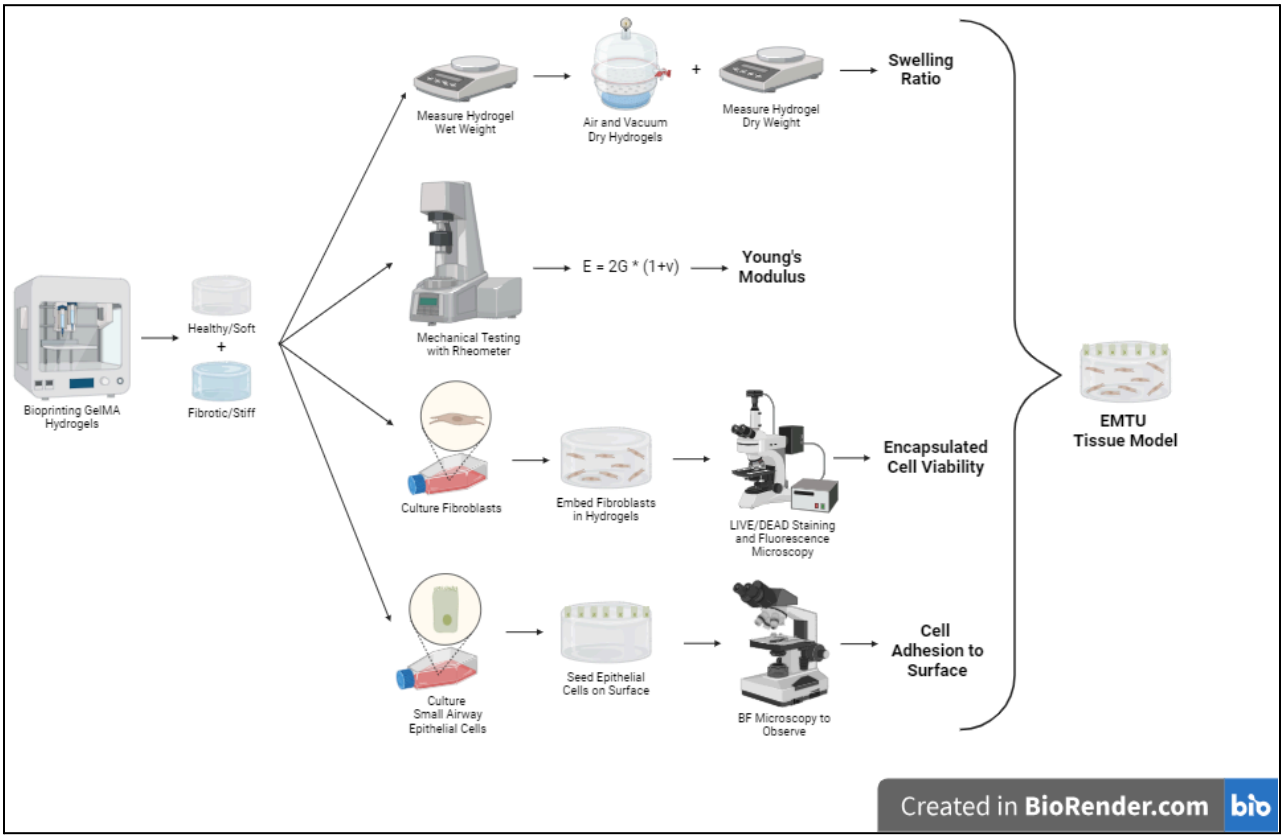
Graphical Abstract



Abstract

Chronic lung diseases such as pulmonary fibrosis, asthma, and chronic obstructive pulmonary disease (COPD) can cause significant damage to the epithelial tissues of the lungs. Currently, no existing scaffolds accurately model the lung extracellular matrix (ECM) and its changes during cell injury from inflammatory diseases. Specifically, no scaffold accurately models the change in mechanical stiffness while also incorporating relevant biochemical cues. Hence, GelMA scaffolds with variable stiffnesses that allow for co-culturing of lung epithelial

cells and fibroblasts were created to model the epithelial-mesenchymal trophic unit for the study of lung tissue inflammation. The scaffold's biocompatibility, mechanical properties, and effect on cell behavior were characterized to elucidate its ability to mimic the *in vivo* tissue environment. The understanding of disease mechanisms gained from using this tissue model would support translational research on developing treatments for inflammatory lung diseases.

1. Introduction

A multitude of chronic lung diseases such as pulmonary fibrosis, asthma, and chronic obstructive pulmonary disease (COPD) can cause damage to epithelial tissues of the lungs. This tissue injury triggers a fibrotic response in subepithelial fibroblasts that results in further fibrosis. According to the World Health Organization, COPD was the 3rd leading cause of death globally in 2019 and over 120,000 people die from COPD annually in the United States alone [1,2]. While there are some treatments to alleviate symptoms, such as bronchodilators and oral steroids, there is no cure for COPD[1]. An accurately mimetic and tunable EMTU tissue model would allow for a greater understanding of the effects of inflammatory diseases on lung epithelium. This knowledge has subsequent applications in translational research, such as developing therapies that target disease-induced changes to lung tissue.

The epithelial-mesenchymal trophic unit (EMTU) is a region of lung tissue composed of small-airway epithelial cells, subepithelial fibroblasts, and the surrounding extracellular matrix (ECM)[3]. The ECM is a complex network of proteins and other macromolecules that provides structural support as well as mechanical and biochemical cues to surrounding cells [4]. When chronic diseases cause epithelial injury, an inflammatory response causes the fibroblasts to increase the production of ECM proteins such as collagen, a fibrous structural protein, and fibronectin, a key binding protein [3]. The interactions between the fibroblast and the ECM are needed for the reconstruction of functional tissue; in lung diseases, this remodeling can become abnormal [5]. One result of this abnormal remodeling is an increase in the stiffness of fibrotic tissue in comparison to healthy tissue. Typically, the Young's Modulus (E) for the healthy and fibrotic tissues are in the range of 2-5 kPa and $\geq$16.5 kPa, respectively [6]. The development of pathogenesis during injury or disease is not fully understood and an *in vitro* model that accurately recapitulates the tissue environment will allow for investigation of these mechanisms.

Due to the lack of effective treatments to reverse fibrosis, understanding the ECM's influence on cellular responses in a multitude of lung diseases has become a focus of therapeutic research studies. Commonly, two-dimensional (2D) monolayer cell-culture systems are used to understand the mechanism behind changes in cellular behavior in a diseased state, but these systems lack the complex cell-ECM interactions of the lung microenvironment. This interaction plays an integral role in defining cell signaling and function, thus the inability for 2D to mimic the biochemical and mechanical complexity of human tissue can lead to inaccurate data [7]. Furthermore, animal models do not accurately represent the mechanisms of human disease [8]. As a result, three-dimensional (3D) hydrogel models that mimic the biochemical and mechanical

microenvironment of the lung provide a more accurate *in vitro* system to study the ECM's influence on cell behavior in a diseased state.

Existing three-dimensional models of the human lung extracellular matrix have been utilized to study the behavior and function of fibroblasts in various conditions. Marinkovic et al. developed a hydrogel model composed of polyacrylamide (PA) at varying stiffnesses and growth factor conditions to study fibroblast behavior [9]. It was found that lung fibroblasts exert lower forces on softer matrices and greater forces on stiffer matrices. Additionally, smooth muscle actin expression on stiffer matrices suggested an interaction between stiff lung ECM and fibrotic mediators in fibroblast force generation. Similarly, Liu et al. engineered a collagen I functionalized PA matrix system, again with a range of stiffnesses [10]. CCL-151 lung fibroblast morphology was analyzed, and significant differences were observed. Lower stiffnesses corresponded to cells with rounded morphology, whereas stiffer gels gave rise to spindle-shaped cells [5]. Through these and other studies, it has been well established that lung ECM has a bioactive role in physiological and pathological processes, such as the phenotype and function of fibroblasts. In the case of chronic lung diseases and injury, these lung fibroblast interactions with the ECM become abnormal and result in tissue remodeling. It has been demonstrated that hydrogel scaffolds can be used to model the complex 3D bioactive environment of the lung ECM in the study of how these conditions affect cellular behavior.

The aim of the current study is to develop a 3D bioprinted GelMA hydrogel as an *in vitro* model to examine the influence of fibrotic mechanical properties on co-cultured lung fibroblasts and epithelial cells. Characterization of the scaffold via rheology, biocompatibility, and cell viability was conducted to determine the ability of the scaffold to accurately mimic the *in vivo* environment.

2. Results and Discussion

2.1. Optimization of Bioprinted GelMA Hydrogels

GelMA is an engineered gelatin-based biomaterial that is produced through the reaction of gelatin with methacrylic anhydride. GelMA is able to be photocrosslinked with UV light, retains natural cell adhesion motifs, and contains matrix metalloproteinase-degradable sequences, which makes it an attractive choice. GelMA 10% w/w with a concentration of 0.25% LAP was used to bioprint hydrogels on the BIO X printer from CELLINK.

There are a series of parameters that must be considered when conducting each bioprinting trial. First, one must decide the shape of the structure being printed, the dimensions, infill density, and number of layers. Next, the dissolution (liquefaction of the cold GelMA to a fluid) temperature, printing temperature, equilibrium time, and extrusion pressure must be selected. Finally, the crosslinking parameters of UV wavelength, duration, and distance must be decided.

A cylindrical structure of 10 x 1mm with a concentric infill of 35% was selected as the final print structure. The dissolution temperature was set at 37℃ for 45 minutes and changed to a print temperature 25.8°C with an equilibrium time of 20 minutes to let the GelMA adjust

temperature. The extrusion pressure was set to 28 kPa and gels were photocrosslinked with 405 nm UV light for 10 seconds following print. *This section will be expanded as the semester progresses and as parameters are further determined.*

2.2. GelMA Hydrogel Stiffness Characterization

Rheometry testing allowed for the GelMA hydrogels to be tested and categorized based on their mechanical properties. This test gave the storage modulus (G') of the gels, which could be used to approximate the shear modulus (G*) at very low frequencies (0.1 Hz). The Young's modulus was calculated using the following equation: $E = 2G^*(1+\nu)$, where ν is Poisson's ratio. For gelatin, $\nu = 0.5$, classifying it as an incompressible material that is deformed elastically at small strain percentages. Young's Modulus could then be used to quantify varying stiffness values between the normal and fibrotic ECM hydrogels.

Two sets of hydrogel batches were able to be successfully printed and tested following the rheometry protocol. The first trial of gels - Group 1 - were printed with varying parameters and structures but yielded similar stiffness values at a 30 sec UV crosslinking time. Group 1 Young's modulus values ranged from 14.97-20.814 kPa with an average of 17.71 ± 2.39 kPa. These values meet the design specifications for the fibrotic tissue range but will need to be further investigated due to varying print parameters and gel structure. The second trial of gels - Group 2 - were printed with identical printing parameters and more similar structure. Group 2 was UV crosslinked for 10 seconds. The Young's modulus of Group 2 was slightly more variable than Group 1 ranging from 9.32-15.90 kPa and had an average of 12.31 ± 2.75 kPa. The structure obtained from trial 2 does not currently meet the fibrotic range for stiffness of over 16.5 kPa *but can be optimized to reach this*. Group 1 and Group 2 results can be seen in Tables 1 and 2 respectively.

Table 1: Group 1 Printing parameters and Young's Modulus (stiffness) measurements of GelMA bioprinted hydrogels

Sample	Extrusion Pressure (kPa)	Young's Modulus (kPa)	Size (Diam. x Thickness)
1	35	17.565	14mm x 3.6mm
2	28	17.508	11.8mm x 3.1mm
3	26	14.970	10.67mm x 2.9mm
4	23	20.814	8.67mm x 1.8mm
Average ± S.D.		17.71 ± 2.39	

Table 2: Group 2 Printing parameters and Young's Modulus (stiffness) measurements of GelMA bioprinted hydrogels

Sample	Extrusion Pressure (kPa)	Young's Modulus (kPa)	Size (Diam. x Thickness)
1	35	9.321	10mm x 0.75mm
2	35	15.903	10mm x 0.80mm
3	35	12.585	10mm x 1.0mm
4	35	11.421	10mm x 1.0mm
Average ± S.D.		12.31 ± 2.75	

2.3. Epithelial Cell Surface Confluency

This section will contain data on the confluency of epithelial cells seeded atop hydrogels and discuss implications of any observed trends. The methodology involves assessing epithelial cell coverage using light microscopy and subsequent analysis in ImageJ to calculate the area fraction of cells to background. The data will be presented in the form of representative microscopy images depicting cell coverage and a bar chart showing the calculated confluency percentages.

2.4. Cell Viability of Encapsulated Fibroblasts

This section will contain data on the fibroblast viability of the soft and stiff GelMA gels and discuss the implications of any observed trends. This data will likely be presented in the form of representative fluorescence microscopy images showing LIVE/DEAD staining, as well as bar charts showing the calculated cell viability based on the amount of live vs dead cells.

2.5. Swelling Ratio

This section will contain data on the swelling ratios of soft and stiff GelMA gels and discuss possible implications of any observed trends. This data will likely be presented in the form of a table listing the gel conditions, the measured wet and dry weights, and the calculated swelling ratio.

3. Materials and Methods

3.1. Cell Culture

MC3T3 cells were cultured using Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen-strep). Cells were cultured in a 37°C, 5% CO2 humidified incubator. The medium was changed every 24 to 48 hours to maintain cell viability and growth. Morphological assessments were performed regularly under a microscope to monitor cell adherence and confluency. Subculture was conducted as needed to

prevent overgrowth. Before encapsulation within hydrogels, cells were passaged within at most 3 days.

Once we begin work with epithelial cells the details of their culture will be added to this section

3.2. Preparation of GelMA Hydrogels

CELLINK BioX 3D Bioprinter provided by Dr. Allan Brasier to make the tissue model. The scaffold is printed from CELLINK GelMA bioink with LAP 0.25% as a photoinitiator. A CELLINK Temperature-Controlled Printhead is used to warm the bioink to a printable consistency. To prevent the bioink from cooling and clogging the nozzle while printing, a thermal insulator nozzle cover has been added to the temperature-controlled printhead.

The bioprinting protocol provided by CELLINK was utilized as the baseline for printing parameters, with optimization conducted to achieve the desired structure as detailed in the results section.

3.3. Hydrogel Characterization

3.3.1. Rheometry

The use of rheology testing on the GelMA hydrogels allowed for the gels to be examined based on their elastic properties. The mechanical properties of the hydrogels needed to be assessed, due to stiffness being a vital component for creating an accurate mimetic of both normal and fibrotic ECM. The properties of the hydrogels were tested by frequency sweep rheometry. Young's Modulus was derived using the equation described in the results section above. The hydrogels were categorized based on their given stiffness values and if they were accurately able to mimic their respective ECM environment.

After the hydrogels were formed, a swelling time of 24 hours was given before testing. Gels were then placed in respective well plates based on their assumed cooling and UV crosslinking time. Testing temperatures also varied between the pipette-based hydrogels and the bioprinted gels. The pipette-based gels were left to sit at room temperature before being tested, while the bioprinted gels were tested immediately after removal from the 37 ℃ incubator. For this test, the Malvern Kinexus Ultra+ Rheometer was used, and a frequency sweep was performed. First, it was ensured that the bottom plate of the rheometer was in the locked position by sliding the lever, located directly below the bottom plate, all the way to the right. The next step was to open the rSpace application on the computer and select “Test 0035; Frequency sweep, strain controlled” as the specific test required for this experiment. After this test was selected, the user was then prompted to enter a gap value pertaining to the thickness (in mm) of the hydrogel being tested. Before plugging this value in, the hydrogel was centered on the bottom parallel plate. Only then was the gap value entered, after which the top parallel plate decreased the gap to this thickness value. At this time, it was confirmed that the hydrogel remained centered and that the upper parallel plate made contact with the top of the hydrogel without compressing the gel.

The next step involved entering the following testing parameters: temperature, start frequency, end frequency, shear strain %, and samples per decade. In this specific test, the values

were as follows: Start Frequency = 0.1 Hz, End Frequency = 10 Hz, Room Temperature = 25 ℃, shear strain = 1%, and 10 samples per decade. Once the testing parameters were entered, the user was allowed to start the test. Once the test began, a 5-minute calibration occurred before the frequency sweep could begin. After the calibration was finished, the frequency sweep started and took approximately 10 minutes to complete. Following completion, test results were copied into an Excel spreadsheet. Next, a gap value larger than the thickness of the gel was entered, so the hydrogel could be removed, and the next hydrogel could be tested. In between tests, it was ensured that the upper and lower parallel plates were cleaned with 70% ethanol. Once all hydrogels were tested, results were interpreted using Microsoft Excel.

3.3.2. Epithelial Cell Confluency

The assessment of epithelial cell confluency atop hydrogels aims to characterize cell coverage on the hydrogel substrates. The procedure will start with the data collection of high-resolution light microscopy images of the cell-seeded hydrogels. These images will be captured using the available microscope under controlled conditions, ensuring uniformity across samples. Subsequently, quantitative analysis will be performed using ImageJ software. The images will undergo thresholding to distinguish cells from the background. The area fraction of cells will then be calculated and normalized to the total image area, providing the confluency percentage. Consistency will be maintained throughout the application of this methodology across all experimental conditions. Following completion of the seeding experiments and data collection, representative microscopy images and quantitative analyses will be presented. These will offer insights into the confluency percentages of epithelial cells seeded on different hydrogel conditions, aiding in the evaluation of hydrogel suitability for supporting epithelial cell growth and adhesion.

3.3.3. Cell Viability of Encapsulated Fibroblasts

Encapsulated fibroblasts were labeled as live using calcein AM and dead using ethidium homodimer-1. Imaging of the stained cells was performed using fluorescence microscopy (NIKON) under FITC and RFP filters. The acquired images were then analyzed to quantify cell viability by counting the number of live and dead cells in each field of view. The percentage of live cells relative to the total number of cells provides a measure of cell viability.

3.3.4. Swelling Ratio

Swelling ratios were calculated to further characterize the hydrogels. This ratio describes the amount of liquid that can be contained within a gel's polymer network; it can be calculated by dividing the difference between the hydrogel's wet weight and dry weight by the dry weight [11]. A higher ratio means the gel swelled to a greater extent.

The wet weight of the hydrogels was obtained after first swelling the gels in cell culture media for 24 hours. The gels were then removed from the media, the excess liquid was wicked away, and the hydrogels were weighed. The dry weight was obtained by first air drying the hydrogels for 24 hours, followed by vacuum drying for another 24 hours. After a total of 48 hours, the dried gels were weighed.

4. Conclusion

In an effort to address the need for a tissue model representative of the *in vivo* environment of the epithelial tissue of the lungs in the study of its diseased state, a 3D cell culture model of the epithelial mesenchymal trophic unit was developed as a research tool. The scaffold was fabricated using CELLINK gelatin methacryloyl hydrogel, whose tunability is relevant in generating GelMA scaffolds with low and high stiffness so that lung epithelial cells can be studied under normal and fibrotic conditions, respectively. Rheological mechanical testing confirmed that scaffold stiffness could be manipulated through fabrication parameters, namely UV crosslinking time.

This section will be expanded by commenting on the significance of LIVE/DEAD staining of cell-laden hydrogels, as well as swelling ratio novelties. This section will also include the significance (if any) of what was observed through 3T3 encapsulation and epithelial cell culture: the adhesion and proliferation observations. Eventual culturing of epithelial cells will be done, and cell viability assays carried out to see how well epithelial cells survive on the scaffold; this can be accomplished by either flow cytometry or immunohistochemical analysis.

In the future, this 3D, *in-vivo* mimicking lung ECM tissue model has applications in research motivated by understanding how the diseased tissue state of chronic lung disease and injury affects the small airway epithelium.

Conflicts of Interest

The authors declare that this work was completed for their senior capstone design project as a part of the University of Wisconsin-Madison's Biomedical Engineering Undergraduate Program's design curriculum, and as such the authors' work was graded.

Acknowledgments

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Appendices

A. Protocol for Bioprinting GelMA Hydrogels

Below is the protocol followed for bioprinting GelMA hydrogels:

Materials:

1. GelMA in amber cartridge from fridge
2. CELLINK Bioprinter
    1. Temperature controlled printhead attachment
    2. Temperature maintaining nozzle attachment
3. 22 gauge nozzle attached to cartridge (Many in case of clog)
    1. Smallest needle for declogging
4. Pressure hose
5. Luer lock connections
6. Petri dish

Method:

1. Place the GelMA cartridge into the connected temperature controlled print head
2. On the bioprinter interface select the type of print you would like to conduct, in our scenario we will be doing a structured print
    1. Go to bioprint → Simple shapes → scroll down to cylindrical and select the 10x06mm.stl
    2. Go to surface → select your surface (for us petri dish)

3. Printer → tool 1 (or whichever you are using/what the print head is connected to) → tool type: temperature controlled ; temperature: 37 degrees for 45 min to make a liquid
    1. At this point place the GelMA cartridge with a 22 gauge nozzle and the tube attached to the machine
    2. Once the time has passed pull the cartridge out and move it up and down to make sure it is a liquid
4. Bioink profile: Cellink GelMA 1
5. Pre-flow: -100 ms
6. Photocrosslinking: 405 nm for 10 seconds

3. Layers
    1. Select concentric for infill pattern
    2. For infill density select 35%
4. On the Print screen
    1. Press the settings symbol and go to Tools - for temperature change to printing temp (25.8) and wait for 20 minutes for it to equilibrate
    2. Set the extrusion pressure to 28-35 kPa
5. Once in the print screen → go to the print option which will then take you to calibration
6. Calibrate first with be leveling and the probe in the back
7. Next manual calibration to provide the printer information on how far you want the nozzle to be from the dish while printing and in what region of the dish
    1. UV light requires it to be in the back half
8. Now the machine should be ready to print, but you should check to make sure there are no bubbles in the cartridge nozzle by flicking and make sure the tip isn't clogged with one of the cell link needles
9. Then you press start to do the print trial
    1. If issues arise adjust the temperature or extrusion pressure, unclog the nozzle, or flick the tube
    2. If continual issues you can test the extrusion pressure with test flow until you reach a point of consistent extrusion of a filament

B. Protocol for Rheometry Testing

Below is the protocol followed for mechanical testing of hydrogels with a rheometer:

Frequency Sweep Rheometry Protocol

1. Once GelMA hydrogels have been formed and allowed to set and swell for approximately 24 hours, rheometry testing may be performed.

2. Carefully remove 3-4 hydrogels of each type; healthy lung ECM and fibrotic lung ECM, keeping the gels of the same type in their respective petri dish.
3. Once gels are in two separate weighing dishes, make your way over to the rheometer testing machine (Malvern Rheometer - Kinexus Ultra+)
4. Make sure that the bottom plate is locked on the rheometer by pushing the level, located on the front of the machine below the bottom parallel plate, all the way to the right
5. Open rSpace application on the computer and when prompted to select a certain test, select the 0035 test; Frequency Sweep Strain controlled.
6. When this specific test is selected, the user will then be prompted to enter a Gap value. This value will pertain to the thickness (mm) of the hydrogel being tested. Center the hydrogel on the bottom parallel plate. Measure the thickness (mm) of the hydrogel and enter the gap value. The upper plate will then move to this gap value.
7. Once making sure that the upper plate makes contact with the top of the hydrogel and the thickness is the correct value, enter values for various testing parameters such as room temperature, start frequency, end frequency, shear strain %, and samples per decade. In this specific test, the values were as follows: Start Frequency = 0.1 Hz, End Frequency = 10 Hz, Room Temperature = 25 ℃, shear strain = 1%, and 10 samples per decade.
8. Once the various testing parameters are entered, the user will then be able to start the test. A 5-minute calibration will be performed before the actual test begins. Once this calibration has been completed, the frequency sweep test will take approximately 10 minutes.
9. When the test is completed, the results table can be copied into an excel spreadsheet. Enter a gap value that is greater than the thickness of the hydrogel to remove the hydrogel from the machine. Clean upper and lower parallel plate surfaces with ethanol.
10. Repeat steps 5-9 for remaining hydrogels. In this specific test, 3-4 hydrogels of each type were tested for a total of 6-8 separate frequency sweeps.
11. When testing is completed, results can be interpreted in Excel.

C. Protocol for LIVE/DEAD Staining

Below is the protocol followed for LIVE/DEAD staining of cells encapsulated in hydrogels:
Materials:

1. LIVE/DEAD Viability Kit (Thermofisher) containing calcein AM and ethidium homodimer-1
2. 15mL conical tube
3. 20µL pipette + tips
4. 1000µL pipette + tips
5. 10mL serological pipette
6. Phosphate-buffered saline (PBS)

7. Fluorescence microscope

Method:

1. Use the serological pipette to add 10mL of PBS to the conical tube
2. Add 5µL calcein AM and 20µL ethidium homodimer-1 to the PBS
    a. Homogenize the staining solution by inverting the tube gently several times
3. Remove media from cells and wash twice with PBS
4. Add 200µL of staining solution directly to cells
5. Cover with aluminum foil and let it incubate for 30 minutes at room temperature
6. Image the cells under a fluorescence microscope
    a. Use the FITC filter for calcein-AM (live cells)
    b. Use the RFP filter for ethidium homodimer-1 (dead cells)

D. Materials and Expenses

Below is a table of materials and expenses the authors used for this project:

Date	Item	Description	Vendor	Unit Cost	Total Cost
–	Fall 2022/Spring 2023 Items	Unusable PEG materials and GelMA materials	–	$1,041.00	$1,041.00
02/17/2023	GelMA	Lyophilized GelMA gifted by Dr. Masters	–	Provided by former advisor	$0.00
03/20/2023	Molds	Silicone molds to make GelMA hydrogels by hand	CELLINK	$50.00	$50.00
09/08/2023	Bioprinter	CELLINK Bio X 3D Bioprinter	CELLINK	Provided by client	$0.00
09/08/2023	GelMA Bioink	CELLINK GelMA Bioink LAP 0.25% 3mL cartridge x3	CELLINK	Provided by client	$0.00
09/08/2023	Temperature-Controlled Printhead	CELLINK Temperature-Controlled Printhead	CELLINK	Provided by client	$0.00
09/08/2023	Nozzle Cover	CELLINK Thermal Insulator Nozzle Cover	CELLINK	Provided by client	$0.00
11/02/2023	GelMA Bioink	CELLINK GelMA Bioink LAP 0.25% 3mL cartridge x3	CELLINK	$108.33	$325.00
11/02/2023	Collagenase	25 mg collagenase to degrade GelMA gels	Santa Cruz Biotech	$70.00	$70.00
02/19/2024	GelMA Bioink	CELLINK GelMA Bioink LAP 0.25% 3mL cartridge x3	CELLINK	$108.33	$325.00
Total					$1,811.00

E. Product Design Specification

Below is the Product Design Specification the authors aimed to follow for this project:

Function:

Dr. Allan Brasier and his research team have a need for a 3D model of the small airway of the lung that varies in extracellular matrix (ECM) thickness and composition. This tissue model will be designed with fibroblasts that facilitate ECM production and effector cells during injury response. The model will include an air-liquid interface (ALI) that allows for *in vitro* research of the small airway to explore how the ECM, epithelium, and fibroblasts orchestrate reparations after damage. This model will be produced as a bioprinted 3D scaffold that has mechanical and biochemical properties that will be compatible with the lung epithelial cells used for experimentation. The ECM scaffold will allow for cellular communication and function similar to that of an *in vivo* environment.

Client Requirements:

- The product should be amenable to analysis via various techniques such as microscopy and should allow for the encapsulation of lung fibroblasts and cell culture of epithelial cells. Provide a suitable microenvironment for cell-cell interactions and ECM remodeling, as well as enable comprehensive analysis of changes in cell behavior.
- Model should include an air-liquid interface to reflect the polarization of the epithelium in the presence of air.
- Scaffold should have tunable mechanical properties to reflect that of native ECM.
- Product needs to be capable of cell encapsulation and be cell adhesive.
- The product needs to provide an environment that allows for ECM remodeling by encapsulated cells and/or cells coating the scaffold surface.
- The products must be replicable and fabricated using a Cellink bioprinter.

Design Requirements:

1. **Physical and Operational Characteristics**

a. Performance Requirements:

The tissue model will consist of a bioprinted scaffold, encapsulated fibroblasts, and seeded human small airway epithelial cells (hSAECs). The scaffold must be able to function as a cell culture platform; therefore, it must provide the proper biochemical and mechanical signals for cell growth and viability. Additionally, the tissue model system must be sustained for as long as the client needs to run experiments on the cultured cells which will take an estimated two weeks.

b. Safety:

The tissue model of the EMTU will include human small airway epithelial cells as well as fibroblasts. Since human cells will be used, all cell culturing and scaffold seeding must be conducted in a BSL-2 lab. When working with human cells, the concept of "Universal Precautions" is used to reduce the risk of bloodborne pathogens. This concept states that all unfixed tissues and cells are assumed to be infectious, which requires them to be handled using BSL-2 practices and procedures. Proper PPE must be worn while handling the cells. Additionally, anyone who works with the cells must have completed UW-Madison's Biosafety Required Training, as well as any other additional training required by the BSL-2 lab in use [1].

c. Accuracy and Reliability:

i. Mechanical Properties:

The scaffold will undergo testing to ensure it meets the mechanical properties necessary to accurately represent the lung ECM. Measuring the Young's Modulus (E) quantifies the stiffness of the hydrogel and can be used to determine whether the hydrogel will accurately mimic its respective tissue properties. The scaffold must have a tunable Elastic Modulus ranging from 3.5-16.5 kPa to reflect the environment that fibroblasts

experience through healthy lung tissue to fibrotic lung tissue. The scaffold will be considered mimetic of healthy lung ECM if the mechanical properties are within 5% of the values of native tissue.

ii. Cell Adhesion and Viability:

The scaffold will have cell culture of hSAECs cultured upon it for ideally two weeks. To allow for this, the scaffold must mimic the small airway ECM and allow for cell adhesion necessary for proliferation. Beyond this, fibroblasts will then be encapsulated to make the model further resemble the *in vivo* environment of the EMTU. Furthermore, the composition of the scaffold along with the process involved in making the model must be replicable in order to build confidence in the merit of results obtained from scaffold use. To ensure that the scaffold is capable of providing an environment for viable cell adhesion, testing will be performed on the initial sets of scaffolds. This will involve microscopic imaging of the cells to study their attachment to the scaffold, as well as their shape and viability. The shape of the cell within the scaffold can be compared to their shape in the native state to gain insight to their functioning within the hydrogel. Additionally, cell viability will be monitored to ensure the success of the hydrogel to support cell culture. The scaffold will be considered capable of providing an adequate microenvironment if at least 80% of seeded cells are able to proliferate.

d. Life in Service:

The bioprinted tissue-model product will be able to be maintained for at least one month. During this period, the product will continue to be compatible with and allow researchers time to implement various microscopy techniques for in-depth analysis while facilitating the encapsulation and culture of lung fibroblasts and epithelial cells within an ALI that encourages cell-cell interactions and ECM remodeling. Importantly, the 3D tissue model will remain replicable, consistently fabricated using a CELLINK bioprinter, following the client's technology requirement.

e. Operating Environment:

Once the 3D scaffold is assembled in sterile conditions, the testing will be performed in a cell culture environment. The scaffold will be left to swell for at least 24 hours in DMEM incubating at 37 ℃ and the air inside will have a 5% CO_2 concentration. Cell seeding will be conducted in a sterile environment and will incubate at 37 ℃ and the air inside will have a 5% CO_2 concentration.

f. Ergonomics:

A clear, concise protocol must be written so that bioprinting the scaffolds will be easily replicable as to increase the efficiency of fabrication and make use of the product easy for the client.

g. Size:

The cylindrical scaffold will have a diameter of 9 mm and should be at least 10 microns thick to allow for the embedding of fibroblasts into the scaffold. The cells will then be cultured in a 12-well or 24-well plate with diameter of 22.4 mm [4].

h. Materials:

The tissue scaffold must support three main criteria. First, it must be able to produce a variable range of stiffnesses, as described in the mechanical properties section (1.c.i). The material must also be conducive to cell adhesion, contain RGD peptides to allow for integrin binding. The material must also be conducive to matrix remodeling, containing motifs which are sensitive to matrix metalloproteinases.

The hydrogel will be fabricated from Gelatin Methacrylate (GelMA). Work completed in the previous semester has demonstrated GelMA is capable of spanning a range of stiffnesses, mimicking the mechanical microenvironment of both healthy and fibrotic lung tissue. The team has access to a 3D bioprinter from CELLINK, a company which also sells GelMA bioinks. The team will fabricate the tissue model using CELLINK bioink such that we can be consistent with the recommendation of CELLINK for effective prints.

i. Aesthetics, Appearance, and Finish:

The scaffold should have an overall appearance that resembles the small airway ECM. Additionally, the scaffold must be translucent for optical clarity – the scaffold will be imaged in order to visualize the cell culture. As it is intended to accurately model the stiffness and composition of the ECM, the main focus of the scaffold will be for the tensile strength to be similar to *in vivo* environments as well as allowing for the incorporation of fibronectin and collagen to mimic a natural state. These functional properties take priority over the aesthetic aspects of the design. This will allow for the epithelial cells to attach to the scaffold with a normal morphology in order to create a realistic model of the EMTU.

2. Production Characteristics

a. Quantity:

Models of both healthy and fibrotic lung tissue are desired, so scaffolds with mechanical properties matching the aforementioned conditions must be fabricated. Therefore, at least 6 scaffolds are needed, three of each condition, for the purpose of replicability and statistical analysis.

b. Target Product Cost:

The materials for the scaffold should cost no more than $500. In the prior semesters, the team used $1091 of the $5000 budget, so there is $3909 left to spend. The new scaffold design

will be made using the client's bioprinter, so the only cost should be the GelMA bioink cartridges.

3. Miscellaneous

a. Standards and Specifications:

FDA approval is required for synthetic 3D scaffolds when they are brought to market. The standards and regulations for these products are governed by ASTM F2150-19: Standard Guide for Characterization and Testing of Biomaterial Scaffolds Used in Regenerative Medicine and Tissue-Engineered Medical Products [5]. However, the FDA does not have specific standards or specifications for the use of 3D synthetic scaffolds to study epithelial-mesenchymal transition (EMTU). Therefore, proper protocols for biocompatibility, sterilization, and labeling must still be followed. The standards and regulations for the use of bioinks and extrusion-based bioprinting are still under development, but there are some relevant standards that are currently being developed, such as ASTM WK72274: New Test Method for Printability of Bioinks for Extrusion-based Bioprinting and ASTM WK65681: New Guide for Bioinks and biomaterial inks used in bioprinting.[6] These standards do not yet provide specific guidance on logistics or compliance criteria. Additionally, there are many FDA requirements surrounding the use of cell and tissue culture products, which are outlined in Standard 21CFR864 [7]. The purpose of these regulations is to ensure that all research is conducted ethically and with appropriate oversight.

b. Customer:

The client has recently purchased a 3D bioprinter from CELLINK, intended for the team's development of the tissue model. As such, the client would like us to make use of the bioprinter. The client has also expressed a desire to use commercially available GelMA from CELLINK, rather than GelMA synthesized and characterized by the team. This will make replication and fabrication much simpler for the client once the team is no longer available.

c. Competition:

Tissue engineering models to provide in vitro means to study the body has in recent years created many impressive novel designs. For models looking specifically at the lung epithelium, there are currently both 2D and 3D models that mimic the in vivo environment. Unfortunately, these models are oversimplified and do not provide accurate research results from experiments done on these models.

Looking into 2D models, these are typically layers of cells on top of polymer or glass dishes. In the past several years, many experiments have been conducted on these 2D models, but while they have allowed some study into cell function, disease, and the microenvironment, the models greatly lack the typical native environment cell behavior. For reference, the 2D models have a stiffness range of 2-4 GPa while the human lung ranges from .44-7.5 kPa [8]. The

differences in stiffness significantly change the behavior of the cells, and thus the experimental data found on them are reflective of in vivo behavior.

While there are many varieties of 3D models on the market, one of the most favorable is a co-culture model using ECM protein gel (matrigel). These models are produced by embedding cells in matrigel and culturing them directly on the surface [9]. These 3D models have variations in methods for each experiment, but generally all involve an ECM gel 3D environment that is more similar (with some limitations) to in vivo than the 2D models. Matrigel is a mouse tumor extracellular matrix mixture, so there are variations for every batch and consists of proteins that don't accurately represent healthy ECM. Matrigel ECM is much similar to the ECM of a tumor with significantly more laminin glycoproteins, which can cause the microenvironment to be unlike native tissue.

The gaps between these models and the in vivo environment result in a lack of data and findings that accurately represent what is happening in the body. As a result, a bio-scaffold of the lung ECM is a model that would bridge the gap between in vitro studies and in vivo actions at the cellular level.

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F. Prior Preliminary Designs

Below are descriptions of different hydrogel scaffold designs considered early in the project (As it is an excerpt from prior work, citation numbers are without context):

Design 1: Gelatin Methacryloyl (GelMA)

GelMA is an engineered gelatin-based material that is produced through the reaction of gelatin with methacrylic anhydride (MA). The addition of these MA groups allows for modified gelatin to be photocrosslinked via UV light and a photoinitiator [8]. After GelMA is constructed the adhesive protein motifs present within gelatin are retained. This motif is arginine-glycine-aspartic acid, or RGD, which is an amino acid sequence naturally present in gelatin that allows for cell adhesion [8]. This adhesion is beneficial because to mimic the ECM environment accurately there must be adhesion of the epithelial cells being cultured to allow for proliferation. Additionally, matrix metalloproteinase (MMP)-sensitive sequences are also naturally found in gelatin. The sequences can be degraded by MMPs, which allows for cell migration and reconstruction of the ECM by fibroblasts within the GelMA hydrogel. One downside to GelMA is the mechanical variation between batches when using the same concentrations. When GelMA is formed by functionalizing gelatin with MA, the degree of MA substitution varies batch to batch. This results in hydrogels with differing properties when using the same concentration of GelMA, but from different batches.

Design 2: Polyethylene Glycol (PEG)

PEG is a synthetic polymer that, when crosslinked, forms a hydrophilic and bio-inert hydrogel that can be a scaffold for cell culturing. PEG is capable of having cells seeded within it by adding proteins such as fibronectin and collagen or RGD peptide sequences to promote cell adhesion and degradation, which this synthetic material does not naturally possess [7]. Young's modulus of PEG can fit within the range of native healthy lung tissue or fibrotic tissue based on the percent concentration added to the solution [7]. PEG was the design choice of the prior semester and the benefits and difficulties during that semester are factored into the design matrix discussion.

Design 3: Lung ECM

Hydrogel scaffolds can be made from native lung ECM. The hydrogel is formed by decellularizing lung tissue with several surfactants, freeze-drying the resulting ECM, solubilizing the ECM with several proteolytic enzymes, and allowing the solution to gel [9]. This whole process requires many materials and takes several days. While the main biological / biochemical properties of the lung ECM are retained by the hydrogel, the mechanical / viscoelastic properties of native lung ECM are lost. The hydrogel is cell adhesive and MMP-degradable, but it is less stiff and less viscoelastic than lung ECM [9] Additionally, the mechanical properties, such as the elastic modulus, can not be tuned.

G. Prior Design Matrix

Below is a Design Matrix the authors used to guide early decision making for this project:

		Design 1: Gelatin Methacrylate (GelMA)		Design 2: Polyethylene Glycol (PEG)		Design 3: Lung ECM	
Design Criteria	Weight	Score	Weighted Score	Score	Weighted Score	Score	Weighted Score
Mechanical Properties	20	4/5	16	4/5	16	2/5	8
Biochemical Properties	20	4/5	16	3/5	12	5/5	20
Ease of Fabrication	15	4/5	12	2/5	6	1/5	3
Ease of Use	15	2/5	6	1/5	3	1/5	3
Mechanical Tunability	10	4/5	8	4/5	8	1/5	2
Biochemical Tunability	10	3/5	6	4/5	8	1/5	2
Cost	10	5/5	10	3/5	6	1/5	2
Total:	**100**		74		59		40

Winner	Tie

*A Gelatin Methacryloyl hydrogel won as the best choice with a total of 74/100, while a Polyethylene Glycol hydrogel scored 59/100, and a Lung ECM derived hydrogel scored 40/100.

Explanation of Criteria

Biochemical properties are defined as the ability for the scaffold to mimic the biocompatibility, porosity, adhesiveness, and cellular differentiation capabilities that are similar to the native lung extracellular matrix (ECM). The suitability of any synthetic or semi-synthetic scaffold is based on the properties found where it is intended to mimic. This means that the scaffold must be compatible with collagen, fibronectin, and other cells that are found in the native lung environment. The porosity of the scaffold is similar to that of native lung tissue which will allow for cellular communication or migration of cells just as native ECM has properties that allow for cell adhesion to the ECM and further communication.

Biochemical tunability is specifically how tunable the cell adhesive properties, porosity, and degradation is for each gel. For synthetic hydrogels this tunability is dependent on the addition of peptides or native cells while natural or synthetic hydrogels may already contain the necessary properties to achieve the desired cell adhesion, degradability, and porosity.

The mechanical properties of the synthetic scaffold will need to reflect those of the native and fibrotic lung environment. ECM changes in mechanical properties such as tension or stiffness can cause major responses in cells which alter their ability to function properly. Due to the cells being sensitive to the mechanical microenvironment around them the mechanical properties must accurately mimic those of native tissue. Given that our client wants varying mechanical properties to model both native and fibrotic lung tissues, the design should be able to produce hydrogels of elastic modulus from 2 kPa to 16.5 kPa [1]. The viscoelastic properties for healthy lung tissue must include the storage modulus (G'= 500 Pa) and loss modulus (G'' = 50 Pa). Fibrotic lung tissue viscoelastic properties must also be met by the storage modulus (G' = 5 kPa) and loss modulus (G'' = 500 Pa).

Mechanical tunability describes the ability of the material's mechanical properties to be tuned according to healthy lung tissue and fibrotic lung tissue modulus values. This can involve changing the degree of crosslinking and concentration of material components.

Another criterion, ease of fabrication, is important when choosing a synthetic material due to the wide range of methods when constructing the scaffold. The fabrication will need to be done in the BME labs and the material must be accessible by students so that a model can be constructed in a timely manner. Given that both the fabrication processes will result in variable properties, and an intention to fabricate hydrogels of varying properties, the ability for the team

to test mechanical and biochemical properties of fabricated hydrogels will also be considered under this category.

Ease of use is weighted slightly lower but is still a vital component to the design process. This is particularly important during the testing stages for cell cultures. Providing the scaffolds that can be constructed in a mold and transferred to an Air Liquid Interface (ALI) is a necessary aspect of the design. The ability for the client to create the designed hydrogels within his own lab also falls under the ease of use. Additionally, in the context of the client's lab, the hydrogel must not interfere with cell culturing, imaging, fibroblast incorporation, or any other assay performed.

Lastly, cost is a factor when determining the material to be used because it will need to be used for multiple trials and cannot be out of the clients price range.

Justification of Scoring

PEG:

- Doesn't have any adhesive or biochemical properties that promote desired cell functions or ECM remodeling, this requires further purchasing of RGD peptides and MMP sequences [2].
    - While PEG can be created to have the biochemical specifications required, its tunability requires much more fabrication with MMP sequences and RGD peptides. While PEG has a wide range of biochemical properties that can be achieved, its process of achieving this tunability is difficult resulting in a lower score for fabrication while tunability remains.
- PEG allows for very fine tuned mechanical properties due to the crosslinking chemistry involved[2].
    - PEG allows for a wide range of mechanical properties to be achieved based on differing molecular weight, arm length, and cross linking make it very tunable but the process of this becomes challenging.
- The ability to crosslink during the fabrication process is very feasible with UV light being available in the team labs but prior semester's issues with the photoinitiator decreases the ease of fabrication score.
- This also increases the cost because more I2959, a photoinitiator will need to be purchased

GelMA:

- Unlike PEG, GelMA is inherently biochemically active. Naturally occuring arginine-glycine-aspartic acid (RGD) peptide sequences promote cell adhesion to the proposed hydrogel [3]. Furthermore, GelMA's structure contains matrix metalloproteinase (MMP) targeted sequences, which allow for ECM remodeling following seeding of fibroblasts into the hydrogel [3]. These amino acid motifs are not significantly affected during methacrylation or cross-linkage. While a derivative of denatured collagen [4], GelMA experiences less antigenicity than collagen based scaffolds [3] [4].
- The elastic modulus for GelMA is directly proportional to the degree of methacryloyl substitution within the gel. As a result, the mechanical composition of the hydrogel can be altered to mimic the elastic modulus of lung epithelium. Common MA substitutions for the synthesis of GelMA result in elastic moduli of 2.0 ± 0.18 KPa (49.8%), 3.2 ± 0.18 KPa (63.8%), and 4.5 ± 0.33 KPa (73.2%).[5] When gelatin is modified with the presence of methacryloyl groups and crosslinked with UV light it gives rise to GelMa hydrogels that have great thermostability [6].
- The fabrication of GelMA is relatively simple as there is no need to incorporate peptide sequences for adhesion or motifs sensitive to matrix metalloproteinases[3]. Fabrication consists of solubilizing gelatin in PBS, methacrylation, and crosslinking via photopolymerization [7]. All processes are capable of being performed in the BME labs.
- A GelMA hydrogel is fabricated in a mold and can be transferred into the clients ALI, or into other culture flasks. Reproducibility of the hydrogel within the client's lab contributed to a low ease of use score given that there is a degree of batch to batch variability. Despite this, ease of use was rated higher for GelMA than PEG or Lung ECM, as the fabrication process is easier.
- Without additional modifications, GelMA is drastically cheaper compared to PEG or Lung ECM, with Gelatin powder available for $116/kg and Methacrylic anhydride available for $.56/ml. [8]

Lung ECM [9]:

- Hydrogels made from native lung ECM would have the best biochemical properties since the natural peptide sequences/domains are present. However, some soluble factors would likely be lost in the formation of the gel.
- Mechanical properties of the lung hydrogels are reduced with respect to the native lung ECM. Stiffness is reduced and there is little mechanical tunability. Furthermore, the relaxation time after compression is increased in the gels; the viscoelastic properties of the hydrogels and the native ECM are different. Hydrogels made from Lung ECM also failed to accurately represent the macromolecular structure of native lung tissue.
- The fabrication of the lung ECM hydrogel is a long process that involves processing lung tissue with many surfactants and enzymes. The tissue needs to be decellularized and then enzymatically solubilized, which takes days to do.
- Ease of use is ranked lower due to the difficulty for the client to reproduce and use this hydrogel.
- Cost of native tissue is very expensive and oftentimes rare to find. Native tissue is very sensitive to exterior environments and many chemicals processes are needed to maintain the integrity of the tissue

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H. Protocol for Pipette-Based GelMA Hydrogels

Below is the protocol followed for fabricating GelMA hydrogels with the pipette-based method:

1. Set water bath to 50 ℃
2. Measure out 50 mg of GelMA and place in 5ml sterile tube
3. Add 850µL of embedding media and place in water bath
4. While GelMA is dissolving, prepare a 20 million cells/ml stock solution
5. Sanitize molds with ethanol and UV light
6. Press silicone molds to a petri dish, making sure that the seal is tight to prevent polymer solution from leaking
7. Get dissolved GelMA solution from water bath
8. Moving quickly to prevent temperature dependent gelation of the GelMA solution, add 50 µL of LAP and 100 µL cell solution and mix well
9. Place 100 µL of solution into each 9mm silicone mold
10. Place gels in 4 ℃ fridge for 15 minutes
11. Place gels under UV light for 5 minutes
12. Place gels in 24 well plate with 400 µL of media

Protocol above will make ten 100 µL gels

I. Pipette-Based GelMA Hydrogel Data

Below is preliminary rheological data and LIVE/DEAD staining data from the authors' early work with pipette-based hydrogels (includes excerpts from prior report explaining issues with the staining data):

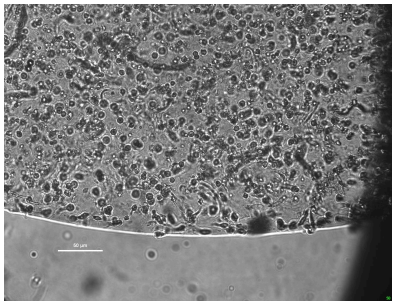
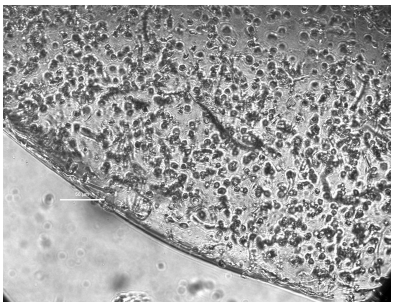
Pipette-Based Hydrogels		
Gel Batch	**Condition (4 °C, UV)**	**Young's Modulus (kPa)**
Normal Batch 1	3 min, 5 min	4.2 ± 0.92

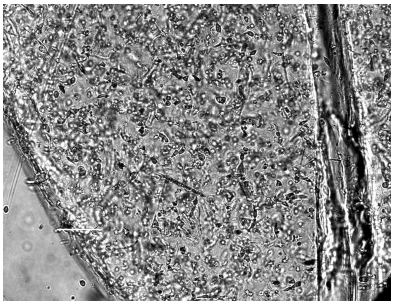
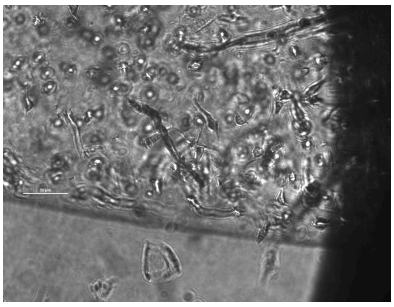
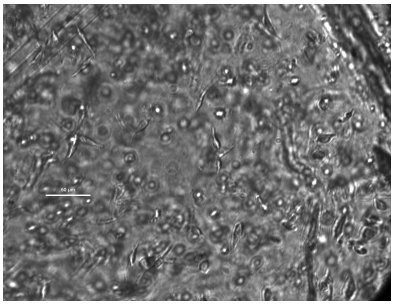
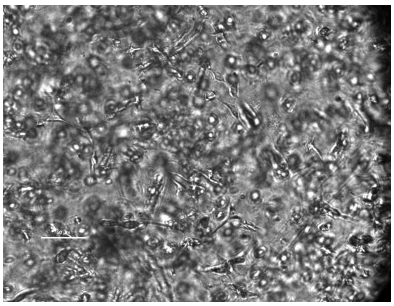
Normal Batch 2	5 min, 5 min	6.63 ± 2.6
Normal Batch 3	10 min, 5 min	13.24 ± 2.8
Fibrotic Batch 1	5 min, 7 min	49.65 ± 22
Fibrotic Batch 2	5 min, 10 min	277 ± 155

Table 1: Young's Modulus Data from pipette-based hydrogels with specified cooling and crosslinking conditions

Observations of morphological signatures of fibroblasts such elongation, spindle-like morphology were limited to the topmost and bottom most planes of the gels, as well as toward the sides of the gels. See Table 2 for brightfield images of encapsulated fibroblasts

Table 2: Brightfield images of cell-encapsulated gels

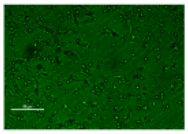
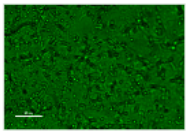
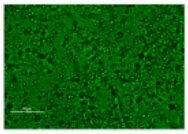
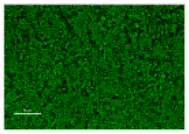
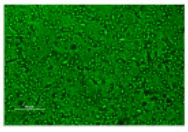
Zoom	**Timepoint**	**Image**
10x	24 hours	
	48 hours	

Zoom	Timepoint	Image
	72 hours	
20x	24 hours	
	48 hours	
	72 hours	

LIVE/DEAD staining was inconclusive, as background noise during FITC imaging of calcein. Images of gel planes at different depths were used to quantify the number of cells in a given plane as a surrogate for cell density. Given the background noise, bright dots of in-focus calcein stain were identified as cells, but as elaborated on under discussion, improper morphology leads to doubts about whether this is a valid measure (see Table 3). Either by this

quantitation, or by qualitative assessment, over all timepoints, as gel stiffness increases, cell density increases.

Table 3: LIVE/DEAD staining of cell-encapsulated gels

Stiffness (kPa)	24hr Cell Count	24hr Images	48hr Cell Count	48hr Images
4.2 ± 0.92	275		430	
6.63 ± 2.6	398		696	
13.24 ± 2.8	644		965