11-27-2002

Characterization of engineered human lung tissue

Benjamin Boytor

Florida International University

DOI: 10.25148/etd.FI14051818

Follow this and additional works at: <https://digitalcommons.fiu.edu/etd>

Part of the [Biomedical Engineering and Bioengineering Commons](#)

Recommended Citation

Boytor, Benjamin, "Characterization of engineered human lung tissue" (2002). *FIU Electronic Theses and Dissertations*. 1783.
<https://digitalcommons.fiu.edu/etd/1783>

This work is brought to you for free and open access by the University Graduate School at FIU Digital Commons. It has been accepted for inclusion in FIU Electronic Theses and Dissertations by an authorized administrator of FIU Digital Commons. For more information, please contact dcc@fiu.edu.

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

CHARACTERIZATION OF ENGINEERED HUMAN LUNG TISSUE

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL ENGINEERING

by

Benjamin Boytor

2002

To: Dean Vish Prasad
College of Engineering

This thesis, written by Benjamin Boytor, and entitled Characterization of Engineered Human Lung Tissue, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

Richard T. Schoephoerster

James E. Moore

Eric T. Crumpler, Major Professor

Date of Defense: November 27, 2002

The thesis of Benjamin Boytor is approved.

Dean Vish Prasad
College of Engineering

Dean Douglas Wartzok
University Graduate School

Florida International University, 2002

© Copyright 2002 by Benjamin Boytor

All rights reserved.

ACKNOWLEDGMENTS

This research would not have been possible if not for the support of numerous individuals. I would first like to thank Dr. Eric T. Crumpler for the advice, patience, and guidance that he exhibited through the duration of this project. I would also like to thank Martha Vallejo, Ronald Gutierrez, and Brijesh Kadam for their collaboration on a number of experiments. Further, I would like to thank Jon DeDiego, Amit Datye, and FCAEM for their help with the SEM imagery, Andres Aguirre for help with tensile analysis, and Rosy Verona, Karen Zapata, and Mike Anzelmo for their administrative help in expediting my many requests.

This research was funded by a grant from the Whitaker foundation.

ABSTRACT OF THE THESIS

CHARACTERIZATION OF ENGINEERED HUMAN LUNG TISSUE

by

Benjamin Boytor

Florida International University, 2002

Miami, Florida

Professor Eric T. Crumpler, Major Professor

Characterizing engineered human lung tissue is an important step in developing a functional tissue replacement for lung tissue repair and *in vitro* analysis. Small tissue constructs were grown by seeding IMR-90 fetal lung fibroblasts and adult microvascular endothelial cells onto a Polyglycolic acid (PGA) polymer template. Introducing the constructs to dynamic culture conditions inside a bioreactor facilitated three-dimensional growth seen in scanning electron microscopy images (SEM).

Characterization of the resultant tissue samples was done using SEM imagery, tensile tests, and biochemical assays to quantify extra-cellular matrix (ECM) composition.

Tensile tests of the engineered samples indicated an increase in the mechanical properties when compared with blank constructs. Elastin and collagen content was found to average 3.19% and 15.49% respectively in relation to total mass of the tissue samples. The presence of elastin and collagen within the constructs most likely explains the mechanical differences that we noted.

These findings suggest that the necessary ECM can be established in engineered tissue constructs and that optimization of this procedure has the capacity to generate the load bearing elements required for construction of a functional lung tissue equivalent.

TABLE OF CONTENTS

CHAPTER	PAGE
1. STATEMENT OF PROBLEM.....	1
2. INTRODUCTION.....	3
3. SIGNIFICANCE AND BACKGROUND.....	6
4. METHODOLOGY AND MATERIALS.....	15
4.1 Cell Seeding of Polymer Scaffolds.....	15
4.2 Tensile Test Protocol.....	18
4.3 SEM Imagery.....	18
4.4 Biochemical Analysis.....	18
4.5 Materials.....	19
4.5.1 Human Microvascular Endothelial Lung Cells (HMVELC).....	19
4.5.2 EGM-2 Growth Media for HMVELC.....	19
4.5.3 Human Fetal Lung Fibroblasts (IMR-90).....	19
4.5.4 EMEM Supplemented Growth Media for IMR-90 cells.....	20
4.5.5 Bioreactors.....	20
4.5.6 PGA Polymer Scaffolding.....	20
4.5.7 Analytical Assays.....	20
5. RESULTS.....	22
6. DISCUSSION.....	42
7. CONCLUSION.....	47
REFERENCES.....	49
APPENDICES.....	52

LIST OF FIGURES

FIGURE	PAGE
1.1 SEM of 3-day blank polymer.....	22
1.2 SEM of 7-day blank polymer.....	23
1.3 SEM of 7-day blank polymer.....	23
2.1 Elastic modulus comparison of blank scaffolds.....	24
2.2 Yield Stress Comparison of blank scaffolds.....	25
3.1 SEM of endothelial construct at 3-hours.....	26
3.2 SEM of endothelial construct at 3-days.....	27
3.3 SEM of endothelial construct at 3-days.....	27
3.4 SEM of endothelial construct at 3-days.....	28
3.5 SEM of endothelial construct at 7-days.....	28
3.6 SEM of endothelial construct at 10-days.....	29
4.1 Collagen standard for endothelial constructs.....	30
4.2 Elastin standard for endothelial constructs.....	30
5.1 SEM of fibroblast construct at 1-day.....	31
5.2 SEM of fibroblast construct at 1-day.....	31
5.3 SEM of fibroblast construct at 10-days.....	32
5.4 SEM of fibroblast construct at 10-days.....	33
5.5 SEM of fibroblast construct at 10-days.....	33
6.1 Elastic modulus of IMR-90 samples.....	34
6.2 Yield stress of IMR-90 samples.....	34

6.3 Yield strain of IMR-90 samples.....	35
6.4 Elastic modulus comparison.....	36
6.5 Yield stress comparison.....	36
6.6 Yield strain comparison.....	37
7.1 Elastin standard for IMR-90 constructs.....	38
7.2 Collagen standard for IMR-90 constructs.....	38
7.3 Elastin and collagen content of batch 1.....	40
7.4 Elastin and collagen content of batch 2.....	40
7.5 Average elastin and collagen content.....	41

LIST OF ACRONYMS

PGA	Poly-glycolic acid
SEM	Scanning electron microscopy
ECM	Extra-cellular matrix
COPD	Chronic obstructive pulmonary disease
LVRS	Lung volume reduction surgery
3D	Three dimensional
EGF	Epidermal growth factor
TGF- α	Transforming growth factor alpha
AR	Amphiregulin
IGFs	Insulin-like growth factors
FGFs	Fibroblast growth factors
HGF	Hepatocyte growth factor
PDGF	Platelet-derived growth factor
TGF- β	Transforming growth factor beta
GAGs	Glycosaminoglycans
pO ₂	Partial oxygen
pCO ₂	Partial carbon dioxide
HARVs	High aspect ratio vessels
DNA	Deoxyribonucleic acid
HMVELC	Human micro-vascular endothelial lung cells
RCCS	Rotary cell culture system
EGM-2 MV	Endothelial cell basal medium-2
ATCC	American type culture collection
EMEM	Eagle's minimal essential medium w/ Earle's BSS and 2mM L-glutamine
NEAA	Non-essential amino acids
FBS	Fetal bovine serum

1. STATEMENT OF PROBLEM

This project approached the problem of mimicking the load-bearing matrices associated with the gas-exchange barrier of the lung. The difficulty was in the fact that this barrier is composed of numerous cell types with differing functions. Initially one cell type was chosen, though future attempts may include co-cultures or other cell types.

Two aspects were observed to be most important in addressing functionality: ventilation and perfusion. Ventilation includes the expansion of the lung during inspiration. In order for proper ventilation to occur, the tissue must exhibit the ability to be elastic. Perfusion is the exchange of gases, particularly oxygen and carbon dioxide, between the air and blood separated by the gas-exchange barrier. Perfusion requires vascularization of the tissue for this process to occur.

Cell types that are included in the gas-exchange barrier were chosen for this study. Endothelial cells and fibroblasts were studied because each of these cells is thought to provide the necessary elements for elasticity and vascularization. Endothelial cells provide the layer separating the capillaries from the alveolar walls and may provide the necessary interface for angiogenesis. Fibroblasts, on the other hand, are found within these walls and account for a majority of the elastic framework. Both cell types were characterized for their contributions in re-creating the gas-exchange barrier.

Experimental protocols followed an iterative process. Ultimately, we described the tissue constructs both morphologically and mechanically. This initial

characterization lead to further optimization of the tissue construct toward the end result of a functional tissue equivalent.

2. INTRODUCTION

For the last decade, attention has been focused on the possibility of engineering cell-derived functional replacements for numerous tissues within the body. The majority of this tissue engineering research has focused on recreating cartilage, bone, skin, and cardiac tissue equivalents. Because of early successes within the field, research has come to include many other types of tissues. This study has expanded on these early works and incorporated established techniques to engineer lung tissue constructs, which were then evaluated for mechanical and morphological properties associated with them.

By creating functional lung tissue equivalents, it can be possible to provide treatments, through replacement patches, for patients inflicted with emphysema, alpha-1 antitrypsin deficiency, or numerous other lung diseases in which there is an associated loss of lung tissue functionality. Currently there are over 16 million patients inflicted with chronic obstructive pulmonary disease (COPD) and this disease is the fourth leading cause of death worldwide (National Heart, Lung, and Blood Institute). In the cases of emphysema and chronic bronchitis, the walls between the alveoli are damaged and a loss of lung tissue elasticity occurs. There are no known cures for these types of diseases and treatment is only supportive. One current treatment is lung volume reduction surgery (LVRS), which involves the removal of a diseased region of lung tissue (National Heart, Lung, and Blood Institute). Removal of the inflicted region within the lung only satisfies eliminating the diseased area and not restoring lung functionality. The estimated direct and indirect costs for this class of diseases reported for 2000 totaled nearly \$30.4 billion

(National Heart, Lung, and Blood Institute). Nine billion dollars accounted for hospital costs and another \$5.7 billion accounted for other direct costs (National Heart, Lung, and Blood). Morbidity costs for 2000 were \$6.5 billion and mortality costs were \$9.2 billion (National Heart, Lung, and Blood Institute). Functional replacements would not only eliminate the need for LVRS, but could restore full or partial functionality to the inflicted tissue.

Engineered tissue replacements must be capable of load bearing during ventilation and perfusion of oxygen and carbon dioxide between air and blood. This requires the tissue to have two important components: A functional respiratory membrane and a functional extra-cellular matrix (ECM).

Recreating the respiratory membrane involved choosing cell types that were representative of those found within the membrane layers. In addition, cell types that were used offered contributions toward ECM production and tissue vascularization.

ECM recreation is the most important factor in reproducing the mechanical characteristics of native lung tissue (Yuan, et al, 2000). The individual ECM components are responsible for the majority of load bearing and elastic recoil; therefore, percentages of these ECM components should correspond to that of native tissue.

We identified two cell types that should be useful in this study, human fetal lung fibroblasts and human microvascular endothelial lung cells. Fetal lung fibroblasts were studied because these cells are found within the interstitial layer of the respiratory membrane. They also offer numerous population doublings and

have been shown to secrete proteins needed for ECM production. Human microvascular lung endothelial cells were also studied because of their role in the vasculature of native lung tissue. These cells line the lumen of the lung capillaries and compose the endothelial layer of the respiratory membrane.

Templates are needed to facilitate cell adhesion and tissue organization. PGA polymer scaffolding has been proven to provide the desired results for generating different types of 3D tissues. We used these scaffolds to induce cell proliferation and ECM production under static growth conditions. Following cell seeding and attachment, dynamic growth conditions were employed to facilitate 3D growth.

Following the development of these tissue constructs, it was important to establish the mechanical and morphological characteristics associated with them. Characterizations included SEM imagery, tensile tests, and biochemical analysis of the ECM.

3. SIGNIFICANCE AND BACKGROUND OF THE STUDY

The respiratory system provides a protected interface for diffusion of nutrients between air and blood. Diffusion occurs within the alveoli, or air-filled pockets that fill the lungs. These exchange sites are connected to the interstitial fluids through the cardiovascular system, which transports oxygen to peripheral tissues and carbon dioxide to the lungs. The total surface area of the alveoli, estimated to be 35 times greater than the surface area of the body, accommodates the peripheral tissues nutrient requirements.

The human body consists of a right and left lung. The lungs are made up of individual lobes separated by deep fissures, the right having three lobes, and the left having two. The bronchial tree distends into the lung structure and branches further into each individual lobe and still further to the terminal bronchioles(Starcher, 2000). Terminal bronchioles give way to pulmonary lobules that contain the alveoli. Microstructure of the alveoli consists of capillaries surrounded by a network of elastic fibers. These elastic fibers are responsible for holding alveolar shape and reducing the lumen size during exhalation. The alveolar epithelium is composed of squamous epithelial cells or *Type I cells* and septal cells or *Type II cells* which are responsible for surfactant production that reduces the surface tension within the alveoli (Starcher, 2000). Gas exchange occurs at this level across the respiratory membrane containing a squamous epithelial cell lining, endothelial cells lining the lumen of the adjacent capillary, and a fused basement membrane that lies between the two. The entire respiratory membrane has an average thickness of 0.5 μ m.

There are two structural factors that are fundamentally important to successful gas-exchange within the lung: the gas exchange surface area and the thickness of the air-blood barrier in the pulmonary parenchyma. These factors are dependent upon the dimensions and morphology of the lung capillary network, its total volume, the density of its meshes, and its 3D structure.

The gas-exchange region of the lung is composed of three main compartments: air, blood, and tissue (Starcher, 2000). The tissue provides the framework for the two dynamic and continuously moving compartments of blood and air. It also keeps the blood and air compartments separate, but allows for intimate contact to ensure rapid uptake of oxygen and release of carbon dioxide. The tissue compartment, also known as the septal interstitium, is comprised of two different phenotypes of myofibroblasts.

Lung morphogenesis depends on the interaction between two tissue layers, epithelium and mesenchyme. More specifically, the characteristic branching morphogenesis of the bronchial epithelial buds depends on interaction with bronchial mesenchyme (Keyzer and Post, 1999). In conjunction with branching, numerous different cell phenotypes are formed along the anterior-posterior axis of the epithelial tubules and mesenchyme (Keyzer and Post, 1999). Each of these cell types exhibits different morphologies and patterns of gene expression (Keyzer and Post, 1999).

There are numerous growth factors that act as inductive signals mediating tissue interactions and regulating branching morphogenesis including: Epidermal

growth factor (EGF), Transforming growth factor alpha (TGF- α), Amphiregulin (AR), Insulin-like growth factors (IGFs), Fibroblast growth factors (FGFs), Hepatocyte growth factor (HGF), Platelet-derived growth factor (PDGF), and Transforming growth factor beta (TGF- β).

The EGF, TGF- α , and AR proteins are found in the bronchial epithelium and expressed locally in the mesenchyme, suggesting that the growth factors are produced in the mesenchyme and act on the epithelium (Keyzer and Post, 1999). These growth factors are thought to influence growth and branching morphogenesis. IGFs may play a role in lung development, but do not appear to be a major determinant during morphogenesis (Keyzer and Post, 1999). FGFs have been shown to play key roles in growth and morphogenesis of the developing lung. Members of this growth factor family are thought to be responsible for mesoderm patterning, limb pattern formation, and stimulating proliferation; FGFs can be found in most types of developing lung cells (Keyzer and Post, 1999). HGFs are produced by fetal lung fibroblasts and stimulate proliferation of adult type II cells. These growth factors are thought to transduce mesenchymal-epithelial signaling. However, these cells do not seem to play a role in branching morphogenesis (Keyzer and Post, 1999). PDGFs are implicated in embryonic development, but further studies need to be done to determine their specific roles (Keyzer and Post, 1999). TGF- β exhibits a variety of biological effects including regulation of ECM and production of integrin expression (Keyzer and Post, 1999).

Although many growth factors have been characterized in their relation to lung development, caution must be taken when including these factors to cell cultures. Exogenous growth factors have been shown to cause inhibitory effects on branching morphogenesis and proliferation. In addition, other problems may arise due to unknown effects of one or more growth factors on another. In this study no additional growth factors were considered outside those used in standard protocols.

The ECM of lung tissue regulates the behavior of cells that interact with it and ECM content is an important factor in the mechanical properties that a tissue exhibits (Keyzer and Post, 1999). The ECM can be defined as a structural network of collagen, elastin, glycoproteins, proteoglycans, fibronectin, and integrins that surround the different cells of the lung (Starcher, 2000). The ECM also functions as a dynamic modulator of various biological processes including: development, migration, proliferation, morphology, and metabolic functions (Keyzer and Post, 1999). This is accomplished through selective binding and subsequent release of growth factors and cytokines, and through its interaction with cell surface receptors (Starcher, 2000). Development of the lung requires cellular multiplication, differentiation, and an integrated system of signals to modulate these processes. The ECM provides the necessary framework for the expansion of each lung compartment (Starcher, 2000). The major stress-bearing constituents of lung tissue are elastin and collagen fibers. These fibers have considerably different individual properties, but work in conjunction with each other to handle the stresses associated with normal lung function.

Collagen fibers are the major constituent of the ECM and account for 60-70% of the lung connective tissue protein mass and 15-20% of the total tissue mass (Keyzer and Post, 1999). These fibers can be found in many different types throughout the lung tissue. Some examples include collagen type I and III, which are associated with mesenchymal connective tissue and collagen type IV that can be found in the basement membranes (Keyzer and Post, 1999). Collagen fibers have a stiffness that is four orders of magnitude higher than the stiffness of elastin fibers. Because of this, the collagen fibers are primarily responsible for load bearing at high strains.

Elastin is the other major component of the connective tissue matrix. Estimates put its content anywhere between 1.3-47% of the total dry-weight of the human lung and approximately 27-34% of the lung connective tissue (Chrzanowski et al., 1980). Elastin organization begins during the pseudoglandular stage of fetal development and its synthesis increases during the canalicular and saccular stage (Starcher, 2000). Elastin fibers are arranged uniformly throughout the lung tissue, which is important because the lung requires an elastic symmetry to function properly (Starcher, 2000). Tropoelastin, a monomeric soluble precursor of insoluble elastin, is produced primarily by cells of mesenchymal origin (Mariani and Pierce, 1999). Development of the alveoli is directly related to the elastic fiber network. Elastic fibers are generally concentrated in areas of high stress, forming a ring around the alveoli openings and concentrating at the alveoli junctions (Starcher, 2000). Elastin fibers within the ECM are responsible for the passive recoil of those compartments that undergo repeated cycles of expansion and

contraction (Mariani and Pierce, 1999). The alveolar walls, alveolar ducts, conducting airways, pleura, and vascular tissue are all dependent on elastin to absorb and store energy during the expansion of the lung and then use the stored energy to return to the lung to its original volume (Mariani and Pierce, 1999). In contrast to collagen fibers, elastin fibers can be stretched to >250% of their original length, and allow for load bearing at low strains. Elastogenesis appears to be an important factor in the development and maintenance of normal lung structure and function; interference with elastin formation has shown to result in severely abnormal alveolar architecture (Mariani and Pierce, 1999).

Glycosaminoglycans (GAGs) are important for the binding of other ECM components, cell adhesion molecules and growth factors; the theory is that GAGs may mediate cell adhesion, structural organization, and proliferation during lung development (Keyzer and Post, 1999). They are composed of unbranched carbohydrate chains that bind to proteoglycans and are not considered to be stress-bearing constituents.

Integrins have been identified as the receptors for attachment between ECM molecules. In fetal rat lung fibroblasts, and number of integrins have been shown to function as collagen-binding receptors.

Another important element within the matrix appears to be the glycoprotein microfibrils. These fibrils are 10-12 nm in length and are thought to provide the scaffold necessary for elastin assembly (Starcher, 2000).

This study focused on elastin and collagen content of the ECM and the interaction between the two fibers in relation to tissue biomechanics. It has been

shown that changes in collagen-elastin matrix contribute to changes in the viscoelastic properties. Therefore, percents of elastin and collagen must be measured to help explain the results obtained from the mechanical tests.

Tissue construct development begins by seeding cells onto a scaffold or template. The use of PGA polymer has been shown to offer the desired properties of the platform needed to produce 3D tissue equivalents. PGA polymer is composed of small-diameter fibers that can be aligned or randomly entangled to form a strong, flexible and porous 3D matrix. This matrix also allows for spatially uniform attachment of cells while minimizing diffusional constraints of nutrient transfer and gas exchange during cultivation. This is a result of the porous nature of the material, which creates large surface areas for increased mass transfer of nutrients and cell attachment. PGA is also biodegradable and biocompatible. Degradation times can vary but should degrade in parallel with the accumulation of tissue components. Its biocompatibility permits stimulation of cell proliferation and ECM creation without inducing an inflammatory response or side effects. Byproducts from the polymer during degradation are shown to be non-toxic *in vivo*. This study considered the use of non-aligned PGA polymer as a suitable template for engineering human microvascular endothelial lung cells (HMVELC).

Surface modifications of polymer scaffolds have been shown to increase cell-seeding densities and improve cell attachment to polymer fibers (Gao, Niklason, Langer, 1998). Hydrolysis of PGA in 1M NaOH transforms ester groups on the surface of PGA polymer fibers to carboxylic acid and hydroxyl groups (Gao, Niklason, Langer, 1998). This modification promotes individual cell adhesion and

serum protein adsorption on the scaffolding without significantly sacrificing the molecular weight or thermal properties of the original polymer (Gao, Niklason, Langer, 1998). Serum protein adsorption onto the fibers correlated with an increase in cell seeding density (Gao, Niklason, Langer, 1998). It is important to optimize attachment of the cells in order to increase proliferation and create uniform tissue growth.

Because of construct thickness associated with 3-D tissue growth, it is imperative that inner cells are receiving proper nutrition to keep them viable. Bioreactors provide the following properties that stimulate internal and external cell growth. Uniform mixing of the internal microenvironment is accomplished leading to high rates of mass transfer (Vunjak-Novakovic, et al, 1997). These reactors also allow for the regulation and maintenance of pH, gas partial pressures (pO_2 , pCO_2), nutrient levels, and shear stresses at the tissue surfaces (Vunjak-Novakovic, et al, 1997). These high aspect ratio vessels (HARVs) show a marked improvement over static culturing conditions in proliferation and DNA content. In addition, a more aerobic cell metabolism was noted which directly related to a less acidic and less hypoxic environment (Vunjak-Novakovic, et al, 1997). The rotating vessels suspend the constructs freely within the microenvironment. The average settling velocity is 2-4 cm/s for speeds of 15-28 rpm (Vunjak-Novakovic, et al, 1997). The settling of the constructs is responsible for the mixing of the nutrients within the bioreactor. Reynold's numbers within the bioreactor are between 114-207 and average shear stress is 1.5 dyn/cm^2 (Vunjak-Novakovic, et al, 1997). In this study bioreactors were

used to facilitate the 3D growth of the constructs approximately 3 days after initial seeding.

4. METHODOLOGY AND MATERIALS

4.1 Cell Seeding of Polymer Scaffolds:

The first step in creating a functional tissue equivalent lies in the initial introduction of the cells to the polymer scaffolding. There are many important factors that must be met for rapid tissue growth to occur. First, there must be a high yield of cells to maximize utilization. In addition, these cells must show a high kinetic rate of attachment to the polymer in order to minimize the time in suspension for shear-sensitive and anchorage dependent cell types. Finally, cells must be seeded in a spatially uniform distribution within the polymer to provide for uniform cell proliferation and homogeneous tissue growth. This uniform distribution is necessary because the cells must interact with each other to create the framework for a functional tissue.

PGA polymer scaffolds were cut to the desired shapes and sizes. For tensile tests, scaffolds were cut into dog-bone shapes measuring 11mm X 5mm X 2mm. Scaffolds for SEM and biochemical analysis were cut into discs measuring 5mm in diameter. Each scaffold was modified by the hydrolysis protocol (Appendix A).

Seeding density was established to be $3-4 \times 10^6$ cells/scaffold. Cells were trypsinized and removed from the culture flasks. After centrifuging ($125 \times g$ for 5 min), the cells were resuspended in 1 ml of the appropriate warmed growth media.

Modified scaffolds were placed into a 20ml syringe. The cell suspension was drawn up and subsequently dispensed to force cells through the scaffold matrix. This was repeated 3 times for each scaffold. Each scaffold was then removed and placed into 1 ml of the cell suspension in a 50 ml centrifuge tube for 2 hours in an

incubator (37°C and 5% CO₂) to allow for cell attachment. The cap on the centrifuge tube was vented to allow for gas exchange. After 2 hours, the scaffolds were transferred to 25cm³ flasks containing 15 ml of growth media (37°C and 5% CO₂). Constructs remained in this static environment for 2 days. Media (100%) was changed daily to ensure optimal cell nutrition. After 2 days, the constructs were transferred to a dynamic environment within the bioreactor.

Dynamic culturing has been shown to greatly increase the cell distribution, proliferation within the polymer matrix, and metabolic activity of the cells (Vunjak-Novakovic, et al, 1997). Although a dynamic environment appears to be beneficial, at high speeds can prove to be detrimental when turbulence can cause accelerated mechanical degradation, polymer fragmentation, and shear stress forces that adversely affect the cells. Therefore, it was appropriate to monitor each specific case and determine the optimal dynamic parameters. The bioreactor was set at the highest possible speed where the construct did not contact the vessel walls or become turbulent in nature. For most cases this was between 11-17rpm. This ensured that the highest possible shear forces would be acting on the construct, promoting increased mass transfer and secretion of proteins required for ECM production. Constructs were cultured in this dynamic environment for up to 10 days. Constructs were sampled at different time periods to be tested for mechanical and morphological properties. Constructs that were sampled were fixed in Hexamethyl Disilazane (Appendix A). Characterizations were done within 24 hours to obtain the best possible results and limit any effect the fixative may have to the tissue properties.

4.2 Tensile Test Protocol

Mechanical testing was done using the Bose® ELeCtroForce ELFT™ 3200. Samples were measured for length, width, and thickness prior to testing. For blank samples the initial length was set to 6.35mm and for IMR-90 samples the initial length was 9.00mm. The initial lengths were determined by the size of the samples to be tested. Every sample tested was stretched a maximum of 12mm. This stretching was done at a rate of 5mm/minute. Values for stress and strain were calculated from the results and graphing stress vs. strain will allow for calculations of the Elastic Modulus for each sample. Averages for samples subjected to the same culture conditions were also calculated. Yield stresses were also calculated for each sample and averaged for each sample group.

Tensile measurements were made using the Bose ELeCtro Force 3200, which was connected to a PC for data collection. Stress T and strain ϵ values can be found from equation 1.

$$\epsilon(t) = \left[\frac{l(t) - l_o}{l_o} \right]; T(t) = \frac{F(t)}{A_o} \quad (1)$$

where l is length, l_o is initial length of strip, F is force, t is time, and A_o is initial cross-sectional area determined by measurements using a Bernier caliper.

Although these samples may not be comparable to native lung tissue at this time, it does offer insight into cell morphology and ECM recreation within the tissue samples.

4.3 SEM

Scanning electron microscopy (SEM) was performed on individual tissue strips to determine morphological properties. Pictures were taken of blank polymer scaffolds as well as those seeded with cells. This offered a comparison in order to distinguish biological components from non-biological components. In addition, polymer degradation can be characterized in accordance with culture time and conditions. Analyzing tissue samples allowed for detection of cell attachment, ingrowth, and organization. ECM set-up and organization was also noted.

4.4 Biochemical Analysis

Endothelial and Fibroblast constructs were sampled after 7 days, and the total wet weight of the samples was acquired. The samples were divided into four equal parts to be used in the biochemical assays. The four parts were used to obtain α -elastin, salt-soluble collagen, acid-soluble collagen, and pepsin-soluble collagen (Appendix A). These four extracts were then quantified using a dye-binding method (Appendix A). Data was compared against a standard curve to give values for total elastin and collagen content.

4.5 MATERIALS

4.5.1 Human Microvascular Endothelial Lung Cells (HMVELC)

HMVELC were obtained (Clonetics, Walkerville, MD) in an ampule containing 572,500 cells. The cells were acquired from a female donor, age 45. Cell viability was 77% and had a seeding efficiency of 25%. HMVELC were of passage 4 and had a 34-hour doubling time for a guaranteed 15 population doublings.

4.5.2 EGM-2 MV Growth Media for HMVELC

Endothelial Cell Basal Medium-2 (EGM-2 MV) was obtained (Clonetics, Walkerville, MD) and combined with the following growth supplements: Human endothelial growth factor, Gentamicin sulfate amphotericin-B, Fetal bovine serum, Vascular endothelial growth factor, Human fibroblast growth factor, Long R Insulin-like growth factor-1, and Ascorbic acid.

4.5.3 Human Fetal Lung Fibroblasts (IMR-90)

IMR-90 cells were obtained (American Type Culture Collection (ATCC), Manassas, Virginia) in a 1ml ampule containing 1.6 million cells with a viability of 1.5 million cells. These cells were acquired from the lungs of a 16-week female fetus. Expected viability of these cells is 91%-97%. These cells are of passage 10 and 24.2 population doublings. The cells are reported to be capable of obtaining 58 population doublings before the onset of senescence.

4.5.4 EMEM Supplemented Growth Media for IMR-90 Cells

Eagle's Minimal Essential medium with Earle's BSS and 2mM L-glutamine (EMEM) was obtained (Fisher Scientific, Atlanta, GA) and modified to contain: 1.0mM sodium pyruvate, 0.1mM nonessential amino acids (NEAA), 1.5g/L sodium bicarbonate, and 10% fetal bovine serum (FBS).

4.5.5 Bioreactors

A rotary cell culture system (RCCS) was obtained from Synthecon Inc., Houston TX. The system incorporates a 4-station rotator base capable of rotating at 4 independent speeds. Each rotator base held a single 50 ml disposable culture vessel, also known as a high aspect ratio vessel (HARV).

4.5.6 PGA Polymer Scaffolding

PGA polymer scaffolds were obtained (Synthecon Inc.) in 10 cm x 10 cm squares. The nature of the polymer is unaligned and has a thickness of 2mm. Individual scaffolds were cut from these pieces in the same direction.

4.5.7 Analytical Assays

Elastin and Collagen content of tissue samples were done using commercially available assay kits obtained from Accurate Chemical and Scientific Corp., Westbury, NY. Samples were divided into equal parts and quantified for elastin, salt soluble collagen, acid soluble collagen, and pepsin soluble collagen. Results obtained were compared against a standard curve for known samples to determine

quantitative values. These results were then averaged to determine average elastin and collagen content. In addition, ratio of elastin to collagen was also determined.

5. RESULTS

Blank polymer control samples were analyzed using tensile tests and SEM photos. Tensile tests were done on samples at varying time periods. These included tests on original untreated polymer, hydrolyzed polymer, and polymer that had been hydrolyzed and soaked in growth media for 24 hours. Further tensile tests were conducted on samples cultured for 2, 3, 4, 5, and 7 days.

SEM photos of the controls show the degradation of the polymer with relation to time in culture (Fig 1.1-1.3). Photos were taken from samples that had been hydrolyzed and soaked in media for 24 hours and then cultured for 3 or 7 days.

Fig 1.1 SEM photo of 3-day old blank polymer. Polymer fiber has been cleaved at one end and further degradation is noted by striations along fiber.

Fig 1.2 Degradation of individual polymer fiber from 7-day old blank sample.

Fig 1.3 Degradation of polymer mesh network from 7-day old sample.

Degradation of the PGA polymer fibers is noted by striations in the individual strands. As time proceeds, degradation continues along the fissures of the striated regions until the polymer is cleaved off at this site. Degradation of polymer also occurs as a loss in diameter over time. Original polymer fibers have diameters varying from 15-25 μm . Loss of diameter can vary based on location of polymer strand, cell attachment, and culture conditions.

Blank samples tested under static conditions maintained their structural integrity for longer than 21 days. Blank samples cultured in static for 2 days then dynamically for 5 to 8 days maintained their structural integrity up to day 6 (Fig2.1).

Fig 2.1 Elastic Modulus comparison of statically cultured (S) and dynamically cultured (D) blank polymer blank polymer scaffolds. Statistical analysis was done using Welch's t-test, and significant differences were found between these two groups (*).

We found that original, hydrolyzed, and hydrolyzed-soaked polymer scaffolds, that were subjected to static culture conditions (S), showed a significantly higher ($p<0.005$) (Appendix C) elastic modulus than hydrolyzed-soaked polymer scaffolds that had been cultured dynamically (D) for >2days (*).

Fig 2.2 Yield Stress comparison of statically cultured (S) and dynamically cultured (D) blank polymer blank polymer scaffolds. Statistical analysis was done using Welch's t-test, and significant differences were found between these two groups (*).

When we plotted yield stress for the same sample groups we found a similar pattern. Statistically, polymer in static culture had a significantly higher ($p<0.005$) (Appendix C) yield stress compared to polymer cultured dynamically (*).

Early experiments included seeding HMVELC onto PGA polymer fibers. Initial problems arose with cell attachment of the endothelial cells to the polymer.

This was overcome by subjecting the polymer scaffolding to hydrolysis prior to seeding. Improved cell attachment was noted on samples photographed after 3 hours of seeding (Fig 3.1). This photo shows how cells have attached to the PGA polymer fiber and have begun to aggregate between strands.

Fig 3.1 SEM photograph at a magnification of 550X shows endothelial cell growth between polymer fibers. This picture was taken 3 hours post-seeding.

Additional tissue constructs were seeded with HMELC and cultured for periods up to 12 days. Under phase contrast microscopy, cell attachment and some ingrowth between polymer fibers was noticed. SEM photos were taken of the samples to further indicate these characteristics. The most common morphology noticed was a monolayer of endothelial cells covering individual polymer fibers (Fig

3.2). Here the endothelial cells can be seen lying down on the polymer fibers (Fig 3.3).

Fig 3.2 and Fig 3.3 Endothelial cell attachment and growth along polymer fiber. These pictures were taken from 3-day old samples cultured under static conditions.

Under static conditions the endothelial cells did show some ability to attach between fibers. Cell growth in the above samples was strictly seen as monolayers attaching to the polymer fibers.

Samples that were cultured for 3 days or longer showed endothelial cell attachment across cleavage sites created by polymer degradation (Fig 3.4-3.6).

Fig 3.4 3-day old sample showing endothelial cell growth between polymer fibers.

Fig 3.5 7-day old sample showing endothelial cell growth between polymer fibers. Polymer strands have degraded to a diameter of approximately 3-5µm.

Fig 3.6 10-day old sample showing complete endothelial cell growth between fibers. Fiber diameter is approximately 3-5µm.

Tensile tests could not be done on any endothelial constructs at any time period. Although cell growth can be seen in SEM photos, constructs lacked the necessary structural integrity to perform these tests. These samples maintained their shape in culture, but when removed from the media could not hold together.

Biochemical assays were done on endothelial constructs that had been cultured for 7-days. Neither elastin nor collagen was present in any of the construct samples that were tested. Fig 4.1 and Fig 4.2 are the collagen and elastin standards that were generated for the biochemical tests on the endothelial constructs. Values from collagen and elastin samples were obtained and amounts were calculated using these standard curves. In this case all values obtained were at or below values of

blank samples. Therefore it was concluded that no elastin or collagen was present in any endothelial constructs.

Fig 4.1 Collagen standard curve for endothelial constructs.

Fig 4.2 Elastin standard curve for endothelial constructs.

SEM photos of fibroblast cell constructs cultured under static conditions for 3 days show cell attachment to PGA polymer fibers (Fig.5.1). Also noted is

connective elements that are visible between the cell mass and the fiber strands (Fig.5.2). These elements are most likely collagen fibers.

Fig 5.1 Fibroblast cell attachment to polymer fibers under static conditions.

Fig 5.2 Fibroblast cells and extra-cellular matrix components.

Fig 5.3 Fibroblast cell attachment to polymer fibers and cell ingrowth. Connective elements of the ECM can also be seen in cell-cell and cell-polymer interactions.

The fibroblasts showed an increased amount of cell proliferation and cell ingrowth when moved to dynamic culture conditions. The morphology of the fibroblasts indicates an elongated shape. SEM picture is of a 10 day-old lung tissue-polymer construct (Fig.5.3). Magnification at 700X shows fibroblast growth around polymer fibers. The polymer fiber in this picture is approximately 10µm in diameter, which corresponds to expected sizes of degraded polymer strands. Fibroblast cells in this picture have aligned in the same direction as dynamic flow and connective elements are seen extending between cells and the polymer fiber.

Increased magnification of 7-day old samples indicates the formation of a structural matrix within the polymer network (Fig. 5.4). Magnification at 10,000X shows this matrix in more detail (Fig. 5.5.).

Fig 5.4 Magnification at 1900X shows fibroblast cell proliferation and structural matrix.

Fig 5.5 High Magnification at 10,000X shows cell and ECM components.

Tensile tests results from 7-day samples of IMR-90 fibroblast constructs demonstrate an average Elastic modulus of 390077 ± 482294 Pa. Individual sample moduli were 946352 Pa, 134880Pa, and 88999 Pa (Fig 6.1). All 3 samples showed an Elastic modulus greater than the average modulus for 2-to-5-day blank polymer groups.

Fig 6.1 Comparison of IMR-90 (fibroblast) construct Elastic moduli.

Fig 6.2 Comparison of IMR-90 (fibroblast) construct yield stresses

Fig 6.3 Yield strain for individual IMR-90 (fibroblast) samples

Yield stress (Fig 6.2) and yield strain (Fig 6.3) comparisons were also done. In sample number one we noticed a yield stress that was approximately twice as much as in samples two or three. However, the corresponding yield strain was 31 percent lower than sample 2 and 55 percent lower than sample 3.

We then averaged these results for elastic modulus, yield stress, and yield strain and compared them statistically to unseeded polymer samples under multiple culture conditions (Fig 6.4-6.6).

Fig 6.4 Elastic modulus comparison of blank-static (S), blank-dynamic (D), and IMR-90 (fibroblast) construct. Statistical comparison was done using Welch's t-test (Appendix C), suitable for small sample groups.

Fig 6.5 Yield Stress comparison of blank-static (S), blank-dynamic (D), and IMR-90 (fibroblast) constructs. Statistical comparison was done using Welch's t-test (Appendix C), suitable for small sample groups.

Fig 6.6 Yield stress comparison of blank-static (S), blank-dynamic (D), and IMR-90 constructs. Statistical comparison was done using Welch's t-test (Appendix C), suitable for small sample groups.

We cannot say statistically that the elastic modulus or the yield stress of the fibroblast constructs were significantly different from the dynamically cultured blank polymer samples (D). This is partially due, in both cases, to the high variance that is seen between the elastic moduli and yield stresses of the three individual fibroblast samples. However, we did note a significant decrease in yield strain from both the static and dynamic blank samples. This indicates that the IMR-90 samples are much more fragile than the blank polymer.

Biochemical analysis quantitatively proved the existence of both elastin and collagen within the IMR-90 constructs. Results from the assays were compared against standard curves of known elastin (Fig 7.1) and collagen (Fig 7.2) samples to

determine actual values of elastin and collagen in the tissue samples. These values were reported as a percentage (%) of total wet weight for the IMR-90 samples.

Fig 7.1 Elastin Standard curve for soluble elastin from IMR-90 constructs.

Fig 7.2 Collagen Standar curve for collagen content of IMR-90 constructs.

IMR-90 constructs were evaluated and duplicate samples were assayed. Group one contained an average of 94.2µg of elastin/50µl of elastin extraction. This group had a total of 110µl of extraction. The total elastin content for group one was 207.24µg, which was approximately 2.59% of the total wet weight. Sample group two contained an average of 81.1µg of elastin/50µl of elastin extraction. This group had a total of 140µl of extraction. The total elastin content in group two was 719.08µg, which was approximately 3.78% of the total wet weight. The standard error for these groups was 4.07%. Therefore, the average elastin content of the two sample groups was 3.19% of the total wet weight.

The IMR-90 groups were also tested for collagen. Duplicate samples were assayed to determine contents for salt soluble, acid soluble, and pepsin soluble collagens. These parts were then added to determine total collagen content of the samples. Sample group one contained an average of 26.99µg of salt soluble collagen/100µl, 30.30µg of acid soluble collagen/100µl, and 33.35µg of pepsin soluble collagen/100µl. Total extract volumes for the group was 980µl, 920µl, and 930µl respectively. Amounts were extrapolated and determined to be 1428.30µg total salt soluble collagen, 1505.52µg total acid soluble collagen, and 930.60µg total pepsin soluble collagen. Total collagen content of group one was calculated to be 3864.42µg, which was 14.31% of the wet weight. Group two contained an average of 24.81µg of salt soluble collagen/100µl, 35.79µg of acid soluble collagen/100µl, and 21.44µg of pepsin soluble collagen/100µl. Total extract volumes for the group was 980µl, 950µl, and 960µl respectively. Amounts were extrapolated and determined to be 770.08µg total salt soluble collagen, 1615.2µg total acid soluble collagen, and

782.44 μ g total pepsin soluble collagen. Total collagen content of group two was calculated to be 3167.72 μ g, which was 16.67% of the wet weight.

The average collagen content for the two sample groups was 15.49% of the total wet weight.

Fig 7.3 Total amounts (ug) of elastin and collagen in group one.

Fig 7.4 Total amounts (ug) of elastin and collagen in group two.

Fig 7.5 Average values of total elastin and collagen content in batch 1 and 2.

6. DISCUSSION

Mechanical and morphological analysis was done to characterize our engineered tissue constructs. We used SEM imagery to provide us with a description of the organization of cellular components within the tissue constructs. With this type of analysis we can observe how cell attachment occurs and what type of organization the cells undergo. In addition, these images provide insight on formation of ECM components. We used biochemical assays to quantitatively measure ECM components, specifically elastin and collagen. After quantification of ECM components, we measured mechanical properties of the samples in order to differentiate between the contributions of the tissue and polymer components. It also becomes necessary to understand cell and ECM contribution to the mechanical properties of the engineered constructs and how we can optimize these constructs to support the loads required for lung function.

We analyzed both endothelial and fibroblast constructs, but only fibroblast constructs provided results in all three characterizations. SEM images of these constructs at different time periods showed cellular outgrowth, forming a network of cells across individual fiber strands. In addition, we observed evidence of ECM expansion (elastin or collagen fibers) stretching between cells and polymer fibers. This was concluded based on the relative size of the strands. These strands are very small (in diameter) relative to polymer fiber diameters. We also compared the sizes of these strands to images taken of blank polymer constructs at comparable magnifications and time in culture. We surmised that these strands are a part of the

fibroblast cell due to the regularity of shape and relatively large length. Fibroblast cells that are seen in static culture flasks have very irregular shapes.

By extracting the solubilized elastin and collagen from the fibroblast constructs, we were able to verify the presence of ECM components, which strengthens our conclusions based on the SEM images. Quantitative results show that the average elastin content for the engineered constructs cultured for 10 days was $3.19 \pm 0.8\%$ of total wet weight of the original samples. Average collagen content was $15.49 \pm 1.67\%$ of total wet weight for the same samples. We cannot compare these percentages to those obtained from native tissue because the total weight of our constructs also includes polymer that has not yet degraded. However, we are able to compare the elastin-collagen ratio that we obtained with the elastin-collagen ratio from the ECM of native tissues. In our constructs there was an approximate ratio of 1:5, meaning there was five times the amount of collagen present. Native tissue ratios are on the order of 1:2. We hypothesize that there is a lack of elastin instead of too much collagen production. This is most likely due to the relative age of the fetal lung fibroblasts. The IMR-90 cells that we used are 16 weeks in age, and elastin production in fetal lungs has been shown to peak during the later stages of fetal development. What is of importance though is that we were able to produce both component fibers within a relatively short period of time. With extended culture periods we expect to see a ratio closer to that of native ECM.

Knowing that some semblance of an ECM has been created and that this will directly affect tissue mechanical properties, we subjected these samples to tensile tests and compared to previously tested blank polymer scaffolds. Initially, tensile

tests were done on blank scaffolds at different time periods to determine the effects of modification and degradation on the mechanical properties. Because there are a limited number of samplings per time period, more samples will need to be tested to get more accurate results. However, we can describe the general trends associated with polymer modification and degradation. In the test, we calculated yield stress, elastic modulus, and yield strain for each of the samples. Statistically, we noticed a significant difference between blank polymer scaffolds that were cultured under static conditions and blank polymer scaffolds that were cultured dynamically. Both elastic modulus and yield stress were higher in the statically cultured samples. However, we could not statistically determine any significant difference in yield strain between static and dynamic blank polymers. This shows that all of the polymer samples could be stretched to relatively the same length. These tests indicate that dynamic conditions greatly increase polymer degradation resulting in a loss of some mechanical properties. Surface degradation of polymer fiber strands was also noticed when examining SEM images. Original diameter sizes were approximately 15-25 μm compared with 7-10 μm diameters that were seen in dynamically cultured samples. In addition, striations along the polymer fibers confirm bulk degradation of the polymer.

We can see that the average elastic modulus and yield stress from the IMR-90 samples is higher than that of the dynamically cultured blank polymers. However, it should be noted that we couldn't statistically determine if these values are significantly different due to the high variance associated with the three individual IMR-90 samples. We are most interested in this comparison because the

properties of the polymer under dynamic conditions should closely relate to the polymer properties in the IMR-90 constructs. Therefore, any differences between the samples should be attributed to any cellular components that may be present. There was a significant decrease in the yield strain of the IMR-90 constructs and both static and dynamic blank samples. This indicates that the IMR-90 samples were much more fragile. Although the tissue components may add strength they did not contribute to elongation. This finding coincides with the elastin and collagen content that was noted. With such a high ratio of collagen to elastin, it is expected that the tissue constructs would not show a high elasticity.

When we compare the individual IMR-90 samples to the blank polymer averages we can see that sample 1 has a much higher elastic modulus and yield stress than the blank samples. With samples 2 and 3 these values are only slightly higher. This variance is most likely due to non-uniform surface modification during hydrolysis. This phenomena creates regions on the scaffolding where cells may not be likely to attach in large numbers ultimately lowering cell seeding density. If this is the case then we will get constructs that have lower tissue masses and non-uniform growth. Mechanical properties that we see may not be much different than that of samples 2 and 3. However, if we achieve uniform hydrolysis and high seeding density then we may see mechanical properties like sample 1. If we can duplicate the mechanical properties seen with this case, we may be able to provide the properties needed for load bearing under physiological conditions.

SEM photos were also taken of endothelial cell constructs and studied for cell attachment and organization. Attachment of the cells to the polymer fibers was

noted, however, these cells did not show the branching that was seen with the fibroblasts. Endothelial cells are characteristically monolayer type cells, and this may account for the limited ingrowth on the scaffolds. However, these cells were able to proliferate and grow under dynamic culture conditions suggesting that this cell type may still be suitable for use within co-culture constructs. Quantitative values from tests for elastin and collagen were negative. This may be the result of the relative age of the cells. Since these are adult cells and not fetal cells, they may not be active in producing the needed ECM proteins. We attempted mechanical tests on 7-day old endothelial constructs, but these cells lacked the structural integrity necessary to comply. This corresponds to the results that we saw from the elastin and collagen tests.

7. CONCLUSION

We have grown fibroblast (IMR-90) and endothelial lung tissue constructs using PGA polymer scaffolds as templates. These constructs were cultured dynamically and characterized to determine their morphological and mechanical properties. Each of these constructs has offered results that can be used to determine future directions related to this study.

During these experiments we were able to facilitate fibroblast and endothelial cell attachment to polymer strands through hydrolysis of the polymer. This successfully led to the growth of tissue constructs that could then be characterized.

Fibroblast construct characterization proved to be most noteworthy. Excellent cellular organization and ingrowth were seen in SEM images and ECM components, elastin and collagen, were quantified by biochemical analysis. In addition, some of these tissue constructs showed increased elastic modulus and yield stress in relation to blank polymer samples that had also been cultured dynamically. These results prove that fibroblast lung cells will provide, in part, the necessary cellular components for load bearing at physiological strains.

In contrast, endothelial constructs did not provide the mechanical properties that were seen in the fibroblast constructs. These cells failed to yield any ECM components and lacked the structural integrity necessary for tensile testing. However, we do know that these cells are an essential component of the respiratory membrane, and will need to be studied further to determine their role in membrane recreation.

With this project we were able to do some initial characterizations that will guide future work. However, it should be noted that these characterizations were done on a very limited number of samples due to time constraints of growing individual samples and the cost associated with it. More samples should be tested to gather more accurate results.

Future directions related to this study will have to include the co-culture of multiple cell types to determine their interaction and organization with respect to each other. Further experiments may also explain the affects of the dynamic culture environment on cell proliferation versus ECM formation. As the respiratory membrane begins to come together, tests must also be done to quantify the functionality in relation to the exchange of oxygen and carbon dioxide.

REFERENCES

1. Agarwal, A. et al. Two-Photon Laser Scanning Microscopy of Epithelial Cell-Modulated Collagen Density in Engineered Human Lung Tissue. *Tissue Engineering*. 7, 2, 2001.
2. Atala, A. et al. *Synthetic Biodegradable Polymer Scaffolds*. Birkhauser Boston, 1997.
3. Burg, K.J.L. et al. Comparative Study of Seeding Methods for Three-Dimensional Polymeric Scaffolds. *J. Biomed. Mater. Res.* 51: 642-649, 2000.
4. Bursac, P.M. et al. Mass Transfer Studies of Tissue Engineered Cartilage. *Tissue Engineering*. 2, 2, 1996.
5. Carrier, R.L. et al. Cardiac Tissue Engineering: Cell Seeding Cultivation Parameters, and Tissue Construct Characterization. *Biotechnology and Bioengineering*. 64(5): 580-589, 1999.
6. Chrzanowski, P. et al. Elastin Content of Normal and Emphysematous Lung Parenchyma. *Am. J. Med.* 69(3): 351-359, 1980.
7. Dolhnikoff, M. et al. Extracellular Matrix and Oscillatory Mechanics of Rat Lung Parenchyma in Bleomycin-induced Fibrosis. *Am. J. Respir. Crit. Care Med.* 160: 1750-1757, 1999.
8. Fung, Y.C. *Biomechanics: Mechanical Properties of Living Tissues*. Second Edition, Springer, 1993.
9. Fournier, R. *Basic Transport Phenomena in Biomedical Engineering*. Taylor and Francis, 1998.
10. Galban, Craig J. et al. Analysis of Cell Growth Kinetics and Substrate Diffusion in a Polymer Scaffold. *Biotechnology and Bioengineering*. 65(2): 121-132, 1999.
11. Gao, J. et al. Surface Hydrolysis of poly(glycolic acid) meshes increases the Seeding density of vascular smooth muscle cells. *J. Biomed. Mater. Res.* 42: 417-24, 1998.
12. Gosiewska, A. et al. Development of a Three-Dimensional Transmigration Assay For Testing Cell-Polymer Interactions for Tissue Engineering Applications. *Tissue Engineering*. 7, 3, 2001.

13. Hallab, Ph.D., Nadim J. et al. Evaluation of Metallic and Polymeric Biomaterial Surface Energy and Surface Roughness Characteristics for Directed Cell Adhesion. *Tissue Engineering*. 7(1): 55-71, 2001.
14. Hance, A. R.G. Crystal. The connective tissue of the lung. *Am. Rev. Respir. Dis.* 112: 657-661, 1975.
15. Karlinsky, Joel B. et al. Stress-strain characteristics of normal and emphysematous hamster lung strips. *Respiratory Physiology*. 90: 251-260, 1992.
16. Khan, A.S. et al. Strain Measurements and Stress Analysis. Prentice Hall, 2001.
17. Magee, John C., et al. Isolation, culture, and characterization of rat lung microvascular endothelial cells. *J. Appl. Physiol.* L433-441, 1994.
18. Maksym, G.N. et al. A Distributed Nonlinear Model of Lung Tissue Elasticity. *The American Physiological Society*, 0161-7567/97, 1997.
19. Maksym, G.N. et al. Force heterogeneity in a two-dimensional network model of lung tissue elasticity. *J. Appl. Physiol.* 1223-1230, 1998.
20. Suki, Béla et al. Lung tissue viscoelasticity: a mathematical framework and its molecular basis. *J. Appl. Physiol.* 2749-2759, 1994.
21. Sweigart, M.A. et al. Toward Tissue Engineering of the Knee Meniscus. *Tissue Engineering*. 7, 2, 2001.
22. Tanaka, R. et al. Changes in Viscoelastic Properties of Rat Lung Parenchymal Strips with Maturation. *J. Appl. Physiol.* 87(6): 2081-2089, 1999.
23. Tummalapalli, Chandra M. et al. Responses of Vascular Smooth Muscle Cell to Extracellular Matrix Degradation. *Journal of Cellular Biochemistry*. 75: 515-527, 1999.
24. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, National Heart, Lung, and Blood Institutes, Published May 2001
25. Vunjak-Novakovic, G. et al. Bioreactor Cultivation Conditions Modulate the Composition and Mechanical Properties of Tissue-Engineered Cartilage. *J. of Orthopaedic Research* 17: 130-138 The Journal of Bone and Joint Surgery, Inc. 1999.

26. Vunjak-Novakovic, G. et al. Cell-polymer-bioreactor system for tissue engineering. J. Serb. Chem. Soc. 62(9): 787-799, 1997.
27. Yuan, H. et al. Effects of Collagenase and Elastase on the Mechanical Properties of Lung Tissue Strips. Journal of Applied Physiology. 89, 3-14, 2000.
28. Zeltinger, Ph.D., J. et al., Development and Characterization of Tissue-Engineered Aortic Valves. Tissue Engineering 7(1): 9-21, 2001.

APPENDIX A

Protocols:

PGA Polymer Preparation Protocol

Scaffold samples were cut from an original piece of a 10cm x 10cm PGA polymer square. Dogbone scaffolds measured ~10mm in length and all were cut in the same orientation in relation to the original polymer square. Disc scaffolds were cut to measure ~ 5mm in diameter.

Hydrolysis Protocol

Scaffolds were modified by hydrolysis using a 1M NaOH solution (Gao, Niklason, Langer, 1998). First, scaffolds were washed in hexane to remove any contaminants from the fibers. Scaffolds were then filtered using multiple Kimwipes. Once dry, they were soaked in 100% Ethyl Alcohol, placed into a 15ml centrifuge tube containing 10ml of distilled water, and shaken for 5 minutes to allow for solvent exchange. Scaffolds were immediately moved to a 15ml centrifuge tube containing 10ml of the NaOH solution and shaken for 1 minute, 15 seconds. Scaffolds were then removed from the tubes and placed into 5 consecutive beakers of filtered water (5 minutes each) to ensure that a neutral pH is reached. The scaffolds were again soaked in 100% Ethyl Alcohol and transferred to a biohood where they were filtered using multiple Kimwipes. Transferring the scaffolds to a biohood reduced the chance that contamination would occur. Scaffolds were then placed into the appropriate media, depending on cell type, overnight in an incubator to allow for serum absorption into the fibers. This process also allowed the scaffold to equilibrate to 37°C and 5% CO₂.

HMVELC Protocols

Subculturing Protocol

(Stated volumes are for 75cm³ flask)

We removed and discarded the culture medium. We then rinsed the cell layer with Delbecco's Phosphate-Buffered Saline (PBS) to remove traces of serum that contain trypsin inhibitor. We added 6ml of 0.025% (w/v) Trypsin-0.01% EDTA that had been warmed to 37°C. We observed the cells under an inverted microscope and tapped bottom of flask to encourage cell detachment. When all cells looked to be rounded and contained within the trypsin solution, we added 12ml of PBS to neutralize the trypsin. We removed the cell suspension and centrifuged (125 x g for 5 minutes) to concentrate the cells. We then aspirated the growth media by gently pipeting off the supernatant. We resuspended the cells into the appropriate amount of EGM-2 MV growth media (37°C) and aliquoted into new 75cm³ flasks. Subcultivation ratio was 1:2 to 1:8.

Media Renewal Protocol

Media was changed 100% every 2 days according to following schedule: 1.0ml/5cm³ at 0%-30% confluence, 1.5ml/5cm³ at 30%-60% confluence, and 2.0ml/5cm³ at 60%-90% confluence.

Initial Cell Culture and Cryopreservation

Human microvascular endothelial lung cells were obtained in 1ml ampules. Frozen cells were removed and thawed by placing into a water bath at 37°C. Vials were gently swirled until just thawed. Cells were then divided into 3-25cm³ culture flasks containing 10ml of EGM-2 MV supplemented growth media that was equilibrated at 37°C and 5% CO₂. At 90% confluence cells were subcultured into 2-75cm³ flasks containing 15ml of EGM-2 MV growth media. Flasks were then subcultured, centrifuged (125 x g for 5 minutes), and resuspended in a prepared solution of 95% EGM-2 MV growth media/5% DMSO at a concentration of ~20,000cells/ml. Aliquots of 1ml were placed into 1.5ml cryovials and placed into a -80°C for 24 hours then immediately transferred into liquid nitrogen storage. Vials were labeled with date, cell type, and passage number. These cells were of passage 5.

IMR-90 Protocols

Subculturing Protocol

(Stated volumes are for 75cm³ flask)

We removed and discarded the culture medium, then rinsed the cell layer with Delbecco's Phosphate-Buffered Saline (PBS) to remove traces of serum that contain trypsin inhibitor. We then added 6ml of 0.25% (w/v) Trypsin-0.03% EDTA that had been warmed to 37°C. We observed the cells under an inverted microscope and waited for cells to round up and detach (usually within 5 minutes). When all cells looked to be rounded and contained within the trypsin solution, we added 12ml of PBS to neutralize the trypsin. We then removed the cell suspension and centrifuged (125 x g for 5 minutes)

to concentrate the cells. We then aspirated the growth media by gently pipeting the supernatant. We resuspended the cells into appropriate amount of EMEM supplemented growth media (37°C) and aliquoted into new 75cm³ flasks. Subcultivation ratio was 1:2 to 1:8. EMEM supplemented media was renewed 100%, every 2-3 days.

Initial Cell Culture and Cryopreservation

IMR-90 cells were obtained in 1ml ampules. Frozen cells were removed and thawed by placing into a water bath at 37°C. Vials were gently swirled until just thawed. Cells were then divided into 3-25cm³ culture flasks containing 10ml of EGM-2 MV supplemented growth media that was equilibrated at 37°C and 5% CO₂. At 90% confluence cells were subcultured into 2-75cm³ flasks containing 15ml of EMEM supplemented growth media. Flasks were then subcultured, centrifuged (125 x g for 5 minutes), and resuspended in a prepared solution of 95% EMEM supplemented growth media/5% DMSO at a concentration of ~800,000cells/ml. Aliquots of 1ml were placed into 1.5ml cryovials and placed into a -80°C freezer for 24 hours and then immediately transferred into liquid nitrogen storage. Vials were then labeled with date, cell type, and passage number. These cells were of passage 11.

Cell Seeding Protocol

Seeding density was established to be 3-4 x 10⁶ cells/scaffold. Cells were trypsinized and removed from the culture flasks. After centrifuging (125 x g for 5 min), the cells were resuspended in 1 ml of the appropriate warmed growth media. Hydrolyzed and presoaked scaffolds were placed into a 20ml syringe. The cell suspension was drawn

up and subsequently dispensed to force cells through the scaffold matrix. This was repeated 3 times for each scaffold. Each scaffold was then removed and placed into 1 ml of the cell suspension in a 50 ml centrifuge tube for 2 hours in an incubator to allow for cell attachment. We made sure the cap on the centrifuge tube is vented to allow for gas exchange. After 2 hours the scaffolds were transferred to 25cm³ flasks containing 15 ml of growth media (37°C and 5% CO₂). Constructs remained in this static environment for 2 days. Media was changed daily to ensure optimal cell nutrition. After 2 days the constructs were transferred to a dynamic environment within the bioreactor.

Bioreactor Parameters

The bioreactor was set at the highest possible speed where the construct did not contact the vessel walls or become turbulent in nature. For most cases this was between 11-17rpm. This ensured that the highest possible shear forces would be acting on the construct, promoting increased mass transfer and secretion of proteins required for ECM production. Constructs were cultured in this dynamic environment for up to two weeks. Adjustments to bioreactor speed were done when necessary. Samples were taken at different time periods to be tested for mechanical and morphological properties.

Bioreactor Media Renewal Protocol

It was necessary to change the media within the disposable vessels on the bioreactor every 2-3 days. Renewal was done by adding and removing media via the syringe ports on the disposable vessel. A 20ml syringe and needle was used to execute renewal.

Fixation Protocol

Samples that were taken from the bioreactors were fixed before they were tested. Samples were first dehydrated through a dilution series of EtOH (50/70/90/100%) for 10 minutes each and then placed in Hexamethyldisilazane twice for 5 minutes and airdried overnight.

Tensile Test Protocol

Mechanical testing was done using the Bose® ELeCtroForce ELF™ 3200. Tissue strips were placed in the machine and stretched at a rate of 5mm/minute. Values for stress and strain were calculated from the results of these tests. Samples were measured for length, width, and thickness prior to testing.

Elastin Extraction Protocol

The Fastin Elastin assay kit was used to measure in-soluble elastin. Tissue-polymer constructs were solubilized in hot oxalic acid for two-1 hour intervals at 95°C. The samples were then placed on a orbital shaker and precipitated overnight in a 5°C refrigerator with Fastin precipitating reagent. The samples were then centrifuged using a microcentrifuge tube fitted with a filter membrane that has a molecular weight cut-off of ~10,000 MW. The oxalic acid free extract contained the tissue elastin in the form of α -elastin which had an average molecular weight of ~8000 MW. Extracts were saved for elastin analysis.

Elastin Assay Protocol

Test samples of 50ul were then aliquoted into 1.5ml microcentrifuge tubes, brought up to 100ul by the addition of 0.05M acetic acid solution, and centrifuged (8000 x g for 10 minutes) to pack the precipitated elastin. Following centrifugation, the acid solution was drained. The microcentrifuge tubes were carefully inverted and gentle tapping onto an absorbent paper towel ensured removal of all liquid contents. The tubes now contained only the precipitated elastin. Fastin dye reagent was added to each tube in 1ml quantities followed by addition of 100ul of 90% saturated ammonium sulphate. A vortex mixer was used to bring the elastin precipitate into solution with the dye reagent. Two short periods were used instead of one long period. The elastin and dye reagents were then allowed to interact for 60 minutes on orbital shaker at room temperature. The elastin-dye complex was then collected through centrifugation (8000 x g for 10 minutes). The supernatants were again discarded in the same manner as stated above. The dye bound to the elastin pellet was solubilized with the destain reagent and brought into solution using two short periods on a vortex mixer. Aliquots of 100ul were plated onto a 96-well plate and the absorbance was measured at a wavelength of 530 nm using a microplate reader (TECAN, NC). A calibration standard containing α -elastin was used to obtain the standard curve for this experiment.

Collagen Extraction Protocol

Sircol Collagen assay kit was used to measure the total soluble collagen within the tissue samples. Three different samples were used to quantify the salt-soluble, acid-

soluble, and pepsin-soluble collagens. Combination of these results yielded the total soluble collagen content.

Salt-Soluble Extraction Protocol

Salt-soluble collagen was extracted by placing a tissue sample in 1ml of 1M NaCl, 0.05M TRIS buffer solution containing protease inhibitors (20mM EDTA, 1mM PMSF, 2.0mM NEM, 1ug/ml of pepstatin). Tissue and buffer solution was incubated overnight on a mechanical shaker at 5°C. Samples were then centrifuged (2000 x g for 10 minutes) to remove particulate matter and the solution was collected for latter analysis.

Acid-Soluble Extraction Protocol

Acid-soluble collagen was extracted by placing a tissue sample in 1ml of 0.5M acetic acid solution. Samples were incubated overnight on an orbital shaker at 5°C. Solution was centrifuged (2000 x g for 10 minutes) and the clear solution was collected for latter analysis.

Pepsin-Soluble Extraction Protocol

Pepsin-soluble collagen was extracted by placing a tissue sample in 1ml of 0.05M acetic acid solution containing pepsin (1mg/10mg tissue sample; Sigma, St. Louis, MO). Samples were incubated overnight on an orbital shaker at 5°C. Solution was centrifuged (2000 x g for 10 minutes) and the clear solution was collected for latter analysis.

Collagen Assay Protocol

Two test samples of 100ul were taken from each extraction solution and placed into a 1.5ml microcentrifuge tube. One ml of Sircol dye reagent was added to each tube and gently shaken on an orbital shaker at room temperature for 30 minutes. Tubes were then centrifuged (8000 x g for 5 minutes) and the supernatants drained and discarded. Additional tapping onto an absorbent paper towel ensured the removal of all dye solution. The collagen bound dye remained as a pellet at the bottom of the tubes. Addition of 1ml of Alkali reagent was now added to each tube and brought into solution using a vortex mixer and two short mixing periods. Aliquots of 100ul were plated onto a 96-well plate and the absorbance was measured at a wavelength of 530 nm using a microplate reader (TECAN, NC). A calibration standard of acid-soluble type I collagen was used to obtain the standard curve for this experiment. ECM contents were normalized to the wet mass of the new tissues for all assays.

Experimental Protocol

Organizing experiments is an iterative process that ultimately leads to optimization of the parameters necessary for identifying engineered tissue characteristics.

Preliminary experiments were done to determine parameters for seeding conditions and optimization of cell attachment to the polymer fibers. Polymer mesh was cut into a disc, 1cm in diameter and placed in a 25cm³ culture flask containing a cell suspension of 20,000 HMVELC cells. Very little attachment was seen after 1 day when analyzed using a phase contrast microscope. Most cells had adhered to the bottom of the

flask and not the scaffold. These results lead to the development of a hydrolysis protocol to improve attachment to the polymer scaffold.

Further experiments were carried out to characterize polymer degradation and properties of engineered tissue constructs. Initially, multiple samples were analyzed over time periods of up to 10 days. Bioreactor conditions ultimately determined the number of samples that we could culture for analysis. Multiple samples were grown for each type of characterization. This required that the same parameters were established to eliminate experimental variations.

APPENDIX B

Original Polymer Samples

Sample	Yield Stress (Pa)	Yield Strain	Elastic Modulus	R-squared
1	131681	0.8611	176651	0.9881
2	124506	0.7972	181309	0.9859
Average	128093 ± 5073	.8292 ± .0418	178980 ± 3293	

Sample 1 (Original Polymer)

Linear region of sample 1

$$y = 176651x - 4821.9$$

$$R^2 = 0.9881$$

Sample 2 (Original Polymer)

Linear region of sample 2

$$y = 181309x - 8391.1$$

$$R^2 = 0.9859$$

Hydrolyzed Polymer Samples

Sample	Yield Stress (Pa)	Yield Strain	Elastic Modulus	R-squared
1	211817	1.168	346324	0.9915
2	234863	1.2407	245858	0.9979
3	301506	1.0815	344697	0.9912
4	160742	1.185	156664	0.991
Average	227232 ± 58404	1.1688 ± .0660	273385 ± 90897	

Sample 1 (Hydrolysis)

Linear region of sample 1

$$y = 346324x - 49367$$

$$R^2 = 0.9915$$

Sample 2 (Hydrolysis)

Linear region of sample 2

$$y = 245858x - 47869$$

$$R^2 = 0.9979$$

Sample 3 (Hydrolysis)

Linear region of sample 3

$$y = 344697x - 53070$$
$$R^2 = 0.9912$$

Sample 4 (Hydrolysis)

Linear region of sample 4

$$y = 156664x - 8709.3$$
$$R^2 = 0.991$$

Hydrolyzed and Media Soaked Polymer Samples

Sample	Yield Stress (Pa)	Yield Strain	Elastic Modulus	R-squared
1	323161	1.1593	367570	0.9979
2	179282	0.8473	264376	0.9936
3	409560	1.2997	391135	0.9954
4	197675	0.8318	301955	0.99
Average	277419 ± 108847	$1.0345 \pm .2324$	331259 ± 58411	

Sample1 (Hydrolyzed and Soaked)

Linear region of sample 1

$$y = 367570x - 57151$$

$$R^2 = 0.9979$$

Sample 2 (Hydrolyzed and Soaked)

Linear region of sample 2

$$y = 264376x - 34072$$

$$R^2 = 0.9936$$

Sample 3 (Hydrolyzed and Soaked)

Linear region of sample 3

$$y = 391135x - 92792$$

$$R^2 = 0.9954$$

Sample 4 (Hydrolyzed and Soaked)

Linear region of sample 4

$$y = 301955x - 37870$$

$$R^2 = 0.99$$

2-Day Dynamic Cultured Blank Polymer Samples

Sample	Yield Stress (Pa)	Yield Strain	Elastic Modulus	R-squared
1	48335	0.7589	88027	0.9698
2	26936	0.7765	57473	0.8747
3	37362	0.5529	52392	0.7012
4	20872	0.7543	64005	0.8728
5	32200	0.4406	69979	0.8856
Average	33141 ± 10471	0.6566 ± .1515	66375 ± 13803	

Sample 1 (2-Day polymer)

Linear region of sample 1

$$y = 88027x - 18046$$

$$R^2 = 0.9698$$

Sample 2 (2-Day polymer)

Linear region of sample 2

$$y = 57473x - 8394.3$$

$$R^2 = 0.8747$$

Sample 3 (2-Day polymer)

Linear region of sample 3

$$y = 52392x - 9242.4$$
$$R^2 = 0.7012$$

Sample 4 (2-Day polymer)

Linear region of sample 4

$$y = 64005x - 2493.2$$
$$R^2 = 0.8728$$

Sample 5 (2-day polymer)

Linear region of sample 5

$$y = 69979x + 3505.7$$
$$R^2 = 0.8856$$

3-Day Dynamic Cultured Blank Polymer Samples

Sample	Yield Stress (Pa)	Yield Strain	Elastic Modulus	R-squared
1	25329	0.4391	58650	0.7715
2	35682	0.5756	70152	0.938
3	38265	0.4814	59115	0.879
4	27002	0.5882	59161	0.9425
5	39607	0.7872	51831	0.8913
Average	33177 ± 6580	.5743 ± .1346	59781 ± 6574	

Sample 1 (3-Day polymer)

Linear region of sample 1

$$y = 58650x + 484.76$$

$$R^2 = 0.7715$$

Sample 2 (3-Day polymer)

Linear region of sample 2

$$y = 70152x - 3448.8$$

$$R^2 = 0.938$$

Sample 3 (3-Day polymer)

Linear region of sample 3

$$y = 59115x - 683.91$$
$$R^2 = 0.879$$

Sample 4 (3-Day polymer)

Linear region of sample 4

$$y = 59161x + 4296.5$$
$$R^2 = 0.9425$$

Sample 5 (3-Day polymer)

Linear region of sample 5

$$y = 51831x - 3000.2$$
$$R^2 = 0.8913$$

4-Day Dynamic Cultured Blank Polymer Samples

Sample	Yield Stress (Pa)	Yield Strain	Elastic Modulus	R-squared
1	38945	0.9817	42412	0.9328
2	42643	0.5332	94443	0.9153
3	25761	0.6321	48091	0.7986
4	42877	0.5081	121614	0.9192
5	46341	0.5708	107006	0.9553
Average	39313 ± 8015	0.6452 ± .1938	82713 ± 35580	

Sample 1 (4-Day polymer)

Linear region of sample 1

Sample 2 (4-Day polymer)

Linear region of sample 2

Sample 3 (4-Day polymer)

Linear region of sample 3

$$y = 48091x - 5204.5$$
$$R^2 = 0.7986$$

Sample 4 (4-Day polymer)

Linear region of sample 4

$$y = 121614x - 18936$$
$$R^2 = 0.9192$$

Sample 5 (4-Day polymer)

Linear region of sample 5

$$y = 107006x - 5014.3$$
$$R^2 = 0.9553$$

5-Day Dynamic Cultured Blank Polymer Scaffold Samples

Sample	Yield Stress (Pa)	Yield Strain	Elastic Modulus	R-squared
1	55552	0.6148	87294	0.9679
2	46992	0.5756	121423	0.8283
3	44247	0.4893	96396	0.8684
4	59482	0.7496	96757	0.9085
5	34478	0.5614	61504	0.7812
Average	48150 ± 9832	$0.5981 \pm .0960$	92674 ± 21549	

Sample 1 (5-Day polymer)

Linear region of sample 1

$$y = 87294x - 336.97$$

$$R^2 = 0.9679$$

Sample 2 (5-Day polymer)

Linear region of sample 2

$$y = 121423x - 11335$$

$$R^2 = 0.8283$$

Sample 3 (5-Day polymer)

Linear region of sample 3

$$y = 96396x - 2210.3$$
$$R^2 = 0.8684$$

Sample 4 (5-Day polymer)

Linear region of sample 4

$$y = 96757x - 1939.6$$
$$R^2 = 0.9085$$

Sample 5 (5-Day polymer)

Linear region of sample 5

$$y = 61504x - 1473.2$$
$$R^2 = 0.7812$$

7-Day Cultured Blank Polymer Scaffold Samples

Sample	Yield Stress (Pa)	Yield Strain	Elastic Modulus	R-squared
1	52164	0.1505	309052	0.6729
2	63323	0.3587	221161	0.702
3	19775	0.102	0	0
4	18696	0.1946	0	0
5	73072	0.2614	460543	0.5001
Average	45406 ± 25012	0.21344 ± .10017	198151 ± 200128	

Sample 1 (7-day polymer)

Linear Region of sample 1 $y = 309052x - 419.68$
 $R^2 = 0.6729$

Sample 2 (7-Day polymer)

Linear region of sample 2 $y = 221161x - 31855$
 $R^2 = 0.702$

Sample 3 (7-Day polymer)

NO LINEAR REGION $E=0$

Sample 4 (7-Day polymer)

NO LINEAR REGION $E=0$

Sample 5 (7-Day polymer)

Linear region of sample 5 $y = 460543x - 59927$
 $R^2 = 0.5001$

IMR-90 Tissue Construct Samples

Sample	Yield Stress (Pa)	Yield Strain	Elastic Modulus	R-squared
1	55057	0.0794	946352	0.9841
2	8614	0.1144	134880	0.6971
3	13021	0.1776	88999	0.8884
Average	25564 ± 25636	.1238 ± .0498	390077 ± 482294	

Sample 1 (IMR-90 7 Day)

Linear region of sample 1

Sample 2 (IMR-90 7 Day)

Linear region of sample 2

Sample 3 (IMR-90 7 Day)

Linear region of sample 3

$$y = 88999x + 606.63$$
$$R^2 = 0.8884$$

APPENDIX C

Elastic Modulus Data and Welch's t-tests

org	hyd	treat	imr90	2	3	4	5	7	
176651	346324	367570	946352	88027	58650	42412	87294	309052	
181309	245858	264376	134880	57473	70152	94443	121423	221161	
	344697	391135	88999	52392	59115	48091	96396	0	
	156664	301955		64005	59161	121614	96757	0	
				69979	51831	107006	61504	460543	
	o	h	t	2	3	4	5	7	imr
o	X	A	R	R	R	R	R	A	A
h	A	X	A	R	R	R	R	A	A
t	R	A	X	R	R	R	R	A	A
2	R	R	R	X	A	A	A	A	A
3	R	R	R	A	X	A	R	A	A
4	R	R	R	A	A	X	A	A	A
5	R	R	R	A	R	A	X	A	A
7	A	A	A	A	A	A	A	X	A
imr	A	A	A	A	A	A	A	A	X

Elastic Modulus Welch's t-tests

ACCEPT

	O	H
Mean	178980	273385.8
Variance	10848482	8.26E+09
Observatio	2	4
Hypothesiz	0	
df	3	
t Stat	-2.074471	
P(T<=t) on	0.064843	
t Critical or	2.353363	
P(T<=t) tw	0.129686	
t Critical tw	3.182449	

ACCEPT

	O	IMR
Mean	178980	390077
Variance	10848482	2.33E+11
Observatio	2	3
Hypothesiz	0	
df	2	
t Stat	-0.758081	
P(T<=t) on	0.263776	
t Critical or	2.919987	
P(T<=t) tw	0.527553	
t Critical tw	4.302656	

ACCEPT

	T	IMR
Mean	331259	390077
Variance	3.41E+09	2.33E+11
Observatio	4	3
Hypothesiz	0	
df	2	
t Stat	-0.210079	
P(T<=t) on	0.426532	
t Critical or	2.919987	
P(T<=t) tw	0.853064	
t Critical tw	4.302656	

ACCEPT

	H	T
Mean	273385.8	331259
Variance	8.26E+09	3.41E+09
Observatio	4	4
Hypothesiz	0	
df	5	
t Stat	-1.071257	
P(T<=t) on	0.166508	
t Critical or	2.015049	
P(T<=t) tw	0.333016	
t Critical tw	2.570578	

ACCEPT

	7	IMR
Mean	198151.2	390077
Variance	4.01E+10	2.33E+11
Observatio	5	3
Hypothesiz	0	
df	2	
t Stat	-0.656195	
P(T<=t) on	0.289551	
t Critical or	2.919987	
P(T<=t) tw	0.579102	
t Critical tw	4.302656	

ACCEPT

	H	IMR
Mean	273385.8	390077
Variance	8.26E+09	2.33E+11
Observatio	4	3
Hypothesiz	0	
df	2	
t Stat	-0.413597	
P(T<=t) on	0.35965	
t Critical or	2.919987	
P(T<=t) tw	0.719301	
t Critical tw	4.302656	

REJECT

	0	4
Mean	178980	82713.2
Variance	10848482	1.27E+09
Observatio	2	5
Hypothesiz	0	
df	4	
t Stat	5.986139	
P(T<=t) on	0.001958	
t Critical or	2.131846	
P(T<=t) tw	0.003916	
t Critical tw	2.776451	

REJECT

	H	4
Mean	273385.8	82713.2
Variance	8.26E+09	1.27E+09
Observatio	4	5
Hypothesiz	0	
df	4	
t Stat	3.959665	
P(T<=t) on	0.008341	
t Critical or	2.131846	
P(T<=t) tw	0.016682	
t Critical tw	2.776451	

REJECT

	T	4
Mean	331259	82713.2
Variance	3.41E+09	1.27E+09
Observatio	4	5
Hypothesiz	0	
df	5	
t Stat	7.473016	
P(T<=t) on	0.000339	
t Critical or	2.015049	
P(T<=t) tw	0.000678	
t Critical tw	2.570578	

REJECT

	O	3
Mean	178980	59781.8
Variance	10848482	43216814
Observatio	2	5
Hypothesiz	0	
df	4	
t Stat	31.78042	
P(T<=t) on	2.92E-06	
t Critical or	2.131846	
P(T<=t) tw	5.84E-06	
t Critical tw	2.776451	

REJECT

	H	5
Mean	273385.8	92674.8
Variance	8.26E+09	4.64E+08
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	3.889665	
P(T<=t) on	0.015064	
t Critical or	2.353363	
P(T<=t) tw	0.030128	
t Critical tw	3.182449	

REJECT

	T	3
Mean	331259	59781.8
Variance	3.41E+09	43216814
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	9.248605	
P(T<=t) on	0.001337	
t Critical or	2.353363	
P(T<=t) tw	0.002675	
t Critical tw	3.182449	

ACCEPT

	2	5
Mean	66375.2	92674.8
Variance	1.91E+08	4.64E+08
Observatio	5	5
Hypothesiz	0	
df	7	
t Stat	-2.297926	
P(T<=t) on	0.02758	
t Critical or	1.894578	
P(T<=t) tw	0.055159	
t Critical tw	2.364623	

ACCEPT

	5	4
Mean	92674.8	82713.2
Variance	4.64E+08	1.27E+09
Observatio	5	5
Hypothesiz	0	
df	7	
t Stat	0.535484	
P(T<=t) on	0.30445	
t Critical or	1.894578	
P(T<=t) tw	0.608899	
t Critical tw	2.364623	

ACCEPT

	3	IMR
Mean	59781.8	390077
Variance	43216814	2.33E+11
Observatio	5	3
Hypothesiz	0	
df	2	
t Stat	-1.186115	
P(T<=t) on	0.178694	
t Critical or	2.919987	
P(T<=t) tw	0.357388	
t Critical tw	4.302656	

ACCEPT

	2	7
Mean	66375.2	198151.2
Variance	1.91E+08	4.01E+10
Observatio	5	5
Hypothesiz	0	
df	4	
t Stat	-1.468864	
P(T<=t) on	0.107898	
t Critical or	2.131846	
P(T<=t) tw	0.215796	
t Critical tw	2.776451	

ACCEPT

	4	7
Mean	82713.2	198151.2
Variance	1.27E+09	4.01E+10
Observatio	5	5
Hypothesiz	0	
df	4	
t Stat	-1.269894	
P(T<=t) on	0.136485	
t Critical or	2.131846	
P(T<=t) tw	0.27297	
t Critical tw	2.776451	

REJECT

	O	T
Mean	331259	178980
Variance	3.41E+09	10848482
Observatio	4	2
Hypothesiz	0	
df	3	
t Stat	5.197514	
P(T<=t) on	0.006918	
t Critical or	2.353363	
P(T<=t) tw	0.013837	
t Critical tw	3.182449	

ACCEPT

	5	IMR
Mean	92674.8	390077
Variance	4.64E+08	2.33E+11
Observatio	5	3
Hypothesiz	0	
df	2	
t Stat	-1.067414	
P(T<=t) on	0.198782	
t Critical or	2.919987	
P(T<=t) tw	0.397563	
t Critical tw	4.302656	

REJECT

	0	2
Mean	178980	66375.2
Variance	10848482	1.91E+08
Observatio	2	5
Hypothesiz	0	
df	5	
t Stat	17.06653	
P(T<=t) on	6.32E-06	
t Critical or	2.015049	
P(T<=t) tw	1.26E-05	
t Critical tw	2.570578	

REJECT

	H	3
Mean	273385.8	59781.8
Variance	8.26E+09	43216814
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	4.690087	
P(T<=t) on	0.009163	
t Critical or	2.353363	
P(T<=t) tw	0.018325	
t Critical tw	3.182449	

ACCEPT

	4	IMR
Mean	82713.2	390077
Variance	1.27E+09	2.33E+11
Observatio	5	3
Hypothesiz	0	
df	2	
t Stat	-1.10203	
P(T<=t) on	0.192667	
t Critical or	2.919987	
P(T<=t) tw	0.385335	
t Critical tw	4.302656	

ACCEPT

	0	7
Mean	178980	198151.2
Variance	10848482	4.01E+10
Observatio	2	5
Hypothesiz	0	
df	4	
t Stat	-0.21413	
P(T<=t) on	0.420459	
t Critical or	2.131846	
P(T<=t) tw	0.840918	
t Critical tw	2.776451	

REJECT

	H	2
Mean	273385.8	66375.2
Variance	8.26E+09	1.91E+08
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	4.513372	
P(T<=t) on	0.010164	
t Critical or	2.353363	
P(T<=t) tw	0.020328	
t Critical tw	3.182449	

ACCEPT

	H	7
Mean	273385.8	198151.2
Variance	8.26E+09	4.01E+10
Observatio	4	5
Hypothesiz	0	
df	6	
t Stat	0.749508	
P(T<=t) on	0.240947	
t Critical or	1.943181	
P(T<=t) tw	0.481893	
t Critical tw	2.446914	

REJECT

	T	2
Mean	331259	66375.2
Variance	3.41E+09	1.91E+08
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	8.873528	
P(T<=t) on	0.001509	
t Critical or	2.353363	
P(T<=t) tw	0.003018	
t Critical tw	3.182449	

ACCEPT

	3	4
Mean	59781.8	82713.2
Variance	43216814	1.27E+09
Observatio	5	5
Hypothesiz	0	
df	4	
t Stat	-1.417146	
P(T<=t) on	0.114701	
t Critical or	2.131846	
P(T<=t) tw	0.229403	
t Critical tw	2.776451	

ACCEPT

	T	7
Mean	331259	198151.2
Variance	3.41E+09	4.01E+10
Observatio	4	5
Hypothesiz	0	
df	5	
t Stat	1.413861	
P(T<=t) on	0.108267	
t Critical or	2.015049	
P(T<=t) tw	0.216535	
t Critical tw	2.570578	

ACCEPT

	2	3
Mean	66375.2	59781.8
Variance	1.91E+08	43216814
Observatio	5	5
Hypothesiz	0	
df	6	
t Stat	0.964288	
P(T<=t) on	0.186071	
t Critical or	1.943181	
P(T<=t) tw	0.372141	
t Critical tw	2.446914	

REJECT

	5	3
Mean	92674.8	59781.8
Variance	4.64E+08	43216814
Observatio	5	5
Hypothesiz	0	
df	5	
t Stat	3.264575	
P(T<=t) on	0.011166	
t Critical or	2.015049	
P(T<=t) tw	0.022332	
t Critical tw	2.570578	

ACCEPT

	3	7
Mean	59781.8	198151.2
Variance	43216814	4.01E+10
Observatio	5	5
Hypothesiz	0	
df	4	
t Stat	-1.54519	
P(T<=t) on	0.098595	
t Critical or	2.131846	
P(T<=t) tw	0.197191	
t Critical tw	2.776451	

ACCEPT

	2	IMR
Mean	66375.2	390077
Variance	1.91E+08	2.33E+11
Observatio	5	3
Hypothesiz	0	
df	2	
t Stat	-1.162216	
P(T<=t) on	0.182542	
t Critical or	2.919987	
P(T<=t) tw	0.365084	
t Critical tw	4.302656	

REJECT

	O	5
Mean	178980	92674.8
Variance	10848482	4.64E+08
Observatio	2	5
Hypothesiz	0	
df	4	
t Stat	8.704768	
P(T<=t) on	0.00048	
t Critical or	2.131846	
P(T<=t) tw	0.000959	
t Critical tw	2.776451	

ACCEPT

	5	7
Mean	92674.8	198151.2
Variance	4.64E+08	4.01E+10
Observatio	5	5
Hypothesiz	0	
df	4	
t Stat	-1.171731	
P(T<=t) on	0.153167	
t Critical or	2.131846	
P(T<=t) tw	0.306335	
t Critical tw	2.776451	

ACCEPT

	2	4
Mean	66375.2	82713.2
Variance	1.91E+08	1.27E+09
Observatio	5	5
Hypothesiz	0	
df	5	
t Stat	-0.957252	
P(T<=t) on	0.191201	
t Critical or	2.015049	
P(T<=t) tw	0.382403	
t Critical tw	2.570578	

REJECT

	T	5
Mean	331259	92674.8
Variance	3.41E+09	4.64E+08
Observatio	4	5
Hypothesiz	0	
df	4	
t Stat	7.757653	
P(T<=t) on	0.000744	
t Critical or	2.131846	
P(T<=t) tw	0.001488	
t Critical tw	2.776451	

Yield Stress Data and Welch's t-test Results

org y.s.	hyd y.s.	treat y.s.	imr90y.s.	5dayys	4dayys	2dayys	3dayys	7dayys	
131681	211817	323161	55057	55552	38945	48335	25329	52164	
124506	234863	179282	8614	46992	42643	26936	35682	63323	
	301506	409560	13021	44247	25761	37362	38265	19775	
	160742	197675		59482	42877	20872	27002	18696	
				34478	46341	32200	39607	73072	
	org	hyd	treat	2	3	4	5	7	IMR90
org	x	a	a	r	r	r	r	r	r
hyd	a	x	a	r	r	r	r	r	r
treat	a	a	x	r	r	r	r	r	r
2	r	r	r	x	r	r	r	r	a
3	r	r	r	r	x	r	r	r	a
4	r	r	r	r	r	x	r	r	a
5	r	r	r	r	r	r	x	r	a
7	r	r	r	r	r	r	r	x	a
imr90	r	r	r	a	a	a	a	a	x

Yield-Stress Welch's t-test results

accept

	<i>org</i>	<i>hyd</i>
Mean	128093.5	227232
Variance	25740313	3.41E+09
Observatio	2	4
Hypothesiz	0	
df	3	
t Stat	-3.369537	
P(T<=t) on	0.021713	
t Critical or	2.353363	
P(T<=t) tw	0.043426	
t Critical tw	3.182449	

accept

	<i>org</i>	<i>treat</i>
Mean	128093.5	277419.5
Variance	25740313	1.18E+10
Observatio	2	4
Hypothesiz	0	
df	3	
t Stat	-2.737815	
P(T<=t) on	0.035735	
t Critical or	2.353363	
P(T<=t) tw	0.071469	
t Critical tw	3.182449	

accept

	<i>hyd</i>	<i>treat</i>
Mean	128093.5	277419.5
Variance	25740313	1.18E+10
Observatio	2	4
Hypothesiz	0	
df	3	
t Stat	-2.737815	
P(T<=t) on	0.035735	
t Critical or	2.353363	
P(T<=t) tw	0.071469	
t Critical tw	3.182449	

reject

	<i>hyd</i>	<i>imr90</i>
Mean	227232	25564
Variance	3.41E+09	6.57E+08
Observatio	4	3
Hypothesiz	0	
df	4	
t Stat	6.159816	
P(T<=t) on	0.001763	
t Critical or	2.131846	
P(T<=t) tw	0.003525	
t Critical tw	2.776451	

reject

	<i>treat</i>	<i>imr90</i>
Mean	277419.5	25564
Variance	1.18E+10	6.57E+08
Observatio	4	3
Hypothesiz	0	
df	3	
t Stat	4.465467	
P(T<=t) on	0.010459	
t Critical or	2.353363	
P(T<=t) tw	0.020919	
t Critical tw	3.182449	

reject

	<i>org</i>	<i>5day</i>
Mean	128093.5	48150.2
Variance	25740313	96675446
Observatio	2	5
Hypothesiz	0	
df	4	
t Stat	14.08701	
P(T<=t) on	7.37E-05	
t Critical or	2.131846	
P(T<=t) tw	0.000147	
t Critical tw	2.776451	

reject

	<i>org</i>	<i>imr90</i>
Mean	128093.5	25564
Variance	25740313	6.57E+08
Observatio	2	3
Hypothesiz	0	
df	2	
t Stat	6.732148	
P(T<=t) on	0.01068	
t Critical or	2.919987	
P(T<=t) tw	0.02136	
t Critical tw	4.302656	

reject

	<i>hyd</i>	<i>4day</i>
Mean	227232	39313.4
Variance	3.41E+09	64243977
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	6.38709	
P(T<=t) on	0.003886	
t Critical or	2.353363	
P(T<=t) tw	0.007772	
t Critical tw	3.182449	

accept

	<i>2day</i>	<i>imr</i>
Mean	33141	25564
Variance	1.1E+08	6.57E+08
Observatio	5	3
Hypothesiz	0	
df	2	
t Stat	0.48807	
P(T<=t) on	0.336882	
t Critical or	2.919987	
P(T<=t) tw	0.673764	
t Critical tw	4.302656	

reject

	<i>hyd</i>	<i>3day</i>
Mean	227232	33177
Variance	3.41E+09	43307350
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	6.611669	
P(T<=t) on	0.003522	
t Critical or	2.353363	
P(T<=t) tw	0.007045	
t Critical tw	3.182449	

accept

	<i>3day</i>	<i>imr</i>
Mean	33177	25564
Variance	43307350	6.57E+08
Observatio	5	3
Hypothesiz	0	
df	2	
t Stat	0.504472	
P(T<=t) on	0.33201	
t Critical or	2.919987	
P(T<=t) tw	0.664021	
t Critical tw	4.302656	

reject

	<i>treat</i>	<i>7day</i>
Mean	277419.5	45406
Variance	1.18E+10	6.26E+08
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	4.175792	
P(T<=t) on	0.012506	
t Critical or	2.353363	
P(T<=t) tw	0.025012	
t Critical tw	3.182449	

accept

	5day	imr90
Mean	48150.2	25564
Variance	96675446	6.57E+08
Observatio	5	3
Hypothesiz	0	
df	2	
t Stat	1.462778	
P(T<=t) on	0.14053	
t Critical or	2.919987	
P(T<=t) tw	0.28106	
t Critical tw	4.302656	

reject

	org	2day
Mean	128093.5	33141
Variance	25740313	1.1E+08
Observatio	2	5
Hypothesiz	0	
df	4	
t Stat	16.09603	
P(T<=t) on	4.36E-05	
t Critical or	2.131846	
P(T<=t) tw	8.71E-05	
t Critical tw	2.776451	

reject

	org	7day
Mean	128093.5	45406
Variance	25740313	6.26E+08
Observatio	2	5
Hypothesiz	0	
df	5	
t Stat	7.03899	
P(T<=t) on	0.000447	
t Critical or	2.015049	
P(T<=t) tw	0.000894	
t Critical tw	2.570578	

reject

	hyd	2day
Mean	227232	33141
Variance	3.41E+09	1.1E+08
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	6.56255	
P(T<=t) on	0.003598	
t Critical or	2.353363	
P(T<=t) tw	0.007196	
t Critical tw	3.182449	

reject

	org	3day
Mean	128093.5	33177
Variance	25740313	43307350
Observatio	2	5
Hypothesiz	0	
df	3	
t Stat	20.45518	
P(T<=t) on	0.000128	
t Critical or	2.353363	
P(T<=t) tw	0.000255	
t Critical tw	3.182449	

accept

	7day	imr90
Mean	45406	25564
Variance	6.26E+08	6.57E+08
Observatio	5	3
Hypothesiz	0	
df	4	
t Stat	1.069496	
P(T<=t) on	0.172545	
t Critical or	2.131846	
P(T<=t) tw	0.345091	
t Critical tw	2.776451	

reject

	<i>hyd</i>	<i>5day</i>
Mean	227232	48150.2
Variance	3.41E+09	96675446
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	6.064063	
P(T<=t) on	0.0045	
t Critical or	2.353363	
P(T<=t) tw	0.008999	
t Critical tw	3.182449	

reject

	<i>treat</i>	<i>3day</i>
Mean	277419.5	33177
Variance	1.18E+10	43307350
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	4.481232	
P(T<=t) on	0.010361	
t Critical or	2.353363	
P(T<=t) tw	0.020722	
t Critical tw	3.182449	

accept

	<i>4day</i>	<i>imr</i>
Mean	39313.4	25564
Variance	64243977	6.57E+08
Observatio	5	3
Hypothesiz	0	
df	2	
t Stat	0.902835	
P(T<=t) on	0.230951	
t Critical or	2.919987	
P(T<=t) tw	0.461903	
t Critical tw	4.302656	

reject

	<i>hyd</i>	<i>7day</i>
Mean	227232	45406
Variance	3.41E+09	6.26E+08
Observatio	4	5
Hypothesiz	0	
df	4	
t Stat	5.814431	
P(T<=t) on	0.002178	
t Critical or	2.131846	
P(T<=t) tw	0.004355	
t Critical tw	2.776451	

reject

	<i>treat</i>	<i>4day</i>
Mean	277419.5	39313.4
Variance	1.18E+10	64243977
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	4.365568	
P(T<=t) on	0.011113	
t Critical or	2.353363	
P(T<=t) tw	0.022225	
t Critical tw	3.182449	

reject

	<i>treat</i>	<i>2day</i>
Mean	277419.5	33141
Variance	1.18E+10	1.1E+08
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	4.471917	
P(T<=t) on	0.010419	
t Critical or	2.353363	
P(T<=t) tw	0.020838	
t Critical tw	3.182449	

reject

	<i>treat</i>	<i>5day</i>
Mean	277419.5	48150.2
Variance	1.18E+10	96675446
Observatio	4	5
Hypothesiz	0	
df	3	
t Stat	4.198974	
P(T<=t) on	0.012324	
t Critical or	2.353363	
P(T<=t) tw	0.024648	
t Critical tw	3.182449	

reject

	<i>org</i>	<i>4day</i>
Mean	128093.5	39313.4
Variance	25740313	64243977
Observatio	2	5
Hypothesiz	0	
df	3	
t Stat	17.50608	
P(T<=t) on	0.000203	
t Critical or	2.353363	
P(T<=t) tw	0.000406	
t Critical tw	3.182449	