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The Effect of Pepsin Digestion on Protein Composition in Decellularized Lung Alveolar Extracellular Matrix Hydrogels

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The Effect of Pepsin Digestion on Protein Composition in Decellularized Lung Alveolar Extracellular Matrix Hydrogels

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ABSTRACT

Pulmonary diseases, including Chronic Obstructive Pulmonary Disease (COPD) and Idiopathic Pulmonary Fibrosis (IPF), remain common causes of death, yet still have no cure. Recently, research of lung tissue, both healthy and diseased, has begun utilizing hydrogels as 3-dimensional (3D) environments for cell culture studies as well as *in vivo* therapeutics to more closely mimic the *in vivo* tissue environment. Synthetic hydrogels, such as Matrigel, have been commonly utilized as a 3D scaffold in which stem cells can be cultured and their behavioral patterns can be observed. The origin of Matrigel, however, is tumorigenic mouse tissue and it is not an optimal model for studying human cells, including lung cells. Recently, hydrogels formed from human decellularized tissues are becoming increasingly utilized in cell culture studies as physiologically relevant extracellular matrices (ECM). These hydrogels are thought to closely resemble the respective tissue environment, therefore providing specific conditions to the cells and positively influencing their growth and behavior due to the presence of tissue-specific ECM proteins. However, the formation of such hydrogels requires harsh processing steps, including 72-hour ECM pepsin digestion. Importantly, while several studies have analyzed the impact of tissue decellularization on ECM protein composition, the impact of the other hydrogel processing steps have been overlooked, particularly the effects of pepsin digestion. Additionally, several studies have utilized these tissue-specific hydrogels to analyze cell proliferation and behavior. Yet, the protein composition of the final hydrogels remains unknown. As ECM composition has significant influence on cellular behavior, we here aimed to determine the impact of pepsin digestion on the final protein content of decellularized lung hydrogels. Utilizing mass spectrometry analysis, total protein staining, and western blotting, we identified an overall decrease in total protein and an overall concentrating effect of Collagen I protein subunits following pepsin digestion.

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ABBREVIATIONS

ATS	American Thoracic Society
AT1s	Alveolar Type I Cells
AT2s	Alveolar Type II Cells
aECM	Alveolar Extracellular Matrix
B-ECM	Brain Extracellular Matrix
COPD	Chronic Obstructive Pulmonary Disease
dECM	Decellularized Extracellular Matrix
DI	Deionized
ECL	Enhanced Chemiluminescence
ECM	Extracellular Matrix
ESI	Electrospray Ionization
FWHM	Full Width Half Maximum
GAGs	Glycosaminoglycans
HCl	Hydrochloric Acid
IPF	Idiopathic Pulmonary Fibrosis
IPI	International Protein Index
iAT2s	Human Induced Pluripotent Stem Cell Derived Alveolar Type 2 Epithelial Cells
LC	Liquid Chromatography
LDS	Lithium Dodecyl Sulfate
LTQ	Linear Ion Trap Quadrupole
MLSB	Mild Laemmli Sample Buffer
MS	Mass Spectrometry
m/z	Mass to Charge Ratio
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NH_4HCO_3	Ammonium Bicarbonate
PBS	Phosphate Buffered Saline
POSTN	Periostin
PVDF	Polyvinylidene Fluoride
SDC	Sodium deoxycholate
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
TBS-T	TBS with Tween
TGFβ,	Transforming Growth Factor β
USB	Urea Sample Buffer
UVMMC	University of Vermont Medical Center
3D	Three-Dimensional

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INTRODUCTION

1.1 The Lungs and Disease

Respiratory diseases remain one of the leading causes of death in the world.[1,2] Diseases of the lung, including Chronic Obstructive Pulmonary Disease (COPD), Idiopathic Pulmonary Fibrosis (IPF), lung cancer, and asthma affect millions worldwide. COPD specifically remains the third leading cause of death worldwide and these numbers increase yearly. [2] While respiratory diseases can be caused by many factors, the most common cause is the inhalation of smoke, including air pollutants and tobacco smoke. Yet, some mechanisms of disease remain unknown, as in the case of asthma which affects millions worldwide and is rising each year. [2] Despite this widespread cause of death and commonality of these conditions, respiratory diseases remain without cures. The treatment options available typically include the use of bronchodilators as temporary relief and control mechanisms. Yet these diseases progressively worsen overtime and significantly hinder the quality of life of those suffering with these diseases.

Aside from medications to control the progression of respiratory diseases and temporarily provide relief, the only option left for end-stage disease is whole lung transplantation.[1,3] Whole lung transplantation, however, is accompanied by a variety of obstacles and is only available to select candidates. For example, those with a history of active smoking are not suitable recipients of donor lungs according to the American Thoracic Society (ATS) recipient criteria.[2] This is of particular importance considering smoking is a leading cause of respiratory diseases and therefore death. Additionally, for those who receive whole lung transplantation, there is an increased risk of lung rejection and lifelong immunosuppression is required.[2,3] While lung transplantation provides relief to some suffering from respiratory diseases, there is a limited availability of viable and transplantable human lungs which is a leading limiting factor.[2,3]

1.2 Previous Research and Significance

With significant limitations in both the availability of donor lungs and tissue rejection related to organ transplantation, the field of tissue engineering has taken to a new approach of whole lung decellularization and recellularization.[4] Whole lung decellularization and recellularization provides the potential to increase availability of transplantable lungs by decellularizing lungs and repopulating the lungs with the recipients' cells. Specifically, whole lung decellularization allows for the formation of acellular, three-dimensional (3D) scaffolds consisting of the lung extracellular matrix (ECM).[4] Decellularization is facilitated by a series of detergent and enzymatic washes, in addition to mechanical agitation, to strip the tissue of all cellular and genetic material leaving intact the underlying 3-dimensional ECM scaffold.[4,5] This process is not only limited to healthy lungs. Recently, diseased lungs, including COPD and IPF lungs, have also been decellularized.[6] This process allows researchers to isolate the ECM from the decellularized tissue (dECM) and subsequently use it as scaffolds to be recellularized with relevant cell lines.[7] Overall, this approach can be used for mechanistic studies to better understand the function of cells and effects of abnormal ECM found in diseased lungs on disease pathogenesis. All told, these lines of investigation may eventually provide an alternative method to lung transplantation. In particular, the recellularization of dECM lung scaffolds with patient-derived stem cells may overcome the issue of limited donor lungs available and the problematic tissue rejection that follows transplantation.

The ECM is an important aspect of all tissues, especially in the lungs. Consisting of hundreds of tissue-specific macromolecules (i.e. proteins, glycosaminoglycans (GAGs), and growth factors), the ECM is essential to tissue structure and function.[8,9] In the lungs, the ECM plays a significant role in the flexible structure and unique biomechanical functions while also

interacting and impacting the wide variety of cells at the tissue level.[10] These lung specific proteins and GAGs interact with specific cell lines to influence cell growth, proliferation, differentiation, and function.[8] Therefore, ECM isolation allows researchers to investigate the cellular mechanisms in specific lung environments, including both healthy and diseased lungs.

Following decellularization, dECM hydrogels can be produced to form an *ex vivo* cell culture model, which is thought to closely recapitulate the tissue-specific environment.[11] The formation of hydrogels, defined as three-dimensional (3D) networks of hydrophilic polymers, is driven by the kinetics and entropy of the refolding and self-assembly of collagen fibers.[11,12,13] However, dECM hydrogel formation requires significant processing steps to breakdown proteins, including lyophilization, liquid nitrogen milling to form a powder, and a 72-hour pepsin digestion.[10] Pepsin is a non-specific protease which cleaves peptide bonds in the protein backbone following aromatic residues (i.e., Tryptophan, Tyrosine, Phenylalanine). This protein fragmentation is necessary in order to solubilize the dECM powder due to the insoluble nature of most ECM proteins, specifically collagen I.[10] Collagen I, a prominent protein found in the lung ECM, has a complex and insoluble structure in its natural state. On the most basic level, collagen I is composed of collagen fibrils, consisting of three alpha (α) helices twisted together and connected by telopeptides and cross-linkages (**Figure 1A**). These collagen fibrils are bundled together and several of these bundles are packed together to form one large, fibrous protein**.** During pepsin digestion, pepsin cleaves collagen at the telopeptide region causing the protein to unfold (**Figure 1B**). Ultimately, this increases collagen I's solubility allowing for the formation of a more homogenous solution of dECM powder which allows for the formation of hydrogels.[8] Collagen I, however, is not the only protein broken down during pepsin digestion. Pepsin's non-specific

nature causes other ECM proteins to be broken down as well, of which some may solubilized, and others may not.

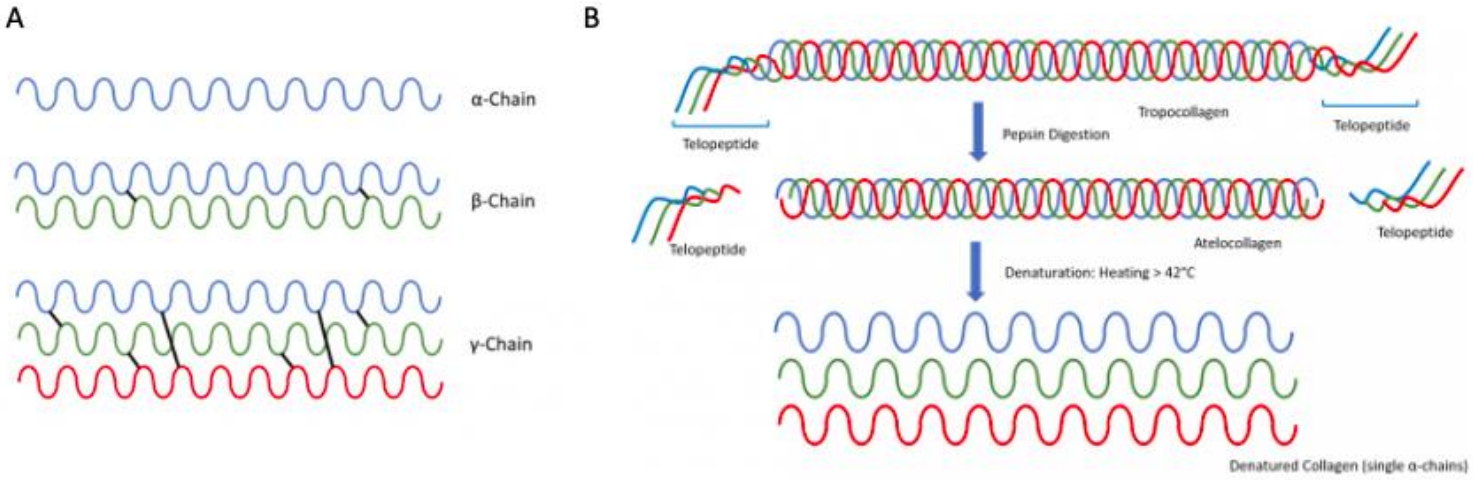

Figure 1 Collagen I chain structure and its Denaturation by Pepsin Digestion. [A] Chain structure of Collagen I, consisting of alpha (α) chains, beta (β) chains, and gamma (γ) chains. Triple helical structure consists of 3 α-chains. [B] Triple helical collagen I digested by pepsin, a non-specific protease, is cleaved at the telopeptides during digestion to release the α-chains. (Figure adapted from Chondrex Inc.)[21]

Although collagen I can reassemble easily and quickly, other ECM proteins may not be able to return from this digested and fragmented state and therefore may not be incorporated into the resulting hydrogel. Recent studies have investigated the mechanical impacts of pepsin digestions on hydrogel formation[10], including the digestion time and subsequently, the impact on mechanical properties including gelation kinetics, mechanical stress, light transmittance, and cell viability, of the resulting hydrogel.[10] 72-hour digestion was found to be optimal for the subsequent gelation and structure of the dECM lung hydrogel according to the mechanical properties, yet the overall impact of pepsin digestion on the total protein composition of the final lung hydrogels remains unknown at present.[10]

dECM hydrogels have become increasingly utilized in many fields of tissue research other than the lung. For example, porcine brain tissue has been decellularized (B-ECM) and used for the formation of hydrogels as a potential human alternative to Matrigel for the study of embryonic stem cell growth and proliferation.[14,15] Specifically, these hydrogels provide a model which mimics the compositional environment closer than any other current *in vivo* cell culture models. In comparison to Matrigel, analysis revealed a significant difference in the protein composition of the B-ECM hydrogels, most likely being a result of the different organism tissue origins.[14] However, understanding protein composition has the potential to optimize these hydrogels and to provide an even closer tissue model for future research. In the B-ECM hydrogels, mass spectrometry analysis revealed that some important B-ECM proteins are lost throughout the hydrogel formation process, with the exception of collagens.[13,14] Additionally, the loss of GAGs was found to be significant throughout the B-ECM decellularization which has also been determined in other porcine tissue studies.[9,14] Thus, while porcine brain dECM hydrogels may not fully reflect the ECM composition of porcine brain tissue, it is still a closer alternative than

Matrigel and other cell culture models utilized today. Additionally, these studies have further highlighted the retention and concentration of collagen fibers with the loss of GAG proteins as a direct result of tissue decellularization.[9,14] However, it is important to consider that each tissue has a different and specific environment in order to properly carryout the specific organ function, and therefore the ECM composition will differ significantly from tissue to tissue. As a result of this, the study of how decellularization and hydrogel processing affects the protein composition is necessary in each tissue of interest.

1.3 iAT2 and aECM Hydrogels

Current research in the Weiss Lab, a pulmonary research lab at the University of Vermont, is focusing on human induced pluripotent stem cell derived alveolar type II epithelial cells (iAT2s) cultured in hydrogels formed from the alveolar region of decellularized lungs (aECM).[16,17] Lung tissue is decellularized and the alveolar region is dissected to form a specific and relevant environment for the culturing of iAT2s (**Figure 2**). Alveolar Type II cells (AT2s) are vital to the function, maintenance, and repair of the alveoli in the lungs.[16,17] Structurally, the alveoli are tiny air sacs located at the end of the bronchioles which perform gas exchange and are commonly injured beyond repair in pulmonary diseases such as COPD. AT2s line the alveoli tissue and function to secrete surfactant proteins which are necessary to maintain the surface tension in the alveoli and prevent them from collapsing during gas exchange.[16,17] Alveolar Type I cells (AT1s) are the other major cell in the alveoli and participate in gas exchange, the critical function of the lung. When damage to the alveoli occurs, AT2s tend to differentiate into AT1s as a mechanism of repair.[16,17]

Current data in the Weiss lab suggests that culturing iAT2s in the physiologically relevant aECM hydrogels has a significant impact on iAT2 proliferation, maturation, and differentiation into AT1s, effects not observed when the AT2s are cultured in Matrigel. This underlies the importance of using aECM in lung hydrogel cultures. To further understand the iAT2 behavior in relation to its environment, a precise understanding of the aECM hydrogel protein composition is required. Therefore, this study is a part of a larger characterization study of protein composition of aECM throughout the hydrogel formation process.

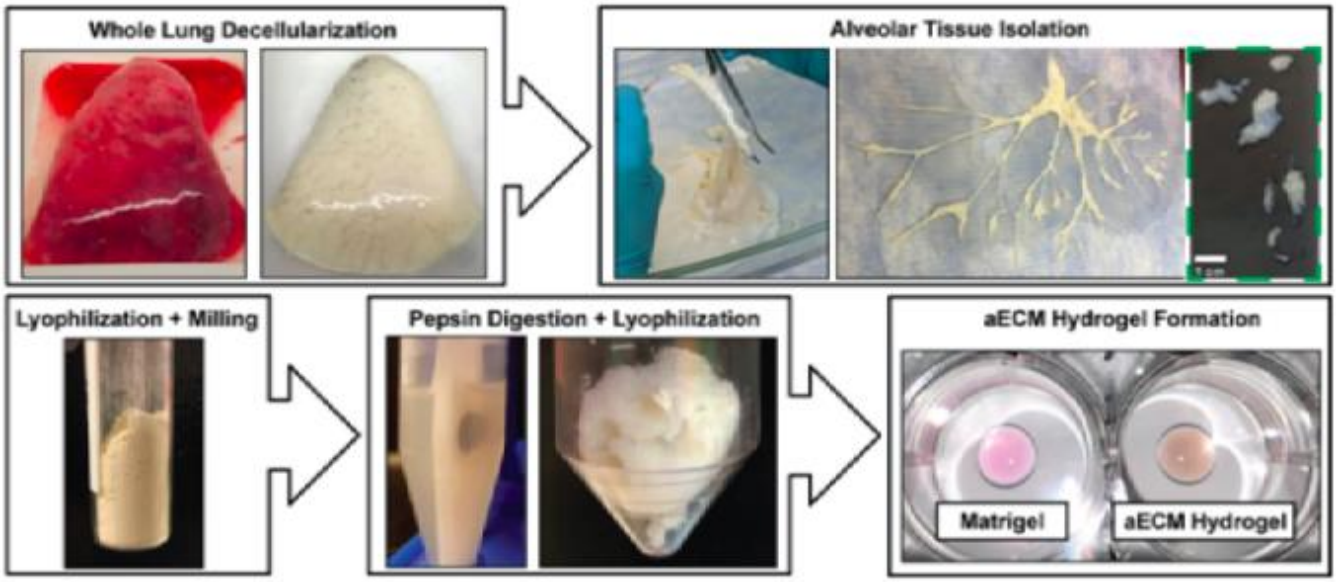

Figure 2 Sequential Steps in Human Lung aECM Hydrogel Formation. Decellularization via a series of washes and manual agitation to strip the lung tissue of all cellular matter. The alveolar region is dissected and subjected to lyophilization, milling, pepsin digestion, lyophilization again and resuspension to form aECM hydrogels.

1.4 Aim and Methodology

The aim of this study was to determine how pepsin digestion of the decellularized human alveolar extracellular matrix (aECM) affects the total protein composition of aECM hydrogels. It was hypothesized that pepsin digestion will result in an overall decrease of total protein in the aECM hydrogel, while enriching larger fibrous proteins such as collagen I due to the solubilization of collagen I and refolding kinetics. While previous studies have identified the loss of proteins via decellularization, a better understanding of how milling, lyophilization, and pepsin digestion affects the final hydrogel protein composition can provide further insight on what may be influencing specific cellular behavior. Specifically, in the Weiss Lab, identifying the composition of the aECM hydrogels will provide insight and potential explanations for iAT2 cell behavior in addition to growth, proliferation, and differentiation patterns. Additionally, identifying the proteins lost throughout the hydrogel formation process will provide the necessary foundation for future studies involving hydrogel supplementation. Hydrogel supplementation has the potential to improve 3D cell culture and allow hydrogels to be more representative of the lung environment.

Mass spectrometry (MS) was utilized to analyze the overall protein composition of aECM samples prior to pepsin digestion, post pepsin digestion, and after hydrogel formation. MS is used for the analysis of proteins by identifying the mass to charge ratio (*m/z*) and the elution time of individual molecules as the sample passes through the mass spectrometer.[18] Proteomic databases then utilize this information to determine the proteins and corresponding genes that can associate with those proteins within the sample.[18] The UVM Proteomics Facility performed this analysis and specific matrisome proteins were classified according to previously published matrisome protein lists.[19]

Additional protein analyses were utilized to gain a relative further understanding of the proteins present in each sample. Specifically, silver staining was utilized following denaturing gel electrophoresis (SDS-PAGE) to relatively compare the total protein found in the pre-digested aECM, pepsin digested aECM, and the aECM hydrogel. The principles of silver staining revolve around the reducing capabilities of silver ions. First, SDS-PAGE was used to separate proteins based on molecular weight, then silver ions present in the stain bind proteins and become reduced in the process to create a visible color change where protein is located.[20] Additionally, western blotting was used to analyze the relative concentration and the subunits of collagen I present in samples of differing stages of hydrogel formation. This technique first utilizes SDS-PAGE to separate proteins based on molecular weight. A transfer step is performed to transfer proteins from the gel onto a porous membrane which can be probed with specific antibodies. These results can be imaged and quantified to determine relative amounts of specific proteins present in each sample, specifically the collagen I subunits. Probing for collagen I is of interest because evidence suggests a concentrating effect of collagen I as a result of pepsin digestion, in addition to the major role collagen I plays during hydrogel formation.

METHODS

Lung Decellularization

Three healthy human lungs (n=3) obtained from patients with no history of lung disease (H62, H65, H66) were obtained from the University of Vermont Medical Center (UVMMC) autopsy services. Whole lung decellularization was performed with a series of sequential washes and manual agitation as optimized by previous members in the Weiss Lab.[5] Briefly, the lobes were rinsed individually with phosphate buffered saline (PBS, Corning) and deionized (DI) water 6 times. Once the excess blood was removed, perfusion of the tracheal airway was manually performed, and perfusion of the vasculature was performed using a peristaltic roller pump (Stockert Shiley, SOMA Technologies). This process utilized 2L rinses of the following in order: 0.1% Triton-X 100 (Sigma), 2% sodium deoxycholate (SDC, Sigma), 1M sodium chloride (NaCl, Sigma), DNase (Sigma), peracetic acid (Sigma), and a DI wash.

Following decellularization, the alveolar-enriched regions were manually dissected and isolated utilizing surgical scissors and forceps. The proximal regions of the airways were exposed and used to dissect further down the airways to the distal airway regions. The alveolar-enriched tissue differed in appearance from the remaining tissue by having a clear and shiny appearance from the final rinse steps of the decellularization process. Once identified and dissected out, the tissue was frozen at -80ºC. The frozen tissue was subjected to lyophilization and further milled (liquid nitrogen mill – Freezer Mill, Spex) into a powder (**Figure 2**). The aECM powder was stored at -20ºC and used for the remainder of the study.

Mass Spectrometry

Decellularized aECM powder was utilized following decellularization as described above. The following steps were all performed in a sterile fume hood to avoid keratin contamination.

Protein Isolation: Stock 4x Mild Laemmli Sample Buffer (MLSB) was prepared (8% SDS, 250 mM Tris HCl) and diluted to 1x MLSB for further use. Approximately 7.5 mg of each aECM sample (lyophilized and milled into aECM powder) was weighed out and resuspended 1:1 w/v in 1X MLSB. Each aECM sample was incubated at 45°C for 30 minutes, with vigorous vortexing interspaced during this time to resuspend the sample. Following incubation, the samples were centrifuged at 10,000 RCF for 10 minutes. The supernatant was isolated and stored at -20°C for future use.

Pepsin Digestion: Approximately 7.5 mg of H62, H65, and H66 aECM samples were resuspended 1:1 w/v in pepsin hydrochloric acid (HCl, 1 mg/mL pepsin, 0.01 M HCl, pH=2). Samples were prepared in 5 mL Eppendorf tubes. A magnetic Eppendorf stir bar was cut and placed in the tube. The samples were placed on a magnetic stir plate and digested for 72 hours at room temperature in the light. Following digestion, the samples were neutralized with sodium hydroxide (NaOH, FisherScientific, 1 M, 1:1). Once samples were neutralized, each sample was incubated at 45°C for 30 minutes, with vigorous vortexing interspaced during this time to resuspend the sample. Then, the samples were centrifuged at 10,000 RCF for 10 minutes and the supernatant was isolated and stored at -20°C for future use.

aECM Hydrogel Preparation: Pepsin digested samples were lyophilized and lyophilized samples were then resuspended in ice cold PBS at a concentration of 8mg/mL.

Protein Quantification: Each sample was quantified using the Pierce™ BCA Protein Assay Kit (Thermofisher Scientific™). The standard samples were prepared according to the manufacturer's instructions using 1x MLSB. 25 uL of each sample, including the prepared standards, were added to the corresponding well of a 96 well plate (ThermoFisher Scientific, 265302). 200 uL of the A+B solution (50:1) was added to each well. The plate was sealed and then mixed on the orbital shaker for 30 seconds. The plate was incubated at 37°C for 30 minutes. The plate was read at a wavelength of 562 nm and the protein concentrations were calculated using Excel software.

SDS PAGE: 20 μg of each sample was prepared for mass spectrometry analysis following the protein quantification. Each sample was prepared with lithium dodecyl sulfate (LDS) sample buffer (pH 8.4, 1x, Invitrogen™ 4x NuPAGE™ LDS Sample Buffer) and 1x sample reducing agent (10X NuPAGE™ Sample Reducing Agent). Samples were brought to 20 μL with 1x MLSB. Samples were briefly centrifuged and incubated at 70°C for 10 minutes. Samples were cooled on ice for 5 minutes prior to loading. 1L of 1x NuPAGE™ Running Buffer was prepared (NuPAGE™ Tris Acetate SDS Running Buffer, 20x, Invitrogen™). The precasted NuPAGE Tris-Acetate Gel (3-8%, Invitrogen) was set up in the mini gel tank (Invitrogen). Running buffer was filled around the gel and 0.5 mL of NuPAGE Antioxidant (Invitrogen) was added to the cathode compartment. All 20 μL of each sample was loaded into a lane of the gel and the gel was run at 150 volts for five minutes.

Gel Staining: The gel was removed from the cassette and stored in a container and fixed for 10 minutes in the fixing solution (40% methanol, 10% acetic acid). Following fixation, the gel was stained with Coomassie blue (0.1% Coomassie Brilliant Blue R-250, 50% methanol, 10% glacial acetic acid) for 20 minutes on an orbital shaker. The gel was then washed in destaining solution (40% methanol, 10% glacial acetic acid) with 1x10 minute wash followed by 3x5 minute washes until the background was destained. Once the protein bands were visualized at the top of the gel, a surgical scalpel was used to cut out 1 mm cubes of gel containing the protein. Each cube was chopped up and placed in an Eppendorf tube and sent to UVM Proteomics Facility to be analyzed by the mass spectrometer.

Mass Spectrometry and Analysis: Samples were dried under vacuum and incubated in 40 μL of 100 mM ammonium bicarbonate (NH_4HCO_3) and 50 mM dithiothreitol (DTT) at 56°C for 1 hour. The samples were cooled, resuspended in 5 μL of 500 mM iodoacetamide in 100 mM NH_4HCO_3, and incubated in the dark at room temperature for 30 minutes. The samples were dried under the vacuum and digested in 50 μL of trypsin (10 ng/μL) in 50 mM NH_4HCO_3 overnight at 37°C. Digestion was stopped by the addition of 5 μL of 10% formic acid. Samples were centrifuged at 14,000g and a ZipTip C18 (P10, Millipore Corporation, Billerica, MA) was used to extract and desalt 15 μL of the supernatant. Samples were dried under the vacuum and resuspended in 20 μL of 0.1% formic acid/2% acetonitrile. Liquid chromatography (LC) was utilized to separate the peptides by using a fused silica microcapillary LC column (12 cm x 100 μm diameter) and C18 reversed-phase resin 5 μm particle size; 20 nm pore size; Magic C18AQ, Michrom Bioresources Inc.). 6 μL of each sample was loaded and eluted with a 3-60% gradient of acetonitrile in 0.1% formic acid. LC was performed over 45 minutes with a constant flow rate of 250 nL/min. Mass

Spectrometry was performed utilizing Nanospray electrospray ionization (ESI) and a linear ion trap quadrupole (LTQ) Orbitrap Discovery mass spectrometer (Thermo Electron, San Jose, CA). The mass spectrometer performed an Orbitrap survey scan from *m/z* 400-2000 (resolution: 30,000 full width half maximum (FWHM) at *m/z* 400) in data-dependent acquisition mode. SEQUEST (Bioworks software, version 3.3.1; Thermo Electron, San Jose, CA) was used to search the International Protein Index (IPI) human protein sequence databases (V 3.75) and identify proteins. The parameters used includes a 100-ppm precursor MS tolerance and 1.0 Da MS/MS tolerance.

Further analysis was performed using Prism 9 software. n=3 was used for each condition (pre-digest, post-digestion, and aECM hydrogel). Mean and SEM was calculated and statistical significance was analyzed using Prism one-way ANOVA function.

Silver Staining

Protein Isolation: Some samples from each lung used for mass spectrometry were also utilized for silver staining. This includes pre-digest, 72-hour pepsin digested samples, and aECM hydrogel samples. Samples were thawed and kept on ice prior to use.

SDS-PAGE: 2.5 μg of protein from each sample was used on precasted tris-acetate gradient gels (NuPAGE Tris Acetate Gel, 3-8%, Invitrogen). Each sample was prepared with 1x lithium dodecyl sulfate (LDS) sample buffer (pH 8.4, 1x, Invitrogen™ 4x NuPAGE™ LDS Sample Buffer) and 1x sample reducing agent (10X NuPAGE™ Sample Reducing Agent). Total volume was brought up to 20 μL with 1x MLSB. Samples were briefly centrifuged and heated to 70°C for 10 minutes, cooled on ice and then loaded into the gel. 10 μL of the SeeBlue™ Plus2 Pre-stained Protein standard (Invitrogen) was run in lane 1. The gel was run at a constant voltage of 150 V for 55

minutes in 1X NuPAGE™ Running Buffer (NuPAGE™ Tris Acetate SDS Running Buffer, 20x, Invitrogen™). Once samples migrated through the gel, the gel was removed from the pre-casted casing and placed in a container for the upcoming staining steps.

Silver Staining: The Pierce™ Silver Stain Kit (ThermoFisher™ Scientific) was utilized according to the manufacturer's instructions. Briefly, the gel was first fixed (30% ethanol, 10 % acetic acid) for 2 x 15 minutes. The gel was washed 2 x 5 minutes in DiH_2O prior to staining. The gel was incubated in the Sensitizer Working Solution (1:500 Silver Stain Sensitizer to DiH_2O) for 1 minute followed by 2 x 1-minute DiH_2O washes. Next, the gel was incubated for 30 minutes in the Stain Working Solution (1:50 Silver Stain Enhancer to Silver Stain). The gel was then developed in the Developer Working Solution (1:50 Silver Stain Enhancer to Silver Stain Developer) for about 1 minute or until bands came to desirable visualization. The Stop Solution (5% acetic acid) was used to wash the gel for 10 minutes prior to imaging.

Imaging: The gel was imaged on the Amersham 600 Imager (GE Healthcare Life Sciences) colorimetric setting with auto exposure.

Western Blotting

Protein Isolation: Pre-digested aECM powder (H62, H65, H66) were weighed out (~7.5 mg) in 1.5 mL microcentrifuge tubes in a sterile environment and resuspended in 1 mL of PBS (pH 7.4). Samples were incubated at 37°C for 30 minutes, vortexed throughout the incubation period. Samples were subjected to centrifugation at 16,000 RCF for 15 minutes and the supernatant was removed and discarded. The pellet was resuspended in 1 mL of 1X MLSB and incubated at 70°C

for 10 minutes. Following incubation, the sample was transferred from the Eppendorf tube into a dismembrator tube with a metal ball (Sartorius Stedim Biotech). The sample was homogenized and broken up at 300 RPM at 1 minute in the Mikro-Dismembrator machine (Sartorius Stedim Biotech). The sample was transferred out of the dismembrator tube and into a 1.5 mL Eppendorf tube. Samples were centrifuged at 16,000 RCF for 15 minutes and the supernatant was removed and saved as the SDS soluble fractions.

The pellets were transferred into 5 mL Eppendorf tubes and resuspended in 1 mL of 1x urea sample buffer (USB, 2% SDS, 67.5 mM Tris, 8 M Urea). Centrifuge stir bars were added and the pellet was left stirring overnight at 4°C in the USB. The stir bar was then removed, the samples were spun down at 16,000 RCF for 15 minutes and the supernatant was removed and saved as urea soluble fractions.

Samples were then subjected to protein precipitation. 100 μL of the SDS soluble fraction and 100 μL of the urea soluble fraction for each sample was combined into a 2 mL microcentrifuge tube. 800 μL methanol (MeOH) was added to the tube, vortexed briefly, and centrifuged for 30 seconds at 9,000 RCF. 600 μL of diH_2O was added to the tube, vortexed briefly, and centrifuged for 90 seconds at 9,000 RCF. The upper phase was removed, leaving the interphase undisturbed. 600 μL MeOH was added, the sample was vortexed and centrifuged for 120 seconds at 9,000 RCF. The precipitated protein pellet was at the bottom and the supernatant was gently aspirated off. The pellet was dried under the vacuum at the back of the hood and the pellet was resuspended in 200 μL of 1X MLSB. The solution was pipetted up and down vigorously, vortexed, and heated at 60°C for 10 minutes to resuspend the pellet. This procedure was performed for each sample.

Protein Quantification: Each sample was quantified following protein isolation using the Pierce™ BCA Protein Assay Kit (Thermofisher Scientific™) according to manufacturer's instructions and the procedure stated above.

SDS-PAGE: 3 μg of protein from each aECM sample was prepared. Pure Human Collagen I (2.5 μg) was loaded as a control. Precasted tris-acetate gradient gels (NuPAGE Tris Acetate Gel, 3-8%, Invitrogen) was utilized. Each sample was prepared with 1x lithium dodecyl sulfate (LDS) sample buffer (pH 8.4, 1x, Invitrogen™ 4x NuPAGE™ LDS Sample Buffer) and 1x sample reducing agent (10X NuPAGE™ Sample Reducing Agent). Total volume was brought up to 20 μL with 1x MLSB. Samples were briefly centrifuged and heated to 100°C for 10 minutes, then centrifuged again prior to loading. Samples were loaded into the gel and run in 1X NuPAGE™ Running Buffer (NuPAGE™ Tris Acetate SDS Running Buffer, 20x, Invitrogen™). 20 μL of the HiMark Pre-stained Protein Standard Ladder (ThermoFisher Scientific) was loaded in lane 1. The gel was run at a constant voltage of 150V for 55 minutes.

Transfer: The gel was transferred in cold 1X NuPAGE transfer buffer (20X, Invitrogen, 0.5% SDS, 10% Methanol) onto a 0.2 μm PVDF membrane (Immuno-Blot PVDF Membrane, Bio-Rad). The transfer was run at 4°C at a constant voltage of 150 V for 90 minutes.

Probing and Washes: All washes and incubations were performed on an orbital shaker plate. Membranes was incubated in blocking solution (5% BSA in 0.05% TBS_{10} $Tween_{20}$) for 1 hour. Following blocking, the membrane was incubated overnight at 4°C in the Anti-collagen I primary antibody (1:2000, Abcam34710). The membrane was subjected to a series of 3 x 10-minute washes

in 0.1% TBS-T. The membrane was incubated for 1 hour at room temperature in the anti-rabbit IgG, HRP-linked secondary antibody (1:2000, Cell Signaling Technologies) in the dark. 2 x 10-minute washes in 0.05% TBS-T was performed, followed by the final 1 x 10-minute wash in 1X TBS.

Imaging: The membrane was developed for 4 minutes in ECL solution (1:1, Clarity Western ECL Substrate, Bio-Rad). The western blot was imaged using chemiluminescence with the colorimetric marker on the Amersham 600 imager (GE Healthcare Life Sciences) using auto exposure.

Quantification: Western blot images were quantified using image J software and normalized to pure Human collagen I. Quantified bands were calculated using Excel and graphs were assembled using Prism software. Prism software analysis was utilized to determine +/- SEM for each data set in addition to statistical significance determined utilizing a one-way ANOVA.

RESULTS

Mass Spectrometry

Mass spectrometry was performed to gain a general insight of proteins found in the aECM powder following lung decellularization, aECM powder following a 72-hour pepsin digestion, and the aECM hydrogels that were subsequently formed. Results indicated a general loss of proteins throughout these steps, particularly during the 72-hour pepsin digestion. Overall protein hits indicated a significant loss of unique matrisome proteins following pepsin digestion from around 250 unique proteins to around 25 unique proteins found in the pepsin digested aECM powder and around 20 unique proteins in the formed hydrogels (**Figure 3A**). Matrisome proteins consists of the ECM-associated proteins. Additionally, analysis revealed that the total protein composition was significantly changed throughout these steps. Hydrogel processing concentrated the total matrisome proteins while removing the other ECM proteins from the mixture. The aECM powder started with a total matrisome percentage under 40%, following pepsin digestion, the total matrisome percentage increased to 60%, which hydrogel formation further increased to over 90% matrisome proteins in the total protein composition (**Figure 3A**). Additionally, a heat map of protein hits was generated, containing the top 25 proteins. As shown in **Figure 3B**, the majority of proteins are lost following pepsin digestion. Collagen I, however, is an exception, in addition to fibrillin I (ECM glycoprotein). Mass spectrometry results indicate a concentration of these proteins following pepsin digestion and hydrogel formation (**Figure 4**). In contrast, proteins including Transforming Growth Factor β (TGFβ, secreted ECM glycoprotein), Periostin (POSTN, ECM glycoprotein), Collagen 6 a6 and a2 subunits are all significantly decreased following pepsin digestion (**Figure 4**). Further analysis indicated the total loss of these proteins in the aECM hydrogels.

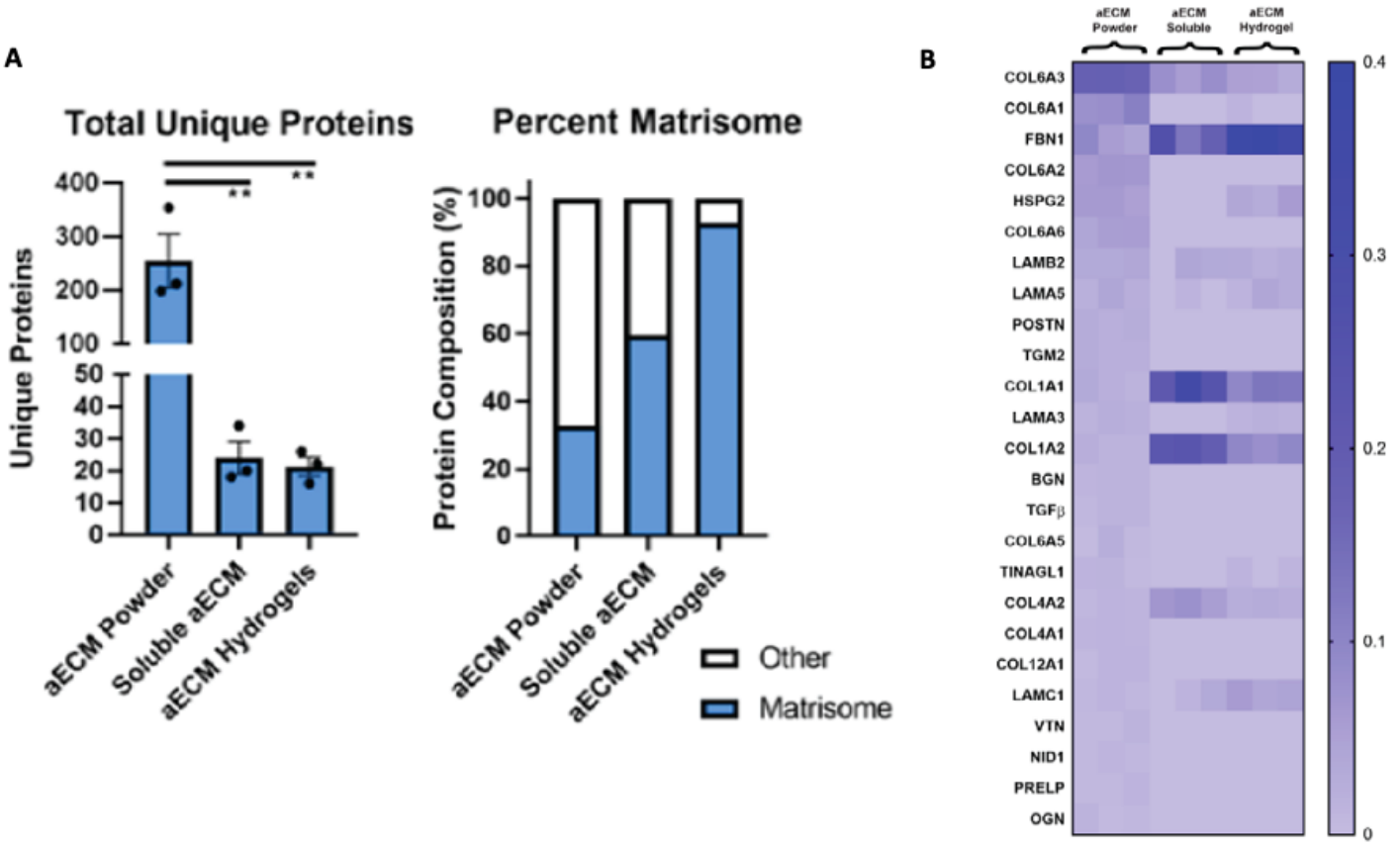

Figure 3 Mass Spectrometry Analysis of Total Protein Composition Pre-Pepsin Digestion, Post-Pepsin Digestion, and After Hydrogel Formation. Proteomic analysis of aECM samples obtained from pre-pepsin digestion aECM powder (n=3), post-pepsin digestion aECM (n=3), and 8 mg/mL hydrogels (n=3). [A] Total unique proteins within each condition. Percent of the total protein composition comprised of matrisome proteins. Error bars represent +/- SEM, n=3 biological replicates. [B] Heatmap of the top 25 proteins across all processing conditions.

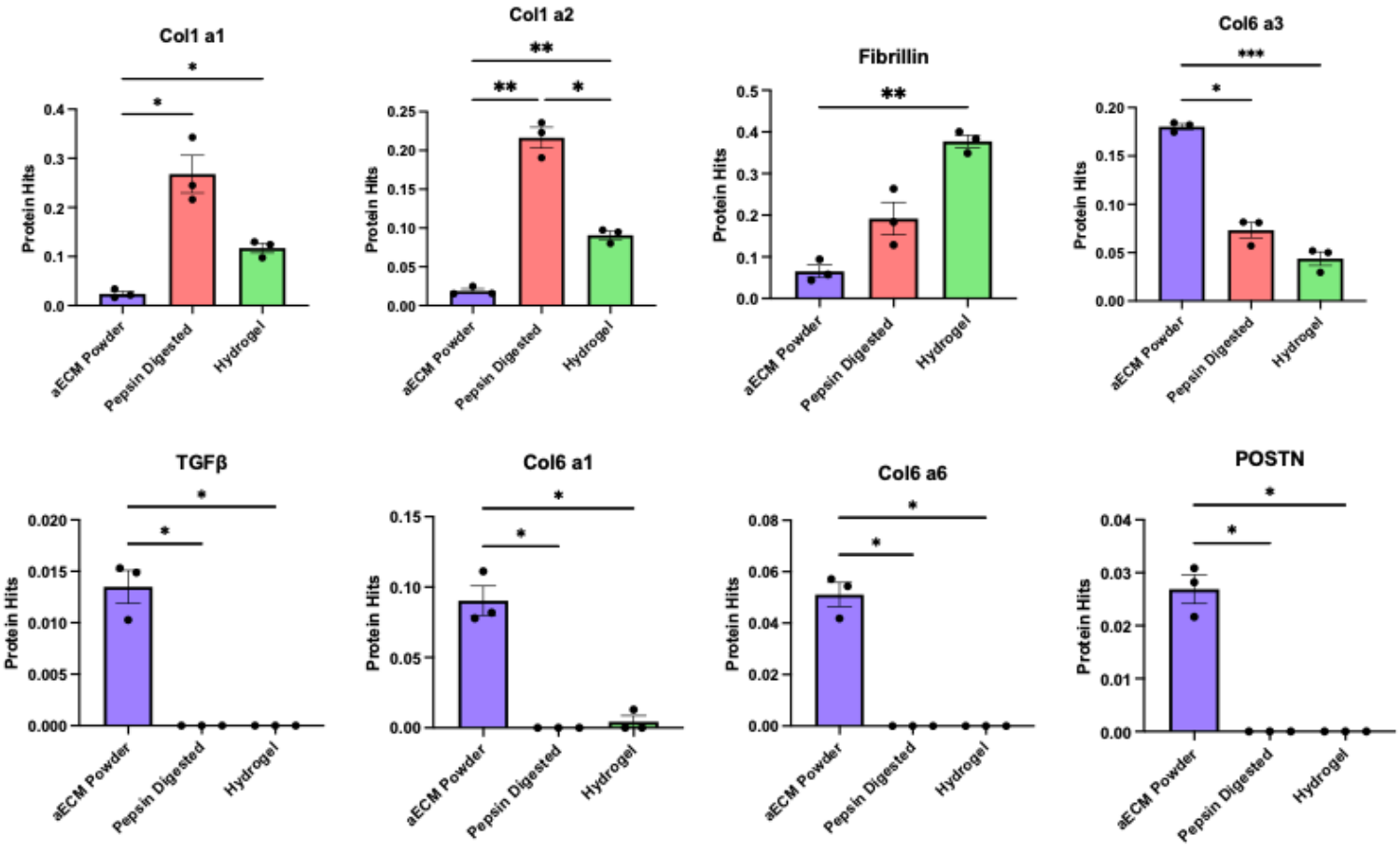

Figure 4 Mass Spectrometry Analysis of Select Important ECM Proteins. Proteomic analysis of aECM samples obtained from pre-pepsin digestion aECM powder (n=3), post-pepsin digestion aECM (n=3), and 8 mg/mL hydrogels (n=3). Error bars represent +/- SEM, n=3 biological replicates.

Silver Staining

Silver staining was used to gain a general insight on the total protein within each sample and where it is relatively distributed according to molecular weight. Silver staining is a qualitative assay which stains the total protein in a gel following SDS-PAGE as the silver ions present in the staining solution binding protein and becoming reduced. As shown in **Figure 5**, the pre-pepsin digested samples for each lung contain the most amount of protein spread across a wide variety of molecular weights, represented by the smearing developed throughout the entire well. A significant decrease in protein was observed following the 72-hour pepsin digestion step and the aECM hydrogel formation. These lanes indicate the presence of multiple distinct bands which have become more concentrated following pepsin digestion and hydrogel formation. Specifically, the band located at a molecular weight of 130 kDa appears to darken, indicating an increase in protein (**Figure 5**). An additional band appears to be concentrated in the aECM hydrogels at a molecular weight of 250 kDa.

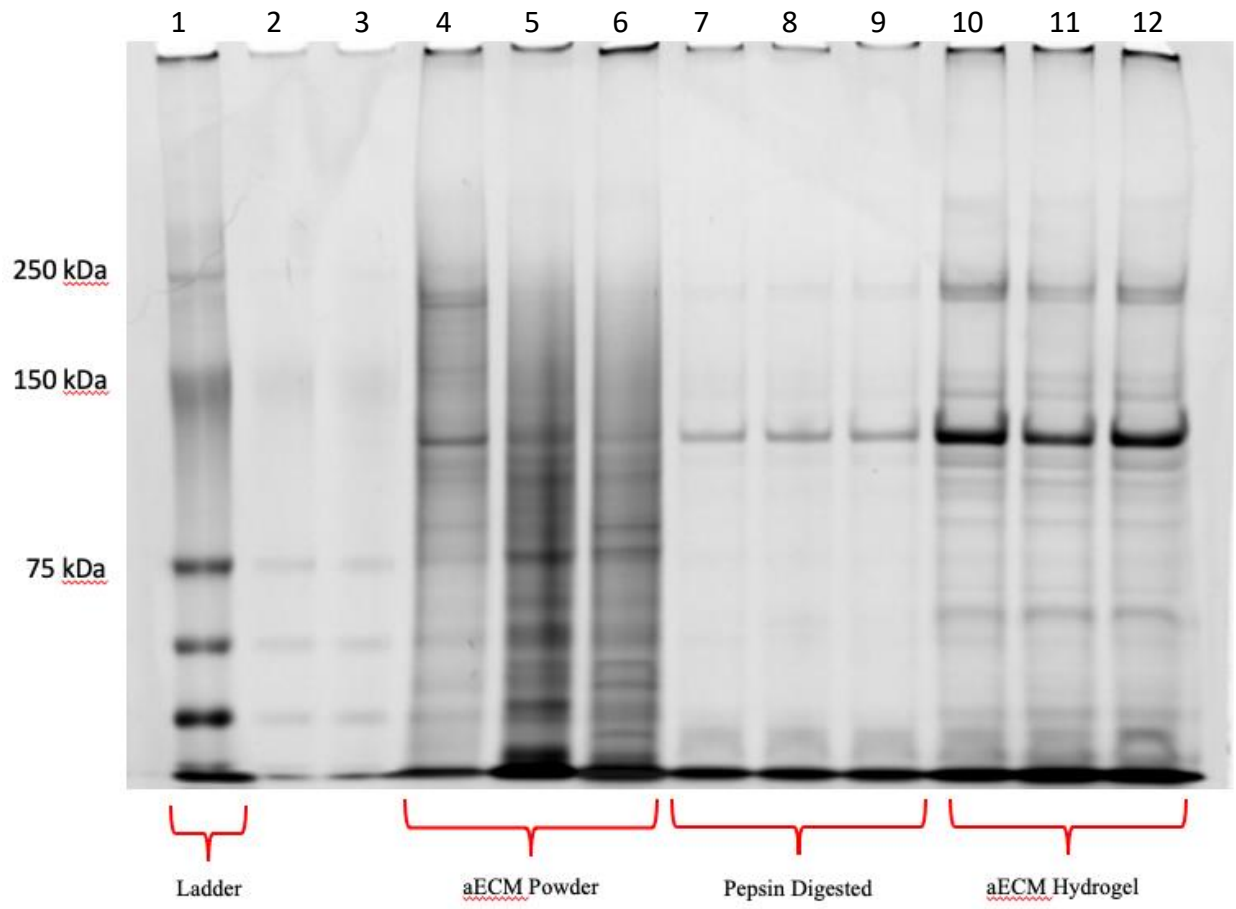

Figure 5 Total Protein Silver Stain of aECM Powder Pre-Pepsin Digestion, After 72-Hour Pepsin Digestion, and After aECM Hydrogel Formation. SDS PAGE was run at a constant voltage of 150V for 55 minutes. Total protein was stained using the Pierce™ Silver Stain Kit (ThermoFisher Scientific). Lane 1: SeeBlue Prestained Protein Standard (10 uL); Lanes 2-3: no samples loaded; Lane 4: H62 aECM powder (2.5 μg); Lane 5: H65 aECM powder (2.5 μg); Lane 6: H66 aECM powder (2.5 μg); Lane 7-9: 72 hour pepsin digested aECM powder (2.5 μg); Lane 10: aECM hydrogel 1 (2.5 μg); Lane 11: aECM hydrogel 2 (2.5 μg); Lane 12: aECM hydrogel 3 (2.5 μg).

Silver Staining was also applied to gels run containing the synthetic matrix, Matrigel and pure human collagen I to directly compare the relative protein amount and distribution to the final aECM hydrogels from H62, H65, and H66. 2.5 μg amount of protein was loaded in each well. As shown in **Figure 6**, the protein distribution is remarkably different between Matrigel and the aECM hydrogels. Matrigel has three large bands of protein, at higher molecular weights. None of these bands line up or correspond to either the aECM hydrogels or pure human collagen I, indicating the presence of different proteins within the hydrogels as a possible result of differing organism origins (i.e., mouse vs human origin). The protein distribution in the aECM hydrogel lanes corresponds to the protein bands found in the pure human collagen I lanes. Specifically, at a molecular weight of 130 kDa, which contains bands for both the aECM hydrogels and collagen I. Additionally, the presence of pure human collagen I points towards the location of one of the main subunits of collagen, identified to be the collagen I alpha (α) subunit. Therefore, the silver-stained gels suggest the presence of the collagen I α subunit being concentrated in the aECM hydrogels.

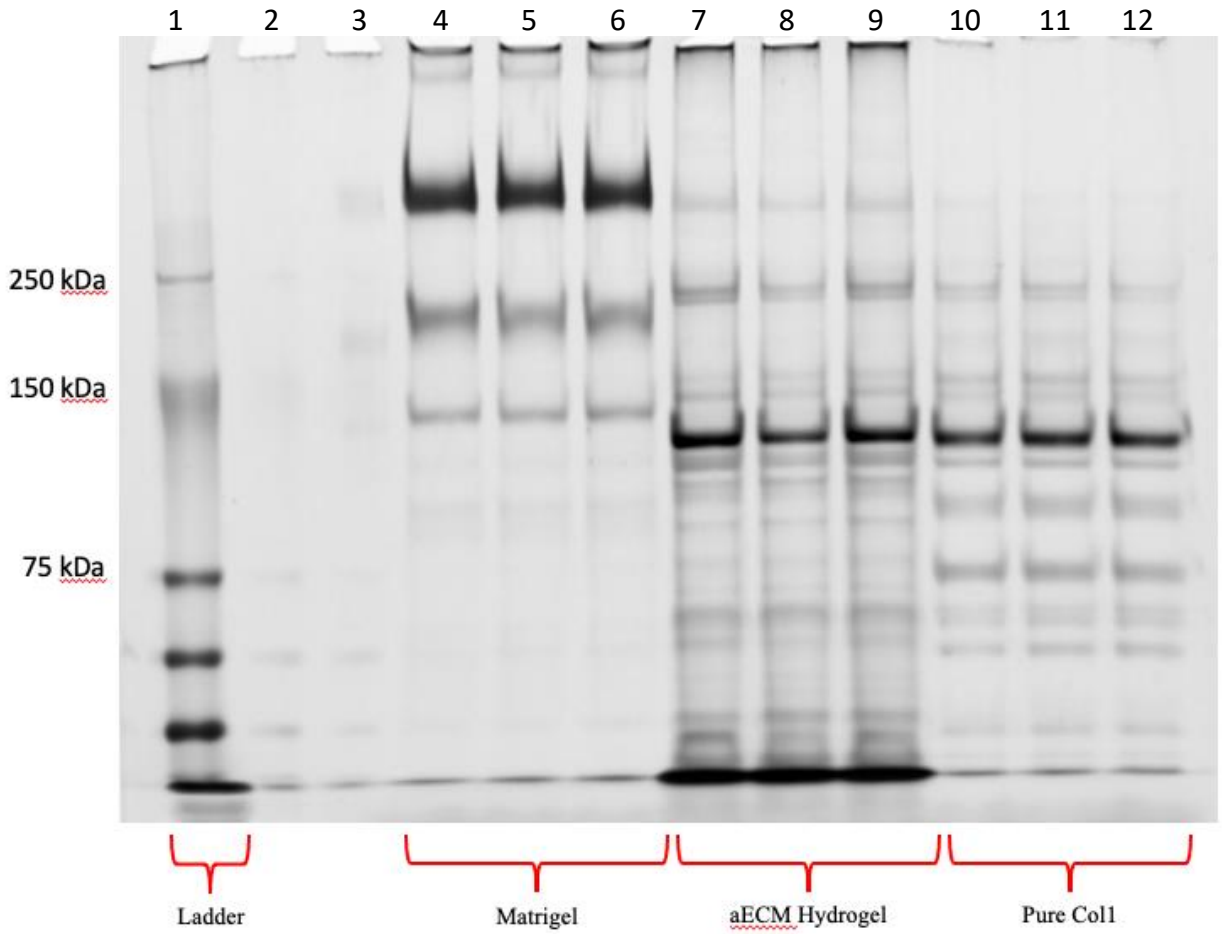

Figure 6 Total Protein Silver Stain of Matrigel, aECM Hydrogel, and Pure Collagen I. SDS PAGE was run at a constant voltage of 150V for 55 minutes. Total protein was stained using the Pierce™ Silver Stain Kit (ThermoFisher Scientific). Lane 1: SeeBlue Prestained Protein Standard (10 uL); Lanes 2-3: no samples loaded; Lanes 4-6: Matrigel (2.5 μg); Lanes 7-9: 8 mg/mL aECM Hydrogel (2.5 μg); Lanes 10-12: Pure Human Collagen I (2.5 μg).

Western Blots

Western Blots were utilized to investigate the effect of pepsin digestion on the aECM powder and resulting hydrogel protein composition by probing for specific proteins. Time only permitted for the probing of samples for Collagen I. 3 μg of protein from each sample was loaded and run on an SDS-PAGE gel, denaturing the proteins. **Figures 7A-C** illustrates three technical replicates. When the membrane was probed for collagen I, many bands were visualized. In lanes 1-3, containing the aECM powder, there is some smearing of collagen I on the sides of the well around 460 kDa. Additionally, faint bands are visualized at 55 kDa and 41 kDa. Lanes 4-6, containing the pepsin digested aECM powder, forms distinct bands around 460 kDa and 130 kDa in addition to faint bands below at 55 kDa and 41 kDa. Lanes 7-9, containing the 8 mg/mL aECM hydrogel, show no bands except for faint signal at 55 kDa. Pure human collagen I was run in lane 10, showing bands with strong signal at 200 kDa, 130 kDa, 117 kDa, and 55 kDa. While this appears counterintuitive for pure collagen I, previous research has identified these bands to represent specific subunits of collagen I.[10]

A separate western blot was performed utilizing solely the aECM hydrogels to visualize the location of the bands present in the sample (**Figure 7D**). Lanes 1-3 represent aECM hydrogel 1 in triplicate. Lanes 4-6 represent aECM hydrogel 2 in triplicate. Lanes 7-9 represent aECM hydrogel 3 in triplicate. The last lane is pure collagen I. Results indicate strong signal across all hydrogels around 460 kDa, faint bands around 260 kDa, and distinct bands at 130 kDa.

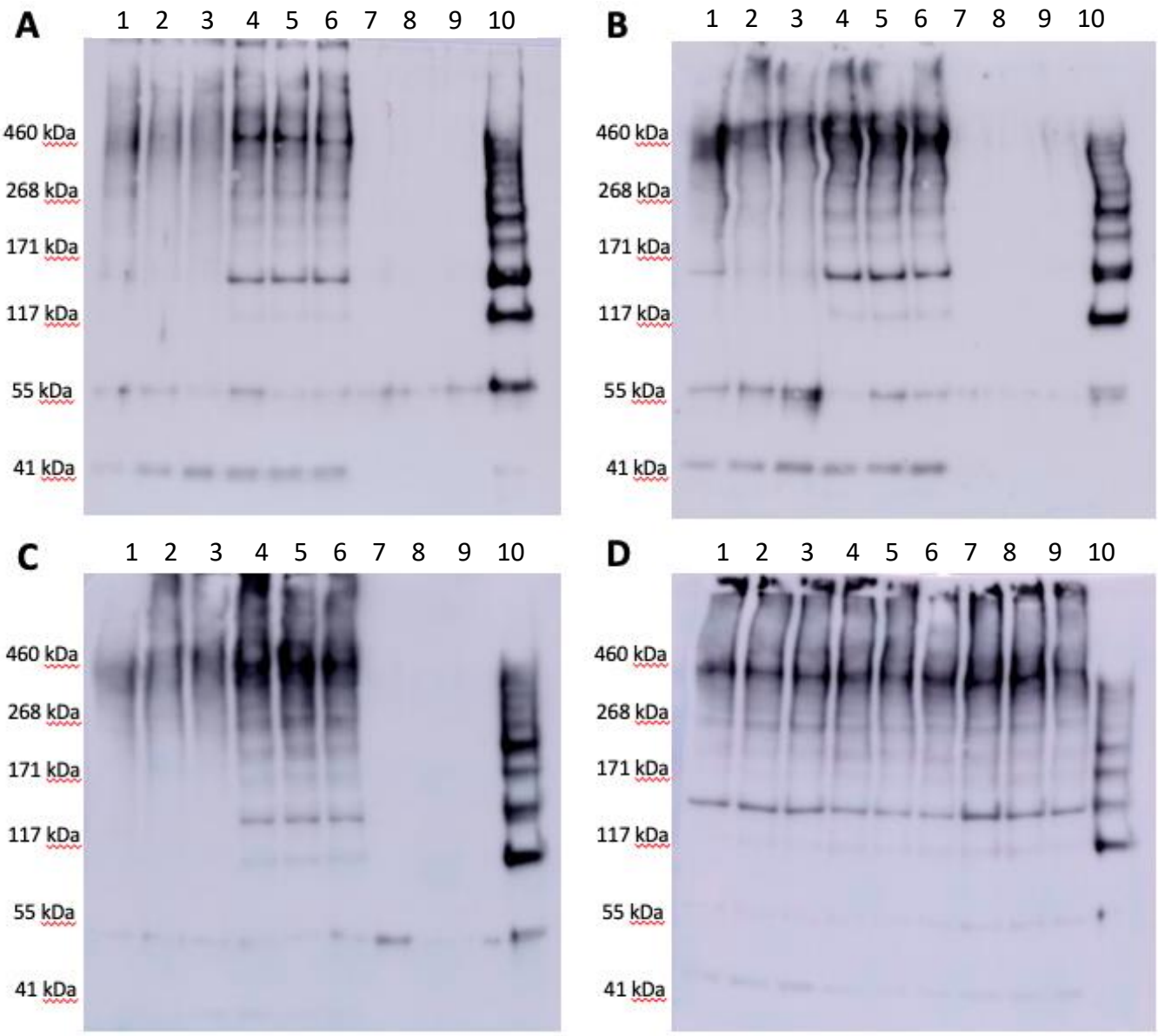

Figure 7 Western Blots Probed for Collagen I. SDS-PAGE was run at a constant voltage of 150V for 55 minutes. Transfer to PVDF membrane occurred at 4°C at a constant voltage of 150V for 90 minutes. Primary antibody utilized was anti-Collagen I (1:2000, Abcam 34710). Secondary antibody utilized was anti-rabbit IgG, HRP-linked (1:2000, Cell Signaling Technologies). [A-C] Technical replicates of pre-digested aECM powder, post-digested aECM powder, and aECM hydrogels. All samples loaded at a protein amount of 3 μg. Lane 1: H62, aECM powder; Lane 2: H65, aECM powder (pre-digestion); Lane 3: H66, aECM powder (pre-digestion); Lane 4: 72-hour pepsin digested aECM powder 1; Lane 5: 72-hour pepsin digested aECM powder 2; Lane 6: 72-hour pepsin digested aECM powder; Lane 7: aECM hydrogel 1; Lane 8: aECM hydrogel 2; Lane 9: aECM hydrogel 3; Lane 10: pure human collagen I. [D] Western blot of aECM hydrogels 1-3 in technical replicates. 40 μg of protein loaded. Lanes 1-3: aECM hydrogel 1; Lanes 4-6: aECM hydrogel 2; Lanes 7-9: aECM hydrogel 3; Lane 10: pure human collagen I.

Quantification of the gamma, beta, alpha1, and alpha2 subunit bands from the triplicate western blots were normalized to collagen. **Figure 8A** represents the gamma subunit around 460 kDa. Results indicate a relative doubling of the gamma subunit following pepsin digestion in comparison to the aECM powder. Very little signal in the aECM hydrogel lanes corresponded to a low quantified value. The beta subunit (~260 kDa) is represented in **Figure 8B**, exhibiting a similar phenomenon. The quantified value across all aECM powder is significantly lower than the value following pepsin digestion. In **Figure 8C**, the alpha1 subunit (~130 kDa) is visualized, which also demonstrates the same pattern to a greater extent. The relative amount of the alpha1 subunit significantly increases following pepsin digestion in comparison to the aECM powder. **Figure 8D** represents the alpha2 subunit (~115 kDa), which shows very little protein present in any sample. However, the largest amount still corresponds to the samples which were pepsin digested.

The aECM hydrogel western blot was quantified and normalized to collagen to visualize the distribution of subunits within the hydrogel (**Figure 9**). The gamma subunit was the most prominent across all aECM hydrogels, while the beta and alpha1 subunits were relatively similar. The alpha2 subunit was barely present. Collagen I subunits were identified based on molecular weight from previous research findings (**Table 1**).[21]

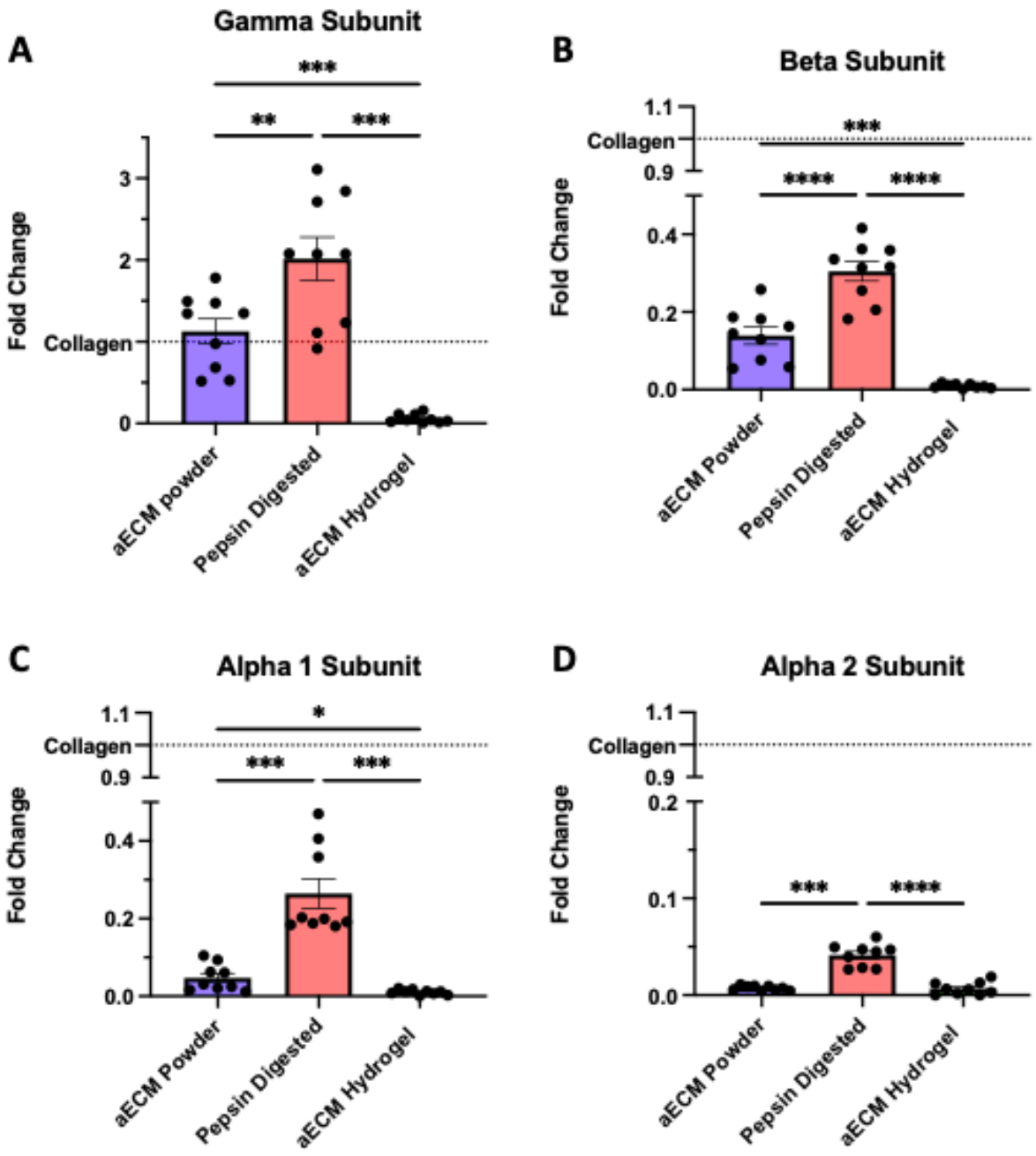

Figure 8 Collagen I Western Blot Quantification According to Subunit. Quantification was performed with ImageJ software. All values are normalized to collagen I. Error bars represent +/- SEM, n=9 technical replicates. [A] Quantification of the collagen I gamma subunit across conditions. [B] Quantification of the collagen I beta subunit across conditions. [C] Quantification of the collagen I alpha1 subunit across conditions. [D] Quantification of the collagen I alpha2 subunit across conditions.

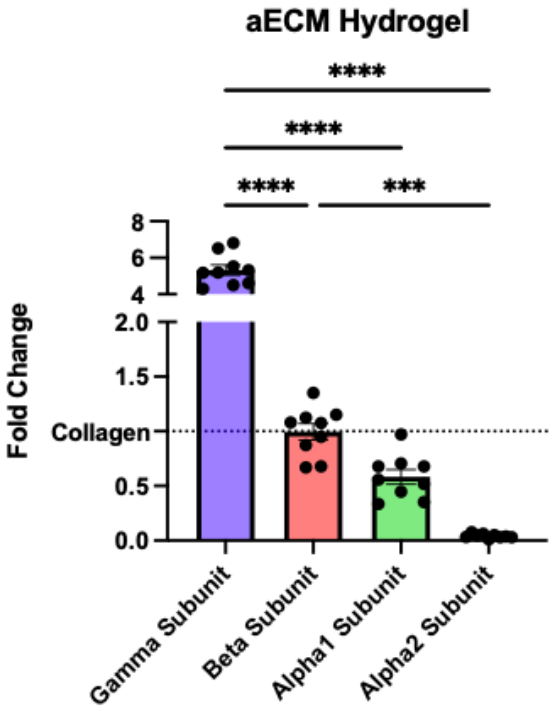

Figure 9 Collagen I Western Blot Quantification of aECM Hydrogels According to Subunit. Quantification was performed using ImageJ software. All values are normalized to collagen I. Error bars represent +/- SEM, n=3 technical replicates.

Table 1 Collagen I Subunits and Physical Properties.

Subunit	Composition	Molecular Weight (kDa)
gamma (γ)	3 α subunits	~460
beta (β)	2 α subunits	~260
alpha1 (α1)	1 α1 subunit	~140
alpha2 (α2)	1 α2 subunit	~115

DISCUSSION

With pulmonary disease on the rise, research has increasingly incorporated new methods, such as tissue specific hydrogels, to study the mechanisms of disease. Additionally, the use of hydrogels has permitted the growth of pluripotent induced stem cells, such as iAT2s, which have immense potential for cellular therapies. However, a comprehensive understanding of the total protein composition within these aECM hydrogels is necessary for understanding cellular growth, proliferation, and behavior.

Specifically, previous research in many organ systems, not limited to the lungs, has utilized the synthetic matrix, Matrigel. Organoids, which are 3D tissue systems produced by stem cells which closely recapitulate the tissue environment of interest, have been studied in both Matrigel and corresponding dECM matrices. Of particular interest, cellular growth, differentiation, and gene expression varied dramatically between the organoids grown in Matrigel and dECM hydrogels.[14] The differences in growth and behavioral patterns observed, most likely stems from the differing protein composition within these 3D matrices, as the cellular environments heavily impacts the behavior of cells. Recent research has identified significant differences between the protein composition of Matrigel and porcine brain tissue.[14] Here, we utilized silver staining to gain a relative understanding of protein composition in Matrigel in comparison to the aECM hydrogels. Silver staining revealed relative protein composition according to band molecular weights. One major finding suggests a significant difference in protein band molecular weights between the aECM hydrogels and Matrigel. Particularly, none of the three major protein bands concentrated in Matrigel correspond to the major protein bands which are concentrated in the aECM hydrogels or the pure human collagen I.

These results suggest a significantly different protein composition within Matrigel than what is found in aECM hydrogels. Specifically, the ECM plays an essential role in cellular behavior due to the vast array of proteins and secreted factors which can interact with and influence the cell physiology and behavior. Therefore, it is suggested that the observed differences in behavioral patterns observed in iAT2s grown in Matrigel and aECM hydrogels in the Weiss Lab are correlated with the difference in protein composition between the two 3D matrices.

In addition to the substantial protein differences found between Matrigel and the aECM hydrogels, there was also a significant difference in the protein composition found in the aECM powder, the pepsin digested aECM powder, and the formed aECM hydrogels. Silver staining revealed that the aECM powder, prior to pre-pepsin digestion, has a wide array of proteins with varying molecular weights. However, following pepsin digestion, it becomes apparent that the majority of these different proteins are removed as a result of the fragmentation of proteins which occurs during this 72-hour digestion. This is further apparent in the formed aECM hydrogel, which displays a few distinct bands of protein with increased saturation, indicating that some proteins are concentrated during the process while the majority of other proteins are lost. While comparing the total protein in the aECM hydrogels to the pure human collagen I, it becomes apparent that collagen I is a protein being concentrated in these aECM hydrogels.

The mass spectrometry results further solidified the observation in the silver staining data. Overall, the total protein composition found in the aECM powder before pepsin digestion is found to significantly change following pepsin digestion. The total decrease in 225 unique proteins was found to occur during the 72-hour pepsin digestion. Additionally, it was seen that during the hydrogel processing steps, the matrisome proteins become a greater percentage of the total protein composition. Matrisome proteins include ECM-specific proteins such as collagens, glycoproteins,

and proteoglycans, all of which play an important role in structure. Specifically, an increase of approximately 60% was observed. However, this causes a reciprocal decrease in other proteins found in the aECM hydrogels in comparison to the aECM powder. This observation is represented in the heatmap in **Figure 2**, which shows the top 25 proteins decreasing throughout the hydrogel processing steps. However, results also point towards a few proteins being enriched and concentrated over this process as well. This includes proteins such as fibrillin, collagen I alpha1, collagen 1 alpha2, and LAMC1. In contrast, we observed a significant decrease in the majority of the top 25 proteins during the hydrogel processing steps. This includes the decrease in collagen 6 subunits, TGFβ, POSTN, BGN, VTN, and many more, all of which were observed to be barely present, if at all, in the final hydrogel. Indications point towards the loss of these proteins during the pepsin digestion step.

Western blot analysis provided insight on the collagen I subunits present within each step in the hydrogel processing: aECM powder pre-pepsin digestion, aECM post-pepsin digestion, and the final hydrogel. This reveals the same concentrating phenomenon seen both in the mass spectrometry results and in the silver staining results. The aECM powder has significantly less collagen alpha1 and gamma subunits in comparison to the pepsin digested aECM. However, one unexpected finding in the western blot data was the absence of any bands in the aECM hydrogel samples across all technical replicates. Potential explanations include a lack of protein present in the samples or low protein concentrations utilized. When run on a separate western blot with a greater amount of total protein loaded (40 μg), the bands were highly visible and comparable across all samples and technical replicates. While this would need to be further optimized, the bands are in the locations expected, specifically the gamma subunit and alpha1 subunit. One other phenomenon observed on the collagen I western blots is the bands around 55 kDa and 41 kDa,

neither of which are subunits of collagen I. One explanation of these bands includes degraded collagen subunits, which has been observed in other studies.[22] Of particular interest, we observed a significant decrease in these two degraded collagen bands in the aECM hydrogels, which may indicate the removal of these degraded proteins during the pepsin digestion and hydrogel formation.

Overall, Collagen I is an important ECM protein which works to maintain structure and support of the environment. Specifically, the monomeric collagen I usually consists of three alpha strands connected by intra-strand cross-linkages. Further inter-strand cross-linkages leads to the production of polymeric collagen which is the phenomenon which drives hydrogel formation. This is apparent in the western blot results, where the gamma subunit (3 alpha subunits) is the most prominent subunit in the aECM hydrogels.

Probing for other proteins with western blotting was planned for the completion of this project. This includes proteins such as TGFβ and fibrillin-1. However, a large amount of time was spent optimizing the western blot assay. ECM proteins are large and insoluble by nature, and therefore, difficulty separating proteins during SDS-PAGE was encountered. The first western blot trials were performed with samples isolated only with SDS and a 30-minute incubation at 45°C. During preparation for the SDS-PAGE, the samples were heated to 70°C for 10 minutes and cooled on ice prior to electrophoresis. This led to the clumping of the sample at the top of the well, and no protein migration down the gel. Ultimately, this problem was solved by a harsher protein isolation in which both SDS and urea were utilized to solubilize the ECM proteins. Additionally, a chloroform phase separation was required to remove the soluble fractions. Therefore, the insoluble lipids and other contaminating, larger species were removed which facilitated the migration of proteins down the gel during electrophoresis. More optimization was necessary

following the re-isolation technique in order to further facilitate the migration of the proteins and resolve the smearing of protein down the side of the wells. This was resolved by an optimization of both the amount of protein loaded into each well and the temperature at which the samples were denatured at prior to electrophoresis. Trouble shooting required multiple western blots performed with a titration of protein amount and the heating temperature of either 70°C or 100°C. This revealed that 3 μg protein loaded in each well and a heating temperature of 100°C for 10 minutes was optimal for protein migration and signal acquisition. However, the aECM hydrogel samples were not present on the western blots when loaded at 3 μg of protein. Due to a lack of time, the only option was to run these samples at a larger protein amount, 40 μg, rather than entirely forming new hydrogels. While this was not optimal, it allowed for the observation of the collagen I subunits present in the aECM hydrogels. However, further analysis would be necessary utilizing newly formed hydrogels to determine whether this was a technical error or the protein present in the hydrogels is truly this low.

CONCLUSIONS AND FUTURE DIRECTIONS

Overall, results suggest the significant loss of many ECM proteins following pepsin digestion. Furthermore, this suggests that the resulting aECM hydrogel does not contain the majority of the ECM proteins which are found in the aECM powder, with the exception of a few proteins, including collagen I and fibrillin I. This has a significant impact on future cell studies, specifically iAT2 studies. Knowledge to date has suggested that decellularization results in the loss of GAG proteins, however the field has taken to assume that the protein composition in the aECM powder directly transfers to the protein composition in the aECM hydrogel.[9] The result of this study suggests otherwise and therefore, more studies will be necessary in the future to confirm this observation. Future directions include examining the effects of hydrogel supplementation with proteins lost during the pepsin digestion and hydrogel processing on cell physiology. Since pepsin digestion is required for the formation of hydrogels, supplementation can be utilized to optimize the hydrogels to closely recapitulate the tissue environment, which has significant potential to positively influence the field of 3D cell culture and tissue engineering.

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