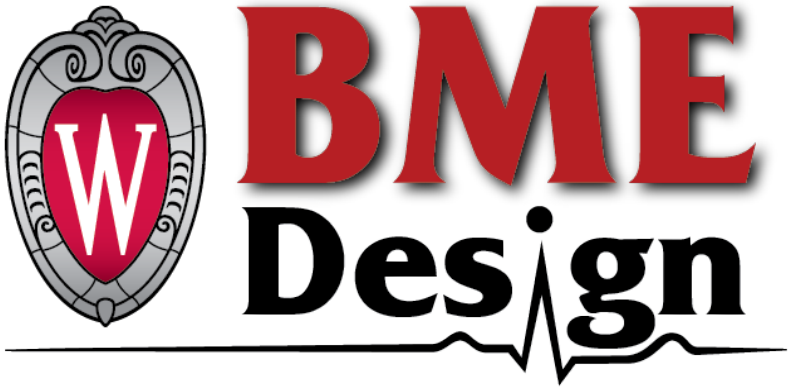
Tissue Model of The Epithelial Mesenchymal Trophic Unit



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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. This presents a problem because when this tissue is damaged, a fibrotic response is triggered in sub-epithelial fibroblasts that result in further disease and fibrosis. Currently, no scaffolds exist that accurately model the lung extracellular matrix (ECM) and its changes due to cell injury. Specifically, incorporating the following in combination: varying mechanical stiffness and tension, incorporation of collagen and fibronectin into the ECM, and cell adhesive properties [1]. As the research on lung diseases evolves, the need for a synthetic scaffold that accurately mimics the ECM increases. This project aims to create a replicable synthetic scaffold with uniform composition that allows for culturing of lung epithelial cells. A synthetic material of polyethylene glycol seeded with collagen and fibronectin was decided to be a viable option for the design with another alternative being a geometrically and practically simpler material: gelatin. In this design iteration, gelatin hydrogels were fabricated and tested within the BME teaching lab at UW-Madison.

Introduction

Problem Statement

A multitude of chronic lung diseases such as pulmonary fibrosis, asthma, and COPD can cause damage to epithelial tissues of the lungs. This presents a problem because when this tissue is damaged a fibrotic response is triggered in sub-epithelial fibroblasts that results in further disease and fibrosis. There are currently no scaffolds that accurately model the lung extracellular matrix and its changes due to cell injury, specifically the following properties in combination: varying mechanical stiffness and tension, porosity, incorporation of collagen and fibronectin within ECM, and cell adhesive properties. Dr. Brasier of the UW School of Medicine and Public Health requires a scaffold that meets these criteria while having a uniform and replicable composition that allows for epithelial cell culture at an air-liquid-interface (ALI).

Background/Motivation

The lung extracellular matrix is a highly complex system of proteins and macromolecules that function as a support system for lung tissue, provide a track for cell migration, and aid in injury repair. The two major compartments found in the lungs are basement membranes and interstitial spaces [2]. The ECM is found in the interstitial spaces compartment and contains fibroblasts, a cell vital to the creation of connective tissue. In times of epithelial injury, fibroblasts are activated and secret ECM proteins such as fibronectin and collagen [3]. Fibronectin functions as a scaffolding protein to maintain and direct tissue organization, while collagen has the ability to direct tissue development and regulate cell adhesion [4]. Together, these cells provide the basis for the repair of tissue in the ECM and are vital to injury repair in the lungs.

Scaffolds are structures found in 3D cell cultures, commonly made from biopolymers. [5]. Cell scaffolds are used to provide attachment and subsequent support for cell development and can be modified to mimic *in vivo* ECMs [6]. Furthermore, for respiratory research, cell cultures are grown at an air-liquid interface. This involves the basal surface (usually the bottom surface) being submerged in liquid medium while the apical surface will be exposed to air [7].

Competing Designs

Tissue engineering models provide *in vitro* means to study the body that have enabled the creation of many amazing and novel designs. For models looking specifically at the lung epithelium, there are currently both 2-D and 3-D models on the market that seek to 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 2-D 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 2-D 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 2-D models have a stiffness range of 2-4 GPa while the human lung ranges from .44-7.5 kPa [8]. The differences in stiffness greatly 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* [9].

While there are many varieties of 3-D 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 [10]. These 3-D models have variations in methods for each experiment, but generally all involve an ECM gel 3-D environment that is more similar (with some limitations) to *in vivo* than the 2-D models. Matrigel is a mouse tumor extracellular matrix mixture, so there are variations for every batch and consists of proteins that may or may not be present in healthy ECM which reduces the accuracy of the model. Matrigel ECM is more similar to the ECM of a tumor with significantly more laminin 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 requirement of the project is the ability to mimic the ECM environment of the small airway of the lung. This will be defined by the ability to emulate the mechanical stiffness of the lung (0.44-7.5 kPa) [8], epithelial cell adhesion once culture is applied, and the scaffold's degradability. The model must be degradable in order for fibroblasts to produce their own fibronectin and collagen to remodel the matrix and create a native ECM environment. Additionally, the storage modulus ($G' = 2.12 \pm 0.61$ kPa) and loss modulus ($G'' = 0.212 \pm .061$ kPa) of lung tissue and that of the hydrogels should be within a similar range[11]. The model must take the form of an air-liquid interface to emulate the conditions which epithelial cells experience in the lungs. All mechanical and biochemical properties of the scaffold, including any incorporation of peptides, must be compatible with the cells applied during experimentation, again in an effort to maintain cell viability. To promote high cell viability the model will include RGD peptides over a range of 2.6-5 mM [12]. Lastly, for reconstruction of the gel (over a month of cell culturing) via fibroblasts, matrix metalloproteinase (MMP) peptides will be incorporated so the gel will be degradable [13].

Preliminary Designs & Evaluation

Design 1: Gelatin Methacryloyl (GelMA)

GelMA is an engineered gelatin-based material with naturally occurring adhesive properties. This is due to the presence of arginine-glycine-aspartic acid, which are RGD sequences that allow for cell adhesion as they are derived from cell-binding sites on fibronectin, a native ECM protein [14]. While this natural adhesion is very beneficial, studies have shown that when constructing the GelMA hydrogel, there can be variations in composition from batch to batch. This variation can happen during the GelMA formation process because if the methacrylic anhydride doesn't attach correctly to the gelatin, the resulting hydrogel after crosslinking will not be viable for cell culture [15].

Design 2: Polyethylene Glycol (PEG)

PEG is a synthetic polymer, when cross linked 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, which this synthetic material does not naturally possess. The Young's modulus of PEG can fit within the range of native healthy lung tissue based on the percent concentration added to the solution [10]. PEG has a multitude of different reactive end groups, each with its own benefits and drawbacks which will be discussed in further sections.

Design 3: Alginate

Alginate has been used for many biomedical applications including wound healing, bone graft substitutes, and cell therapy. Alginate is a naturally occurring polysaccharide that can be extracted from brown seaweed. Highly purified alginate samples are essential for 3-D tissue scaffolds due to the impure samples disrupting cell signaling pathways. These polysaccharides consist of linear, unbranched 1,4 residues of β-D-mannuronic acid (M) and α-L-guluronic acid (G). The chemical composition and distribution of these blocks in the alginate molecule play a crucial role in forming ionic gels. The blocks can vary considerably in length and distribution depending on what species and part of the seaweed that alginate is extracted from. Gel elasticity, porosity and stability can be increased by using samples with increased G content and length of G-blocks in the 3-D model. Alginate itself shows little to no cell adhesion and cellular interactions but can be improved with peptide coupling. The coupling of an RGD (Arginine-Glycine-Aspartic Acid) peptide can greatly increase cell adhesion, cell proliferation and cell differentiation. Increased cell adhesion can also be achieved by the addition of biologically active materials such as collagen [16].

Design Matrix

Table 1: Design Matrix Comparing Three Scaffold Materials

		Design 1: Gelatin Methacrylate (GelMA)		Design 2: Polyethylene Glycol (PEG)		Design 3: Alginate	
Design Criteria	Weight	Score	Weighted Score	Score	Weighted Score	Score	Weighted Score
Biochemical Properties	30	4/5	**24**	3.5/5	21	3.5/5	21
Mechanical Properties	25	4/5	20	5/5	**25**	3/5	15
Ease of Fabrication	20	3/5	12	4/5	**16**	2/5	12
Ease of Use	15	5/5	**15**	5/5	**15**	3/5	9
Cost	10	4/5	**8**	3/5	6	2/5	4
Total:	**100**		**79**		**83**		**61**

Design Matrix Criteria

Biochemical properties are defined as the ability for the scaffold to adhere to the biocompatibility, porosity, adhesiveness, and cellular differentiation capabilities that are similar to native lung ECM. This category also includes the ability for the scaffold to degrade via fibroblast remodeling of the ECM. The suitability of any synthetic scaffold is based on the properties found where it is intended to mimic. This means that the scaffold must be compatible with the collagen, fibronectin, and must allow for adhesion of any cells that need to be cultured on it. The porosity of the scaffold is similar to that of native lung tissue which will allow for cellular communication or migration of cells. This porosity will be focused mainly on water soluble molecules due to those being typically involved. Native ECM has adhesive properties to it that allow for cell adhesion to the ECM and further communication. Synthetic materials lack these adhesive sites and do not have the biochemical signals to communicate. As a result, the synthetic scaffold must be able to be crosslinked with peptides or proteins that allow for cell adhesion in order for it to have cells embedded within it and for those cells to communicate via biochemical signals and receptors such as integrins. Therefore, the scaffold must not cause a toxic or immune response in the cells being seeded. The scaffold must then also be able to degrade in order for embedded fibroblasts to produce proteins such as collagen and fibronectin so that the model can eventually evolve to a native ECM produced by the fibroblasts to create

ideal conditions for epithelial cell culture. The degradability of the scaffold must be able to be accomplished via MMP peptides or natural degradation abilities of the polymer.

The mechanical properties of the synthetic scaffold will need to reflect those of the native lung environment. The ECM changes in mechanical properties such as tension 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. This means that the Young's Modulus of the scaffold must fall within that of healthy lung tissue between .44-7.5 kPa [8]. However, Young's Modulus doesn't provide a complete depiction of the mechanical environment sensed locally by cells in the lungs due to the viscoelastic characteristics that the lungs possess[11]. Taking this into consideration, the storage modulus (G') and loss modulus (G'') offer a more accurate representation of these properties sensed by cells. Therefore, the viscoelastic properties of the storage modulus (G' = 2.12 ± 0.61 kPa) and loss modulus (G'' = 0.212 ± .061 kPa) need to be met.

Another criterion, ease of fabrication, is important when choosing a synthetic material due to the wide range of methods when constructing the scaffold. Crosslinking is the means used to form hydrogels and this is the process of the reactive chain ends forming together. This reaction can occur through a free radical polymerization (chain growth) approach or through step growth polymerization. This crosslinking method is the main factor in ease of fabrication due to the ability for the materials and tools to be procured vary.

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 scaffold already built into the 24-well plates and made of a translucent material so that imaging during cell culture can be done is mandatory.

Lastly, cost is a factor when determining the synthetic material, type of crosslinking, and molds to set the gel inside, all of which cannot fall outside of the client's desired budget.

Design Ratings/Selection

In the three considerations discussed above, polyethylene glycol(PEG) hydrogel scored the highest. To evaluate each category, the biochemical properties were first considered. PEG does not have the natural adhesive properties as GelMA does causing it to be rated slightly lower. While it is lower than GelMA, previous experiments in literature indicate that PEG has high cell viability (>90%) and adhesion when natural components such as collagen or fibronectin are seeded [17]. This high cell viability is possible because of PEG being non-toxic and hydrophilic. As an added benefit, PEG is also capable of being crosslinked with MMP peptides to increase its degradability over time [18]. Due to these beneficial properties, PEG received a score of 21. The varying composition of alginate causes unpredictable degradation rates that are not ideal for a controlled environment, and thus PEG is preferred as the degradability is highly controllable and modifiable.

In the mechanical properties criterion, PEG ranked the highest due to its wide range of Young's modulus from 5-300 kPa which can be fine tuned by the percent concentration and

molecular weight chosen [8]. While both Alginate and GelMA can be adjusted to fit within this range, literature has shown that both have varying batch-to-batch compositional outcomes. For Alginate this is due to being a naturally derived polymer from brown seaweed [16]. Because it is naturally derived there are differences in its molecular weight and chain sequencing which can cause large variabilities in stiffness and stability for each scaffold made. For GelMA, the instability is due to the reaction mechanism not always producing the desired product and thus forming gels that are unusable. Due to the shortcomings of GelMA and Alginate with mechanical properties and reliability to reproduce the same properties repeatedly they received a lower score while PEG was highest at 25.

PEG received the highest rating for ease of fabrication on account of the multiple means of crosslinking being possible. Within PEG hydrogels there are different reactive end chains that can be chosen from to create the scaffold. To connect these reactive chains is a process called crosslinking that can be completed via a step-growth reaction or free radical polymerization reaction (chain growth). For our project, we will be using a chain growth method with PEG-norbornene (PEG-NB) and PEG-dithiol (PEG-DT) being crosslinked with UV light at 365 nm [10]. As a result of the feasibility to accomplish crosslinking with PEG, it received the highest ease of fabrication.

Both GelMA and PEG ranked similarly for ease of use due to them having translucency that permits for imaging during cell culture and degradability which can last for a month of cell culturing needed by the client. Due to the variability of alginate's composition discussed earlier, its varying degradation rates cause it to be unpredictable and receive a lower score for ease of use.

Lastly, for cost GelMA ranked higher because all of its components can be commercially purchased as a kit or inexpensive separate parts to create the final product. PEG receives a slightly lower score because while it is more expensive than GelMA it is within the clients price range.

Final Design

Polyethylene Glycol Hydrogel

In this model, polyethylene glycol synthetic materials are cross linked via photopolymerization with UV light. This allows for fibroblasts to be encapsulated within the gel [19]. This model allows for very precise selection of stiffness based on PEG-NB and PEG-DT concentrations detailed in the protocol table. Synthetic hydrogels such as PEG do not have any natural adhesive properties [10]. As a result, RGD sequences are used to create binding sites with the selected concentration of between 2.6-5 mM, as stated below. Lastly, to allow for fibroblast reconstruction of the ECM, fibroblasts are added at concentrations of 100,000-200,000 cells/mL [12] and MMP peptides are incorporated at a concentration of 3% (weight/volume) when degradation by enzymes at 1 nM follows to allow for the synthetic matrix degradation timeline to match that of reconstruction [20].

Gelatin Hydrogel

The secondary design created was a gelatin based hydrogel due to the ease of fabrication needed to conduct testing. This gelatin hydrogel can be created with controlled stiffness based on powder concentration that fits within the necessary range for native tissue mimicking. Additionally, gelatin hydrogels are translucent, and biocompatible/non-toxic to fibroblasts [21]. This causes the design to not only meet the specifications needed to be an accurate mimic of lung ECM but also has high ease of fabrication.

Testing

Translucency

Translucency was tested qualitatively, a design that allows for a simple determination of whether or not the hydrogels were translucent enough for imaging purposes. When proteins and cells are incorporated into the hydrogel scaffold later on, they can be seen under the microscope if the gels are translucent.

To test this property, a light was shone from the bottom of the gel. For test replication purposes, this could have been performed with any significant light source, such as a microscope bulb or a flashlight. If light could be observed going through the gel and out of the surface of the gel, then that gel passed translucency testing.

Rheology

The use of rheology testing on the gelatin hydrogels allowed for the gels to be tested based on their viscoelastic characteristics. Three mechanical properties were compared to native lung tissue to determine whether or not the hydrogels accurately modeled the in-vivo mechanical properties of the lungs: Young's Modulus (E), Storage Modulus (G'), and Loss Modulus (G''). The storage modulus relates to the elasticity of the gel while the loss modulus more accurately measures the viscosity or fluid-like properties of the gels. Hydrogels comprise of a mixture of these two properties which can be accurately represented by these corresponding moduli.

Once the gelatin hydrogels were formed and allowed to sit for 12-24 hours, rheometry testing was performed. Three hydrogels of each concentration (3.15% and 2.36%) were removed from a 48 well plate and placed into two weighing boats, making sure that the gels of varying concentration were kept in separate weigh boats. 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. Thus, using a caliper, the thickness of the hydrogel was measured (thickness of the hydrogels in this experiment ranged from 3.45-4 mm). 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 was still 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 test could begin. Once the calibration was finished, the frequency test started and took approximately 10 minutes to complete. Once completed, 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 in MatLab with a loglog plot.

Diffusion

The motivation for diffusion testing was to determine whether or not the team's fabricated hydrogels allowed for diffusion of media to supply nutrients to the fibroblasts and epithelial cells that are to be cultured by the client.

To begin, four gelatin hydrogels were selected for testing: two with a concentration of 3.15% weight per volume and two with a concentration of 2.36% weight per volume. A total of four aluminum mesh plates were then fabricated by molding over square-shaped aluminum wire mesh sheets over four petri dishes in a concave shape, mimicking a transwell insert for ALI. Each mesh plate was then massed.

After obtaining initial images of the gels, the second iteration of diffusion testing protocol involved transferring each gel onto the center of its corresponding mesh plate and measuring the mass of each gel and mesh plate together. Each petri dish was then filled with 5mL of Dulbecco's Modified Eagle Medium (DMEM), after which the mesh plates and gelatin hydrogels were replaced to their corresponding petri dishes such that the bottom half of the gels were in contact with the media and the top half exposed to air. The four petri dishes, mesh plates, and gels were transferred as a unit to a 4℃ refrigerator.

After 1 hour and 23 hours, these units were removed briefly from the refrigerator. The second iteration of diffusion testing protocol involved removing each mesh plate and gel together from their petri dish, pat-drying the bottom of each mesh plate with a paper towel to remove any excess media, and then measuring the mass of each mesh plate and gel as a unit. After imaging the four gels to visually document the diffusion of media at each time increment, each mesh plate

and gel unit was replaced onto their respective petri dish and transferred back to the 4℃ refrigerator.

Using the masses of each individual mesh plate and the masses of each mesh plate and gel unit after 0 hours, 1 hour and 23 hours, the masses of each of the four gels at all three time increments were calculated; these data provided a quantitative representation of media diffusion into the gels. Moreover, images of the gels taken at each time increment provided a visual representation of the progression of media diffusion into each gel.

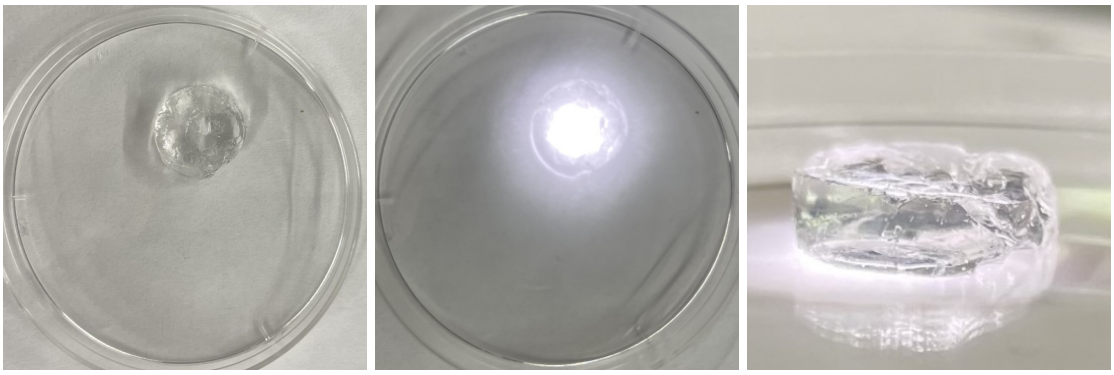
It is important to note that rather than measuring the mass of each mesh plate and hydrogel as a unit, the first iteration of diffusion testing protocol involved measuring the mass of each gelatin hydrogel individually prior to the diffusion and after each time interval.

Results

Translucency

After gelatin hydrogel formation, two gels of each concentration were tested. Both the 3.15 w/v% and the 2.36 w/v% gels appeared clear after initial removal from the 24-well plate. When each gel was tested with the described protocols of shining the light on the underside of the gel, all displayed the desired property of allowing light to pass through the gel and out of its surface. By exhibiting translucency, gels are able to be imaged through for cell culture purposes and therefore pass the translucency test.

Figure 1: Gel before shining light (left), and gel during translucency testing viewed from the top (middle) and from the side (right)



Rheology

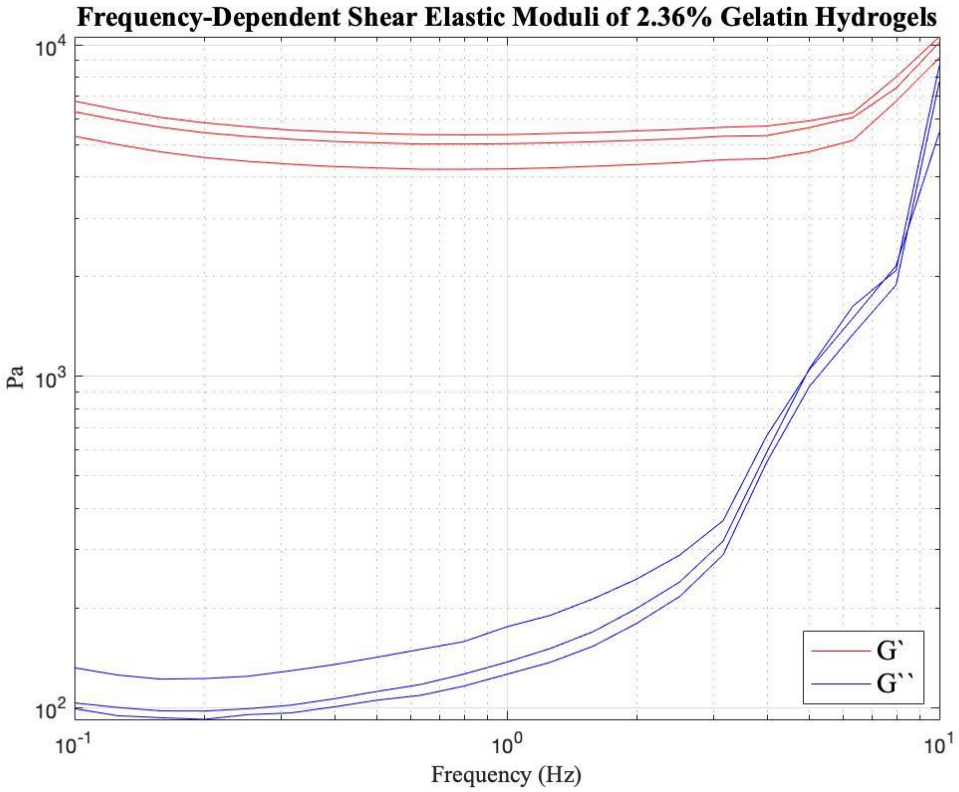
The results from the frequency test with the parameters described earlier in the testing section are summarized in table 2. The Young's Modulus, Storage Modulus, and Loss Modulus of both concentrations of hydrogels are included as well as the values for native lung tissue. As

the gelatin concentration increased in the hydrogels, the Young's Modulus, Storage Modulus, and Loss Modulus increased. This follows the pattern that increasing the degree of crosslinking leads to an increased hydrogel stiffness. Young's Modulus was derived from the equation $E=2G(1+\mu)$ where μ=Poisson's ratio. For gelatin, $\mu=0.5$, classifying it as an incompressible material that is deformed elastically at small strain. At very low frequencies, G can be approximated as G' and the Young's Modulus can be derived using the storage modulus and Poisson's ratio at 0.1 Hz. In graphs #1 and #2, the storage modulus can be observed as constant until about the 5-10 Hz value, while the loss modulus appears to be constant until about the 1 Hz value. This follows the idea that at larger strains, these values will be much more prominent, especially the loss modulus. These constant regions seen in the graphs also correspond with that of a chemically linked hydrogel where time dependence plays no factor and the crosslinking of the gels does not change in a given concentration.

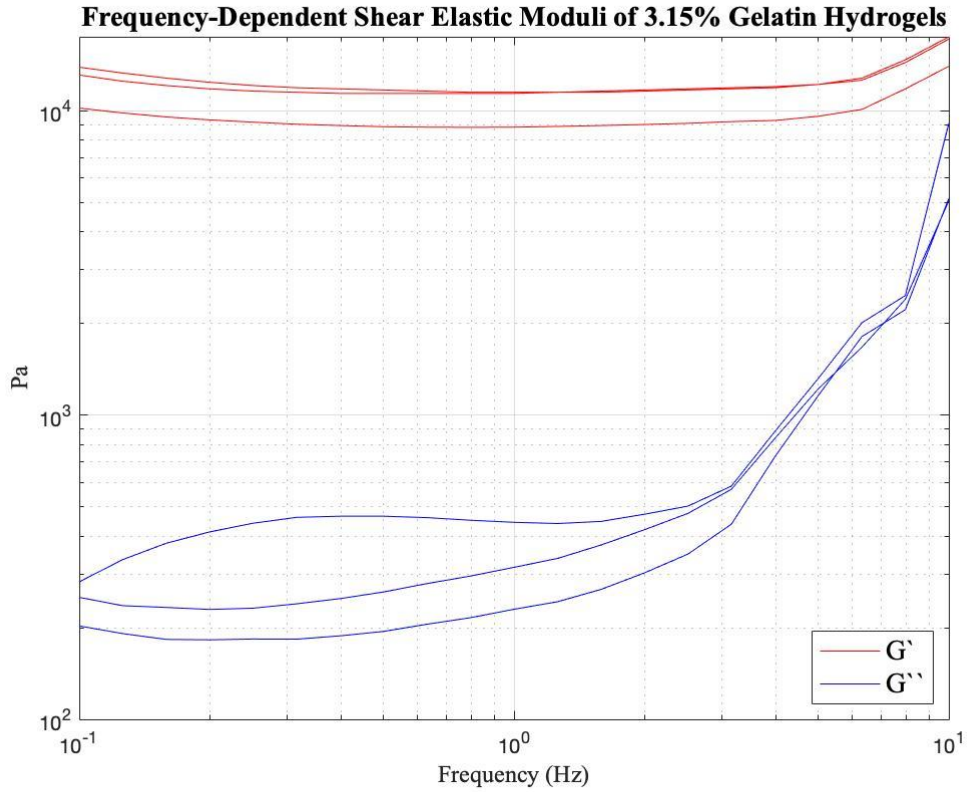
Table 2: Rheology Testing - (All results derived at 0.1 Hz)

Materials	Young's Modulus (E)	Storage Modulus (G')	Loss Modulus (G'')
Gelatin Hydrogel - 2.36%	18.375 ± 2.24 kPa	6.125 ± 0.747 kPa	0.112 ± 0.018 kPa
Gelatin Hydrogel - 3.15%	37.24 ± 5.82 kPa	12.41 ± 1.94 kPa	0.246 ± 0.04 kPa
Native Lung Tissue	0.44-7.5 kPa	2.12 ± 0.61 kPa	0.212 ± 0.061 kPa

Graph 1: LogLog plot of the Frequency-Dependent Shear Elastic Modulus of the 2.36% Gelatin Hydrogels



Graph 2: LogLog plot of the Frequency-Dependent Shear Elastic Modulus of the 3.15% Gelatin Hydrogels



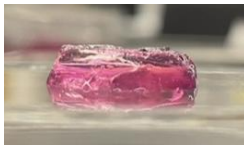
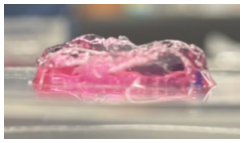
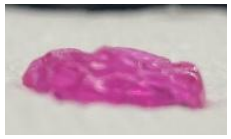
Diffusion

After completing testing using the initial protocol for measuring diffusion which involved measuring the mass of the gels individually rather than with the mesh plate, it was found that mass decreased over time for all gels (2.36 w/v% and 3.15 w/v%) when media was diffused through the air-liquid interface setup. Notably, the gels of lower concentration (2.36 w/v%) had a steeper rate of mass decrease. Loss of mass in gels was determined to be likely due to transfer of the gels back and forth between the testing apparatus and the scale as well as due to the natural degradation of gelatin. Mass values are listed below and summarized in Table 3 and Graph 3, respectively. However, while mass of gels decreased, diffusion still clearly took place as displayed by the gradual darkening of the pink shade of media in the gels (Table 3). Overall, these results indicate that when used in a cell culture experiment, media would be able to diffuse through a gelatin hydrogel in order to supply nutrients to the embedded fibroblasts and epithelial cells at the ALI. While this experiment was only conducted with the gelatin hydrogel, the process of executing a diffusion test sets up the protocol for testing diffusion of media through the original model of a PEG-NB/PEG-DT hydrogel and similar results could likely be obtained using the defined protocol.

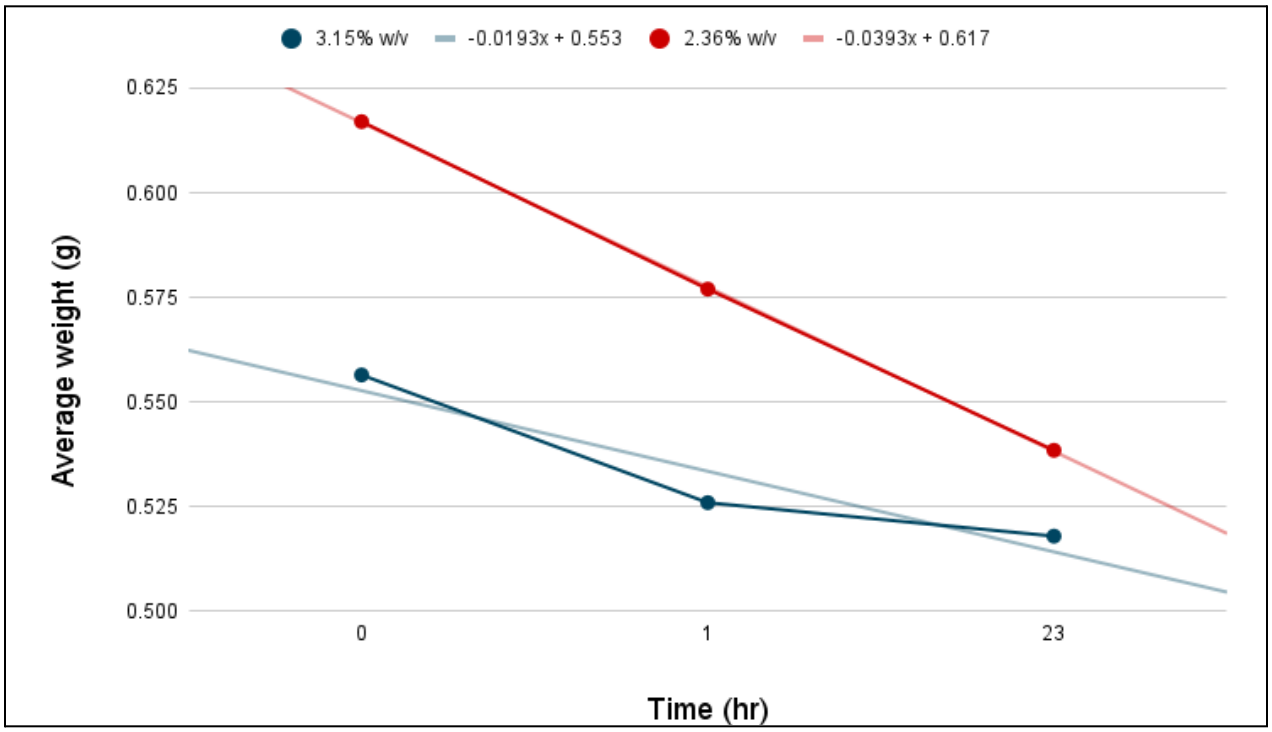
Table 3: Mass of Gelatin Hydrogels with Diffusion of Media

Gel #	Concentration (w/v%)	Volume of Gelatin Solution	Initial Mass (g)	Mass after 1 hour	Mass after 23 hours
1	3.15	600 µL	0.583	0.552	0.544
2	3.15	600 µL	0.530	0.500	0.492
3	2.36	600 µL	0.598	0.552	0.514
4	2.36	600 µL	0.636	0.602	0.563

Table 4: Visual Results of Diffusion of Media Through Gelatin Hydrogels

Concentration of Gel (w/v%)	Gel Prior to Diffusion	Gel after 1 Hour	Gel after 23 Hours
3.15			
2.36			

Graph 3: Mass of Gelatin Hydrogels Over Time



Discussion

Through the progression of the design process, a major limitation of fabrication was that of time. Given additional time to complete comprehensive troubleshooting of PEG hydrogel formation, it would not be unreasonable to say that a model more representative of the *in vitro* conditions of the ECM of the small airway of the lung could have been fabricated and tested. Given that this testing could not occur, however, it cannot be experimentally determined whether the PEG model would have better met the design specifications outlined under the *Specifications* section. While research suggests the matrix of the PEG model would be higher in complexity and more homogeneous in composition, resulting in a model more representative of native lung tissue, comparison between the gelatin and the PEG models would allow for conclusions to be drawn about whether these features of PEG override the ease of fabrication found with the gelatin model. Furthermore, additional time would allow for completed testing of the epithelial cell response to the final model, one of the primary indicators for success of the design. Without observing epithelial cell interactions, testing remains somewhat incomplete as the final success of the design is dependent upon cell viability upon application to the model.

Further, the absence of transwell inserts was a significant design limitation this semester as it became impossible for the model to take the form of a true air-liquid interface. While it is reasonable to predict that future iterations of the model could be fabricated within the transwell

inserts fit to 24-well plates, this geometry could not be demonstrated or tested in the exact form of transwell inserts this semester as the inserts were unavailable due to a manufacturing shortage. However, the use of an aluminum mesh sheet molded into a concave surface on a petri dish as described in the diffusion testing protocol enabled the team to bypass this obstacle and create a mimic of the structure of an air-liquid interface.

In addition to the desired design additions outlined above regarding each limitation experienced, future versions of the model would incorporate MMPs, peptides, and fibroblasts at the defined concentrations to better emulate the environment of the small airway. These elements could be added upon accomplishment of a reliable gel which yields replicable results, meets all design criteria, and supports epithelial cell growth upon application to the model.

Conclusion

In summary, the team was asked to create a 3-D synthetic scaffold with easily modifiable concentrations to mimic the range of stiffnesses found in the epithelial tissue of the small airway of the lung. Additionally, the team needed to incorporate appropriate fibroblast, MMP peptide, and fibronectin and collagen concentrations to facilitate ECM degradation and remodeling. Further, the client requested for the model to have cell adhesion properties, to allow for epithelial cell culture at an air-liquid interface (ALI), and to have sufficient translucent properties for microscope imaging purposes. This 3-D synthetic scaffold was requested in order to combat the client's current issues with utilizing models composed of materials like Matrigel, as such models have significant batch-to-batch variation that do not allow for appropriate mimicry of the composition of the small airway ECM.

To address the client's requests to the greatest extent possible, the team initially decided to design a PEG-DT and PEG-NB hydrogel model. This model was meant to be fabricated by photocrosslinking polyethylene glycol (PEG) synthetic materials, adding RGD sequences to create binding sites on the gel and promote cell adhesion, and incorporating appropriate fibroblast and MMP peptide concentrations to allow for ECM degradation and remodeling. However, upon fabrication, the aliquot and photocrosslinking steps for this model proved difficult due to the process' high sensitivity to slight discrepancies and the team's time, material, and expertise limitations.

As a result, an alternative gelatin model was fabricated by varying the ratio of powdered gelatin to water. This phantom model was chosen particularly for its ease of fabrication. These gels were created at a range of stiffnesses that fit within the client's requested range and possessed translucency, diffusion, and fibroblast biocompatibility properties similar to the team's original PEG-DT and PEG-NB model. Additionally, these gelatin hydrogels were successful at confirming that our testing protocols for rheology, translucency, and diffusion were feasible. While it is important to note that gelatin will dissolve when raised to the temperature of 25°-40°C, it is possible to overcome this issue. Literature has shown that crosslinking with squaric acid can decrease thermal degradation of gelatin hydrogels while still preserving a

Young's Modulus within a range of 25-53.96 kPa [22]. Though the team executed diffusion testing at 4°C due to time and resource limitations, this research indicates that a crosslinked gelatin model could overcome thermal degradation and may still be a viable option at 37°C, the temperature at which cells are cultured.

Despite the challenges faced, the team was successful at addressing the client's requirements in a variety of avenues. The outline of materials and protocol required for the PEG-DT and PEG-NB model provide the client with a 3-D synthetic scaffold that accurately mimics the intricacies of the small airway ECM and epithelial tissue, yet the model's complex fabrication highlights the need for the client to consider collaborating with individuals experienced in PEG hydrogel fabrication. On the other hand, the alternative gelatin model provides the client with a simplified yet much more easily replicable scaffold that still possesses the necessary properties for fibroblast incorporation, epithelial cell culture, and microscope imaging.

Future Work

There are several goals the team hopes to achieve together with the client to further advance the project. Firstly, PEG-DT and PEG-NB hydrogels must be correctly formulated so that translucency, rheology, and diffusion tests can be performed on such hydrogels. The team also wishes to incorporate the transwell insert specifically requested by the client for ALI (which was backordered for the semester and could not be obtained) into the diffusion testing protocol and for future cell culture purposes. The team also wishes to work with the client in incorporating the appropriate fibroblast concentrations into the hydrogels, as well as incorporating MMP peptides to facilitate fibroblasts' ECM degradation. Further, the plan is to have the client apply epithelial cells to the surface of the hydrogels for cell culture, as well as use these models for imaging purposes.

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Appendices

Appendix A - Materials & Expenses

Item	Price	Link
PEDGA	$58.00	https://www.sigmaaldrich.com/US/en/search/pegda?focus=products&page=1&perpage=30&sort=relevance&term=pegda&type=product_name
PEG-4AC	$518.00	https://www.sigmaaldrich.com/US/en/search/polyethylene-glycol-acrylate?focus=products&page=1&perpage=30&sort=relevance&term=polyethylene%20glycol%20acrylate&type=product_name
Phosphate Buffer Saline(PBS)	$25.00	https://www.sigmaaldrich.com/US/en/product/sigma/806552
Ethanol	$50.50	https://www.sigmaaldrich.com/US/en/product/supelco/48075
Teflon Mold	$27.50	https://fluorolab.com/product/pfa-well-plates/

Appendix B - Product Design Specifications

Function:

Dr. Allan Brasier and his research team have a need for a 3-D 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 by scientists to explore how the ECM, epithelium, and fibroblasts orchestrate reparations after damage. This model will be produced as a 3-D 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:

- Product should allow for the exploration of cell-cell interactions and the effects of changes in the extracellular matrix due to respiratory virus on the activity and state of fibroblasts within the lung.
- Would like to explore how to reconstitute the normal airway – normal epithelium with a normal extracellular matrix embedded with the fibroblasts – through the use of microfluidic devices, 3D printing, or other approaches.
- Model should include an air-liquid interface to reflect the polarization of the epithelium in the presence of air. A submerged culture system is not desirable. Epithelial cell lining on the top should be exposed to air and the bottom should have some source of nutrients or matrix.
- Matrix should be synthetically made and should be able to incorporate different types of fibronectin and should be of various stiffnesses.
- Will need to be able to measure tension on the scaffolds.

Design Requirements:

1. **Physical and Operational Characteristics**

a. *Performance Requirements:* The scaffold has both biochemical and structural factors that affect its success at providing an environment that is similar to the native lung ECM. Looking into structure, the scaffold must be in similar tension to that of native tissue because even slight differences can affect how the cells function. For example, tensioned ECM will induce a stretching of the cells' cytoskeleton, and compression of the ECM will result in an altered local charge of cells [1]. Using synthetic ECM materials allows the fine tuning of mechanical and other biophysical properties but has limitations with cell-cell communication which is vital for ECM functionality. As a result, the model will include the ability to coat and inject collagen and fibronectin to facilitate the biochemical communication aspect of the ECM. This model must meet these requirements of biochemical and mechanical properties to best mimic the native tissue environment.

b. *Safety:* Working with human epithelial cells and cell culturing requires chemical training as cell or tissue cultures can be associated with human pathogens. The following cell cultures and tissues require biosafety level 2 (BSL-2) practices and procedures:

1. All cultured cells derived from human sources, including immortalized and “well established” cell lines.
2. All cultured cells derived from nonhuman primate tissue.
3. All cultured cells exposed to, or transformed by, a primate oncogenic virus.
4. All human clinical materials, such as samples of human tissue, obtained from surgery, biopsy, or autopsy.
5. All primate tissue.
6. All virus-containing primate cultured cells.
7. All mycoplasma contains cultured cells.

When working with human and tissue 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. Lab personnel must also receive annual OSHA bloodborne pathogens training. The adherence of these standards is key to ensuring safety of all laboratory personnel [2].

c. *Accuracy and Reliability*: The scaffold will undergo tension and compression testing to ensure it meets the mechanical properties necessary to accurately represent the lung ECM. Additionally, it will be tested with active cell cultures grown for one month to ensure it can mimic the ECM and that the cells attach normally. Beyond this testing, other cell layers and components will then be added to make the model further resemble the *in vivo* environment of the EMTU.

d. *Life in Service*: The tissue model should be maintained for a minimum of 1 month to perform the human ALI cell culture method. This month of time will allow for optimal cell culturing on the scaffold so that any testing done will be most accurate to in vivo.

e. *Operating Environment* : Once the 3-D scaffold is assembled in sterile conditions, the testing will be performed in a cell culture environment. This environment will include HEPA filtered air. The filter will remove 99.97% of dust and air borne particles with a size of 0.3–10 microns [3]. The cell environment will be kept at 37 degrees celcius and the air inside will have a CO2 concentration of 5%. When not being used for research, the scaffold will be stored on a 1’ x 10” x 1’ cell culture rack [2].

f. *Ergonomics*: The model should mimic the tension and morphology of the extracellular matrix (ECM) as closely as possible. The model should also allow for the epithelial cells to attach well to it, and it should be air-exposed so the ALI method can be performed. Further, the model should be biocompatible, so as to not cause harm to living tissue that will be experimented on.

g. *Size*: The scaffold will have an area of 1 sq. cm and should be at least 10 microns deep 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 client did not give specific requirements for the material to be used for the project in an effort to not bias the design process. In the beginning stages, the scaffold should consist of the extracellular matrix. A potential future goal is to be able to embed fibroblasts into the scaffold. The chemistry of the scaffold needs to allow for proteins, specifically fibronectin and collagen, to be added under both stressed and normal environments. The scaffold cannot be made out of plastic material, as the tension in these materials is too high and does not mimic the *in vivo* environment. Further, the scaffold must be biocompatible to allow for testing of the cells from Dr. Brasier's lab.

i. *Aesthetics, Appearance, and Finish*: The scaffold should have an overall appearance that will mimic the small airway ECM as closely as possible. As it is intended to accurately model the stiffness and composition of the ECM, the main focus of the scaffold will be for the tension to be similar to in vivo environments as well as allowing for the incorporation of fibronectin and collagen to mimic a natural state. 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*: Each scaffold should have 10^4 to 10^5 cells for DNA isolation and flow cytometry. Currently, a particular quantity of the scaffolds is not a requirement by the client but in the future there should be enough scaffolds to do testing in a 12 well-plate.
b. *Target Product Cost*: The cells and ECM proteins needed for the development of this model will be provided by the client, so there will be approximately no cost for the product excluding the synthetic material used to create the scaffold.

3. Miscellaneous

a. *Standards and Specifications*: FDA approval is required for these types of synthetic 3-D scaffolds. The standard and regulations of these products fall under ASTM F2150-19: Standard Guide for Characterization and Testing of Biomaterial Scaffolds Used in Regenerative Medicine and Tissue-Engineered Medical Products [5]. Before reaching the market, the design must abide by these FDA standards and address any risk that the device may have. There are also many FDA requirements surrounding the use of cell and tissue culture products which fall under Standard 21CFR864 [6].
b. *Customer*: As of the initial meetings, the client does not have specific preferences for how to proceed through the design process, provided that the requirements outlined above are met. Preliminary meetings suggest the use of microfluidic devices or 3-D

printing of a scaffold as potential design pathways, but the client has no initial preference, and the design is also not limited to these two possible methods.

c. *Competition*: Tissue engineering models to provide in vitro means to study the body has in recent years created many amazing and novel designs. For models looking specifically at the lung epithelium, there are currently both 2-D and 3-D models on the market 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 2-D 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 2-D 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 2-D models have a stiffness range of 2-4 GPa while the human lung ranges from .44-7.5 kPa [7]. The differences in stiffness greatly change the behavior of the cells and thus the experimental data found on them are not as accurate as *in vivo*.

   While there are many varieties of 3-D 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 [8]. These 3-D models have variations in methods for each experiment, but generally all involve an ECM gel 3-D environment that is more similar (with some limitations) to *in vivo* than the 2-D 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.

Appendix C - Fabrication Protocols

PEG Hydrogel Formation Protocol

Hydrogel Stiffness	PBS (uL)	PEG-DT (uL)	PEG-NB (uL)	I2959 (uL)
2 kPa	122.4	13.8	61.9	49.5

4 kPa	100.7	23.1	74.3	49.5
5 kPa	68.2	30.8	99	49.5
6 kPa	118.8	99	29.7	49.5

RGD sequence mM	2.6	3.0	3.4	4.0	4.6	5.0
uL of RGD	9.6	11.1	12.6	14.8	17	18.5

Protocol Overview

1. When taking out thaw aliquots on ice
2. Make sure to vortex everything before adding and in between each step
3. Add calculated amount if PBS
    a. For all of the below additions go slow, super viscous so need to make sure no air bubbles
4. Add calculated crosslinker to solution
5. Add calculated PEG-NB to solution
6. Add photoinitiator last
7. Leave to set in UV light for 5 minutes
    a. Before setting the gels give the UV light 5 minutes to warm up
8. After the gels are set need to be submerged in PBS
    a. This is to allow for swelling, 24 hours needed

[for all of the below protocols use 1x PBS and make sure to vortex between steps]

Forming Aliquots

PEG-DT

1. For our experiment, we will striving for 200 mM crosslinker aliquots (400 mM more accurately because DI-thiol)
    a. This means that we will add 1 gram of crosslinker into 1.47 mL of PBS
    b. This is based on calculations on separate document
2. To form this we will weigh out 1 gram of PEG-DT {halved it bc only 1 gram total in vial}
3. Then we will add this to a 15 mL conical tube with 1.47 mL of PBS

4. Vortex the solution
5. Leave on ice to till clear (roughly 30 minutes)

PEG-NB

1. For this PEG it is the same exact process as above just with 300 mg of PEG into 800 uL of PBS

Photo-Initiator [CANNOT BE EXPOSED TO LIGHT-TINFOIL]

1. We want to have a .5% (wt/v) of I2959 this means that we will need 5mg/ mL
    a. This means that we will add 20 mL of PBS with 100 mg of I2959
2. Place 20 mL of PBS in a glass bottle
3. Place on heated platform at 50C
4. Add 100 mg of 12959 to the bottle (wrapped in aluminum foil)
5. Let dissolve on the heated platform at 50C and mixing for over an hour
6. Store in Eppendorf tubes at -20C

Gelatin Hydrogel Formation Protocol

Gelatin Concentration (w/v%)	Volume of Water (mL)	Gelatin Added (g)
2.36	300	7.087
3.15	225	7.087

Protocol Overview

1. Measure desired quantity of water and reserve approximately 50 mL to keep cold.
2. Add one packet of gelatin (7.087 g) to the reserved 50 mL of cold water and stir.
3. Use a hot plate to bring the remaining quantity of water to a boil in a beaker. Once boiling, remove the beaker from the hot plate and pour over the cold water and gelatin mixture. Stir until uniformly combined and all gelatin is dissolved.
4. Using micropipette, transfer 600 µL of each solution to wells of a 24 or 48-well plate. Cover and refrigerate at 4°C for at least three hours to ensure complete gels are completely set.

Appendix D - Testing Protocols

Translucency Test Protocol

1. Line the bottom of the gels (could either be separated in a petri dish or in plates) with a piece of paper.

2. Turn on a phone flashlight and put it under the paper.
3. Image gels as light was shone underneath.

Attentive Qualitative Translucency Test Protocol

This is an update to the previous translucency test so that quantitative results can be obtained. This test has not been done during this project due to time and resource limitations but can be implemented and updated in the future.

1. Find a light source, such as a phone flashlight or microscope light bulb; a laser pointer would be recommended so that the light going out of the source is not as dispersed.
2. Turn off all other light sources (block them out if necessary) then turn on that light source and find the light intensity of that light source using a lux meter.
3. Put the gel on that light source and find the intensity of the light going through the gel using the lux meter.
4. Find the percentage of how much light passes through the gel using the calculation:

$$\frac{intensity\ of\ light\ passing\ through\ gel}{intensity\ of\ light\ source} \times 100$$

Frequency Sweep Rheology Protocol

1. Once gelatin hydrogels have been formed and allowed to set for approximately 12-24 hours, rheometry testing may be performed.
2. Carefully remove 3 gels of each concentration (3.15% and 2.36%) from 48 well cell culture plates, keeping the gels of the same concentration in the same weighing 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 hydrogels of each concentration (3.15% and 2.36%) were tested for a total of 6 separate frequency sweeps.
11. When testing is completed, results can be interpreted in MatLab.

Diffusion Test Protocol

1. Remove gels from 24-well plates using a spatula.
2. Prior to experiment, image each gel with camera.
3. Transwell inserts are not readily available, so instead, mold sheet of thin aluminum wire mesh over top of petri dish with concave-down shape but make sure the mesh is not touching the bottom of the petri dish.
4. Remove and measure mass of mesh wire form.
5. Place gel in the center of mesh and measure total mass of the mesh and gel. Calculate the initial mass of the gel by subtracting the mass of mesh from the total mass of mesh and gel.
6. Place mesh back onto petri dish with gel on top. Fill petri dish with 5 mL of DMEM, just enough so that the bottom of gel is in contact with the media but the top is exposed to air.
7. Leave gel in place for specified time increments (1 hour, 23 hours (or overnight)) inside refrigerator at 4°C.
8. After each increment, remove mesh from petri dish.
    a. Pat bottom of mesh dry with paper towel to remove excess media.
    b. Measure mass of mesh with gel still on top.
    c. Take picture of gel to document diffusion of pink-colored media.
    d. Replace mesh and gel back onto petri dish and refrigerate.
9. Using subtraction, determine masses of gels at each time interval.
10. Use images taken throughout time increments to display a visual representation of media diffusion into gels.

Appendix E - Rheology Data Analysis MATLAB

Definition of data sets

```
F225a = [0.1 0.1259 0.1585 0.1995 0.2512 0.3162 0.3981 0.5012 0.631 0.7943 1 1.259 1.585 1.995 2.512 3.162
3.981 5.012 6.31 7.943 10];
```

F225b = [0.1 0.1259 0.1585 0.1995 0.2512 0.3162 0.3981 0.5012 0.631 0.7943 1 1.259 1.585 1.995 2.512 3.162 3.981 5.012 6.31 7.943 10];

F225c = [0.1 0.1259 0.1585 0.1995 0.2512 0.3162 0.3981 0.5012 0.631 0.7943 1 1.259 1.585 1.995 2.512 3.162 3.981 5.012 6.31 7.943 10];

F300a = [0.1 0.1259 0.1585 0.1995 0.2512 0.3162 0.3981 0.5012 0.631 0.7943 1 1.259 1.585 1.995 2.512 3.162 3.981 5.012 6.31 7.943 10];

F300b = [0.1 0.1259 0.1585 0.1995 0.2512 0.3162 0.3981 0.5012 0.631 0.7943 1 1.259 1.585 1.995 2.512 3.162 3.981 5.012 6.31 7.943 10];

F300c = [0.1 0.1259 0.1585 0.1995 0.2512 0.3162 0.3981 0.5012 0.631 0.7943 1 1.259 1.585 1.995 2.512 3.162 3.981 5.012 6.31 7.943 10];

Gp225a = [1.31e4 1.25e4 1.21e4 1.18e4 1.16e4 1.15e4 1.14e4 1.14e4 1.14e4 1.14e4 1.14e4 1.15e4 1.15e4 1.16e4 1.17e4 1.18e4 1.19e4 1.22e4 1.28e4 1.47e4 1.75e4];

Gp225b = [1.02e4 9.83e3 9.54e3 9.33e3 9.17e3 9.03e3 8.94e3 8.86e3 8.83e3 8.82e3 8.83e3 8.88e3 8.94e3 9.01e3 9.09e3 9.21e3 9.29e3 9.59e3 1.01e4 1.18e4 1.40e4];

Gp225c = [1.39e4 1.33e4 1.28e4 1.24e4 1.21e4 1.19e4 1.18e4 1.17e4 1.16e4 1.15e4 1.15e4 1.15e4 1.16e4 1.17e4 1.18e4 1.19e4 1.20e4 1.22e4 1.26e4 1.44e4 1.72e4];

Gp300a = [6.29e3 5.94e3 5.65e3 5.44e3 5.30e3 5.20e3 5.12e3 5.07e3 5.03e3 5.03e3 5.04e3 5.07e3 5.11e3 5.16e3 5.22e3 5.31e3 5.33e3 5.64e3 6.05e3 7.42e3 1.02e4];

Gp300b = [5.31e3 5.01e3 4.76e3 4.58e3 4.46e3 4.37e3 4.30e3 4.26e3 4.22e3 4.22e3 4.23e3 4.26e3 4.31e3 4.36e3 4.42e3 4.51e3 4.54e3 4.77e3 5.16e3 6.77e3 9.16e3];

Gp300c = [6.78e3 6.38e3 6.05e3 5.83e3 5.67e3 5.54e3 5.47e3 5.41e3 5.37e3 5.36e3 5.37e3 5.41e3 5.45e3 5.51e3 5.57e3 5.65e3 5.70e3 5.91e3 6.25e3 8.01e3 1.06e4];

Gpp225a = [2.83e2 3.35e2 3.79e2 4.13e2 4.41e2 4.61e2 4.65e2 4.65e2 4.60e2 4.51e2 4.44e2 4.40e2 4.47e2 4.72e2 5.02e2 5.84e2 8.83e2 1.32e3 2.01e3 2.47e3 9.10e3];

Gpp225b = [202.7 191.3 183.1 182.5 183.6 183.4 188.1 194.5 205.4 215.8 230 243.5 267.3 302.9 349.2 437.8 729.3 1.16e3 1.81e3 2.22e3 5.15e3];

Gpp225c = [251.7 236.3 233.2 229.7 231.9 239.7 249.3 262.2 279.4 295.7 315.9 337.9 374 419.8 475.1 570 838 1.22e3 1.67e3 2.40e3 5.06e3];

Gpp300a = [103.4 100.2 97.84 97.71 99.29 101.8 106.4 112 117.5 126.3 137.3 150.9 169.8 199.4 239.9 318.4 587.5 1.06e3 1.63e3 2.09e3 8.68e3];

```
Gpp300b = [99.33 94.6 93.3 92.26 95.3 96.44 100.6 105.4 109 116.2 126.2 137.1 153.5 179.7 216.9 289.5 548.6
934.4 1.34e3 1.89e3 7.76e3];

Gpp300c = [132 125.5 122 122.5 124.4 129.3 134.9 142.1 150 158.3 175.6 190  213.4 244.5 289.2 366.9 659.4
1.05e3 1.50e3 2.16e3 5.46e3];
```

32 g/L Graph

```
figure(1)
loglog(F225a,Gp225a,'red',F225a,Gpp225a,'blue',F225b,Gp225b,'red',F225b,Gpp225b,'blue',F22

title("Frequency-Dependent Shear Elastic Moduli of 32 g/L Gelatin Hydrogels",'FontName','Times New
Roman','FontSize',14);

xlabel("Frequency (Hz)",'FontName','Times New Roman','FontSize',12)
ylabel("Pa",'FontName','Times New Roman','FontSize',12)
legend({'G`','G``},'Location','southeast','FontName','Times New Roman','FontSize',14)
grid on;
```

24 g/L Graph

```
figure(2)
loglog(F300a,Gp300a,'red',F300a,Gpp300a,'blue',F300b,Gp300b,'red',F300b,Gpp300b,'blue',F30

title("Frequency-Dependent Shear Elastic Moduli of 24 g/L Gelatin Hydrogels",'FontName','Times New
Roman','FontSize',14);

xlabel("Frequency (Hz)",'FontName','Times New Roman','FontSize',12)
ylabel("Pa",'FontName','Times New Roman','FontSize',12)
legend({'G`','G``},'Location','southeast','FontName','Times New Roman','FontSize',14)
grid on;
```

Standard Deviations of the G' and G'' Values for each Concentration of Gel

```
A = [1.31e4 1.02e4 1.39e4];
StdGp225 = std(A)

B = [2.83e2 202.7 251.7];
StdGpp225 = std(B)

C = [6.29e3 5.31e3 6.78e3];
StdGp300 = std(C)

D = [103.4 99.33 132];
StdGpp300 = std(D)
```