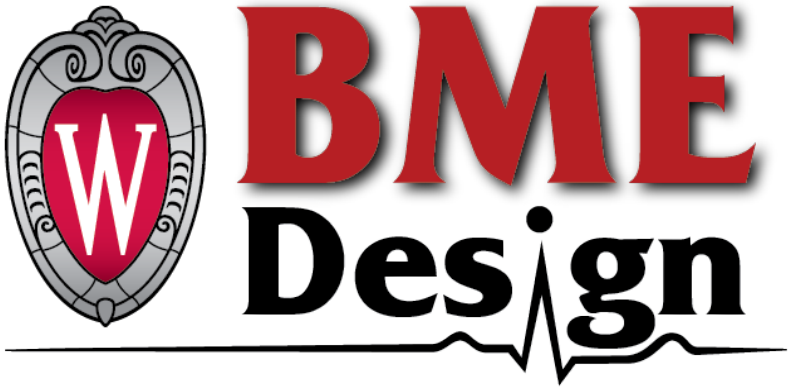
Tissue Model of the Epithelial Mesenchymal Trophic Unit



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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. Developing an in vitro model that accurately mimics the lung's extracellular matrix and its changes during cell injury can provide researchers with a valuable tool to study disease mechanisms and develop potential treatments. Understanding how the lung's mechanical stiffness and porosity change in response to injury – and how these changes affect epithelial cell behavior – can allow researchers to design therapies that target these specific changes. 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 produced as a solution in the previous semester. Initial fabrication procedures, and accompanying methods to test the hydrogel's efficacy were produced. The fabrication of the GelMA hydrogels will be translated to a 3D bioprinter that uses GelMA Bioink LAP 0.25% with 45-55% degree of methacrylation, and tested in the upcoming semester of Fall 2023.

Introduction

Problem Statement

A multitude of chronic lung diseases such as pulmonary fibrosis, asthma, and chronic obstructive pulmonary disease (COPD) can cause damage to epithelial tissues of the lungs. This presents a problem as 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 tissue models that accurately recreate the lung extracellular matrix and its changes as a result of cell injury. Such a model would need to have tunable mechanical stiffness and porosity, as well as be cell adhesive and degradable. Dr. Allan Brasier of the UW School of Medicine and Public Health requires a scaffold that meets these criteria to be fabricated with a bioprinter. The scaffold must have a uniform and replicable composition that allows for epithelial cell culture in an air-liquid-interface (ALI) so that his lab can study the effects of fibrosis on small-airway lung epithelial cells.

Background/Motivation

The extracellular matrix (ECM) is a complex network of proteins and other macromolecules that provides structural support as well as mechanical and biochemical cues to surrounding cells [2]. A key component of the ECM in the small airways of the lungs is the basement membrane that is located below the epithelial cells. Embedded in this basement membrane are subepithelial fibroblasts. These epithelial cells, lung ECM, and fibroblasts comprise the epithelial mesenchymal trophic unit (EMTU) [3]. When chronic diseases cause epithelial injury, an inflammatory response causes the subepithelial fibroblasts to increase production of ECM proteins such as collagen, a fibrous structural protein, and fibronectin, a key binding protein [3]. The increased protein deposition raises the mechanical stiffness of the lung ECM, which causes a positive feedback loop that leads to increased fibroblast activity. It is not fully understood how this increased ECM stiffness affects the epithelial cells.

Tissue models made from biomaterial scaffolds allow for the *in vitro* modeling of biological phenomena that are difficult to investigate *in vivo*. These models can recreate tissue microenvironments more accurately than traditional cell culture [4]. There are no tissue models that recreate the EMTU with tunable stiffness to represent healthy or fibrotic conditions, while maintaining important biochemical properties. Such a model is needed because it would allow for the elucidation of the effects of fibrosis on lung epithelial tissue, which is a complex phenomenon that requires a high degree of control to be represented properly. In addition, the model created may have a number of broader applications in drug delivery/discovery, precision medicine, and related fields of research.

Competing Designs

Tissue engineering models provide *in vitro* means to study the body that have enabled the creation of many novel designs. For models related specifically at the lung epithelium, there are

currently both 2D and 3D models on the market that seek to mimic the *in vivo* environment. Unfortunately, these models are often oversimplified and do not provide accurate research results for cell behaviors obtained from experiments performed on these models.

2D models are typically layers of cells cultured on top of polymer or glass dishes. While several 2D model experiments performed over the years have allowed some study into cell function, disease, and the microenvironment, these models do not reflect typical cell behavior in the native environment. For reference, 2D models have a stiffness range of 2-4 GPa while healthy human lung ranges from 0.44-7.5 kPa [5]. The differences in stiffness significantly affect the function of fibroblasts in the ECM that differentiate into a specialized phenotype when mechanically stressed, resulting in experimental data not representative of behavior *in vivo* [6].

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 [7]. These 3D models have variations in methods for each experiment, but generally all involve an ECM gel 3D environment that is more similar (with some limitations) to *in vivo* than the 2D models. Matrigel is a mouse tumor extracellular matrix mixture, so there are compositional variations present in every batch. It also consists of proteins that may or may not be present in healthy ECM, reducing the accuracy of the model. Matrigel ECM is more similar to the ECM of a tumor with significantly more laminin, and glycoproteins, which can cause the microenvironment to be unlike native tissue.

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

Specifications

The primary goal of the project is to create a model that can mimic the extracellular matrix (ECM) environment of the small airway of the lung in both healthy and fibrotic lung conditions. This requires the ability to emulate the mechanical stiffness of the lung and ensure that the epithelial cells can adhere to the scaffold once it is cultured. It is also important that the scaffold is degradable, as this allows for fibroblasts to produce their own ECM proteins, remodeling the matrix and creating a microenvironment that resembles native lung ECM. In order to achieve these goals, the Young's modulus must emulate a healthy and fibrotic lung ECM environment. That is, to represent the environment that fibroblasts are subjected to in the lungs, the Young's Modulus (E) for the healthy and fibrotic hydrogels should be 3.5 kPa and 16.5 kPa, respectively [8]. Meeting these moduli values is crucial to displaying correct cell phenotype and accurately representing characteristics unique to healthy and fibrotic lung ECM states. This is imperative as the mechanical and biochemical properties of the scaffold, including any incorporation of peptides, must be compatible with the cells used during experimentation to maintain cell viability.

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

Previous Semester

Final Design: Gelatin Methacryloyl (GelMA)

GelMA is an engineered gelatin-based material that is produced through the reaction of gelatin with methacrylic anhydride (MA). GelMA is able to be photocrosslinked with UV light, retains natural cell adhesion motifs, and contains MMP degradable sequences, making it an attractive choice. GelMA hydrogels with a 50% degree of functionalization (DOF) were previously constructed by using a pipette to insert 100 uL into a mold. They were then photocrosslinked with 365 nm wavelength UV and variation of time between 1 and 5 minutes to produce normal and fibrotic tissue respectively. See Appendix F for the prior semester's evaluation of preliminary design which led to GelMA being chosen and Appendix C for the hydrogel synthesis protocol.

Testing & Results

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

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

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

Following calibration, the frequency sweep test was conducted and took 10 minutes for each hydrogel. Test results were documented in an Excel spreadsheet, and thorough cleaning with 70% ethanol was performed between tests. The test yielded the storage modulus (G'), allowing

for the estimation of the shear modulus (G) at very low frequencies (0.1 Hz). Young's modulus (E) was then calculated using the equation $E = 2G(1+\nu)$, with ν representing Poisson's ratio. Young's Modulus served as a valuable metric for quantifying the varying stiffness values between the normal and fibrotic ECM hydrogels.

In addition to the rheometric testing, an increase in cooling time in the fridge was observed to correspond with an increase in hydrogel stiffness. This was particularly evident in the fibrotic hydrogel, which had an average Young's Modulus of 49.2 ± 11.65 kPa. Normal gel batches 2 and 3 exhibited similar mechanical stiffness, averaging approximately 2.35 ± 1.62 kPa, though they showed greater variability compared to normal gel group 1. Normal gel batch 1, with a 30-second cooling time, most accurately represented the normal lung ECM, displaying an average Young's Modulus of 3.42 ± 0.49 kPa. These findings lay the foundation for the ongoing project and serve as a metric to characterize the mechanical properties of the hydrogel scaffold to be printed using the CELLINK bioprinter. Lastly, The study involved the client's laboratory assessing the biochemical properties of the hydrogel scaffolds. The gels were coated with a monolayer culture of lung epithelial cells and were tested for cell adhesion and proliferation. Attempted confluent analysis through ImageJ brought challenges due to poor image quality and time constraints. Additionally, not all necessary images were supplied, limiting the depth of analysis. These findings complement the ongoing project's focus on hydrogel suitability for cellular growth and attachment, alongside mechanical property assessments. See Appendix E for the rheometry testing protocol.

Lessons Learned

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

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

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

New Direction: 3D Bioprinting

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

Materials and Methods

Materials

In the Fall 2022 semester, the client gave the team a $5,000 budget. In the previous 2 semesters, the team used a total of $1,091 of this budget to purchase materials to fabricate PEG hydrogel scaffolds and GelMA hydrogel scaffolds in molds, leaving $3,909 left in the budget. Due to the evolving nature of this project, the majority of the previously purchased materials have been given to the client as they are no longer relevant. The team is using a CELLINK Bio X 3D Bioprinter provided by the client to make the tissue model. While the project's fabrication method has changed, the material has not; the scaffold is printed from CELLINK GelMA bioink with LAP 0.25% as a photoinitiator. A CELLINK Temperature-Controlled Printhead is used in order to warm the bioink to a printable consistency. To prevent the bioink from cooling and clogging the nozzle while printing, a thermal insulator nozzle cover has been added to the temperature-controlled printhead. Additional GelMA bioink cartridges will likely be purchased as the semester continues. A full materials list with expenses can be found in Appendix A.

Methods

The GelMA scaffolds are fabricated by bioprinting. First, the bioink is warmed to a liquid consistency to make it printable. Then, the bioprinter is calibrated and parameters are programmed to the desired settings. A CAD file with a specific print structure can be uploaded to the bioprinter, or one of the preset structures can be used. Finally, the bioprinter prints the scaffold layer-by-layer. The GelMA bioink contains LAP photoinitiator, so the scaffold can be directly photocrosslinked after printing. Several parameters can be optimized to achieve high fidelity prints, including printing pressure and speed. The mechanical stiffness of the printed scaffold can be tuned by adjusting the time and/or distance of the UV photocrosslinking. The full procedure for bioprinting GelMA can be found under Appendix D.

Proposed Testing

Quantification of Hydrogel Stiffness

As done in the previous semesters of this project, the elastic modulus of produced hydrogels will be assessed using rheology. A strain controlled frequency sweep using a Malvern Kinexus Ultra+ Rheometer, available in the teaching lab, will be performed from .1 to 10 Hz. This protocol measures the storage modulus (G') of the material. At low frequencies (.1 Hz) this can be approximated as the shear modulus (G) of the material (Eq.1).

$$\{1\}\ G\ \approx\ G'(.1\ Hz)$$

For an incompressible hydrogel, the Poisson's ratio (ν) is .5 [9]. The Elastic Modulus (E) of a material is directly proportional to twice the Poisson's ratio plus on (Eqn. 2).

$$\{2\}\ E\ =\ 2G(1\ +\ \nu)\ [9]$$

While the storage modulus of the material at .1 Hz is the only frequency relevant to the stiffness calculation, the full sweep will be recorded for thoroughness. A loss modulus that is less than the storage modulus indicates that the phase shift is less than 45°, and the material is mainly elastic. To ensure consistency between tested batches, swelling time prior to testing and temperature will be controlled during testing. The full protocol for rheology testing can be found under appendix E.

Epithelial Confluency

Confluency of epithelial cells seeded atop hydrogels will be assessed using light microscopy. Images will be imported to ImageJ, and an area fraction of cells to background will be calculated.

Live/Dead Staining

To ensure that the scaffold material is biocompatible with seeded cells, quantification of cell mortality must be conducted. Live / dead staining and morphological observation of cells within GelMA hydrogels will be conducted using Calcein-AM, and propidium iodide. A full procedure for live/dead staining can be found under appendix G.

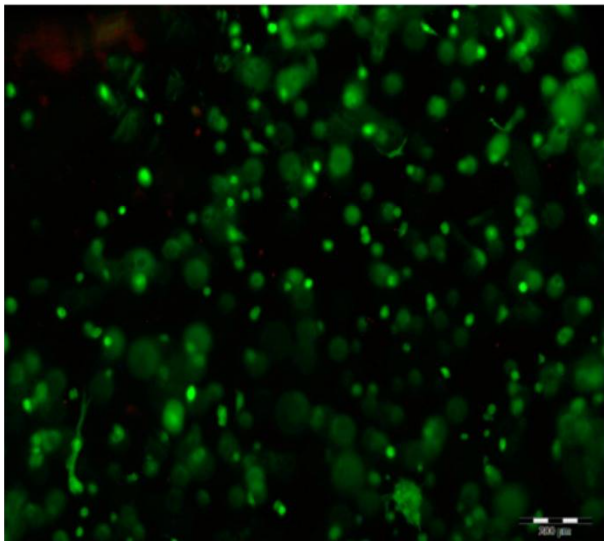

Figure 1: Image of GelMA hydrogel stained with Calcein-AM/ propidium iodide [10]

Western Blot

Assessment of gene expression can be accomplished by measuring protein levels through Western Blotting. The procedure remains the same for cells cultured in GelMA hydrogels from traditional cell culture, with the expectation that the hydrogels should be degraded prior to cell lysis. Degradation of GelMA hydrogels can be accomplished via incubation in collagenase [11].

Following scaffold degradation, cells will be lysed by addition of the lysing buffer radioimmunoprecipitation assay buffer (RIPA). RIPA buffer contains sodium dodecyl-sulfate polyacrylamide (SDS) and will also denature proteins [12]. Lysates will be separated by gel electrophoresis using SDS-PAGE gels. Following electrophoresis, proteins will be transferred to a nitrocellulose or Polyvinylidene Difluoride blotting membrane. Blocking with bovine serum albumin (BSA) will be performed to prevent the nonspecific bindinging of antibodies, as well as reduce background noise during imaging. Membranes will be incubated in primary antibodies specific to the target protein. Following washing, membranes will be incubated in secondary antibodies conjugated with Horseradish Peroxidase, and finally imaged under 428 nm light. Presence of target protein will indicate the phenotypic expression of specific cell types.

Epithelial Subtypes

For an effective tissue model, characteristics of a healthy model must align with its *in vivo* context. Human small airway epithelium is diverse; five characteristic cell types are present, indicated by their phenotype. An intensive model would be able to support the differentiation and growth of each cell type, resulting in expression of relevant marking genes, listed in table 1 below. Expression of a protein encoded by each gene can be detected by using Western Blot.

Cell Type	Relevant Genes	Respective Proteins
Ciliated	FOXJ1, DNAI1, SPAG6, TEKT1	Forkhead box protein J1, dynein, Sperm Associated Antigen 6, Tektin
Secretory	SCGB1A1, SCGB3A1, MUC5B, LYZ	Uteroglobin, Uteroglobin, Mucin, lysozyme
Alveolar	SFTPA1, SFTPA2, SFTPB, SFTPC, SFTPD	Collectin (all)
Neuroendocrine	CHGA, CHGB, CALCA, SCG5	Chromogranin A, Chromogranin B, Calcitonin, Neuroendocrine protein 7B2
Ionocyte	FOXI1, ATP6V1B1, CFTR	Forkhead box protein, V-type proton ATPase subunit B, Cystic fibrosis transmembrane conductance regulator

Table 1 : Small Airway Epithelial Cell Types and Markers [13]

Considering that phenotypic expression is expected to vary under diseased states, for the purpose of model validation western blot for epithelial subtypes will be performed only in hydrogels mimicking healthy tissue. Investigation into how expression is altered in fibrotic states is something that the model is capable of, but is outside the scope of initial validation.

Realistically, due to the cost of material, and time constraints, realistically only a few markers will be assayed. Per the client's request, focus will be placed on the presence of ciliary and secretory cells.

Fibroblast Stiffness-Response

Yes - Associated Protein 1 (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) are mechanoresponsive transcription cofactors in lung fibroblasts [14]. Their expression is associated with pulmonary fibrosis, where in stiff substrates, YAP and TAZ are present in greater concentrations [15]. Comparison between YAP and TAZ expression in healthy and fibrotic stiffnesses can be determined by using immunoblot analysis, as done in the determination of epithelial subtype presence. To determine correlation stiffness and expression, comparison of blot band size can be used to determine relative expression.

Discussion

Implications

Gelatin methacryloyl has been chosen for the production of a 3D scaffold for cell culture based on its ease of use, low cost, and adjustable mechanical and biochemical properties. The goal of the model is to manipulate scaffold stiffness in order to investigate lung epithelial cell behavior under normal and fibrotic conditions. In a previous semester, GelMA hydrogels were produced through the functionalization of gelatin through a reaction with methacrylic anhydride in a solution of PBS. Photoinitiator was added to the warmed solution, and the mixed solution was placed in silicon molds and cooled so that radical crosslinking under UV light could take place (See Appendix C for detailed fabrication protocol). Through this method, appropriate stiffness values were achieved for the model mimicking healthy lung ECM, but the fibrotic young's modulus was not achieved - the stiffness of these hydrogels was too high. In an effort to bridge this gap between *in vivo* and *in vitro* conditions, the proposed fabrication protocol has shifted to utilization of a 3D CELLINK bioprinter. This shift allows for the use of pre characterized GelMA, reducing the compositional variation between fabricated hydrogel batches. Further, this technique has more potential for varied hydrogel architecture, something that will elevate the utility of the model when it comes to achieving cell behavior reflective of the *in vivo* environment.

As the model is improved and further iterations are successful in mimicking the mechanical properties of the epithelial-mesenchymal trophic unit, the objective will shift to how to suspend fibroblasts and collagen in the model. This will include achieving cell culture and successfully studying cell behavior and communication on both the healthy and fibrotic tissue models. The purpose of this project continues to be to provide a more comprehensive and biomimetic surface to model the small airway epithelia. The objective is to achieve this through a three-dimensional culture substrate, the stiffness of which can be varied. Upon successful fabrication, the 3D model that will be developed will have potential to be integral in the study of biological processes such as cell behavior, signaling pathways, and drug responses. Further, achievement of physiological stiffness mimicry will make this model preferable over current simplified models due to the increased accuracy of cell adhesion, migration, morphology, and signaling. The importance of achieving this level of accurate mimicry has implications in allowing researchers to accurately study fibrotic lung diseases, such as pulmonary fibrosis.

Considerations

Once the model has been fabricated, characterization of the gels must be completed to ensure that the microenvironment provided by the hydrogels is reflective of the mimetic accuracy required by the model. The stiffness of the gels fabricated can be influenced by numerous parameters. For example, GelMA concentration, photoinitiator concentration, and UV photopolymerization conditions all contribute to the stiffness of the fabricated model. An effort has been made to reduce some of these variables in the use of pre characterized GelMA and the

CELLINK bioprinter, but even still there exists a potential for variation between batches, resulting in model inconsistencies. Further, the mechanism of the UV light attachment in the current bioprinter lacks a high level of precision. This has an effect on photopolymerization which is influenced by UV light intensity and exposure as a function of time, light power, and distance from the UV light. In order to reduce inconsistencies in achieved model stiffness, it is important that these variables be controlled in each iteration.

Indeed, the hydrogel formation process involves numerous sources of error. Along with UV light variations causing unanticipated changes in mechanical properties, another previous source of error came when hydrogels were being removed from a 24 well plate before crosslinking molds were acquired. The result was an abundance of gel deformities and widths which were not conducive to ALI cell culturing. This source of error remains an anticipated factor in the updated fabrication protocol, where a technique for gel transfer must be developed to reduce gel deformities, tears, and other abnormalities.

Conclusion

In summary, a 3D bioprinted GelMA hydrogel will be fabricated for co-culture of hSAECs and 3T3 fibroblasts. Hydrogels with fibrotic and normal ECM tissue stiffness will be created to explore the implications of increased stiffness on hSAECs. Correct hydrogel stiffness will be verified by comparing values obtained through rheology testing with literature supported *in vivo* tissue values. Upon co-culture completion, cell adhesion will be quantified with ImageJ cell analysis. A live/dead cell staining assay will also be performed to determine cell viability. Determination of correct phenotypic expression of healthy lung tissue fibroblasts will be done with a Western Blot, focusing specifically on genes expressed in ciliated and secretory cells. Continuous optimization of scaffold properties will be implemented and may include characterizations such as surface topology modifications and addition of growth factors to ensure an accurate and biomimetic EMTU model.

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Appendices

Appendix A - Materials & Expenses

Table 2: Materials list

Item	Description	Cost/Quantity	Link (if applicable)
Fall 2022/Spring 2023 Items	Unusable PEG materials and GelMA materials	$1,091	–
Bioprinter	CELLINK Bio X 3D Bioprinter	Provided by client	link
GelMA Bioink	CELLINK GelMA Bioink LAP 0.25% 3mL Cartridge	Provided by client	link
Temperature-Controlled Printhead	CELLINK Temperature-Controlled Printhead	Provided by client	link
Nozzle Cover	CELLINK Thermal Insulator Nozzle Cover	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 bioprinted 3D scaffold that has mechanical and biochemical properties that will be compatible with the lung epithelial cells used for experimentation. The ECM scaffold will allow for cellular communication and function similar to that of an *in vivo* environment.

Client Requirements:

- The product should be amenable to analysis via various techniques such as microscopy and should allow for the encapsulation of lung fibroblasts and cell culture of epithelial cells. Provide a suitable microenvironment for cell-cell interactions and ECM remodeling, as well as enable comprehensive analysis of changes in cell behavior.

- Model should include an air-liquid interface to reflect the polarization of the epithelium in the presence of air.
- Scaffold should have tunable mechanical properties to reflect that of native ECM.
- Product needs to be capable of cell encapsulation and be cell adhesive.
- The product needs to provide an environment that allows for ECM remodeling by encapsulated cells and/or cells coating the scaffold surface.
- The products must be replicable and fabricated using a Cellink bioprinter.

Design Requirements:

1. **Physical and Operational Characteristics**

a. *Performance Requirements:*

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

b. *Safety:*

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

c. *Accuracy and Reliability:*

i. *Mechanical Properties:*

The scaffold will undergo testing to ensure it meets the mechanical properties necessary to accurately represent the lung ECM. Measuring the Young's Modulus (E) quantifies the stiffness of the hydrogel and can be used to determine whether the hydrogel will accurately mimic its respective tissue properties. The scaffold must have a tunable Elastic Modulus ranging from 3.5-16.5 kPa to reflect the environment that fibroblasts experience through healthy lung tissue to fibrotic lung tissue. The scaffold will be considered mimetic of healthy lung ECM if the mechanical properties are within 5% of the values of native tissue.

ii. *Cell Adhesion and Viability:*

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

d. *Life in Service:*

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

e. *Operating Environment:*

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

f. *Ergonomics:*

The model will mimic the stiffness and morphology of the lung ECM as precisely as possible. This involves replicating specific mechanical 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 tissue scaffold must support three main criteria. First, it must be able to produce a variable range of stiffnesses, as described in the mechanical properties section (1.c.i). The material must also be conducive to cell adhesion, contain RGD peptides to allow for integrin binding. The material must also be conducive to matrix remodeling, containing motifs which are sensitive to matrix metalloproteinases.

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

i. *Aesthetics, Appearance, and Finish:*

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

2. Production Characteristics

a. *Quantity:*

Models of both healthy and fibrotic lung tissue are desired, so scaffolds with mechanical properties matching the aforementioned conditions must be fabricated. Therefore, at least 2 scaffolds are needed, one of each condition, though more will be necessary to demonstrate replicability and conduct testing upon.

b. Target Product Cost:

The materials for the scaffold should cost no more than $500. In the prior semesters, the team used $1091 of the $5000 budget, so there is $3909 left to spend. The new scaffold design will be made using the client's bioprinter, so the only cost should be the GelMA bioink cartridges.

3. Miscellaneous

a. Standards and Specifications:

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

b. Customer:

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

c. Competition:

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

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

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

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

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Appendix C - Hydrogel Fabrication Protocols

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

- Protocol above will make ten 100 µL gels

Appendix D - GelMA Scaffold Bioprinting Protocol

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

1. Warm up the GelMA to 37°C to have a liquid formulation in a water bath (~20 minutes, or until visibly liquid)
    a. The temperature-controlled printhead can also be used to warm the GelMA

2. Cap the GelMA cartridge pressure hose end cap and a 22G nozzle then place in a temperature-controlled printhead set at 27°C
    a. Be sure to have the thermal insulator nozzle cover attached to the temperature-controlled printhead to prevent bioink gelling within the nozzle
3. Wait for the GelMA to reach the set temperature (5-10 minutes). While the gel is reaching printability temperature, set up and prepare the BIO X bioprinter for printing
    a. Set the print bed temperature to 15°C
    b. Set up the printing protocol with desired printing surface (well plate or Petri dish), bioink profile, printing pattern, pressure, speed, delay, and photocrosslinking settings
        i. For GelMA bioink, it is recommended to start at 5 mm/s print speed, 23-33 kPa print pressure, 500ms pre-flow delay, 10 s photocrosslinking time with 405 nm UV light, and 5 cm photocrosslinking distance
4. Calibrate the bioprinter such that it will print in the back half of the Petri dish so the 405 nm UV light will reach the print
5. Test the pressure to see if any bioink extrudes
    a. If there is extrusion, preemptively unclog the nozzle with a needle and then start the print
    b. If there is no extrusion, increase the pressure and test again
6. Fine-tune printing settings to achieve desired construct
    a. Adjust printer speed, pressure, and pre-flow delay to get even/consistent printing
    b. Adjust photocrosslinking distance and photocrosslinking time to tune the stiffness of the print

Appendix E - Frequency Sweep Rheometer Protocol

1. Once GelMA hydrogels have been formed and allowed to set and swell for approximately 24 hours, rheometry testing may be performed.
2. Carefully remove 3-4 hydrogels of each type; healthy lung ECM and fibrotic lung ECM, keeping the gels of the same type in their respective petri dish.
3. Once gels are in two separate weighing dishes, make your way over to the rheometer testing machine (Malvern Rheometer - Kinexus Ultra+)
4. Make sure that the bottom plate is locked on the rheometer by pushing the level, located on the front of the machine below the bottom parallel plate, all the way to the right
5. Open rSpace application on the computer and when prompted to select a certain test, select the 0035 test; Frequency Sweep Strain controlled.
6. When this specific test is selected, the user will then be prompted to enter a Gap value. This value will pertain to the thickness (mm) of the hydrogel being tested. Center the hydrogel on the bottom parallel plate. Measure the thickness (mm) of the hydrogel and enter the gap value. The upper plate will then move to this gap value.

7. Once making sure that the upper plate makes contact with the top of the hydrogel and the thickness is the correct value, enter values for various testing parameters such as room temperature, start frequency, end frequency, shear strain %, and samples per decade. In this specific test, the values were as follows: Start Frequency = 0.1 Hz, End Frequency = 10 Hz, Room Temperature = 25 ℃, shear strain = 1%, and 10 samples per decade.
8. Once the various testing parameters are entered, the user will then be able to start the test. A 5 minute calibration will be performed before the actual test begins. Once this calibration has been completed, the frequency sweep test will take approximately 10 minutes.
9. When the test is completed, the results table can be copied into an excel spreadsheet. Enter a gap value that is greater than the thickness of the hydrogel to remove the hydrogel from the machine. Clean upper and lower parallel plate surfaces with ethanol.
10. Repeat steps 5-9 for remaining hydrogels. In this specific test, 3-4 hydrogels of each type were tested for a total of 6-8 separate frequency sweeps.
11. When testing is completed, results can be interpreted in Excel.

Appendix F - Spring 2023 Preliminary Designs & Evaluation

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

Design 1: Gelatin Methacryloyl (GelMA)

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

Design 2: Polyethylene Glycol (PEG)

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

Design 3: Lung ECM

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

Design Matrix

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

		Design 1: Gelatin methacryloyl (GelMA)		Design 2: Polyethylene Glycol (PEG)		Design 3: Lung ECM	
Design Criteria	Weight	Score	Weighted Score	Score	Weighted Score	Score	Weighted Score
Mechanical Properties	20	4/5	16	4/5	16	2/5	8
Biochemical Properties	20	4/5	16	3/5	12	5/5	20
Ease of Fabrication	15	4/5	12	2/5	6	1/5	3

		Design 1: Gelatin methacryloyl (GelMA)		Design 2: Polyethylene Glycol (PEG)		Design 3: Lung ECM	
Ease of Use	15	2/5	6	1/5	3	1/5	3
Mechanical Tunability	10	4/5	8	4/5	8	1/5	2
Biochemical Tunability	10	3/5	6	4/5	8	1/5	2
Cost	10	5/5	10	3/5	6	1/5	2
Total:	**100**		74		59		40

Winner	Tie

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

Design Matrix Criteria

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

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

The mechanical properties of the synthetic scaffold will need to reflect those of the native and fibrotic lung environment. ECM changes in mechanical properties such as tension or stiffness can cause major responses in cells which alter their ability to function properly. Due to the cell's sensitivity to the mechanical microenvironment around them, the mechanical properties must accurately mimic those of native tissue. Given that our client wants varying mechanical

properties to model both native and fibrotic lung tissues, the design should be able to produce hydrogels of elastic modulus from 3.5 kPa to 16.5 kPa.

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

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

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

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

Design Ratings/Selection

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

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

these incorporated [10]. This results in a more expensive and difficult fabrication process for PEG.

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

Mechanical tunability describes the ability of the material's mechanical properties to be tuned, which both GelMA and PEG rank highest in. Both of these designs have multiple mechanisms for tunability such as degree of substitution, chain length, cooling time, and time under UV light [8][10]. All these mechanisms to achieve the desired stiffness range result in GelMA and PEG ranking higher in comparison to lung ECM which does not allow for tunability to specific mechanical properties.

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

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

Appendix G - Live / Dead Staining Procedure

Adapted from Gelatin-Methacryloyl (GelMA) Hydrogels with Defined Degree of Functionalization as a Versatile Toolkit for 3D Cell Culture and Extrusion Bioprinting [1].

1. Incubate GelMA hydrogels in 3 µM Calcein-AM, 2 µM Propidium iodide supplemented culture medium for 15 minutes at 37 °C.
2. Image under fluorescent microscopy (494, 493) / (517,636) nm absorbance / emission respectively. [2][3]
3. Dead cells will appear red, live cells will appear green.
4. Count live / dead cells, determine mortality

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