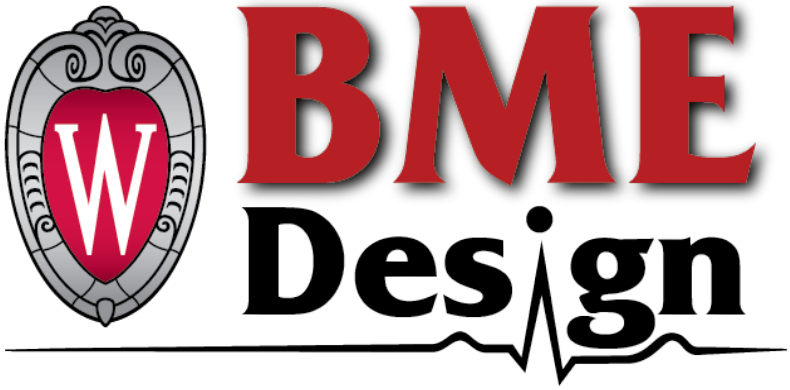
Tissue Model of the Epithelial Mesenchymal Trophic Unit



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BME 400

Preliminary Report

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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 models the change in mechanical stiffness while also incorporating relevant biochemical cues. This project aims to create replicable scaffolds with variable stiffnesses that allow for culturing of lung epithelial cells and fibroblasts to model the epithelial mesenchymal trophic unit so the client can study lung tissue inflammation. The understanding gained from using this tissue model would support translational research on developing treatments for inflammatory lung diseases. The team proposed a bioprinted hydrogel scaffold composed of gelatin methacryloyl (GelMA). The team began optimization of hydrogel bioprinting and started evaluating GelMA cytotoxicity. Over a period of several days, cell viability of fibroblasts encapsulated within the GelMA hydrogels was recorded. It was found that the cells did not die when embedded in GelMA. Further testing and optimization are needed to obtain protocols for bioprinted hydrogels that have stiffnesses which mimic healthy and diseased lung tissue.

Introduction

Problem Statement

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. There are currently no tissue models that accurately recreate the lung extracellular matrix and its changes as a result of cell injury. Such a model would need to have tunable mechanical stiffness, as well as be cell adhesive and degradable. Dr. Allan Brasier of the UW School of Medicine and Public Health requires a scaffold that meets these criteria to be fabricated with a bioprinter. The scaffold must have a uniform and replicable composition that allows for epithelial cell culture in an air-liquid interface (ALI) so that his lab can study the effects of fibrosis on small-airway lung epithelial cells.

Background/Motivation

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) [1]. 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 [2] (see Figure 1).

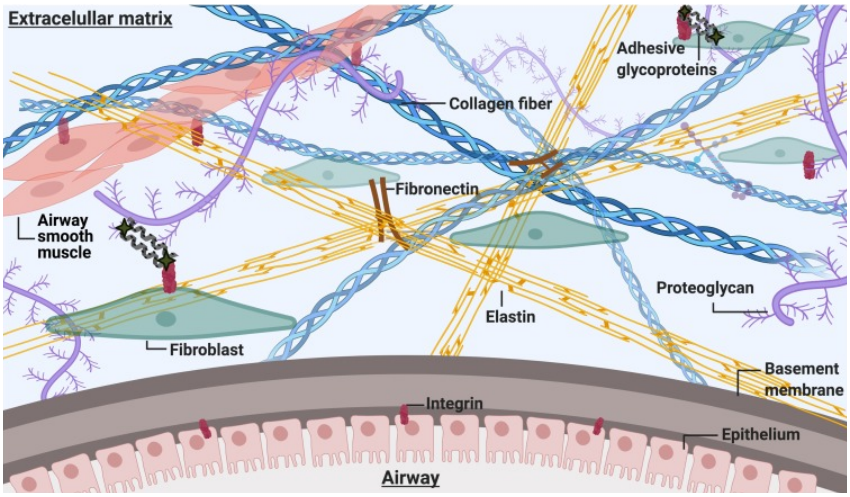


Figure 1: Graphic depicting the epithelial cells, fibroblasts, and ECM that make up the EMTU [3].

When chronic diseases cause epithelial injury, an inflammatory response causes the fibroblasts to increase production of ECM proteins such as collagen, a fibrous structural protein, and fibronectin, a key binding protein [1]. The increased protein deposition raises the mechanical stiffness of the lung ECM, which causes a positive feedback loop that leads to increased

fibroblast activity (see Figure 2). It is not fully understood how this increased ECM stiffness affects the epithelial cells.

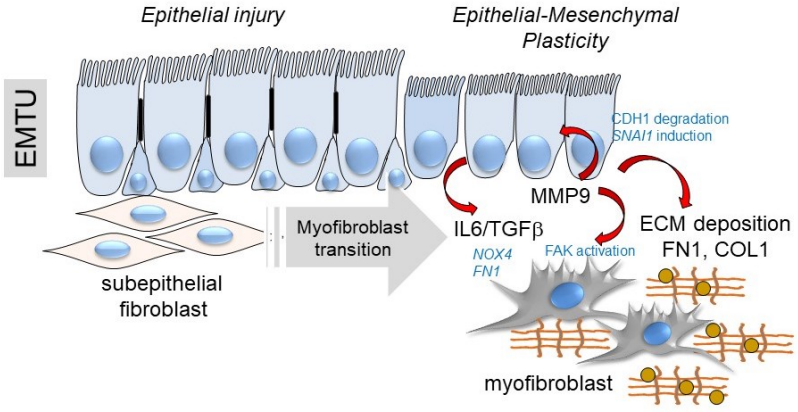


Figure 2: Schematic showing how epithelial inflammation causes fibroblasts to increase collagen production in the EMTU [3].

According to the World Health Organization, COPD was the 3rd leading cause of death globally in 2019 [4]. Over 120,000 people die from COPD annually in the United States alone [5]. While there are some treatments to alleviate symptoms, such as bronchodilators and oral steroids, there is no cure to COPD [4]. An accurately mimetic and tunable EMTU tissue model would allow for greater understanding of the effects of inflammatory diseases on lung epithelium. This knowledge could then be applied to translational research, such as developing therapies that target disease-induced changes to lung tissue.

Competing Designs

Tissue engineering models provide *in vitro* means to study the body that have enabled the creation of many novel designs. For models related specifically at the lung epithelium, there are currently both 2D and 3D models on the market that seek to mimic the *in vivo* environment. Unfortunately, these models are often oversimplified and do not provide accurate research results for cell behaviors obtained from experiments performed on these models.

2D models are typically layers of cells cultured on top of polymer or glass dishes. While several 2D model experiments performed over the years have allowed some study into cell function, disease, and the microenvironment, these models do not reflect typical cell behavior in the native environment. For reference, 2D models have a stiffness range of 2-4 GPa while healthy human lung ranges from 0.44-7.5 kPa [6]. The differences in stiffness significantly affect the function of fibroblasts in the ECM that differentiate into a specialized phenotype when mechanically stressed, resulting in experimental data not representative of behavior *in vivo* [7].

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 gel surface [8]. 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 compositional variations present in every batch. It also consists of proteins that may or may not be present in healthy ECM, reducing the accuracy of the model. Matrigel ECM is more similar to the ECM of a tumor with significantly more laminin, and glycoproteins, which can cause the microenvironment to be unlike native tissue.

The discrepancies 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.

Specifications

The primary goal of the project is to create a model that can mimic the extracellular matrix (ECM) environment of the small airway of the lung in both healthy and fibrotic lung conditions. This requires the ability to emulate the mechanical stiffness of the lung and ensure that the epithelial cells can adhere to the scaffold once it is cultured. It is also important that the scaffold is degradable, as this allows for fibroblasts to produce their own ECM proteins, remodeling the matrix and creating a microenvironment that resembles native lung ECM. In order to achieve these goals, the Young's modulus must emulate a healthy and fibrotic lung ECM environment. That is, to represent the environment that fibroblasts are subjected to in the lungs, the Young's Modulus (E) for the healthy and fibrotic hydrogels should be 2-5 kPa and ≥16.5 kPa, respectively [8]. Meeting these moduli values is crucial for accurately representing characteristics unique to healthy and fibrotic lung ECM states. This is imperative as the mechanical and biochemical properties of the scaffold, including any incorporation of peptides, must be compatible with the cells used during experimentation to maintain cell viability.

The model must also be compatible with an air-liquid interface to emulate the conditions that epithelial cells experience in the lungs; the apical (top) side of the cells is exposed to air while the basal (bottom) side of the cells is exposed to media. Additionally, the scaffold must be fabricated via bioprinting, so that the client can easily reproduce it. This requires careful consideration of the mechanical and biochemical properties of the scaffold, as well as the culture conditions used to maintain the viability and functionality of the cells. See Appendix B for the full Product Design Specifications.

Previous Semester

Final Design: Gelatin Methacryloyl (GelMA)

GelMA is an engineered gelatin-based material 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 made it an attractive choice. GelMA hydrogels with a 50% degree of functionalization (DOF) were previously constructed by using a pipette to insert 100 uL into a mold. They were then

photocrosslinked with 365 nm wavelength UV and variation of time between 1 and 5 minutes to produce normal and fibrotic tissue respectively. See Appendix F for the prior semester's evaluation of the preliminary design which led to GelMA being chosen, and see Appendix C for the hydrogel synthesis protocol.

Testing & Results

In the previous semester, rheology testing was applied to GelMA hydrogels to evaluate their mechanical properties, in order to accurately mimic normal and fibrotic extracellular matrix environments. The hydrogels, prepared at a 5% w/v concentration, were subjected to a 24 hour swelling period and categorized based on their assumed stiffness values. Normal hydrogels exhibited a thickness range of 1.25-1.80 mm, while fibrotic hydrogels ranged from 3.5-4 mm in thickness.

The testing procedure involved ensuring proper alignment of the hydrogel with the rheometer's bottom plate, specifying the gap value according to the hydrogel's thickness, and inputting testing parameters, including start and end frequencies, room temperature, shear strain, and samples per decade. The specific testing parameters were as follows:

- Start Frequency = 0.1 Hz
- End Frequency = 10 Hz
- Room Temperature = 25 ℃
- Shear strain = 1%
- 10 samples per decade

Following calibration, the frequency sweep test was conducted and took 10 minutes for each hydrogel. Test results were documented in an Excel spreadsheet, and thorough cleaning with 70% ethanol was performed between tests. The test yielded the storage modulus (G'), allowing for the estimation of the shear modulus (G) at very low frequencies (0.1 Hz). Young's modulus (E) was then calculated using the equation $E = 2G(1+\nu)$, with ν representing Poisson's ratio. Young's Modulus served as a valuable metric for quantifying the varying stiffness values between the normal and fibrotic ECM hydrogels.

In addition to the rheometric testing, an increase in cooling/setting time at 4 °C was observed to correspond with an increase in hydrogel stiffness. This was particularly evident in the fibrotic hydrogel, which had an average Young's Modulus of 49.2 ± 11.65 kPa. This observation is possibly due to the polymer chains of the hydrogel having more time to entangle, which made photocrosslinking more efficient. Normal gel batches 2 and 3 exhibited similar mechanical stiffness, averaging approximately 2.35 ± 1.62 kPa, though they showed greater variability compared to normal gel group 1. Normal gel batch 1, with a 30 second cooling time, most accurately represented the normal lung ECM, displaying an average Young's Modulus of 3.42 ± 0.49 kPa. These findings lay the foundation for the ongoing project and serve as a metric to characterize the mechanical properties of the hydrogel scaffold to be printed using the CELLINK bioprinter. Lastly, The study involved the client's laboratory assessing the biochemical properties of the hydrogel scaffolds. The gels were coated with a monolayer culture

of lung epithelial cells and were tested for cell adhesion and proliferation. Attempted confluent analysis through ImageJ brought challenges due to poor image quality and time constraints. Additionally, not all necessary images were supplied, limiting the depth of analysis. These findings complement the ongoing project's focus on hydrogel suitability for cellular growth and attachment, alongside mechanical property assessments. See Appendix E for the rheometry testing protocol.

Lessons Learned

One of the main lessons learned during the hydrogel synthesis process was the lack of replicability in the protocols created due to variability in UV distance with a moveable lamp and timing adjustments to reach a certain stiffness. The UV lamp being used in the BME team labs is moveable but cannot be set to a precise height for each trial, leaving room for error when photocrosslinking the gels. As a result, there was large in-batch variability of Young's modulus and raised concern for the replicability and efficacy of the existing protocol. Furthermore, when attempting to achieve a particular stiffness in trials with the same UV time, there were struggles to obtain the same values when repeating the protocol. This suggests the presence of nontrivial sources of error and the need to standardize fabrication methods to obtain batches which show little variability and consistent stiffness values.

Pertaining to the GelMA bioink itself, the use of 365 nm wavelength and 50% DOF provide little room for obtaining both the normal and fibrotic range of stiffness. The use of pre-characterized GelMA aims to eliminate variation caused by inconsistencies in hydrogel fabrication, and utilizing a longer wavelength of 405 nm will allow for tunability. That is, at wavelengths of 405 nm, the energy is low enough such that cell injury will not occur during UV crosslinking. Therefore, it is possible to tune stiffness by adjusting time of exposure to the UV light and/or the distance between the gel and the light without concern of cell injury.

Lastly, the hydrogels in the healthy tissue stiffness range had significantly lower cell adhesion and as a result cell proliferation than that of fibrotic stiffness. To mitigate this, the 24 hours of swelling will be conducted in the cell media to provide serum and proteins that promote adhesion rather than PBS.

New Direction: 3D Bioprinting

Due to the aforementioned complications seen with the pipetting protocol for GelMA hydrogel fabrication and the limitations of the gifted 50% DOF GelMA, the use of a bioprinter with pre-characterized GelMA will allow for the creation of more accurate and replicable scaffolds to be produced.

Materials and Methods

Materials

In the Fall 2022 semester, the client gave the team a $5,000 budget. In the previous 2 semesters, the team used a total of $1,091 of this budget to purchase materials to fabricate PEG hydrogel scaffolds and GelMA hydrogel scaffolds in molds, leaving $3,909 left in the budget. Due to the evolving nature of this project, the majority of the previously purchased materials have been given to the client as they are no longer relevant. The team is using a CELLINK BioX 3D Bioprinter provided by the client to make the tissue model. While the project's fabrication method has changed, the material has not; the scaffold is printed from CELLINK GelMA bioink with LAP 0.25% as a photoinitiator [9]. A CELLINK Temperature-Controlled Printhead is used in order 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. Additional GelMA bioink cartridges were purchased. For cell viability testing, hydrogels were made using molds and lyophilized GelMA that was gifted by the team's former advisor, Dr. Kristyn Masters. Collagenase was purchased to degrade GelMA gels to obtain embedded cells for trypan blue staining for determining cell viability. As an alternative cell viability assay, LIVE/DEAD Viability Kit was used with calcein-AM and ethidium homodimer to stain live and dead cells, respectively. By the end of the semester, the team spent $1,486 and now has $3,514 left in their budget. A full materials list with expenses can be found in Appendix A.

Methods

The pipette-based method employed in this study acted as a surrogate for precisely adjusting the properties intended to be reproduced in the subsequent bioprinted method. Gel properties were fine-tuned by employing controlled cooling and setting at 4 °C for specific durations: 3, 5, and 10 minutes. This step was introduced to modulate the initial gelation process, thereby influencing the subsequent mechanical characteristics of the GelMA hydrogels. After this stage, 365 nm UV light exposure was utilized to ensure consistent photocrosslinking at a predetermined distance of 1 inch. The pipette-based method was not intended to be continued independently, but rather served as a tool for parameter optimization and fine-tuning, guiding the replication of desired properties in the subsequent bioprinted method. This sequential approach allowed for a systematic exploration of gel characteristics, emphasizing the complementary role of the pipette-based method in the overall experimental design.

The bioprinted GelMA scaffolds are fabricated with a CELLINK BioX bioprinter (see Figure 3). First, the bioink is warmed to a liquid consistency to make it printable. Then, the bioprinter is calibrated and parameters are programmed to the desired settings. A CAD file with a specific print structure can be uploaded to the bioprinter, or one of the preset structures can be used. Finally, the bioprinter prints the scaffold layer-by-layer. The GelMA bioink contains LAP photoinitiator, so the scaffold can be directly photocrosslinked after printing. Several parameters

can be optimized to achieve high fidelity prints, including printing pressure and speed. The mechanical stiffness of the printed scaffold can be tuned by adjusting the time and/or distance of the UV photocrosslinking. The full procedure for bioprinting GelMA can be found under Appendix D.

Figure 3: The client provided the team with a CELLINK BioX bioprinter [10].

Testing

Rheology

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 (see Appendix E for the rheometry testing protocol). Young's Modulus was derived using the equation described in the results section below. 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, the team again ensured 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.

Gel Imaging

Upon production of cell laden gels, brightfield and fluorescence microscopy was performed to assess cell morphology and viability. Brightfield images were taken at various depths of the hydrogel using both a Nikon microscope canon, and an Olympus IX71 microscope using NIS elements. Brightfield imaged cells are qualitatively described in terms of fibroblast-like morphology. Brightfield images were taken at 24, 48, 72 hours.

To preliminarily assess cell variability, LIVE/DEAD staining with calcein-AM and ethidium homodimer was employed. Cell laden hydrogels were rinsed twice with 1X PBS prior to 20 minutes of incubation in 3µM, 3µM calcein-AM, and ethidium homodimer supplemented PBS at 37 °C [11][12][13]. A multichannel image was taken using FITC and TRITC filters to capture the green and red emission spectra of calcein-AM and ethidium homodimer respectively. Imaging was performed on an Olympus IX71 microscope using NIS elements. Images were exported as JPEGs to be analyzed in ImageJ. Green emissions are attributed to live cells, red emissions are attributed to dead cells [13]. Fluorescent images were taken at 24 and 48 hours. Planar images of gels were also used as a surrogate for total cell number, until a more quantitative method to assay cell number is identified

Bioprinting Optimization

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 duration and distance must be decided. At the start of the bioprinting process, the initial structure consisted of a 10x10x1 mm cubic

structure with a rectilinear, grid-lattice as the inside component. The infill density was left at the standard of 15% and it was one layer. The dissolution temperature to create a liquid GelMA for printing was set to 37℃ for 30 minutes. The liquid state was ensured by removing the cartridge and visually checking that air bubbles within moved. The printer head temperature was then set to 27°C with an equilibrium time of 2-3 minutes and the extrusion pressure was set to 35 kPa.

A cylindrical structure of 1 x 0.6mm with a concentric infill of 35% was selected as the final print structure. The dissolution temperature was set at 37℃ for 30 minutes and changed to a print temperature 28°C with an equilibrium time of 20 minutes to let the GelMA adjust temperature. The extrusion pressure was set to 22 kPa and once printed it was photocrosslinked with 405 nm UV light for 2 seconds at a distance of 4 cm.

Results

Rheology

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 = 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.

For the pipette-based hydrogel trials, five different groups of gels were created with varying cooling and photocrosslinking times. These parameters were altered over the course of the experiment to fulfill the design requirements for healthy and fibrotic ECM. For the normal batch gels 1, 2, and 3, the UV crosslinking time was kept constant at 5 minutes and cooling time was varied from 3 to 10 min. These results showed that an increase in cooling time before photocrosslinking led to an increase in hydrogel stiffness. For the fibrotic gels shown below, the cooling time was kept constant at 5 minutes and the crosslinking time was increased from 7 to 10 minutes. As can be observed with the fibrotic gels below in table 1, the increasing of UV crosslinking time resulted in a much more drastic effect when varied. These results showed a 3 minute increase in UV time, leading to over a 400% increase in stiffness.

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

Rheometry testing was also performed on the bioprinted gels. Due to complications and bioprinter optimization, variation of cooling time and crosslinking was unable to be explored. However, baseline stiffness measurements were obtained with a trial of gels seen in Table 2 below. Crosslinking was performed with a 405 nm for 2 seconds. Bioprinter bed cooling settings were not investigated. Gels showed an average Young's Modulus of about 1 kPa, being slightly below the healthy lung ECM specified values. Normal batch 1 of bioprinted hydrogels also showed a very high standard deviation of 915 Pa between gels that were tested in this batch.

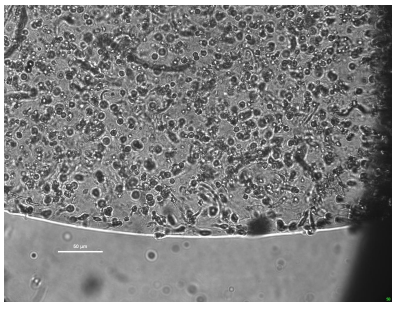
Bioprinted Hydrogels		
Gel Batch	**Condition (UV)**	**Young's Modulus (Pa)**
Normal Batch 1	2 seconds	995 ± 915

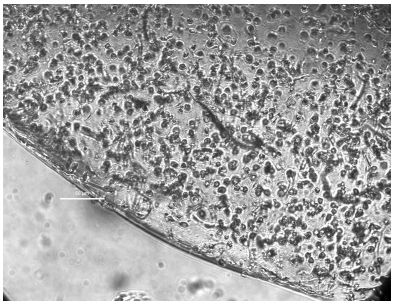
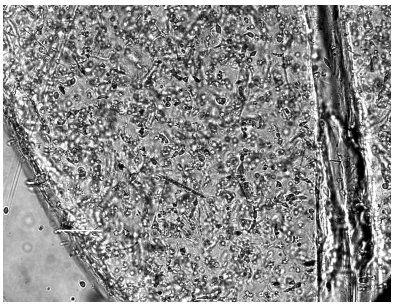
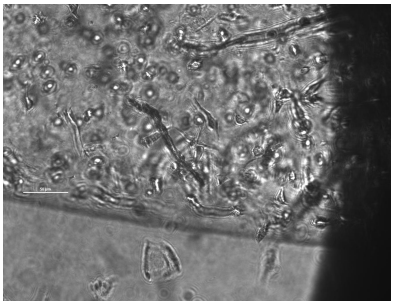
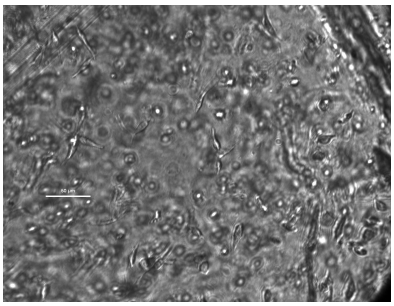
Table 2: Young's Modulus data from bioprinted hydrogels with specified crosslinking conditions

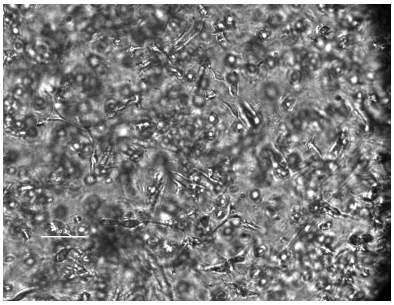
Gel Imaging

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 [14]. See Table 3 for brightfield images of encapsulated fibroblasts

Table 3: Brightfield images of cell-encapsulated gels

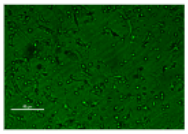
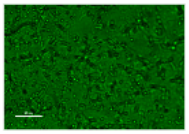
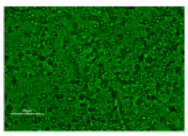
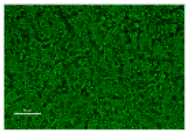
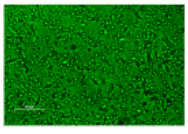
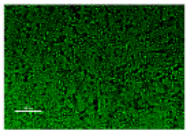
Zoom	**Timepoint**	**Image**
10x	24 hours	

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

Zoom	Timepoint	Image
	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 4). Either by this quantitation, or by qualitative assessment, over all timepoints, as gel stiffness increases, cell density increases.

Table 4: 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	

Bioprinting Optimization

The success of a bioprinter trial this semester was determined by 1) the final photocrosslinked structure matching the idealized design and 2) having stiffness that falls within the range of healthy or fibrotic tissue. The initial trial detailed in the testing section and all others excluding the final print met neither of these criteria (see Table 5). The final print session

resulted in a structure that met the first criterion of success but did not meet the second standard, as its Young's Modulus was 995 +/- 915 Pa.

Table 5: Bioprinter trials with their parameters. Several runs were done for each trial. While not noted in the table, UV time was around 4–5 seconds for each trial except the final trial, which was 2 seconds. Success was determined by the gel having the designed printed structure and by the gel having the desired stiffness. Success in both of these metrics is denoted by +/+ while failure in both is denoted by -/-.

Print Trial	Structure	Infill Density	Print Temperature	Equilibrium Time	Extrusion Pressure	Success
1	Cubic	15%	27℃	2-3 min	35 kPa	-/-
2	Cubic	15%	27℃	2-3 min	35 kPa	-/-
3	Cubic	15%	27℃	2-3 min	27 kPa	-/-
4	Cubic	15%	27℃	10 min	30 kPa	-/-
5	Cylindrical	15%	28℃	10 min	33 kPa	-/-
6	Cylindrical	15%	27℃	10 min	27 kPa	-/-
7	Cylindrical	60%	28℃	15 min	23 kPa	-/-
8	Cylindrical	50%	28℃	20 min	25 kPa	-/-
Final	Cylindrical	35%	28℃	20 min	22 kPa	+/-

Discussion

The rheometry testing conducted on GelMA hydrogels has yielded significant insights into the mechanical properties of both pipette-based and bioprinted hydrogels. The Young's Modulus data, a critical parameter for characterizing hydrogel stiffness, is particularly valuable in the context of biomimetic applications, such as replicating the mechanical properties of healthy and fibrotic extracellular matrices (ECM).

The investigation into variations in cooling and photocrosslinking times for pipette-based hydrogels presented a direct correlation with their mechanical properties, as evidenced by the observed Young's Modulus values. Maintaining a consistent UV crosslinking time at 5 minutes for the normal batch gels allowed for the focused examination of cooling time's influence on stiffness. The positive correlation between increased cooling time and heightened hydrogel stiffness aligns with existing literature indicating that longer cooling times enhance chain

entanglement, optimizing subsequent crosslinking for higher stiffness and highlights the precise control required over these parameters to tailor hydrogel mechanical characteristics [15]. The heightened sensitivity of fibrotic gels to UV crosslinking time is particularly notable, where a 3 minute increase in cooling duration between batches resulted in a substantial 400% rise in stiffness. This heightened sensitivity relays the importance of precise control in the fabrication process, especially when modeling the altered composition and structure of fibrotic ECM. Further, these findings emphasize the critical role of cooling and crosslinking times in achieving specific stiffness profiles for biomimetic applications.

The initial trial conditions detailed in the testing section resulted in the hydrogel dispersing outward into a structureless body. This was likely due to the fluid being extremely low viscosity, as the wait time between setting the temperature from 37℃ to 27℃ was only 2–3 minutes. This did not leave a thorough enough time for the GelMA to change temperature with the printhead. Additionally, this print trial was not conducted using the thermal insulation nozzle cover, which aids in maintaining the temperature of the GelMA through both the cartridge and the printing nozzle. This nozzle in later trials helps prevent clogging of GelMA in the nozzle tip due to a decrease in temperature. Furthermore, this trial and all those up until the most recent were conducted using expired GelMA.

Over the semester, through trial and error, the final print session successfully created cylindrical structures that are of size compatible with an ALI. The change from a cubic to cylindrical structure was due to the need for the final product to be compatible with an ALI. Both of these structures are preloaded on the bioprinter, but the cubic is detailed in the CELLINK bioprinting video, which led it to be the initial choice [16]. Allowing for more time between temperature changes ensured that the temperature of the GelMA matched what the printer head was set to during extrusion. Lowering the extrusion pressure ensured that the volume of ink being extruded did not spread outward once on the surface. This change allowed for the structure being printed to retain its shape from printing till being set by the UV light crosslinking. As mentioned above, the final hydrogels printed had Young's Modulus values of 995 +/- 915 Pa. This stiffness does not fall within the range for either healthy or fibrotic lung tissue, but with the UV crosslinking time being set to 2 seconds, it leaves room for an increase in time so that the necessary ranges can be reached. Others creating similar models with the CELLINK materials have used longer times such as 10 seconds to create complex 3D structures with GelMA [17]. Furthermore, it has been demonstrated that 60 seconds at a wavelength of 405 nm resulted in GelMA hydrogels with a Young's Modulus reaching 98 kPa [18]. Both of these articles instill confidence that these findings would translate well to our model, and UV time adjustment will lead to success of having models of both normal and fibrotic stiffness.

In comparison to the CELLINK GelMA printing protocol, our final parameters for extrusion pressure and temperature fall within the range recommended by the company [19]. This protocol in contrast does not detail the necessary parameters to achieve a particular structure nor does it account for nozzle clogging due to the temperature sensitivity of GelMA which were key challenges overcome this semester.

The calcein-AM / ethidium homodimer LIVE/DEAD staining conducted this semester did not result in images that are up to the standard of the literature, and thus were not quantitatively considered. Notably, and obviously, green background noise under FITC imaging of calcein-AM made it difficult to distinguish what is and isn't a live cell. Furthermore, under both brightfield microscopy and LIVE/DEAD staining, cell morphology in the center of the gels appears spherical, non-fibroblastic, and non adhered. These cells do appear to have a greater intensity under FITC imaging of calcein-AM relative to the confounding background noise, which may indicate that the cells are, in fact, alive, but not morphologically expressive. Sources of possible LIVE/DEAD imaging errors have been identified as leaving the laser on when imaging, improper covering of fluorescent samples, too long incubation in imaging solution, or improper washing of media from gels.

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, two primary models were developed in parallel and will continue to be improved so that a successful 3D cell culture model of the epithelial mesenchymal trophic unit can be implemented as a successful research tool. Both models were fabricated using gelatin methacryloyl hydrogel, a material appropriate for application with its documented ease of use, low cost, and adjustable mechanical and biochemical properties. This 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, however, stiffness values produced by all bioprinted scaffolds as well as pipette-based fibrotic models did not fall within the target range representative of native lung tissue. These results serve as proof of concept and evidence that stiffness can be modified, but further efforts must be made to achieve the required values.

LIVE/DEAD calcein stain imaging of cell-laden gels produced through the pipette model were inconclusive and resulted in images containing a stained background which washed out what would have been stained live cells. Further iterations of cell imaging methods and trials are needed in order to gather data regarding the cell viability of the produced hydrogels. It is predicted that this imaging will first be achieved using the pipette-fabricated hydrogels before being applied to the final models of the bioprinted hydrogels, as the priority for bioprinting remains developing gels displaying appropriate stiffness values.

In the future, the hydrogel fabrication process will continue to be optimized in order to achieve models that have the desired healthy and fibrotic stiffness. The priority should be achieving these values using the bioprinting fabrication technique, as this is ultimately the desired fabrication protocol of the client. This is to be achieved primarily through the modification of crosslinking parameters, such as UV crosslinking duration following print extrusion. Another future objective is to calculate the swelling ratios of produced gels by comparing the weight before and after swelling. As mentioned above, developing successful

protocols capable of imaging the gels and providing information related to cell viability is also a primary concern for upcoming work.

Once the team can reliably produce gels of both stiffness conditions, fibroblasts and collagen can be encapsulated in the gels to incorporate fibers similar to *in vivo* fibrosis. This encapsulation process was initiated this semester with the encapsulation of 3T3 fibroblast cells, but no conclusion could be made regarding cell viability due to the aforementioned complications. Future imaging technique development is needed to quantify the success of cell viability in future scaffold development. Degradation testing can be done on these scaffolds by either adding collagenase or by allowing the fibroblasts to degrade the scaffold on its own (via MMPs) and measuring gel weight over time. 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. Furthermore, the team will look at cell adhesion and proliferation through confluency analysis with ImageJ on high quality images of the cultured epithelial cells

The future work discussed here will allow for a more *in vivo*-mimicking tissue model that the client can use in his research on how the stiffening of the lung ECM caused by chronic lung inflammation affects the small airway epithelium.

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Appendices

Appendix A - Materials & Expenses

Item	Description	Cost/Quantity
Fall 2022/Spring 2023 Items	Unusable PEG materials and GelMA materials	$1,041
Molds	Silicone molds to make GelMA hydrogels by hand	$50
GelMA	Lyophilized GelMA gifted by Dr. Masters	Provided by former advisor
Bioprinter	CELLINK Bio X 3D Bioprinter	Provided by client
GelMA Bioink	CELLINK GelMA Bioink LAP 0.25% 3mL cartridge x3	Provided by client
Temperature-Controlled Printhead	CELLINK Temperature-Controlled Printhead	Provided by client
Nozzle Cover	CELLINK Thermal Insulator Nozzle Cover	Provided by client
GelMA Bioink	CELLINK GelMA Bioink LAP 0.25% 3mL cartridge x3	$325
Collagenase	25 mg collagenase to degrade GelMA gels	$70
LIVE/DEAD Viability Kit	Calcein/Ethidium stains for cell viability	Provided by BME Teaching Lab
Total		$1,486

Appendix B - Product Design Specifications

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:

The bioprinting of the scaffold must be easily replicable so 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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Appendix C - Hydrogel Fabrication Protocols

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

Appendix D - GelMA Scaffold Bioprinting Protocol

This protocol was adapted from CELLINK's GelMA bioprinting protocol as well as notes from a CELLINK field scientist

1. Warm up the GelMA to 37°C to have a liquid formulation in a water bath (~20 minutes, or until visibly liquid)
    a. The temperature-controlled printhead can also be used to warm the GelMA
2. Cap the GelMA cartridge pressure hose end cap and a 22G nozzle then place in a temperature-controlled printhead set at 27°C
    a. Be sure to have the thermal insulator nozzle cover attached to the temperature-controlled printhead to prevent bioink gelling within the nozzle
3. Wait for the GelMA to reach the set temperature (5-10 minutes). While the gel is reaching printability temperature, set up and prepare the BIO X bioprinter for printing
    a. Set the print bed temperature to 15°C
    b. Set up the printing protocol with desired printing surface (well plate or Petri dish), bioink profile, printing pattern, pressure, speed, delay, and photocrosslinking settings
        i. For GelMA bioink, it is recommended to start at 5 mm/s print speed, 23-33 kPa print pressure, 500ms pre-flow delay, 10 s photocrosslinking time with 405 nm UV light, and 5 cm photocrosslinking distance

4. Calibrate the bioprinter such that it will print in the back half of the Petri dish so the 405 nm UV light will reach the print
5. Test the pressure to see if any bioink extrudes
    a. If there is extrusion, preemptively unclog the nozzle with a needle and then start the print
    b. If there is no extrusion, increase the pressure and test again
6. Fine-tune printing settings to achieve desired construct
    a. Adjust printer speed, pressure, and pre-flow delay to get even/consistent printing
    b. Adjust photocrosslinking distance and photocrosslinking time to tune the stiffness of the print

Appendix E - Frequency Sweep Rheometer 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.

Appendix F - Spring 2023 Preliminary Designs & Evaluation

Below is an excerpt from the prior semester's Final Report detailing the preliminary designs and how they were evaluated, which led to the selection of GelMA. As it is an excerpt, citation numbers are without context. See Spring 2023 Final Report for the relevant citations.

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.

Design Matrix

Table 1: Design Matrix for Tissue Model Scaffold consists of eight design criteria to evaluate each design.

		Design 1: Gelatin methacryloyl (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.

Design Matrix 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 and migration of cells just as native ECM has properties that allow for cell adhesion to the ECM and cell movement.

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 cell's sensitivity 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 3.5 kPa to 16.5 kPa.

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 may 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 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 within the client's research.

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.

Design Ratings/Selection

Based on the design criteria as described above, the preliminary design GelMA scored the highest with a score of 74 particularly due to its high rankings for mechanical properties and ease of fabrication. Looking into mechanical properties specifically, GelMA and PEG received the same rankings due to both having the ability to achieve the Young's modulus of 2 kPa and 17 kPa for both normal and fibrotic lung tissue respectively [10][8]. The mechanical properties of lung ECM received a significantly lower ranking due to its inability to retain the mechanical properties of the native tissue it was decellularized from [9].

For biochemical properties, lung ECM ranked the highest under this category because it maintains the natural adhesive and degradation motifs found in the native tissue it was derived from [9]. In comparison, GelMA ranked second in this category because it does have natural cell adhesion and MMP sequences incorporated but not to the same level of native tissue [8][11]. PEG doesn’t have any naturally occurring cell adhesion or degradation components but can have these incorporated [10]. This results in a more expensive and difficult fabrication process for PEG.

Under the ease of fabrication category and ease of use, GelMA ranked the highest due to the ability for no peptides for cell adhesion or degradation needing to be added and also multiple mechanisms to control mechanical properties [12]. These mechanisms of control can include degree of substitution during methacrylation, cooling time at 4 °C, and time under UV light. The downside to GelMA fabrication is its batch to batch variation due to possible differences in substitution during methacrylation which can influence its mechanical properties, this can be remedied by characterizing the GelMA after the reaction [12]. PEG ranked low under these categories due to difficulties in previous semester fabrication processes and the need to incorporate RGD and MMP sequences. Along these lines, the lung ECM hydrogels are difficult to construct and are a laborious process that includes the use of surfactants and enzymes but this can be mediated by purchase of powder and buffer [9]. As a result both PEG and lung ECM would later on, be difficult for the client to reproduce, therefore the ease of use ranks lower for these designs as well.

Mechanical tunability describes the ability of the material’s mechanical properties to be tuned, which both GelMA and PEG rank highest in. Both of these designs have multiple mechanisms for tunability such as degree of substitution, chain length, cooling time, and time

under UV light [8][10]. All these mechanisms to achieve the desired stiffness range result in GelMA and PEG ranking higher in comparison to lung ECM which does not allow for tunability to specific mechanical properties.

Biochemical tunability is the same premise but based on the ability to achieve the necessary cell adhesion and degradation of the hydrogel, which PEG ranks the highest for. While PEG ranked the lowest in biochemical properties due to the lack of them naturally occurring, under tunability it ranks the highest because it is a blank slate. PEG is capable of having RGD and MMP sensitive sequences incorporated which allows for the concentrations to be varied, and as a result, tune the hydrogel biochemically[10]. GelMA has these biochemical properties naturally occurring within the gelatin but is not able to be as controlled as much as PEG resulting in a slightly lower score. Lung ECM is also already biochemically active with the cell adhesion and degradation motifs found in native tissue but during production of the hydrogels these properties are not able to be controlled resulting in a lower biochemical tunability score.

Lastly, GelMA is significantly cheaper than PEG (due to peptides increasing the cost) and lung ECM with gelatin powder available for $116/kg and methacrylic anhydride available for $0.56/mL [13].

Appendix G - Live / Dead Staining Procedure

Staining procedure [1]

Sample preparation

1. Aspirate media from scaffold wells
2. Wash multiple times with PBS

Staining

The following should be done under dark conditions, or shielded from light exposure as best as possible.

1. Add 1µM, 5µM calcein-AM, ethidium homodimer
2. Allow to incubate of 15 minutes at 37°C

Imaging

The following should be done under dark conditions, or shielded from light exposure as best as possible.

1. Image scaffold under using multichannel imaging of FITC and TRITC filters
2. Adjust exposure and filter cutoffs as necessary to reduce background noise,

Works Cited

[1] Cohen, Noam et al. “PEG-fibrinogen hydrogel microspheres as a scaffold for therapeutic delivery of immune cells.” Frontiers in bioengineering and biotechnology vol. 10 905557. 9 Aug. 2022, doi:10.3389/fbioe.2022.905557