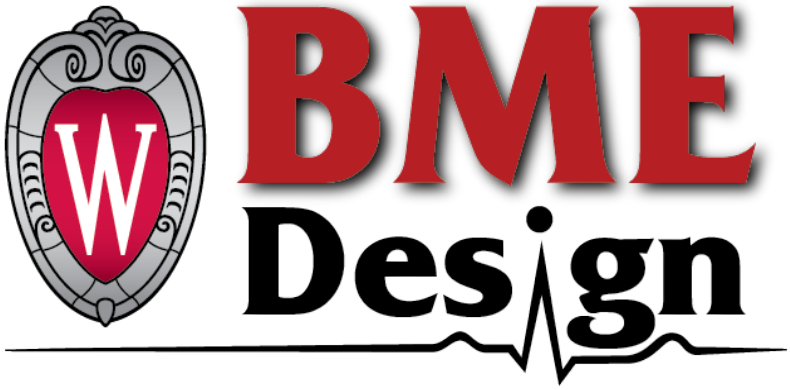
Tissue Model of the Epithelial Mesenchymal Trophic Unit



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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. Specifically, no scaffold models the change in mechanical stiffness and porosity while also incorporating ECM proteins and promoting cell adhesion [1]. In response to a lack of scaffolds to facilitate the modeling of healthy and fibrotic lungs, a hydrogel to accurately mimic the conditions of healthy and fibrotic ECMs is being developed. Based on specifications developed through background research and per client requests, potential designs were proposed, and criteria to evaluate the proposed designs were determined. Meeting necessary specifications, a hydrogel scaffold composed of gelatin methacryloyl / methacrylate (GelMA) was ultimately proposed as a solution. Initial fabrication procedures, and accompanying methods to test the hydrogel's efficacy were produced. The GelMA hydrogels will be fabricated and tested over the remainder of the spring 2022 semester.

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 space 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* ECM [6]. Furthermore, for respiratory research, cell cultures are grown at an air-liquid interface. This involves the basal surface (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 2D and 3D 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.

2D models are typically layers of cells cultured 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 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 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 [10]. 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. 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 (2-16.5 kPa) 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 fibronectin and collagen, which in turn allows them to remodel the matrix and create a microenvironment that resembles native lung ECM. In order to achieve these goals, it is essential that the Young's modulus, storage modulus (G') and loss modulus (G") emulate a healthy and fibrotic lung ECM environment. In order to accurately represent the viscoelastic ECM environment that fibroblasts are subjected to in the lungs, the Young's Modulus (E) for the healthy and fibrotic hydrogels should be 2 kPa and 16.5 kPa, respectively. The G' and G" values mimicking the healthy lung ECM should be 500 Pa and 50 Pa, respectively. For the hydrogel mimicking fibrotic lung ECM, the G' and G" values are increased by an order of magnitude and be 5 kPa and 500 pa, respectively [11]. Meeting these moduli values are crucial to displaying correct fibroblast phenotype and more accurately representing fibroblasts 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 take the form of an air-liquid interface to emulate the conditions that epithelial cells experience in the lungs. 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.

Preliminary Designs & Evaluation

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 [12]. After GelMA is constructed the naturally adhesive protein motifs present within gelatin are retained. This motif is arginine-glycine-aspartic acid, or RGD, sequences that allow for cell adhesion as they are derived from cell-binding sites on fibronectin, a native ECM protein [12]. 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, MMP sensitive sequences are also naturally incorporated in the GelMA hydrogel allowing for cell migration and reconstruction of the ECM by fibroblasts. 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, which this synthetic material does not naturally possess [10]. 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 [10].

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 [13]. 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 [13].

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 2 kPa to 16.5 kPa. The viscoelastic properties for healthy lung tissue must include the storage modulus (G'= 500 Pa) and loss modulus (G'' = 50 Pa). Fibrotic lung tissue viscoelastic properties must also be met by the storage modulus (G' = 5 kPa) and loss modulus (G'' = 500 Pa) [13]. The G' and G" values should be within an order or magnitude of each other to accurately represent the viscoelasticity of in-vivo lung ECM.

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 [14][12]. 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 [13].

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 [13]. 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 [12][15]. PEG doesn't have any naturally occurring cell adhesion or degradation components but can have these incorporated [14]. 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 [16]. 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 [16]. 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 [13]. 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 [12][14]. 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[14]. 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 and lung ECM with gelatin powder available for $116/kg and methacrylic anhydride available for $0.56/mL [17].

Materials and Methods

Materials

The proposed GelMA hydrogel will be fabricated from gelatin that will be functionalized with methacrylic anhydride rather than pre characterized GelMA, both to reduce cost, and allow control of mechanical properties through the degree of methacrylation. The gelatin is sourced from bovine skin with a bloom strength of 225g, and is type B gelatin, in a lyophilized powder form. Liquid methacrylic anhydride will be used to functionalize the gelatin. Phenyl(2,4,6-trimethylbenzoyl)phosphinic acid lithium salt (LAP) will be used as the crosslinking photoinitiator. Methacrylation, cross linking, and swelling will be done in a PBS solution. Finally, the vessels used for hydrogel formation will be silicon molds for crosslinking, and a 24 well plate for swelling. Gelatin and methacrylic anhydride will be purchased from Sigma-Aldrich, LAP from VWR international, Silicon molds from Grace Bio-Labs. PBS and 24 well plates will be provided by the client.

Methods

GelMA hydrogels, from their most basic components, are formed by functionalizing gelatin by reaction with methacrylic anhydride in solution[18]. This can be done in a solution of PBS, which the team will use for cost efficient prototypes for mechanical characterization, or in an embedding media, for cellular applications. Powdered GelMA is added to PBS and warmed at 50 ° C. If cells are being embedded, an embedding media is used in place of PBS. Silicon molds are then sterilized and pressed into the bottom of a petri dish or similar surface. Photoinitator is added to the warmed GelMA solution, and at this time a solution of cells may be added. The fully mixed solution is placed within the silicon molds. The gelatin methacryloyl / methacrylamide polymers are then crosslinked via cooling in a 4°C fridge, and by radical crosslinking using a photoinitiator under UV spectra. Finally, gels are removed from the molds and allowed to swell in a 24 well plate. A stepwise procedure for hydrogel formation can be found under Appendix C.

Proposed Testing

The scaffold needs to be translucent so the client can image the cells, so the GelMA hydrogels will be tested for translucency. This will be accomplished by shining a light through the gel to gauge if light passes through. To characterize the hydrogels, the swelling ratio will be determined by comparing the dry and wet weights of the gels. The mechanical properties of the gel scaffolds need to be assessed since that is one of the most important client requirements. These properties of the hydrogels will be tested by frequency sweep rheology (see Appendix D for the rheology testing protocol). This testing will give the storage and loss moduli of the gels, which can then be used to calculate the Young's modulus. The client's lab will assess the biochemical properties of hydrogel scaffolds; after coating the gel in a monolayer culture of lung epithelial cells in an ALI, the client will test cell adhesion and viability.

Conclusion

To conclude, a gelatin methacryloyl hydrogel will be produced for use as a 3D cell culture model of the epithelial-mesenchymal trophic unit. The selection of GelMA was based on its ease of use, low cost, and amenable mechanical and biochemical properties. The objective is to create GelMA scaffolds with the desired stiffness and ensure they are appropriate for the client's use. Subsequently, fibroblasts will be encapsulated in the gel to allow for the study of lung epithelial cells in normal and fibrotic extracellular matrix (ECM) states. This will result in a model that is more biologically relevant and valuable to the client.

References

[1] E. T. Osei, S. Booth, and T.-L. Hackett, "What Have In Vitro Co-Culture Models Taught Us about the Contribution of Epithelial-Mesenchymal Interactions to Airway Inflammation and Remodeling in Asthma?," Cells, vol. 9, no. 7, p. 1694, Jul. 2020, doi: 10.3390/cells9071694.

[2] E. S. White, "Lung Extracellular Matrix and Fibroblast Function," *Ann Am Thorac Soc*, vol. 12, no. Suppl 1, pp. S30–S33, Mar. 2015, doi: 10.1513/AnnalsATS.201406-240MG.

[3] U. F. O. Themes, "Fibroblasts in Lung Homeostasis and Disease," *Thoracic Key*, Jan. 11, 2017. https://thoracickey.com/fibroblasts-in-lung-homeostasis-and-disease/ (accessed Oct. 11, 2022).

[4] B. Li and J. H.-C. Wang, "Fibroblasts and myofibroblasts in wound healing: force generation and measurement," *J Tissue Viability*, vol. 20, no. 4, pp. 108–120, Nov. 2011, doi: 10.1016/j.jtv.2009.11.004.

[5] B. P. Chan and K. W. Leong, “Scaffolding in tissue engineering: general approaches and tissue-specific considerations,” *Eur Spine J*, vol. 17, no. Suppl 4, pp. 467–479, Dec. 2008, doi: 10.1007/s00586-008-0745-3.

[6] S. A. Langhans, “Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning,” *Frontiers in Pharmacology*, vol. 9, 2018, Accessed: Oct. 11, 2022. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fphar.2018.00006

[7] “Air-Liquid Interface Culture for Respiratory Research.” https://www.stemcell.com/air-liquid-interface-culture-respiratory-research-lp.html (accessed Oct. 11, 2022).

[8] M. E. Smithmyer, L. A. Sawicki, and A. M. Kloxin, “Hydrogel scaffolds as in vitro models to study fibroblast activation in wound healing and disease,” Biomater Sci, vol. 2, no. 5, pp. 634–650, May 2014, doi: 10.1039/c3bm60319a.

[9] D. Kessler et al. "Fibroblasts in Mechanically Stressed Collagen Lattices Assume a “Synthetic” Phenotype." Journal of Biological Chemistry, vol. 276, no. 39, 2001, doi: https://doi-org.ezproxy.library.wisc.edu/10.1074/jbc.M101602200.

[10] J. Zhu, “Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering,” Biomaterials, vol. 31, no. 17, pp. 4639–4656, Jun. 2010, doi:10.1016/j.biomaterials.2010.02.044.

[11] E. Hui, L. Moretti, T. H. Barker, and S. R. Caliari, “The combined influence of viscoelastic and adhesive cues on fibroblast spreading and focal adhesion organization,” Cellular and molecular bioengineering, 02-Jun-2021. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8548477/. [Accessed: 10-Feb-2023].

[12] K. Yue, G. Trujillo-de Santiago, M. M. Alvarez, A. Tamayol, N. Annabi, and A. Khademhosseini, “Synthesis, properties, and biomedical applications of gelatin methacryloyl (gelma) hydrogels,” Biomaterials, vol. 73, pp. 254–271, 2015.

[13] R. H. de Hilster, P. K. Sharma, M. R. Jonker, E. S. White, E. A. Gercama, M. Roobeek, W. Timens, M. C. Harmsen, M. N. Hylkema, and J. K. Burgess, “Human lung extracellular matrix hydrogels resemble the stiffness and viscoelasticity of native lung tissue,” American Journal of Physiology-Lung Cellular and Molecular Physiology, vol. 318, no. 4, Apr. 2020.

[14] Yom-Tov, O., Seliktar, D., & Bianco-Peled, H. (2016). PEG-Thiol based hydrogels with controllable properties. European Polymer Journal, 74, 1-12. https://www.sciencedirect.com/science/article/pii/S0014305715300434#:~:text=Poly(ethylene%20glycol)%2DThiol,drug%20delivery%20and%20regenerative%20medicine.

[15] Y. Piao, H. You, T. Xu, H.-P. Bei, I.Z. Piwko, Y.Y. Kwan, X. Zhao, “Biomedical applications of gelatin methacryloyl hydrogels,” Engineered Regeneration, 2 (2021), pp. 47-56.

[16] D. Grijalva Garces, C. P. Radtke, and J. Hubbuch, “A novel approach for the manufacturing of Gelatin-methacryloyl,” Polymers, vol. 14, no. 24, p. 5424, 2022.

[17] “Milliporesigma,” MilliporeSigma | Life Science Products & Service Solutions. [Online]. Available: https://www.sigmaaldrich.com/US/en. [Accessed: 16-Feb-2023].

[18] Van Den Bulcke, A. I., Bogdanov, B., De Rooze, N., Schacht, E. H., Cornelissen, M., & Berghmans, H. (2000). Structural and rheological properties of methacrylamide modified gelatin hydrogels. Biomacromolecules, 1(1), 31–38. https://doi.org/10.1021/bm990017d

[19] Sun, Mingyue, et al. “Synthesis and Properties of Gelatin Methacryloyl (Gelma) Hydrogels and Their Recent Applications in Load-Bearing Tissue.” MDPI, Multidisciplinary Digital Publishing Institute, 21 Nov. 2018, https://www.mdpi.com/2073-4360/10/11/1290.

Appendices

Appendix A - Materials & Expenses

Table__: Materials list

Name	Description	Cost / Quantity	Link
Gelatin	Gelatin from bovine skin, as detailed in [19]	$34.90 / 100g	https://www.sigmaaldrich.com/US/en/substance/gelatinfrombovineskin123459000708
Methacrylic anhydride	(Liquid)	$56.00 / 100mL	\https://www.sigmaaldrich.com/US/en/product/aldrich/276685
LAP photoinitiator	Water soluble lithium salt (non sterile)	$150.00 / 500 mg	https://us.vwr.com/store/catalog/static_catalog.jsp?catalog_number=103792-908
Silicone Molds	Silicone molds to cure gel	$171/25 molds	https://gracebio.com/products/hybridization-and-incubation/silicone-isolators-hybridization-and-incubation/search/
PBS	1x PBS	Provided by Client	
24 well plates	For swelling	Provided by Client	

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 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 culture of lung fibroblasts while providing 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.

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 have similar stiffness 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 encapsulate collagen and fibronectin to facilitate the biochemical communication aspect of the ECM or provide these interactions via incorporated peptides. 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. Within the scope of the project, 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 human clinical materials, such as samples of human tissue, obtained from surgery, biopsy, or autopsy.

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.When culturing human and tissue cells, including human lung epithelial cells and fibroblasts, the specific PPE required under universal precautions may vary depending on the nature of the work being done and the potential for exposure to infectious agents. However, some common types of PPE required under Universal Precautions include:

1. Gloves: Disposable gloves made of latex, nitrile, or other materials can be used to protect the hands from contact with potentially infectious materials, as well as to prevent contamination of the cell cultures.
2. Lab coat or gown: These can be worn to protect clothing and skin from splashes, spills, and other sources of contamination.
3. Eye protection: Safety glasses or goggles can be worn to protect the eyes from splashes or sprays of infectious materials, as well as to prevent contamination of the cell cultures.
4. Face shield: A face shield may be used in addition to eye protection to provide additional coverage for the face and neck.
5. Respiratory protection: Depending on the nature of the work being done, respiratory protection may be necessary to prevent inhalation of aerosols or other airborne particles.
6. Shoe covers: Disposable shoe covers can be used to prevent contamination of the cell cultures from shoes.

Additionally, it is important to regularly clean and disinfect the PPE to prevent contamination of the cell cultures.

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:

i. Mechanical Properties:

The scaffold will undergo testing to ensure it meets the mechanical properties necessary to accurately represent the lung ECM. In addition to measuring the Young's

Modulus (E), the lungs exhibit viscoelastic properties which require the storage modulus (G') and loss modulus (G'') to also be obtained. The scaffold must have a Young's Modulus ranging from 2-16.5 kPa to accurately mimic the environment that fibroblasts experience in healthy lung tissue and in fibrotic lung tissue. The viscoelastic properties for healthy lung tissue must include the storage modulus (G'= 500 Pa) and loss modulus (G'' = 50 Pa). Fibrotic lung tissue viscoelastic properties must also be met by the storage modulus (G' = 5 kPa) and loss modulus (G'' = 500 Pa) [3]. 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 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. 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 measurements with these first hydrogels will provide knowledge on how well the mechanical properties, porosity, and the overall biochemistry of the hydrogel supports 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 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 reflect not only cell viability but also the degradation and reconstruction of the ECM.

e. *Operating Environment:*

Once the 3D 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 ℃ and the air inside will have a 5% CO_2 concentration.

f. Ergonomics:

The model should mimic the stiffness and morphology of the lung ECM as closely as possible. This involves having the viscoelastic properties as discussed prior, porosity that allows for exchange of material, cell adhesion and viability, and degradation of the matrix to allow for ECM reconstruction.

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. Materials to be used for creating a hydrogel can range from synthetic materials such as polyethylene glycol to a naturally based hydrogel like collagen. Based on the clients requirements for mechanical tunability, creating a synthetic to semi-synthetic hydrogel would best allow for this precision. The chemistry of the scaffold needs to allow for proteins, specifically fibronectin and collagen, to be added under both stressed and normal environments. This means that the material must be biocompatible and allow for cell adhesion to the scaffold.

i. Aesthetics, Appearance, and Finish:

The scaffold should have an overall appearance that will mimic the small airway ECM as closely as possible. 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. 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:

It is intended to produce scaffolds with variable stiffness, beyond that of healthy lung epithelia to model fibrotic or other diseased states. Therefore, as proof of concept to produce variability, a gradient of scaffold stiffnesses will be produced across a 24 well plate. Each scaffold, sized to a well plate as discussed in a prior section, should house 10^4 to 10^5 cells for DNA isolation and flow cytometry [5].

b. Target Product Cost:

The materials for the scaffold should cost no more than $500. In a prior semester, the team used around $1000 of the $5000 budget on the scaffold design, so there is around $4000 left to spend. The new scaffold design will be made from less expensive materials such as gelatin based hydrogels, or the polyethylene glycol materials from the prior semester may be used.

3. Miscellaneous

a. Standards and Specifications:

FDA approval is required for these types of synthetic 3D scaffolds when introduced to the market. 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]. However, the FDA does not have specific standards or specifications for the use of 3D synthetic scaffolds to study EMTU . In that regard, proper protocol regarding biocompatibility, sterilization, and labeling must still be followed. Additionally, there are many FDA requirements surrounding the use of cell and tissue culture products which fall under Standard 21CFR864 [6] to ensure that all research is conducted ethically and with appropriate oversight..

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 a synthetic to semi-synthetic material based on their preference of precise mechanical properties.

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 2D and 3D 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 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 [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 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 [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 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

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 10 100 µL gels

Based on prototype testing of GelMA hydrogels, either degree of methacrylation, time in a 4 ℃ fridge, or time under UV light will be varied to obtain specific mechanical properties requested by the client. Decreasing the degree of crosslinking can be achieved by decreasing the time in the fridge or under UV light, and a softer gel can be obtained. An increase in the degree of crosslinking can be achieved by increasing the time in the fridge or under UV light and a stiffer gel will be the result. Degrees of methacrylation can also be varied to tune the desired mechanical properties. This is achieved by varying the concentration of methacrylic anhydride in

the initial GelMA formation reaction. Besides changing the mechanical properties, varying the degree of methacrylation can also have a direct impact on porosity, pore size, and swelling behavior of GelMA[19]. The methods of changing the degree of crosslinking will first be attempted to tune the mechanical properties. If unsuccessful, varying the degree of methacrylation will then be attempted instead.

Appendix D - Frequency Sweep Rheology Protocol

1. Once GelMA hydrogels have been formed and allowed to set and swell for approximately 12-24 hours, rheometry testing may be performed.
2. Carefully remove 3 hydrogels of each type; healthy lung ECM and fibrotic lung ECM, from 24 well cell culture plates, keeping the gels of the same type 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 type were tested for a total of 6 separate frequency sweeps.
11. When testing is completed, results can be interpreted in MATLAB.