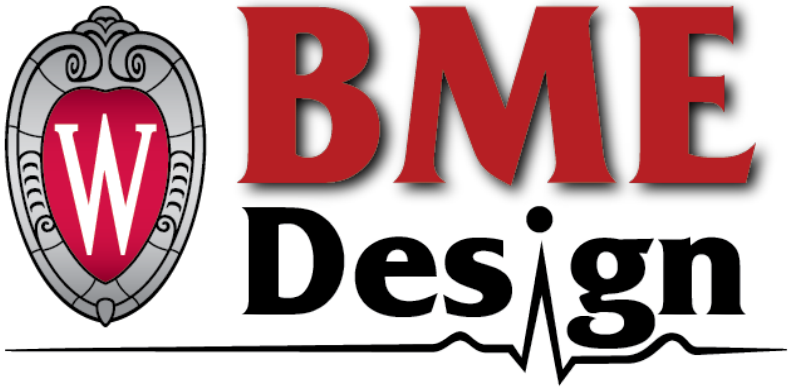
Tissue Model of The Epithelial Mesenchymal Trophic Unit



Date: September 23, 2022
BME 200/300
Lab Section 305

Product Design Specification

Client: Dr. Allan Brasier
Advisors: William Murphy

Team Members:
Carley Schwartz cischwartz@wisc.edu (Leader)
Caitriona Treacy ctreacy2@wisc.edu (Communicator)
Elijah Diederich ediederich@wisc.edu (BSAC)
Sara Morehouse smorehouse2@wisc.edu (BWIG)
Ana Martinez almartinez4@wisc.edu (BPAG)
Althys Cao nvcao@wisc.edu (BWIG)

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Abstract

As the research on lung diseases evolves, the need for a synthetic scaffold that accurately mimics the ECM increases. The goal of the project is to create a replicable synthetic scaffold that can be used for culturing of lung epithelial cells. The scaffold will be tested on mechanical stiffness, porosity, swelling, and translucency before being handed to the client. The client will then cell culture on the model to assess cell viability and adhesiveness. To complete this goal, a synthetic material of polyethylene glycol seeded with collagen and fibronectin was decided upon. This design is possible to create within the BME lab at UW-Madison, won't cause a toxic reaction with the cells, and meets the biomechanical standards that indicate it would work well to replicate the native lung ECM.

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

The lung ECM is a highly complex system of proteins and macromolecules that function as a support system for the lungs, 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 [1]. 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 [2]. 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 [3]. 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. [4]. Cell scaffolds are used to provide attachment and subsequent support for cell development and can be modified to mimic in vivo ECMs [5]. Furthermore, for respiratory research, cell cultures are grown in an ALI. This involves the basal surface (usually the bottom surface) being submerged in liquid medium while the apical surface will be exposed to air [6].

Considerations & 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) [7], epithelial cell adhesion once culture is applied, and the scaffold's permeability to water soluble water molecules. The latter two measurements will increase cell viability once culture is applied. The model must take the form of an air-liquid interface to emulate the conditions which cells experience in the lungs. The mechanical and biochemical properties of the scaffold must be compatible with the cells applied during experimentation, again in an effort to maintain cell viability. Finally, the ability to isolate and incorporate specific proteins such as fibronectin and collagen into the matrix is an important consideration in the context of the goals of the project. That is, the composition of the scaffold must be known and able to be manipulated easily.

Competing Designs

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.

Preliminary Designs and Evaluation

Designs

Design 1: Gelatin Methacryloyl (GelMA)

GelMA is an engineered gelatin-based material with naturally occurring adhesive properties. This is due to there being arginine-glycine-aspartic acid, which are RGD sequences that also occur in native ECM proteins such as fibronectin that allow cell adhesion [9]. 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 [10].

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 such as fibronectin and collagen 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 [11]. PEG has a multitude of different reactive end groups, each with its own benefits and drawbacks which will be discussed in further sections [12].

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 [13].

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	25	4/5	20	5/5	**25**	3/5	15

		Design 1: Gelatin Methacrylate (GelMA)		Design 2: Polyethylene Glycol (PEG)		Design 3: Alginate	
Properties							
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. 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 any other cells needing 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 will need to be capable of having cells planted within it to add this component of biochemical communication. This means that the scaffold must not cause a toxic or immune response in the cells being seeded.

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 [7].

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

Design Ratings/Selection

In the three considerations discussed above, polyethylene glycol(PEG) hydrogel scored the highest. Looking into each category, for biochemical properties PEG doesn't 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 [14]. This high cell viability is possible because of PEG being non-toxic and hydrophilic. As a result of these beneficial properties, PEG received a score of 21.

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 % concentration and molecular weight chosen [13]. 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 [15]. 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-diacrylate being crosslinked with UV light at 365 nm [12]. 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, it can also result in varying degradation rates causing it to be unpredictable and receive a lower score.

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.

Materials and Methods

Materials

The materials needed to construct the hydrogel are PEG-4AC and PEGDA, both with molecular weights of 10kDa. Additionally PBS buffer, ethanol, and deionized water will be needed. Lastly, teflon molds that fit inside the clients 24-well plate are needed.

Methods

PEG-4AC hydrogels are prepared by mixing PEG-4AC (MW, 10 kDa) solutions with PEGDA (MW, 10 kDa) solutions. PEG-4AC powder and PEGDA powder are added to PBS to a final concentration of 10% (w/v) and 5% (w/v) respectively. This solution will be stirred until

complete dissolution is achieved followed by the addition of 1% (v/v) photoinitiator solution (10% (w/v) I2959 in 70% ethanol and 30% deionized water). The 0.5 ml aliquots of the precursor solution will be transferred into a round Teflon mold with a 14 mm diameter. This will then be irradiated with UV light (365 nm, 4–5 mW/m2) for 5 min in order to achieve chemical cross-linking [16].

Conclusion

In conclusion, the team plans to utilize polyethylene glycol (PEG) seeded with collagen and fibronectin in order to fabricate a three-dimensional scaffold for lung epithelial cell culture. This synthetic material was chosen because it is the most feasible to fabricate, is both biocompatible and biomechanically similar to the native lung ECM, and minimizes fabrication variability. Upon the scaffold's success in mechanical stiffness, porosity, translucency, cell adhesiveness, degradability, and swelling measures, this product will assist the client in continuing to research the lung epithelium in diseased states by allowing for cell culture, imaging projects, and experiments that incorporate fibroblasts into the scaffold.

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Appendix

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/

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.

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