A FOUNDATIONAL STUDY OF HEART VALVE TISSUE ENGINEERING:
Exploration of Natural and Synthetic Scaffold Approaches

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ABSTRACT

The objective of this project was to explore the possibilities of two different approaches to heart valve tissue engineering: naturally-derived and synthetic scaffolds. Since it has been shown that the mechanical properties of the native heart valve leaflet derive principally from collagen orientation, this project focused on collagen and cellular structures that were produced through each approach. In order to establish defined goals for the structural properties of the engineered constructs the first objective of this thesis was to obtain the mechanical properties and gain an appreciation of the structural properties of the native heart valve leaflets.

This research investigated the cellular orientation within collagen matrigel scaffolds (i.e. a naturally-derived scaffold) and micromolded poly(glycerol sebacate) scaffolds (i.e. a synthetic scaffold). Fiber tracking software along with fast-Fourier transform-based software was used to analyze these orientations.

In the case of the naturally-derived collagen gel scaffolds, a distinct difference in collagen and F-actin orientations were found between biaxially- and uniaxially-constrained gels. Specifically, a degree of orientation was seen in the uniaxial case, whereas a more random distribution of orientations was seen in the biaxial case. As for the synthetic, microfabricated PGS scaffolds, it was found that cells would preferentially align along the long-axis of the microfabricated scaffold pores. Collectively these results provide a foundation for future studies in heart valve tissue engineering exploring ways to mimic the native collagen and cell orientations in tissue constructs.
# TABLE OF CONTENTS

List of Figures and Tables........................................................................................................ iv
Acknowledgements....................................................................................................................... v

Chapter 1. INTRODUCTION ........................................................................................................ 1
  1.1 Motivation and Background .............................................................................................. 1
  1.2 Objectives .......................................................................................................................... 2
  1.3 Scope of Research .............................................................................................................. 3
  1.4 Thesis Organization ......................................................................................................... 4

Chapter 2. A BRIEF REVIEW OF HEART VALVES, VALVULAR DISEASE, AND VALVE REPLACEMENT .................................................................................................................. 5
  2.1 Anatomy ............................................................................................................................. 5
     2.1.1 General Anatomy ........................................................................................................ 5
     2.1.2 Collagen Structure and Orientation of Semilunar Valves ........................................... 8
  2.2 Heart Valve Disease and Replacement ............................................................................. 10
     2.2.1 Heart Valve Disease .................................................................................................. 10
     2.2.2 Current Valvular Repair Techniques ....................................................................... 11
     2.2.3 Current Replacement Heart Valves ......................................................................... 11
     2.2.4 Failure in Bioprosthetic Replacements .................................................................... 14
  2.3 Approaches to Heart Valve Tissue Engineering ............................................................... 15
     2.3.1 Example 1: Synthetic, Biodegradable Scaffolds ....................................................... 16
     2.3.2 Example 2: Naturally-derived Scaffolds .................................................................. 18
     2.3.3 Example 3: Decellularized Scaffolds ....................................................................... 21
  2.4 Summary ............................................................................................................................ 23

Chapter 3. OBJECTIVE 1: Mechanical Characterization of Native Heart Valve Leaflets .26
  3.1 Materials of Methods ....................................................................................................... 26
     3.1.1 Porcine Heart Valve Leaflet Excision and Mechanical Test Preparation .................. 26
     3.1.2 Mechanical Testing .................................................................................................. 27
     3.1.3 Quantifying Local Collagen Morphology .................................................................. 28
  3.2 Results .............................................................................................................................. 29
     3.2.1 Native Mechanical Properties ............................................................................... 29
     3.2.2 Native Collagen Morphology .................................................................................. 30
  3.3 Discussion .......................................................................................................................... 32
Chapter 4. OBJECTIVE 2: Analysis of Collagen and F-actin Orientations in Naturally-Derived Collagen Gel Scaffolds ................................................................. 34
  4.1 Materials of Methods ........................................................................ 34
    4.1.1 Model System ........................................................................ 34
    4.1.2 Tissue Constructs .................................................................... 35
  4.1.3 Visualization of Collagen and F-actin ........................................... 36
  4.1.4 Quantification of Collagen and F-actin Orientation ....................... 37
  4.2 Results......................................................................................... 37
    4.2.1 Collagen Alignment .................................................................. 37
    4.2.2 F-actin Alignment .................................................................... 39
  4.3 Discussion .................................................................................. 41
    4.3.1 Tissue Constructs .................................................................... 42
    4.3.2 Collagen Imaging ..................................................................... 43
    4.3.3 F-actin Reorientation ............................................................... 43
    4.3.4 Cellular Structures ................................................................. 44

Chapter 5. OBJECTIVE 3: Analysis of Cell Orientation in Synthetic, Micromolded Poly(glycerol sebacate) Scaffolds ............................................. 45
  5.1 Materials and Methods .................................................................... 45
    5.1.1 Micromolding of PGS Scaffolds ................................................ 45
    5.1.2 PGS Curing ............................................................................. 46
    5.1.3 Valvular Interstitial Cell Isolation, Expansion and Seeding ........... 48
    5.1.4 F-actin Imaging of the PGS Scaffolds to Visualize Cell Orientation 50
    5.1.5 Quantifying F-actin Orientation ................................................ 51
    5.1.6 Collagen and DNA Assays ....................................................... 52
    5.1.7 Statically Stretched Scaffolds .................................................... 53
  5.2 Results....................................................................................... 54
    5.2.1 F-actin Orientation ................................................................. 54
    5.2.2 Biochemical Assays ............................................................... 57
    5.2.3 Statically Stretched Scaffolds .................................................... 58
  5.3 Discussion ................................................................................ 59
    5.3.1 F-actin Orientation ................................................................. 59
    5.3.2 Biochemical Assays ............................................................... 62
    5.3.3 Statically Stretched Scaffolds .................................................... 64

Chapter 6. CONCLUSION ...................................................................... 65

References ..................................................................................... 66

Academic Vita
LIST OF FIGURES AND TABLES

Chapter 2
Figure 2.1: Diagram of the Heart ................................................................. 6
Figure 2.2: Anatomy of the Semilunar Valves ............................................. 7
Figure 2.3: Polarized Light Microscopy Images of Fiber Architecture Maps ........ 9
Figure 2.4: Mechanical and Bioprosthetic Heart Valves .............................. 12
Figure 2.5: Various Construct Geometries ................................................ 19
Figure 2.6: Pictures of Porcine Valve Leaflets ............................................ 23

Chapter 3
Figure 3.1: Radial and Circumferential Directions .................................... 28
Figure 3.2: Stress-Strain Plots for Native Porcine Heart Valve Leaflets ........... 29
Table 3.1: Mechanical Data Averages for Native Porcine Heart Valve Leaflets 29
Figure 3.3: Native Collagen Morphology .................................................. 30
Figure 3.4: General Collagen Orientations for a Native Pulmonary Porcine Leaflet 31

Chapter 4
Figure 4.1: Contracted Tissue Before and After Release ............................ 35
Figure 4.2: Time Lapse with CTB and CNA ............................................. 38
Figure 4.3: Bimodal Curves for Collagen in Tissue Constructs ..................... 38
Table 4.1: A Summary of Observations made at Time Points from 4 Tissue Samples 39
Figure 4.4: Time Lapse with Phalloidin and DAPI ................................... 40
Figure 4.5: Bimodal Curves for F-actin in Tissue Constructs ........................ 40
Figure 4.6: CTB Images ........................................................................ 42
Figure 4.7: Initial Collage Orientation in Contracted Tissues ....................... 43

Chapter 5
Figure 5.1: Ceramic Micromold ............................................................... 46
Figure 5.2: Micromolding Technique ....................................................... 48
Figure 5.3: F-actin Orientation Index Averages ........................................... 54
Figure 5.4: Aortic Valve Interstitial Cell Seeded Scaffold Orientation Images .... 55
Figure 5.5: F-actin Orientation Distribution for an Aortic VIC Seeded Scaffold 55
Figure 5.6: Pulmonary Cell Seeded Scaffold Orientation Images ................. 56
Figure 5.7: F-actin Orientation Distribution for a Pulmonary VIC Seeded Scaffold 56
Table 5.1: Averages for Biochemical Assays ............................................ 58
Figure 5.8: Biochemical Assay Graphs ..................................................... 58
Figure 5.9: Light Microscope Images of 10% Stretched Scaffolds .................. 59
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I. INTRODUCTION

1.1 Motivation and Background

Human heart valves (i.e., aortic, pulmonary, mitral, and tricuspid) are vital components of the heart as they regulate and allow the heart to pump blood unidirectionally throughout the circulatory system to specific parts of the body. Unfortunately, these heart valves are often prone to disease and malfunction. In fact, in the United States alone around 99,000 heart valve surgeries are performed each year [1]. While current replacement heart valves, including mechanical and bioprosthetic valves, are lifesaving and perform relatively well, they present several drawbacks and limitations, including thrombogenicity (mechanical) and structural degeneration (bioprosthetic). Thus a major challenge in bioengineering is to find better methods for the replacement of heart valves; one promising approach is tissue engineering. Since heart valves are subject to a cyclic blood flow that induces pressures and mechanical stresses, it is crucial that any replacement be done in such a way that it provides sufficient mechanical and structural support to withstand the shear forces from the blood flow and the mechanical forces of opening and closing. Indeed, the native heart valve leaflet tissue structures and mechanical properties are optimized for this demanding environment [2]. Interestingly, in the context of tissue engineering, these mechanical factors can potentially be used to promote improved tissue structural and associated mechanical properties.

It is critically important that investigators first understand the structural characteristics and mechanical properties of a native tissue before constructing a tissue engineering scaffold. Thus it has been shown that the mechanical properties of the native heart valve leaflets derive principally from collagen orientation [3]. Consequently, scaffolds are often constructed in such a
way as to facilitate new tissue formation that will mimic the native tissue as closely as possible [4]. Structural properties, such as the orientation of the extracellular matrix (i.e. collagen and elastin fibers) and cellular orientations within the tissue are two important areas of interest when designing scaffolds. The motivation behind this research was to determine what type of scaffold might be best suited for mimicking the native heart valve leaflet structure.

1.2 Objectives

Toward understanding the native heart valve leaflet, the first objective of this research was to quantify the anisotropic (i.e. directionally-dependent) mechanical properties of porcine aortic and pulmonary heart valve leaflets. In particular, leaflets were excised from fresh porcine hearts, trimmed into specimens suitable for mechanical testing, and uniaxial tensile stress-strain curves were generated for both the circumferential and radial directions. In addition, an effort was made to apply confocal reflectance microscopy to the native heart valve leaflets in order to gain an appreciation for the collagen morphology (i.e. orientation). The mechanical properties of the native porcine heart valve leaflets provide guidance for the design of tissue engineered heart valve leaflets.

In the second and third objectives of this thesis, two distinct engineered heart valve tissues were investigated. One was based on a naturally-derived scaffold (i.e., collagen gel), and one was based on a synthetic scaffold (i.e., microfabricated poly(glycerol sebacate); PGS). Fiber tracking software and fast-Fourier transform-based software were used to analyze either collagen or F-actin (i.e., cell cytoskeleton) orientations in all of the tissues. In the case of the naturally-derived collagen gel scaffolds, it was hypothesized that distinct collagen and F-actin orientations would
evolve with biaxially- or uniaxially-constrained gel contraction. Specifically, a great degree of orientation was expected in the uniaxial case, whereas a more random distribution of orientations was expected in the biaxial case. In the case of the synthetic, microfabricated PGS scaffolds, it was hypothesized that cells would preferentially align along the long-axis of the microfabricated scaffold pores. In addition, an effort was made to evaluate the additional influence of static stretch on these cell orientations.

This research was conducted in the laboratories of Dr. George C. Engelmayr, Jr. and Dr. Anita Driessen-Mol. Dr. Engelmayr is an Assistant Professor in the Department of Bioengineering at The Pennsylvania State University conducting research in functional engineered tissues and scaffold design; Dr. Driessen-Mol is an Assistant Professor at Eindhoven University of Technology conducting research in Cardiovascular Tissue Engineering.

1.3 Scope of Research
In trying to determine what type of scaffold might be most suitable for mimicking the native heart valve structure, the focus of these studies was on the cell and collagen orientation properties of the two different scaffolds, naturally-derived and synthetic, along with mechanical characterization of native heart valve leaflets. While of potential utility in heart valve tissue engineering, a third type of scaffold, decellularized tissue, was not investigated herein because decellularized tissues has been demonstrated in some cases (i.e., xenogeneic) to fail upon implantation due to host-mediated immune responses [5].
In developing a technique for examining the structural characteristics of the engineered tissues, this study attempted to use various combinations of imaging / image analysis methods. Since it has been shown that collagen orientation is a principal determinant of tissue mechanical properties [3], quantification of the collagen fiber was the initial focus for each of the engineered tissues. However, due to limitations associated with each of the various imaging / image analysis techniques (described in detail in the following sections), this thesis focused principally on F-actin (i.e., cell cytoskeleton) orientation. Note, it has been observed that collagen orientation tends to mirror cell orientation [6], thereby indicating that F-actin orientation can also serve as a marker of collagen orientation.

1.4 Thesis Organization

This thesis begins with a brief overview of the native heart valve followed by common valve diseases and current medical procedures that are available for patients. This is meant to give the reader some background on the structure and function of the heart valve and the importance of its mechanical strength and structure. A following section provides information and background on some different tissue engineering techniques now under consideration, specifically those focused around synthetic, naturally-derived and decellularated scaffolds. Throughout these discussions the shortcomings and places for innovation are highlighted. Once sufficient background has been laid down the methods and results of the experiments are described in the following chapters. One chapter will be designated for the experiments focusing on the native leaflet (i.e. mechanical and structural characterizations). The first two chapters will be used to describe the experiments centered on the collagen gel and the PGS polymer scaffolds. Finally this thesis concludes with a chapter in which all the previous chapters will be addressed.
II. A BRIEF REVIEW OF HEART VALVES, VALVULAR DISEASE, AND VALVE REPLACEMENT

2.1 Anatomy

It is important to obtain a clear background of the heart and the heart valves before understanding how to engineer replacement valves. This section provides a brief overview of the functions of each of the different heart valves and provides micro- and macro- structural descriptions of the aortic and pulmonary valves.

2.1.1 - General Anatomy

The heart is composed of four different heart valve leaflets that control the flow of blood through the heart in a unidirectional way (Figure 2.1). These heart valves are dynamic tissues that respond and remodel to changes in local mechanical forces. They are composed of specialized cells and extracellular matrix that create the unique characteristics of heart valve tissues [7]. Each of these valves are located in the fibrous skeleton of the heart, a portion of the heart that it dense with collagen fibers and remains stationary while the rest of the heart undergoes dynamic movements. This fibrous skeleton secures dynamic valve function between the heart chambers. The tricuspid and mitral valve control the flow of blood from the atrium to the ventricles. These are considered atrioventricular valves. Whereas the pulmonary and aortic heart valves are defined as semilunar valves. The pulmonary valve controls blood flow from the right ventricle to the pulmonary arteries and the lungs. The aortic valve controls the blood flow from the left ventricle to the aorta and the rest of the body [8].
This diagram of the heart clearly depicts the locations of the pulmonary, aortic, mitral and tricuspid valves within the heart [8].

This thesis focuses mainly on the aortic and pulmonary valves and thus will go into more detail on the architecture (i.e. micro- and macrostructure) of each. Principally it has been shown that the aortic and pulmonary valves have the same architecture and only show minor differences in their histological characteristics which stem from their locations in different physiological environments. Within both the aortic and pulmonary roots similar anatomical nomenclature is used to describe the sinuses, the annulus, the commissures and the leaflet sections of the heart valves. The sinus is defined as the space between the aortic or pulmonary wall and the actual heart valves. The annulus and the commissures are fibrous points of attachment between the sinus wall and what we define as the leaflets. The central structures of the valves consist of three
leaflets. The leaflets are the structures that are suspended in the lumen between the commissures. Figure 2.2 depicts these features [9].

**Figure 2.2: Anatomy of the Semilunar Valves**

*This figure depicts the areas of the heart valve defined as the leaflet, sinus and commissure* [9].

Semilunar heart valve leaflets consist of four different components: the hinge, the belly, the coapting surface and the lannula with the nodule of Arantii. The hinge of the leaflet consists of the areas where the leaflets are attached to the annulus. In this area thick collagenous bundles are present to transmit stress on the leaflets to the sinus wall. The belly is the main area of the each leaflet where specific arrangements of collagen structures can be identified. At the midpoint of the free edge of the coapting surface is the nodule of Arantii. The crescent-shaped portion that is located at either side of the nodule of Arantii is called the ‘lannula’.

Two main types of cells comprise semilunar valves; endothelial and interstitial cells. The endothelial cells cover the surface of the leaflets and provide a nonthrombogenic blood-tissue barrier that supports immune and inflammatory reactions. Valvular interstitial cells, VICs, are
most prevalent in the leaflets. They are responsible for synthesizing extracellular matrix, ECM, and the enzymes responsible for matrix remodeling.

Although both of the semilunar valves show similar characteristics they have individual features, such as collagen structure and orientation that are specific to the roles that they play in the cardiac cycle.

2.1.2 - Collagen structure and orientation of semilunar valves

Joyce et al. did research to gain a better understanding of the compositional and structural organization of the extracellular matrix and collagen fibers in heart valves [10]. The focus was on collagen fiber architecture and the valve’s responses to diastolic forces. The lab worked with both pulmonary and aortic valves to gather quantitative information in order to make comparisons. They determined that both pulmonary and aortic valves cusps consist of the three same layers; fibrosa, spongiosa, and ventricularis. Fibrosa is found directly below the pulmonary artery surface and is primarily composed of type I collagen fibers. Ventricularis is found immediately below the ventricular surface and composed of elastin and collagen. Where spongiosa is located between the previous two and is rich in glycosaminoglycans and water [10]. Areas of crimp are found in the collagen architecture of the fibrosa and are used to allow the collagen to adapt to stresses since collagen fibers are inelastic and unable to support large strains. By uncrimping the collagen fibers can stretch by minimal stress and allow the leaflet to open and close [7].
Research also found that although pulmonary and aortic valves do display these structural similarities, they do present different functional and mechanical properties. The pulmonary valves showed overall higher degrees of collagen alignment throughout the cusps of the leaflet than compared to the aortic valves which varied more regionally (Figure 2.3). The areas of crimp in the collagen fibers under stress also varied from the pulmonary valves to the aortic valves. Overall, the pulmonary valves demonstrated a larger increase in crimp periods compared to the aortic valves.

**Figure 2.3: Polarized Light Microscopy Images of Fiber Architecture Maps**

*This image shows the polarized light microscopy images of the collagen fiber architecture maps for A,C) unrestricted and B,D) restricted heart valve leaflets. Well aligned fibers are depicted as purple and red fibers where less aligned fibers are shown in blue and green [10].*

The information uncovered by this research will help develop tissue engineering heart valves by using the pulmonary valves extracellular matrix structure to help define the necessary critical fiber architectural characteristics that should be mimicked in substitute valves [10].
2.2 Heart Valve Disease and Replacement

Due to the high number of failures in native heart valves due to diseases and harsh mechanical stresses heart valve repair and replacement methods have been under research for some time now. As technology advanced heart valve replacements have also advanced and currently there are two main types on the market today for patients, mechanical and bioprosthetic valves.

2.2.1 - Heart Valve Disease

The human heart valve is a vital part of the heart as it regulates and allows the heart to pump blood to specific parts of the body. Unfortunately, these heart valves are prone to disease and malfunction and often need replaced. Failures in the native heart valves, or heart valve diseases, can be caused by a variety of different factors. Some patients are born with an irregularly shaped valve leaflet (i.e. congenital heart disease) while others develop these diseases later in life by means of infection, calcium buildup or severe energy changes in the body (i.e. acquired heart valve disease). These diseases often lead to failure in the forms of regurgitation and stenosis. Regurgitation is when the heart valve is unable to open and close completely and properly. Regurgitation of heart valves can allow blood to flow in the wrong direction in the heart, thus allowing less blood to be pumped to the rest of the body and forces the heart work harder. Stenosis is the thickening or stiffening of the heart valve leaflet which creates a narrow valve opening for blood to flow through. If stenosis occurs, the heart must also work harder to move blood though the rest of the body [8].
2.2.2 Current Valvular Repair Techniques

In the case of early stage or mild valvular disease (for example, calcific nodules could potentially be detected early via high resolution x-ray of the thoracic cavity), in which the progression of deleterious structural alterations in the valve and valve leaflet tissues is not yet irreversible (and especially when the disease manifest via focal lesions, e.g., calcific nodules), one potential approach that may be favored is valve repair instead of frank valve replacement.

Current valve repair procedures will vary depending on the individual patient’s defect. However some common surgeries include commissurotomies, valvuloplasties, reshaping, decalcification and patching. In general commissurotomy is used when the valves are thickened and stuck together. The procedure consists of separating the valves at the points where the leaflets meet. Valvuloplasties are surgeries in which a device is attached around the outside of the valve opening to strengthen leaflets and help a weakened heart valve to close more tightly. Decalcification is preformed to remove any buildup of calcium from heart valves allowing the leaflet to close properly again. Reshaping involves removing a wedged shape section of the leaflet suturing them back together, where patching consists of covering a hole or tear in a leaflet with a tissue patch or stent [8].

2.2.3 - Current Replacement Heart Valves

In light of progressive heart valve disease and ultimate failure, replacement heart valves have been developed. Today there are two main types of prosthetic heart valves; mechanical and bioprosthetic (Figure 2.4). Each type of replacement valve has its own set of pros and cons. Consequently the type of replacement valve is determined on a patient to patient basis.
Mechanical hearts valves were the first type of replacement heart valve on the market and many different designs have been developed over the years. The original mechanical heart valve was the ball and cage valve. It consisted of a silastic ball which was positioned in sewing ring cage and was allowed to move backwards and forwards to close and open the valve. The next mechanical heart valve that was produced was the disc valve. This prosthesis is made out of a single graphite disc coated with pyrolytic carbon and positioned between two struts in a stainless steel housing. The bileaflet valve design is another design for mechanical heart valves which consists of two semicircular leaflets. Having two discs creates one central and two peripheral orifices for more natural blood flow in comparison to the single disc valve. In general mechanical heart valves are very durable but require lifelong anticoagulation drugs for the patients [12].

There are many advantages to bioprosthetic heart valves over mechanical heart valves. For one, bioprosthetic heart valves do not require the anticoagulation treatment that mechanical heart valves do [13]. Some mechanical heart valves produce an audible clicking noise with every opening and closing of the valves. This noise is not present with bioprosthetic heart valves.
Bioprosthetic heart valves can also better mimic the mechanical functions of a human heart valve [11].

Bioprosthetic heart valves are artificial valves carefully crafted from autograft, homograft or heterograft tissues. These tissues can either be actually heart valve leaflets or sections of pericardial sac that are sterilized before implantation. Typically these when the tissues are valves from another human (cadavers) or animal they can either be stent-mounted or stentless. Stented valves are sewn onto a sewing ring and then implanted into the patient whereas stentless valves are sewn into the patient by hand. The stentless valves provide a greater affective orifice area which improves blood flow however they are much more difficult to implant. When the tissues are derived from the pericardium, they are mounted on a stented frame. Unfortunately, bioprosthetic heart valves are currently not very durable and often fail due to degeneration within 10 to 20 years after implantation [12].

Despite the present advantages, bioprosthetic heart valves still bear some serious disadvantages and are currently under much research. Most recipients in today’s world are elderly meaning that the durability of the heart valves is compatible with the natural life span of the recipients. Problems occur however when younger patients receive these valves. Bioprosthetic heart valves are not yet durable enough to provide young patients with lifetime support. Research even shows that the degeneration process seems to occur much faster in younger patients compared older ones. The initial period of bioprosthetic degeneration starts during the initial forty-eight to seventy-two hours of transport form the abattoir to the plant. This pre-implantation damage is caused by serious autolytic changes which lead to loss of tissue integrity even before the tissue
comes into contact with the chemical components of the body. This loss of integrity is severally damaging to the already failure prone bioprosthetic valves.

2.2.4 Failure in Bioprosthetic Replacements

There are four main modes of failure in bioprosthetic heart valves: calcification, inflammation, mechanical damage, and pannus overgrowth. Individually each of these events can cause the heart valve to fail as could any combination of the four. Immediately following implantation, plasma surrounds and enters the bovine or porcine tissue. This leads to harmful deposition of blood components on and inside the newly implanted tissue. These components can then cause separation of collagen fibers and calcification, a buildup of calcium salts within the soft tissue. Inflammation of cells covering the surface of the tissues or within specific parts of the tissues can also cause failure. The giant cells that form have a high potential for erosion. Macrophages form in defense and often invade and cause degrading in particular areas of the prosthetic collagen. Heart valves in general undergo many compressive and tensile stresses while blood pumps through the heart, and therefore have a high potential for mechanical damage. Collagen fibrils have a limited extensibility before breaking. The way bioprosthetic heart valves are presently implanted does not allow for ideal valve mechanics. As a result, the valve bends at an acute angle and buckling leads to failure. Pannus overgrowth refers to the overgrowth with thick anastomotic intimal hyperplasia which is usually present with calcification or inflammation. The failures in wound healing can include a combination of the failures common in that native hearts valves along with those of the prosthetic heart valves. Stenosis however is very common when stitches are involves in the repair process.
James C. Ellsmere et al. studied failure of heart valves due to mechanical fatigue [14]. They found that most cases of failure due to tears and perforations were found in areas of highest stress. Because of this, new valve material and designs are tested through accelerated fatigue testing to help approximate the possibly lifespan. Ellsmere et al. tested the synergistic effects of tensile stress and proteolysis of untreated bovine pericardium. He found that in fact tensile loading does accelerate the proteolysis of the tissue. He also found that dynamic loading is more harmful than an equivalent static load [14]. With discoveries like this, engineers and scientists alike feel that with advances in tissue engineering, bioprosthetic heart valves can take the quantum leap and become the future of prosthetic heart valves [13].

2.3 Approaches to Heart Valve Tissue Engineering

Despite their limitations, currently both mechanical and bioprosthetic heart valve replacements are responsible for saving millions of lives, however advancements in engineered tissues could overcome these limitations and could generate a heart valve replacement that would be nonthrombogenic, infection resistant and a viable for cells. Typically, tissue engineered heart valves consist of some sort of biocompatible scaffold that is seeded with cells, cultivated in vitro and then implanted in vivo. This thesis will provide background on and discuss three of different potential approached that are currently being researched throughout different tissue engineering laboratories; 1) cell seeding of synthetic, biodegradable scaffolds 2) cell seeding of naturally-derived scaffolds and 3) cell seeding of decellularized heart valves as a scaffold [7].
2.3.1 Example 1: Synthetic, Biodegradable Scaffolds

The first tissue engineered heart valve was developed by Shinoka et al. in the laboratory of Dr. John E. Mayer, Jr. at Children’s Hospital Boston in 1995 [15]. That tissue engineered heart valve was based on a synthetic biodegradable polymer (i.e., nonwoven poly(glycolic acid) (PGA)). Ideally synthetic scaffolds are biocompatible, highly porous, resorbable, synthetic materials that will provide a scaffold that has controlled properties and is easily reproducible. These scaffolds must have macrostructure and surface properties that allow for cell attachment, survival, migration, differentiation and proliferation. Synthetic scaffolds are meant to provide a temporary matrix for the seeded cells until the cells can are capable of producing their own matrix that supports the function of the native tissue. The rate of degradation of the scaffold is adjusted to ideally match the application and rate of formation of the new tissue [7]. Typically these types of scaffolds are made from biocompatible polymers.

When implementing a polymer into a patient the mechanical and structural properties of that polymer must be considered. Different polymers can relate to and play an effect on the system in different manners. Currently the most widely used polymers for synthetic scaffolds are poly(glycolic acid) (PGA) and poly(lactic acid) (PLA) and their co-polymers. PGA is characterized by its highly crystalline, linear structure. Since PGA is so hydrophilic it tends to uptake a lot of water and thus is rapidly resorbable. This tends to result in a quick loss of the polymers mechanical strength. PLA is a hydrophobic copolymer of PGA. The hydrophobicity of this polymer reduces the amount of water absorbed by the scaffold as well as the scaffolds degradation time [7]. Poly(glycerol-sebacate), PGS, is another biodegradable polymer that is used in designing synthetic scaffolds [16]. PGS is made of two components that are both known
to be nontoxic and natural. Glycerol is a nontoxic alcohol monomer and a basic building block for lipids. Sebacic acid is a natural metabolic intermediate that has been FDA approved [16]. The structure of PGS resembles the ECM through a tough elastomeric proteinaceous network that can provide mechanical and structural stability. In the case of heart valve repair this type of polymer can closely resemble the native collagen network within each leaflet. The covalent crosslinking and hydrogen-bond interactions provide the mechanical strength needed in a polymer stent for the heart valve. While the network of random coils and trifunctional monomer provides the elasticity needed. The 1:1 polycondensation of glycerol and sebacic acid creates small cross links where hydroxyl groups attach to form a backbone and create a very hydrophilic polymer surface. The 3D network structure of the PGS resembles collagen but behaves like elastin in that it has characteristics of an elastomeric and tough material [16]. It has been shown that the preparation and curing of the polymer has a direct effect on the characteristics of the cross linked network and thus the polymer as well. A denser cross linked network produces a more rigid polymer [17]. Knowledge like this could be useful in producing a stent best fit for the valvular application.

Currently, the biggest challenges that lie within the creation of synthetic scaffolds deal with the mimicking of the mechanical and structural properties of native leaflets. Advances in biodegradable polymers are a key step in the advancement of synthetic scaffolds in tissue engineering.
2.3.2 Example 2: Naturally-derived Scaffolds

Alternatives to the synthetic, polymer based scaffolds are naturally-derived scaffolds. Naturally-derived scaffolds are scaffolds mainly comprised of ECM components, such as collagen or fibrin. These scaffolds support the same cellular and mechanical properties as the synthetic scaffolds as they provide initial ECM components as support. A common method for naturally-derived scaffolds is to encapsulate the cells in a fibrin or collagen gel. This method generally involves combining a soluble, fibrillar collagen with cells. This mixture is then neutralized and allowed to reassemble to create a gel. This gel entraps the cells within the collagen which leads to an interaction between the cells and fibrils that causes the gel to contract [18]. This method allows for any synthesized ECM components to immediately accumulate in the extracellular spaces instead of diffusing through to the surrounding media [7].

There are many research groups actively studying and experimenting with a variety of different mechanical and biological properties of these naturally-derived scaffolds. Shi et al. researched novel geometries for tissue-engineered tendonous collagen constructs [19]. Shi et al. recognized the importance of tissue-engineered heart valves to mimic the complex structure and mechanics of native valves. Native valves consist of a layered topology, meshed networks, and branched collagen fibers. They must also be able to withstand about 40 billion openings and closings per single year and be able to resist pressure while closed. Shi chose to focus on the affects of different multibranched collagen structures on mechanical strength. Collagen gels were casted into branched or rectangular wells that were constrained but allowed contraction to take place along the long axis (Figure 2.5). These constructs were then cultured further while in direct contact with other gels and allowed to form highly compacted and aligned collagen fiber bundles.
within the tissue. The tissues were then tested for mechanical strength. Shi et al. found that the different collagen geometries showed very different results when being tested. Multilinked “net” design failed perhaps due to the lack of sufficient tension due to the geometry. With more testing it was found that by increasing the posts to channels ratio of “cross-branched” structures allowed for a measurable mechanical stiffness. It was also found that constructs became weaker with an increasing number of branches. The strongest constructs fabricated were long and linear. Between Farahani’s and Shi’s research it is becoming ever so clear that geometry will play a key role in finding the ideal prosthetic heart valve [19].

Figure 2.5: Various Construct Geometries

This image depicts various construct geometries created by Shi [19].
With many labs all working with collagen fibers and their effects on the strength of the heart valve many different theories are developed. Frank Baaijens, Carlijn Bouten, and Niels Driessen have been working to summarize key modeling assumptions, like those previously mentioned, together to form a review of recent collagen remodeling analyses. By being able to compile a list of remodeling rules, engineers would be able to create prosthetic tissue engineered heart valves that better resemble the native valves’ ability to adapt to changes when a load is applied. It is generally thought that collagen synthesis is affected by mechanical stimuli. Many different labs have worked through different techniques and theories which have led to very unclear remodeling rules. This group of researchers focuses on distinguishing between the three main hypotheses. Hypothesis 1 assumes that the collagen fibers align in the positive principal stretch directions. Where, hypothesis 2 assumes that collagen fibers align in between the principal strain directions. Hypothesis 3 assumes that the collagen fibers align in between the two largest principal stress directions. In both hypothesis 2 and 3 the typical helical path of collagen fibers was reproduced and modeled. However, in hypothesis 1, remodeling analysis could not reproduce this helical pattern. Hypothesis 2 was also correct in predicting the architecture of the collagen in the articular cartilage whereas hypothesis 1 was incorrect. Although parts of these theories where proven right by this teams research, more in depth quantitative validation needs to be assessed to determine the exact mechanisms in which cells remodel the extracellular matrix so that collagen fibers align with preferred fiber directions [20].
2.3.3 Example 3: Decellularized Scaffolds

The decellularized scaffold approach to tissue engineering of heart valves is another method which takes advantage of native ECM properties. In this method a native heart valve leaflet is decellularized, or stripped of all its cellular material, through a series of solutions that does minimal damage to ECM. Once all of the cellular material is removed an acellular ECM scaffold remains which can then be reseeded with the proper host cells. Decellularized scaffolds seem to be advantageous in that they best mimic the structural and mechanical properties of native leaflets and decrease the risk of calcification however there seems to be a question of immunogenicity. The true challenge with these scaffolds is finding a balance between removing all of the cellular material that could trigger an immune response and maintaining the structural integrity of the ECM.

Baraki *et al.* focused their research on illustrating the function, histological changes, and remodeling capacities of decellularized allogenic aortic valve conduits [21]. The principal of their experiment consisted of attempting to remove all viable tissue from the valve while still reserving the integrity of the extracellular matrix. Their nine month study showed that the decellularized tissues are superior in durability to native homografts. Not only did the decellularized conduits show excellent mechanical strength for surgical implantation, but they also showed excellent functionality [21].

Focusing on the thought that decellularizing tissues would reduce immune response, Zhou *et al.* explored the use of decellularization of xenogeneic heart valve tissue in tissue engineering [22]. The main focus of this research was to compare four different ways of decellularization and their
effects on the integrity of the extracellular matrix. Leaflets were treated with one of the four methods that are listed below:

Group A – 1% sodium deoxycholate in PBS at 37°C for 24 hours
Group B – 1% sodium dedecylsulfate in PBS at 37°C for 24 hours
Group C - 0.05% trypsin in PBS at 37°C for 24 hours
Group D - 0.1% trypsin/0.02% EDTA in PBS at 37°C for 1 hour, hypotonic 0.01 M Trisbuffer pH8.0 containing at 4°C for 4 hours, hypertonic 0.05 M Tris buffer pH8.0 containing Triton X-100 PMSF at 4°C for 4 hours, DNase (Sigma)/RNase (Fluka) incubating at 37°C for 2 hours, and again with hypertonic Tris buffer at 4°C for 4 hours

Each of these methods worked in completely removing the cells. However, in group B, C, and D extracellular components were found to be misaligned. Two-photon laser scanning microscopy was used to determine retention in collagen and elastin structure of the leaflets (Figure 2.6). Group A seemed to hold its structure almost completely while the other groups were less successful. Overall, it was found that the method used on Group A was the most successful in the areas tested here [22].
While decellularized native valves intrinsically possess native like structure and mechanical properties, immune responses may lead to valve failure [5]. Toward recapitulating more native-like properties while avoiding immunological issues, recently investigators have been focusing on scaffolds made with the previously mentioned synthetic and naturally-derived techniques.

2.4 Summary

While they perform a relatively simple mechanical check-valve function, allowing for unidirectional blood flow through the chambers of the heart and through the circulatory system, heart valve leaflets are anatomically quite complex. The primary culprits in heart valve disease are regurgitation and stenosis, both of which effectively force the heart to work harder to pump oxygenated blood to the organs and tissues of the body. When heart valves become diseased they are typically replaced rather than repaired. Current replacement heart valves include both

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**Figure 2.6: Pictures of Porcine Valve Leaflets**

Above shows pictures taken by two-photon laser microscopy. It shows the structure of collagen and extracellular matrix in the leaflet. The letters correspond to the groups previously listed were ‘n’ is the control group [22].
mechanical and bioprosthetic types, each of which exhibit significant limitations such as requiring lifelong anticoagulation (mechanical) and relatively low durability (bioprosthetic). Tissue engineered heart valves are currently under investigation as a potential alternative to mechanical and bioprosthetic replacement valves. In particular, because tissue engineered heart valves are based on living cells, it is anticipated that they will exhibit superior biocompatibility, as well as resistance to infection, self-repair, and potentially a capacity to grow. A variety of approaches have been taken toward developing a tissue engineered heart valve, including use of naturally-derived hydrogels such as collagen, synthetic polymers such as PGA, and even decellularized native heart valve leaflets. To date it remains unclear which, if any, of these approaches will ultimately be translatable into the clinic; each approach has its unique advantages and disadvantages.

The development of a clinically translatable tissue engineered heart valve remains a lofty goal full of numerous technical obstacles and unknowns. The overall goals of the studies comprising this thesis were to develop a better understanding of native heart valve leaflet mechanical properties, as well as to compare two different scaffold materials for their ability to promote orientation of collagen and/or cells, thereby assessing, in part, their capacity to mimic native heart valve leaflet structures. Toward this overall goal I pursued the following specific objectives:
Objectives:

1 - Quantify the anisotropic (i.e., directionally-dependent) mechanical properties of porcine aortic and pulmonary heart valve leaflets (studies conducted at Penn State).

2 - Use fiber-tracking software to analyze collagen and F-actin (i.e., cell cytoskeleton) orientations in naturally-derived collagen gel scaffolds contracted under biaxial- or uniaxial-constraint conditions (studies conducted at Eindhoven University).

3 - Use fast Fourier-transform to analyze F-actin (i.e., cell cytoskeleton) orientations in synthetic, micromolded poly(glycerol sebacate) PGS scaffolds comprised of diamond shaped pores (studies conducted at Penn State).
III – OBJECTIVE 1

Mechanical Characterization of Native Heart Valve Leaflets

The objective of this set of experiments was to determine and define the mechanical properties of the native porcine pulmonary and aortic heart valve leaflets. In addition, an effort was made to apply confocal reflectance microscopy to the native heart valve leaflets in order to gain an appreciation for the collagen morphology (i.e. orientation). This information will be used to provide guidance for the design of tissue engineered heart valve leaflets that will be tested in later experiments.

3.1 Materials and Methods

3.1.1 Porcine Heart Valve Leaflet Excision and Mechanical Test Preparation

Porcine aortic and pulmonary heart valve leaflets, which were obtained immediately following slaughter from Brenneman's Meat Market (Huntingdon, PA), were aseptically excised under a laminar flow hood [23]. Individual leaflets were excised and rinsed exhaustively in a 2% (v/v) solution of antibiotic-antimycotic in Hank’s Balanced Salt Solution (HBSS, Invitrogen) to remove any remaining blood cells. Leaflets that were used for observing collagen morphology were laid out as flat as possible and fixed in 10% buffered formalin prior to confocal reflectance microscopy. The leaflets excised for mechanical testing were cut into radial and circumferential strips and allowed to soak in HBSS (invitrogen) until testing.
3.1.2 Mechanical Testing

Native heart valve tissue samples cut in the radial and circumferential directions were subjected to uniaxial mechanical testing [23] in order to measure the initial modulus (0-10% strain region; equivalent to the Young’s modulus for a linear elastic homogenous isotropic material), the peak tangent modulus (i.e., the modulus in the steepest region of the stress-strain curve), the ultimate tensile strength (UTS) and the strain-to-failure using a LFPlus materials testing machine, a 5N load cell, and Nexygen™Plus software (Lloyd Instruments, Ltd., West Sussex, UK). Samples were cut using a scalpel from the central portion of each leaflet to be, on average, 15mm long and 5 mm wide rectangular strips. The thicknesses of specimens were then measured at 3 equally spaced locations along the length of the specimen using a dial gauge (accuracy 0.01mm; The L.S. Starrett Co., Athol, MA). Once the cross-sectional area information for each specimen was entered into the software (calculated by multiplying the sample width by the sample thickness), they were attached to the LLOYOD equipment through mechanical grips and stretched to failure using a 500N load cell to measure the reaction force. Sandpaper was attached to the faces of the grip to prevent the tissues from slipping off before failure. The samples were loaded at a 10mm/min extension rate. The peak tangent modulus of each tissue specimen was determined by finding the maximum slope on the stress strain curve. Ultimate tensile strength (UTS) and strain-to-failure (ef) were taken as the maximum stress and strain measured at the onset of failure, respectively.
Figure 3.1: Radial and Circumferential Directions
This image depicts the radial and circumferential directions of the heart valve leaflets.

3.1.3 Quantifying Local Collagen Morphology
An effort was made to use confocal reflectance microscopy to image the collagen fibers in different regions of the leaflet (e.g., belly region, commissures, basal attachment). Using an Olympus FluoView 1000 confocal microscope, a wavelength of 488nm was emitted onto the samples and collected at the same wavelength (488nm). This technique was used in order to obtain information about the collagen fibrils within the plane of the fibrosa layer and ventricularis layers of the trilaminar leaflet tissue. The confocal reflectance microscopy was used to image small areas at specific locations of the leaflet at high magnification (20x). In particular, the areas of interest regarding the morphological characterization were the central belly region of the leaflet, the commissures (i.e., the corners where the individual leaflets are attached to the aortic or pulmonary root), and the basal attachment at the bottom of the leaflet where it connects with the aortic or pulmonary root. Multiple pictures (7) were taken in each of these regions to develop a general idea of collagen morphology. Our findings were then compared with those reported by other groups.
3.2 Results

3.2.1 Native Mechanical Properties

Representative uniaxial tensile stress-strain curves for the circumferential and radial strips of native porcine aortic and pulmonary valve leaflet tissues are depicted in Figure 3.2. In particular, Table 3.1 shows the calculated mean ± standard error mechanical property values that were measured for each case. Figure 3.3 summarizes this data in bar chart.

![Stress-Strain Plots for Native Porcine Heart Valve Leaflets](image)

**Figure 3.2: Stress-Strain Plots for Native Porcine Heart Valve Leaflets**
Representative uniaxial tensile stress-strain plots for radial and circumferential strips of native porcine (A) aortic and (B) pulmonary valve leaflet tissues. Peak tangent moduli are denoted.

<table>
<thead>
<tr>
<th></th>
<th>E (Mpa)</th>
<th>UTS (MPa)</th>
<th>Ef (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>0.84766 ± 0.37</td>
<td>0.444 ± 0.24</td>
<td>1.57 ± 0.11</td>
</tr>
<tr>
<td>AC</td>
<td>8.525 ± 1.49</td>
<td>2.02 ±0.48</td>
<td>0.6125 ± 0.04</td>
</tr>
<tr>
<td>PR</td>
<td>0.813 ± 0.22</td>
<td>0.725 ± 0.31</td>
<td>1.725 ± 0.42</td>
</tr>
<tr>
<td>PC</td>
<td>4.1 ± 1.99</td>
<td>1.47 ± 0.70</td>
<td>0.86 ± 0.25</td>
</tr>
</tbody>
</table>

**Table 3.1: Mechanical Data Averages for Native Porcine Heart Valve Leaflets**
The calculated mean ± standard error mechanical property values that were measured for the aortic (A) and pulmonary (P) leaflets in both the radial (R) and circumferential (C) directions.
Figure 3.3: Summary of Mechanical Data for Native Porcine Heart Valve Leaflets

Mechanical data for porcine aortic (A) and pulmunary (P) valve leaflet tissues measured in the circumferential (C) and radial (R) directions. Peak tangent moduli (E), ultimate tensile strength (UTS), and strain-to-failure (ef) are reported. N=6 samples were tested per group.

3.2.2 Native Collagen Morphology

In this research we attempted to use confocal reflectance microscopy to image collagen fibers within native heart valve leaflets. Collagen fibers could be discerned in confocal reflectance micrographs obtained using 488nm wavelength illumination (Figure 3.4).

Collagen orientation can be qualitatively observed in these small areas of the leaflet, and an overall general orientation can be inferred from multiple images (Figure 3.4). Please note that the dark and bright banding is due to the collagen crimp, and the actual orientation of the collagen fibers is orthogonal to the dark and bright bands. It is because of this characteristic of the images that no further image analysis was performed. Instead observations made from the
images that were collected were compared to previously published articles. Below shows one image obtained by the aforementioned method.

Figure 3.4: General Collagen Orientations for a Native Pulmonary Porcine Leaflet
Representative confocal reflectance images from a pulmonary porcine leaflet of the three areas of interest: A,B) The central belly region of the leaflet and C) the commissure. D) DIC image for the commissure shows the collagen crimp.
3.3 Discussion

Mechanical properties measured in the current study for native porcine aortic valve leaflets were consistent with previously reported values in the literature. For example, Mavrilas and Missirlis reported peak tangent moduli, \( E \), values of 7.78 ± 1.7 MPa and 1.28 ± 0.34 MPa for the circumferential and radial directions of fresh porcine aortic valve leaflets, respectively [24]. The corresponding values we measured in the current study were 9.6 % higher (8.525 ± 1.49 MPa) and 33.8 % lower (0.84766 ± 0.37 MPa) than the values reported by Mavrilas and Missirlis. By contrast, Mavrilas and Missirlis reported that human aortic valve leaflets were stiffer, in particular in the circumferential direction, with peak tangent moduli of 14.55 ± 3.7 MPa (circumferential) and 1.57 ± 0.1 MPa (radial) [24]. Comparatively little information is available in the literature regarding the peak tangent moduli of the native porcine pulmonary valve leaflets. In the present study, we found the pulmonary valve leaflet tissue to be significantly less stiff than the aortic leaflet in the circumferential direction and similarly stiff versus the aortic leaflet in the radial direction. Compared with the aortic valve leaflet, the pulmonary valve leaflet was 52 % less stiff in the circumferential direction (4.1 ± 1.99 MPa) and 4 % less stiff in the radial direction (0.813 ± 0.22 MPa). As for the ultimate tensile strength (UTS) and strain-to-failure (\( \varepsilon_f \)), the pulmonary valve leaflet was found to be 27% smaller and 40% larger, respectively, in the circumferential direction (UTS: 1.47 ± 0.70MPa and \( \varepsilon_f \): 0.86 ± 0.25). In the radial direction the UTS was 27% smaller (0.725 ± 0.31 MPa) and the strain-to-failure was 10% smaller (1.725 ± 0.42) compared to the aortic valve leaflet.

Collagen fiber orientations qualitatively deduced from confocal reflectance micrographs were likewise comparable to previously published data. For example, collagen fibers were observed to
be principally oriented in the circumferential direction; this is consistent with a study by Joyce et al. which used small angle light scattering (SALS) to demonstrate preferential circumferential alignment in both the porcine pulmonary and aortic valve leaflets [10]. While Joyce et al. were able to detect subtle differences in collagen fiber morphology between the aortic and pulmonary valve leaflets, such distinctions were not possible in the current study because the undulated (i.e., crimped) collagen fibers observable in confocal reflectance micrographs were not directly amenable to automated image analysis (e.g., by fast Fourier transform-based image analysis).

From this study it was determined that by orienting the collagen fibers in a way to mimic the principally circumferentially oriented fibers in the native leaflets would produce the properties closest to those of native leaflets.
IV – OBJECTIVE 2
Analysis of Collagen and F-actin Orientations in Naturally-Derived Collagen Gel Scaffolds

The object of this set of experiments was to determine the effects of biaxial and uniaxial loading conditions on matrix and cellular orientation in 3D collagen matrigel constructs. Using a model system developed at Eindhoven University of Technology, collagen gels were seeded with human vena saphena cells and allowed to culture under biaxial static loading. After the gels were completely contracted the load was changed to a uniaxial load which was then applied in static culture. Both collagen and cellular structures were observed throughout the culture. It was hypothesized cell orientation and matrix anisotropy would be random at first (under the biaxial load) and then be redirected and align to the direction of the new uniaxial load.

4.1 Materials and Methods

4.1.1 Model System
The model system used in these experiments was adapted after the model system of Jasper Foolen, PhD created at Eindhoven University of Technology. Through an extensive process done by Jasper Foolen, PhD silicon posts were adhered to the membranes of Bioflex culture plates (Flexcell international). Once this process was complete, all of the corner posts were removed along with any excess silicon on the remaining posts. This new design allowed the tissue to contract around the remaining posts to form an octagon shape and allow for uniform biaxial straining. Circular PDMS inlets were created and then applied around the prepared silicon posts to help in the matrigel seeding process. Wells were washed in 70% ethanol followed by sterile PBS in preparation for matrigel seeding.
4.1.2 Tissue Constructs

Collagen matrigel gels were produced using type I collagen (0.45mg/mL, rat tail, BD Bioscience), matrigel (8.5%, BD Bioscience), and NaOH solution (2% to control pH). Once the gel was created it was kept on ice to prevent polymerization before cell seeding. Human vena saphena cells (HVSC) were cultured in Advanced DMEM growth media (with 10% FBS, 1% penicillin/streptomycin, 1% glutamax) and approximately 2 million cells were then suspended in Advanced DMEM growth media (with 10% FBS, 1% penicillin/streptomycin, 1% glutamax, 0.26 mg/ml ascorbic acid) and added to the matrigel solution. 64 µl of the final gel mixture was then added to the inlets of each well of the prepared Bioflex culture plates. These gels were then allowed to polymerize in the incubator for 1 hour at 37 ºC and 5% CO₂ before clear DMEM media (with 10% FBS, 1% penicillin/streptomycin, 1% glutamax, 0.26 mg/ml ascorbic acid, 1% nonessential amino acids, 0.9% pyruvate) was added to each well. Tissues remained in incubator during remainder of the culture. After complete tissue contraction (around 4-6 days) the tissues were released from the posts at 2 opposing sides before being placed back in the incubator for further culture.

Figure 4.1: Contracted Tissue Before and After Release
This figure shows how the contracted tissue will look around the new design of the model system a) before and b) after the tissue is released from the posts on two sides.


**4.1.3 Visualization of Collagen and F-actin**

Real time visualization for cells and collagen was done using cell tracker blue (CTB, 1.5μl/ml) and CNA (20 μl/ml) probes at seven specific time points throughout the experiment. The time points included times immediately before and after the removal of two sides the tissues from supporting posts as well as time points 19, 24, 43, 48 and 67 hours after the release. Images were taken using a multitrack setting on a multiphoton microscope (Zeiss LSM 510 META NLO) in Two-Photon-LSM mode with a Achromplan 40x /0.8NA long distance water objective, attached to a Zeiss Axiovert 200 M. The track for CNA used an excitation source of 488nm with a HFTKP 700/488 filter and a pinhole set at 1μm. The second track used a 760nm excitation source (a Coherent Chameleon Ultra Ti/Sapphire laser) with a HFTKP 700/543 filter and the pinhole settings placed at ‘max’ for visualization of the CTB probe. At every time point additional tissues were fixed in 10% formalin for 30 minutes and later prepared for F-actin and cell nuclei visualization. To prepare these tissues they were first permeabilized with 0.5% Triton-X-100 in PBS for 30 minutes and then allowed to incubate with TRITC-conjugated Phalloidin (1:100) and DAPI (1:500) on a shaking table at room temperature for 20-30 minutes. After incubation the tissues were washed with PBS and stored in PBS in at 4°C. F-actin and cell nuclei images were taken using the same multiphoton microscope and 40x water objective. Excitation sources of 760nm and 543nm were used to visualize the DAPI and TRITC Phalloidin probes respectively. Using these settings, z-stacks were taken at the center of each tissue and later used in observations and quantification.
4.1.4 Quantification of Collagen and F-actin orientations

Fiber tracking software that was made available through Eindhoven University of Technology used Mathematica and a program created by Frans Kanters (based on code provided by Hans van Assen) to determine the F-actin and collagen fiber orientations. The software analyzed individual images of each z-stacks taken on the multiphoton microscope and produced corresponding histograms describing overall fiber orientation. Once obtaining all the information from the histograms in one text file this array was put into a Matlab program which subsequently produced corresponding bimodal curves with disparity and R-squared values.

4.2 Results

4.2.1 Collagen Alignment

Z-stack images that were taken from the real time visualization showed very dense areas of collagen and a general orientation however it was hard to distinguish any true fibers (as seen in Figure 4.2). This being so, the accuracy of the results from the fiber tracking software is to be questioned. The data from these images was sent through both the fiber tacking and bimodal fitting software and no drastic change in orientation was reported. The bimodal curves can be seen in Figure 4.3. Since these results are unreliable, Table 4.1 shows any observations that could be made about the cellular activity and general collagen orientation in the four tissues that were viewed in real time.
Figure 4.2: Time Lapse with CTB and CNA
This figure shows the changes in cell and collagen behavior at the 7 different time points.

Figure 4.3: Bimodal Curves for Collagen in Tissue Constructs
This figure shows that results that were given from the fiber tracking and bimodal fitting software on the images taken with the CNA probe.
<table>
<thead>
<tr>
<th>Time Points</th>
<th>Cellular Activity</th>
<th>Collagen Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>t=0 (6 days after gel seeding)</td>
<td>Cells are attached to the matrix. Appear long and narrow.</td>
<td>Alignment present in the top and bottom. Center seems very random.</td>
</tr>
<tr>
<td>t= 1 (after release of tissues)</td>
<td>Cells start to remove themselves from the matrix. Some are small and round.</td>
<td>Some vertical alignment is seen right away in tissues.</td>
</tr>
<tr>
<td>t=2 (19 hrs after release)</td>
<td>Not many cells seem to be seen here.</td>
<td>Not much change in matrix.</td>
</tr>
<tr>
<td>t=3 (24 hrs after release)</td>
<td>Cells appear to look longer again.</td>
<td>Slight vertical alignment throughout entire tissue.</td>
</tr>
<tr>
<td>t=4 (43 hrs after release)</td>
<td>Cells are once again attached to the matrix (although there are not as many cells as before).</td>
<td>Seems more aligned in the direction of constraint.</td>
</tr>
<tr>
<td>t=5 (48 hrs after release)</td>
<td>No notable change.</td>
<td>No notable change.</td>
</tr>
<tr>
<td>t=6 (67 hrs after release)</td>
<td>No notable change.</td>
<td>No notable change.</td>
</tr>
</tbody>
</table>

### 4.2.2 F-actin Alignment

The z-stack images of the F-actin that were run through the fiber tracking and bimodal fitting software gave results showing a drastic change in the fiber orientation between T=0 and T=1 onward. A random network of fibers is seen in biaxially loaded tissue whereas a primarily uniaxial orientation is seen immediately after the two sides of the tissue are released. This data suggests that cell reorientation happens very fast. Figure 4.4 shows a time lapse of the F-actin fiber network at the different time points. Since fibers can clearly be distinguished in these images it can be assumed that the software can work accurately. Figure 4.5 shows the corresponding bimodal curves.
Figure 4.4: Time Lapse with Phalloidin and DAPI
This figure shows the changes in cell nuclei and f-actin behavior at the 7 different time points.

Figure 4.5: Bimodal Curves for F-actin in Tissue Constructs
This figure shows that results that were given from the fiber tracking and bimodal fitting software on the images taken with Phalloidin stained F-actin. Notice the drastic change in orientation between T=0 and T=1 onward.
4.3 Discussion

Although the data obtained from the CNA probe was inconclusive for quantifying the remodeling of the collagen fibers, the images do suggest that remodeling does occur to some extent. Further experiments must be done in order to quantify this change more precisely. The results from the F-actin images suggest that cell reorientation occurs drastically and quickly after the tissue is released from its biaxial position. CTB images show that immediately after release (i.e. \( T=1 \)) cells in the direction perpendicular to the new constraint direction become small and round and only long, extended cells are found in the direction of the constraint (see Figure 4.6). This strain avoidance characteristic that is seen here is similar to the results found by Ping et al. and Lee et al. in their research [25,26]. In particular, initial studies of 3D constructs done by Grenier et al. show that orientation of fibroblasts and collagen orientation maintain to be random under static loading. However, under uniaxially constrained conditions, cell and collagen fiber alignment was seen in the direction of stress, even upon a change in the direction of stretch [27]. Hu et al. speculates that strain avoidance has to deal with tensional homeostasis. Tensional homeostasis is the phenomenon in which cells aim to maintain a constant stress within the matrix of a tissue. It is apparent that this established tension is crucial for the survival of the cell. Thus as a new stress is applied to the tissue, cells will reorient in such a way as to maintain that constant tension needed for survival [28]. Although it is acknowledged that tensional homeostasis and strain avoidance could be associated with the remodeling of collagen fibers it is unclear of what is actually happening in the F-actin fibers. Do the fibers actually remodel and move into a position aligned with the direction of constraint or do they simply lose all the F-actin fibers that were in the perpendicular direction and later rebuild new fibers in the direction of constraint? More experiments must be conducted before any of these questions can be answered.
or the dominant mechanism of this matrix and cellular remodeling can be determined. Below are some suggestions for future directions to follow up on this study.

4.3.1 Tissue Constructs

Once tissues were prepared and contracted around the posts it was observed that there was normally some preferred orientation present from the beginning. It would be recommended that that initial orientation be noted and that the tissue is then removed from the posts in such a way that the direction of constraint is perpendicular to the initial alignment. This was not consistently done in the above experiments and may have affected the bimodal fitting. It was noted that
changes in orientation were more noticeable in the tissues when the strain was applied in the direction perpendicular to any initial alignment.

![Diagram](image)

**Figure 4.7: Initial Collagen Orientation in Contracted Tissues**

This figure shows how there is some a) initial alignment in the collagen fibers (depicted with red arrows) and how the b) direction of strain (depicted with a black arrow) then be applied perpendicular to that alignment.

### 4.3.2 Collagen Imaging

Being that it was difficult to identify individual fibers when using CNA probes, confocal reflectance microscopy could be used instead. Since the 3D tissue constructs have a high density of collagen the images that are acquired are often too unclear for accurate quantification. Confocal reflectance microscopy should show the larger bundles of collagen fibers instead of individual fibers which will hopefully allow for the fiber tracking software to produce more accurate results. Also performing assays to keep track of the amount of cells and collagen throughout the experiment as well as performing histology on the samples could provide very helpful information. This data could help determine if the collagen is degrading in the direction perpendicular to the constraint or if it is in fact remodeling.

### 4.3.3 F-actin Reorientation

In order to determine if the all the F-actin fibers move into alignment or rather if any fiber not aligned in direction of strain is just lost and later rebuilt, keeping track of the number of fibers
throughout the experiment could be helpful. The histograms produced by the fiber tracking correlate to amount of fibers in each image. This data could be helpful in determining how the amount of F-actin fibers within the tissue changes over time.

4.3.4 Cellular Structures

When examining the real time images with CNA and CTB it was observed that there was a noticeable decrease in the amount of positive CTB staining 19 hours after the tissue had been released from two of its sides. Staining for dead cells could provide some more information on the cellular behavior in this tissue constructs.
V – OBJECTIVE 3
Analysis of Cell Orientation in Synthetic, Micromolded Poly(glycerol sebacate) Scaffolds

The objective of this set of experiments was to determine cell (i.e., F-actin) orientations within the 2:1 aspect ratio diamond shaped pores of the micromolded PGS scaffolds. It was hypothesized that cells would preferentially align along the long-axis of the diamond-shaped pores. For these cell-seeded scaffolds, the content of collagen and DNA were also quantified. In addition, efforts were made to both (1) determine collagen orientation within the diamond shaped pores and (2) to evaluate the additional influence of static stretch on these cell orientations, which effectively elongated the diamond shaped pores in the scaffolds. The details of these pilot experiments are provided in the subsequent discussion section.

5.1 Materials and Methods

5.1.1 Micromolding of PGS scaffolds

PGS scaffolds were fabricated such that they consisted of 2:1 aspect ratio diamond shape pores with approximately 75 micron-thick struts. In the first step of scaffold fabrication, a mold was made from an ultra-high temperature machinable glass-mica ceramic sheet (0.5" thick, 2" x 2", McMaster-Carr), shown in Figure 5.1. A 300 micron-deep border (~0.5") was cut around the edges of the sheet using a milling machine to provide an area for melting the PGS prepolymer. The mold was then made from the ceramic sheet using a dicing cutter machine (Kulicke & Soffa Industries, Inc., 1998) with a 90µm wide saw blade; the machine was used to cut 300 micron-deep channels into the ceramic at angles of 30° and 150°, yielding a lattice of channels which could be used to produce the PGS strut and pore pattern onto the ceramic sheet (Figure 5.1). After the mold was fabricated, the surface was prepared for the polymer curing process by coating it with sucrose (90% w/v sucrose in water) and baking it for 18 hours at 120°C. The
sucrose coating served as a sacrificial layer that could be dissolved away to help release the polymer following curing.

![Figure 5.1: Ceramic Micromold](image)

*This diagram shows a sketch of the ceramic micromold.*

### 5.1.2 PGS Curing

PGS pre-polymer was synthesized through polycondensation of glycerol and sebacic acid (1:1 molar ratio) by adapting the methods of Wang *et al.* per Masoumi *et al.* [16,23]. Specifically, 113.8 g of anhydrous glycerol (Product # 49770; Sigma-Aldrich, Corp., St. Louis, MO) and 202.25 g of sebacic acid (Product # 28,325-8; Sigma) were stirred using a magnetic stirrer at 200-400 RPM while being heated to 120°C for 24 hours under a dry nitrogen blanket using a heating mantle (Model 100A O408; Glas-Col, LLC, Terre Haute, IN) and digital temperature controller (Model 210; J-KEM Scientific, Inc., St. Louis, MO). A high vacuum (< 50mTorr; Model 1405; Welch Vacuum Technology, Niles, IL) was then applied for an additional 24 hours to yield a viscous PGS pre-polymer. The pre-polymer was decanted into a clear glass storage jar and allowed to solidify into a soft waxy consistency upon cooling to room temperature. The PGS pre-polymer was then stored in a dessicator at room temperature.
Once the pre-polymer was prepared it was applied to the sucrose coated ceramic mold. PGS pre-polymer was melted around the edges of the ceramic mold and allowed to flow into the channels of the fabricated mold. The PGS pre-polymer was then cured in a vacuum oven (Model 1470; VWR) under high vacuum (< 50 mTorr; Model 1405; Welch Vacuum Technology) at 160°C for 12 hours. After curing, the mold was soaked in 60°C water overnight in order to dissolve away the sucrose layer and allow for the separation of the PGS scaffold from the ceramic mold. A razor blade was used to help facilitate the separation process which resulted in a 300 micro-thick porous scaffold. To ensure that no ceramic beads were left behind in the diamond pores, the scaffolds were soaked in 60°C water overnight once again. Scaffolds were stored at room temperature until ready for use.
Figure 5.2: Micromolding Technique
This figure depicts the micromolding technique used to create 2:1 aspect ratio diamond shaped pores with the PGS polymer. This image shows the A) dicing cutter machine that was used to cut a pattern in the B) ceramic sheet which produced the PGS scaffolds in C) with ceramic beads in pores and D) without ceramic beads in pores under a light microscope at 20x magnification.

5.1.3 Valvular Interstitial Cell Isolation, Expansion, and Seeding
Valve interstitial cells, obtained from porcine heart valve leaflets, were isolated by either collagenase digestion or by migration from the leaflet by adapting the methods of Butcher et al. [29]. Once the heart valve leaflets were excised (via the previously mentioned methods in section 3.1.1), the leaflets were denuded by wiping them with sterile gauze to remove the
valvular endothelial cells. The valvular interstitial cells were then isolated overnight through digestion of the leaflet tissue in a solution of 0.2% (w/v) type I collagenase (Worthington Biochemical) in HBSS at 37°C. A sterile cell strainer (100 µm average pore size; BD Falcon) was used to remove the tissue debris and the valvular interstitial cells were retrieved by centrifugation at 1000xg for 10 min. The isolated cells were then resuspended and expanded in culture medium comprised of E199 medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic-antimycotic (Invitrogen). In some cases intact heart valve leaflets were placed within sterile tissue culture flasks and cells were isolated by allowing the cells to migrate out of the leaflet and into the flask. This process took several weeks to yield confluent flasks while changing the medium regularly.

In preparation of cell seeding, PGS micromolded scaffolds were first autoclave-sterilized (121°C, 15 PSI, for 30 minutes) and then soaked in culture medium for 6 days to help cell attachment as described by Chen et al. [17]. Once properly prepared, each scaffold was put into a sterile-vented 50 ml bioreactor tube (Product # 87050; TPP Techno Plastic Products AG, Trasadingen, Switzerland) to allow for gas exchange during seeding.

Confluent flasks of porcine pulmonary and aortic valvular interstitial cells were trypsinized (0.25% (w/v) trypsin, 1mM EDTA; Invitrogen) and resuspended in culture medium such that 12 ml of cell suspension was added to each 50 ml bioreactor tube at a density of ~7x10^6 cells/cm². The bioreactor tubes where then placed in a rotisserie rotator (Labquake® hybridization rotator; Thermo-Fisher, Pittsburgh, PA) which rotated at 8 RPM for 24 hours inside a humidified incubator at 37°C and 5% CO₂ (NU-8700; Nuaire, Inc., Plymouth MN). After cell attachment
the scaffolds were transferred to individual wells of a 6 well plate (Costar Ultra Low Attachment; Coming, NY) and cultured statically for 2 or 4 weeks to promote cell proliferation and extracellular matrix secretion. Upon culturing the cell-seeded scaffolds, the E199-based media used for cell expansion was further supplemented with 82 µg/ml of L-ascorbic acid-2-phosphate in order to promote collagen synthesis.

5.1.4 F-actin Imaging of the PGS Scaffolds to Visualize Cell Orientation

Following cultivation, the cell-seeded scaffolds were prepared for nuclei and F-actin visualization [23,30]. Note, F-actin is a filamentous cytoskeletal protein whose orientation is commonly used as a marker of overall cell orientation. Samples were first rinsed in HBSS and then fixed in 10% neutral buffered formalin (Sigma) overnight in the refrigerator (i.e., at 2-8°C). In order to remove any excess formalin, fixed samples were then rinsed in room temperature HBSS on an orbital mixer 3 times for 5 minutes each. The samples were then allowed to incubate at room temperature for 2 hours in 0.2% (v/v) Triton X-100 (Sigma) in HBSS. Following the 2 hours the samples were then rinsed 3 times for 5 minutes each in 0.05% (v/v) Triton X-100 in HBSS and then blocked in 1% (w/v) bovine serum albumin (Sigma) and 0.05% (v/v) Triton X-100 in HBSS for 2 hours. Once the blocking was complete, samples were incubated for 3 hours in Alexa Fluor 488-phalloidin (1:40 (v/v) dilution of stock solution in 1% (w/v) bovine serum albumin and 0.05% (v/v) Triton X-100 in HBSS; Invitrogen). The scaffold samples were then rinsed 5 times for 5 minutes each in HBBS and stored in the refrigerator overnight. The following day the samples were placed on glass slides and cover slipped with a drop of Vectashield mounting media with DAPI (Vector Laboratories, Inc., Burlingame, CA) to counterstain cell nuclei. Samples were imaged with a 25x and 40x water-immersion objectives
on a FluoView FV1000 laser scanning confocal microscope (Olympus America, Inc., Center Valley, PA), with F-actin and nuclei pseudo-coloured green and blue, respectively.

### 5.1.5 Quantifying F-actin Orientation

Once the F-actin images were taken on the confocal microscope, they were cropped into circular portions and saved as JPEG files in preparation for the Fast Fourier Transform (FFT) software to analyze the F-actin orientation. Note, cropping the images using a circular mask was necessary such that the edges of the image did not contribute to the orientation detected by the software. To use this software, first the cropped JPEG image files and MATLAB scripts (i.e., "latest_orient.m" and subscript "circlepoint.m") were transferred into a folder that was then opened within the MATLAB software. The "latest_orient.m" file was executed individually for each collected image, yielding a data file that was subsequently processed to obtain the F-actin orientation distributions.

In brief, the FFT software (written by George C. Engelmayr Jr.; [30]) works by converting the spatial information of the image into a frequency domain, which maps the rate at which the pixel intensities of a grayscale image change in the spatial domain. The software then outputs a distribution pattern on a frequency plot that can be used to determine the degree of fiber alignment present in the original image. Low-frequency pixels are shown around the center of the frequency plot whereas high-frequency signals are found on the periphery of the plot. While the low-frequency pixels mainly represent the background of the image the high-frequency pixels correspond to the details and edges (F-actin fibers) in the image. Thus an image with randomly oriented fibers would produce a frequency plot with a circular distribution pattern. For
images where there is some degree of orientation in the fibers the distribution pattern would be more of an oval shape displayed at an angle orthogonal to the actual fiber orientation. Further analysis of this data will define the Orientation Index (OI) that is the angular increment about the angle of the centroid encompassing 50% of the F-actin filaments. Note that lower OI values indicate a high degree of alignment.

As the MATLAB program produced the frequency plot of each image it also produced a data file that was used to complete the further analysis. This data file was then opened in Microsoft Office Excel (2007) and the data including the intensity values and corresponding angle (from 0° to 180°) was extracted. The intensity data was then normalized and integrated to obtain the area under the curve. Corresponding graphs for each image were made by plotting the normalized intensity corresponding with the fraction of F-actin filaments versus the angle. Finally the angle of the centroid (i.e. where 50% of the area under the curve is on one side of the angle and 50% on the other) and the OI values were determined.

5.1.6 Collagen and DNA assays

In order to quantify the amount of collagen that was synthesized within the scaffolds after 2 and 4 weeks cultivation, the Sircol™ collagen assay kit (Biocolor LTD., United Kingdom) was used per the manufacturer’s instructions using a Genesys 20 spectrophotometer (Thermo Fisher Scientific) [23,31]. Samples (~ 2.5 by 2.5 mm) were cut from the cell-seeded scaffolds and weighed prior to extraction of the collagen. In order to extract the collagen, samples were placed in PCR tubes in 100 μL of extraction solution (0.5M acetic acid and 1 mg/ml pepsin A (sigma) in
water) for 16 hours on an orbital rocker (Orbiton I™; Boekel Scientific, Feasterville, PA). After the 16 hours extraction time the samples were assayed using the Sircol collagen assay kit.

The DNA content of the scaffolds after 2 and 4 weeks cultivation were quantified using the PicoGreen dsDNA quantification kit (Invitrogen) per the manufacturer’s instructions using a Spectramax Gemini XS plate reader (Molecular Devices, Inc., Sunnyvale, CA) [23,31]. Samples (~ 2 by 2 mm) were first cut from the cell-seeded scaffolds and weighed. The samples were then transferred to microcentrifuge tubes with 1ml of buffered 0.125 mg/ml papain solution for 10 hours in a 60°C water bath in preparation for the PicoGreen assay.

5.1.7 Statically Stretched Scaffolds

An additional study was attempted in which the effects of statically stretching the micromolded, cell-seeded scaffolds were observed. For this part of the study, the scaffolds were fabricated and cell seeded under the same afore mentioned processes. They were then allowed to culture statically in individual wells for 1 day to allow for cell attachment. The next day the scaffolds were subjected to a 10% stretch. Small pieces of autoclaved rubber band were placed around the ends of the scaffolds and held in place by Teflon tape. This allowed the ends of the scaffolds to be taped down to a glass slide in a way that statically stretched the scaffolds. The scaffolds were then allowed to culture statically for 5 more days. Every day or two light microscopy images were taken to track any changes in cell orientation and the media was changed. After the 5 days the scaffolds were fixed in 10% neutral buffered formalin (Sigma) while still being stretched. Later the scaffolds were removed from the wire and stained for F-actin. The same previously mentioned procedure was used. These scaffolds were then imaged through confocal microscopy.
5.2 Results

5.2.1 F-actin Orientation

Results from the FFT orientation software showed that F-actin fibers align predominately with the long axis of the 2:1 aspect ratio diamond shaped pores. Please note that for this software 90° is defined as the long axis of the scaffolds and a lower Orientation Index (OI) represents a higher level of orientation. The software showed that scaffolds seeded with aortic valve interstitial cells had an average OI of 51.6 ± 7.4° for a centroid of 94.6 ± 6.0° (Figure 5.3). For scaffolds seeded with pulmonary valve interstitial cells the average OI was 40.5 ± 2.0° for a centroid of 94.2 ± 7.6°. Example images for aortic and pulmonary cell seeded scaffolds are depicted in Figures 5.4 and 5.6, respectively. Corresponding example F-actin orientation distribution graphs are shown in Figures 5.5 and 5.7, respectively.

![F-actin orientation in diamond pores](image)

**Figure 5.3: F-actin Orientation Index Averages**

OI averages for the aortic and pulmonary valve interstitial cell seeded scaffolds. Note that while a statistically significant difference (p < 0.05) was detected between the aortic and pulmonary valve interstitial cell groups (*), we anticipate, based on the similar morphology exhibited by the cells, that this apparent difference would likely vanish upon analysis of increased numbers of images (images of n=6 pores were available for analysis in the current study)
Figure 5.4: Aortic Valve Interstitial Cell Seeded Scaffold Orientation Images
Example F-actin images used and produced in the FFT software to quantify aortic valve interstitial cell orientation including A) the 40x confocal image showing the F-actin and DAPI staining, B) the cropped F-actin image that was input into the software, C) the black and white image that was produced through the software, and D) the FFT image output from the software.

Figure 5.5: F-actin Orientation Distribution for an Aortic VIC Seeded Scaffold
Corresponding graph depicting the amount of fibers orientated at specific angles in the above images. The centroid shows around which angle most of these fibers are oriented around and the orientation index quantifies the amount of fibers.
Figure 5.6: Pulmonary Cell Seeded Scaffold Orientation Images

Example F-actin images used and produced in the FFT software to quantify pulmonary valve interstitial cell orientation including A) the 40x confocal image showing the F-actin and DAPI staining, B) the cropped F-actin image that was input into the software, C) the black and white image that was produced through the software, and D) the FFT image output from the software.

Figure 5.7: F-actin Orientation Distribution for a Pulmonary VIC Seeded Scaffold

Corresponding graph depicting the amount of fibers orientated at specific angles in the above images. The centroid shows around which angle most of these fibers are oriented around and the orientation index quantifies the amount of fibers.
5.2.2 Biochemical Assays

As expected, collagen and DNA assays showed that there was an increase in both DNA (i.e., cell number) and collagen content in the scaffolds that were cultured for 4 weeks compared with 2 weeks (Table 5.1; Figure 5.8). For both cell types, the collagen content was significantly higher at 4 weeks versus 2 weeks culture time (+156% and +190% for the aortic and pulmonary valve interstitial cell seeded scaffolds, respectively). For both culture times, the aortic valve interstitial cell seeded scaffolds were shown to have a trend of higher mean collagen content than the pulmonary valve interstitial cell seeded scaffolds; however, the difference (+25.3%) was statistically significant only at the 2 week time point. By contrast, at the 2-week time point the DNA content was significantly less (-30%) in the aortic versus pulmonary valve interstitial cell seeded scaffolds; at the 4-week time point there was a trend of increased DNA in the aortic versus pulmonary valve interstitial cell seeded scaffolds, but it failed to reach statistical significance. To gain further insight into the collagen synthesis behavior of these cells, the ratio of collagen to DNA was calculated for the aortic and pulmonary valve interstitial cell seeded scaffolds for the different cell types at the different culture times. For the 2-week culture time the aortic and pulmonary valve interstitial cell values were 24.0 ±12.3 μg/g wet weight and 15.1 ±8.6 μg/g wet weight, respectively. These values suggested a trend of +59% in the mean collagen per DNA in the aortic versus pulmonary valve interstitial cell seeded scaffolds, however the difference did not reach statistical significance. For the 4-week time point, the collagen to DNA ratios for the two cell types were similar (30.7 ±6.3 for aortic and 30.5 ±6.2 for pulmonary). The table and graphs below show the average values for the different cell types and culture times.
Table 5.1: Averages for Biochemical Assays

Collagen and DNA contents (mean ± standard deviation) for both aortic and pulmonary valve interstitial cell seeded scaffolds cultured 2 and 4 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Collagen (ug/g wet weight)</th>
<th>DNA (ug/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aortic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 week</td>
<td>266.1 ± 96.7</td>
<td>11.8 ± 5.2</td>
</tr>
<tr>
<td>4 week</td>
<td>681.1 ± 153.9</td>
<td>26 ± 11.1</td>
</tr>
<tr>
<td><strong>Pulmonary</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 week</td>
<td>212.3 ± 50.3</td>
<td>16.9 ± 7.3</td>
</tr>
<tr>
<td>4 week</td>
<td>615.1 ± 203.4</td>
<td>18.9 ± 9.3</td>
</tr>
</tbody>
</table>

Figure 5.8: Biochemical Assay Graphs

These graphs show the differences in collagen and DNA content for both aortic and pulmonary valve interstitial cell seeded scaffolds that were left in culture for 2 and 4 weeks.

5.2.3 Statically Stretched Scaffolds

Light microscope images of the cell seeded scaffolds taken daily show the progression of cell proliferation and growth. It appears as if the cells attach to the acute angle of the pores and then migrate around the edge pore until finally completely filling the pore in the middle of the scaffold. The confocal images that were taken were unable to be used to quantify F-actin
orientations within the stretched pores because the cells became unattached once released from their stretched position. Figure 5.9 shows the cell proliferation and growth over 4 days.

![Image of stretched scaffolds](image)

**Figure 5.9: Light Microscope Images of 10% Stretched Scaffolds**

*These images are representative of the cell growth within the pores of the stretched scaffolds at A,B) one day after seeding (20x and 40x), C) two days (40x), D) three days (40x) and E,F) four days after cell seeding (40x and 20x)*

5.3 Discussion

5.3.1 F-actin Orientation

The results from the F-actin orientation analysis show that there is a preferential alignment of the cells with the long axis of the 2:1 aspect ratio diamond pores. In particular, we found an OI value of 51.6 ± 7.4° for a centroid of 94.6 ± 6.0° for the scaffolds seeded with aortic valve interstitial cells (Figure 5.5) and an OI value of 40.5 ± 2.0° for a centroid of 94.2 ± 7.6° for the pulmonary valve interstitial cell seeded scaffolds (Figure 5.7). The lower OI values of the pulmonary cells show that more of the fibers are aligned in this direction compared to the aortic cells that on average have higher OI values. As highlighted above in the results, while a statistically significant difference (p < 0.05) was detected between the aortic and pulmonary
valve interstitial cell groups, we anticipate, based on the similar morphology exhibited by the cells, that this apparent difference would likely vanish upon analysis of increased numbers of images (images of n=6 pores were available for analysis in the current study). Previous studies have investigated cell and collagen orientation in microfabricated pores and using an FFT-based analysis of F-actin. For example, Engelmayr et al. previously demonstrated that the aspect ratio of microfabricated non-degradable scaffolds comprised of rectangular pores could significantly enhance the orientation of cells (rat skin fibroblasts) and their synthesized collagen along the long axis direction of the pores [32]. In that study, a small angle light scattering (SALS) technique was used to quantify collagen orientation; OI values were calculated from the SALS data using the same definition as in the current study and mapped across the scaffold surfaces yielding OI values ranging from 25 to 65 (i.e. bracketing the values measured in the current study). Cell orientations were deduced qualitatively from phase contrast light micrographs, confocal micrographs of F-actin labeled scaffolds, and scanning electron micrographs. In addition, Engelmayr et al. demonstrated in that study that 2:1 aspect ratio diamond shaped pores could qualitatively promote cell orientation along the pore long axis [32]. This previous finding served as a basis for the current study on biodegradable microfabricated PGS scaffolds; in particular, it was shown previously that the 2:1 aspect ratio diamond shaped pore was more consistent in promoting cell alignment than 2:1 aspect ratio rectangular pores. More recently, Nichol et al. used an early version of the FFT analysis software used in the current study [33]. Nichol et al. used the software to measure OI values in tissue engineered cardiac muscle constructs based on a synthetic, self-assembling peptide nanofiber gel. Neonatal rat cardiomyocytes or cardiomyocytes plus cardiac fibroblasts were seeded within the nanofiber gels, and the resultant engineered tissues were cultivated statically with or without the matrix
metalloproteinase (MMP) inhibitor doxycycline (it was hypothesized that MMPs secreted by the cardiac fibroblasts would enhance cardiomyocyte elongation and alignment). In Nichol et al. [33] the OI index was calculated differently from in the present study. Specifically, OI was calculated directly from the shape of the white circular to elliptical region in the FFT image (i.e., by the expression 1 minus the ratio of the minor-to-major axis length of the FFT image; an OI value of 0 indicated a completely random cell orientation and an OI value of 1 indicated unidirectional cell alignment). Nichol et al. reported OI values ranging from approximately 0.15 to 0.55, finding that co-cultured cardiomyocytes plus cardiac fibroblasts resulted in enhanced alignment that correlated with expression of MMP-2 [33]. The version of the FFT-based image analysis software used in the current study was first introduced in Engelmayr et al. [30]. In that study, PGS scaffolds with elongated, accordion-like honeycomb pores, 2:1 aspect ratio rectangular pores, and square pores were fabricated by laser microablation and seeded with a mixed population of heart cells, including cardiomyocytes and cardiac fibroblasts. The OI was calculated the same as in the current study, as determined from FFT-based image analysis of F-actin in confocal micrographs. OI values ranged from 85 ± 5° for square pores, 54 ± 1° for accordion-like honeycomb pores, and roughly 40° for adult rat right ventricular myocardium (a control tissue) [30]. Of note, the OI value measured in the current study for pulmonary valve interstitial cell seeded scaffolds were very similar to that measured previously for native cardiac muscle (i.e., 40.5 ± 2.0°), suggesting that native-like degrees of cell alignment can be achieved by appropriate control of scaffold microstructure. In the current study, the OI value for native heart valve leaflet could not be determined due to the aforementioned problems with confocal reflectance microscopy as applied to the native leaflet; F-actin staining could have been performed on the native leaflet, however the significantly lower cell density of the native leaflet
versus native cardiac muscle may have made it difficult to apply the FFT image analysis program. In the context of microfabricated PGS scaffolds, another recent study by Guillemette et al. investigated the orientation of C2C12 skeletal muscle cells on laser microfabricated PGS scaffolds comprised of rectangular or square pores with or without a supplementary contact guidance grooves [34]. C2C12 cells were found to preferentially align along the long axis of the rectangular pores (as measured from scanning electron micrographs), with a significantly greater degree of alignment observed in scaffolds exhibiting the contact guidance grooves. In particular, approximately 60% of the C2C12 cells were aligned within ± 10° of the rectangular pore long axis with the supplementary contact guidance grooves. By contrast, less than 20% of the C2C12 cells were aligned in the case of the square pore without the contact guidance grooves. Results of Guillemette et al. [34] suggest that the addition of contact guidance features to the 2:1 aspect ratio diamond shaped pores could be expected to enhance preferential alignment of heart valve interstitial cells.

5.3.2 Biochemical Assays
The results from the biochemical assays confirm that valve interstitial cells are capable of (1) proliferating on the microfabricated PGS scaffolds, as evidenced by the increased DNA content observed in the aortic valve interstitial cell seeded scaffolds at 4 versus 2 weeks, and (2) VICs are capable of synthesizing collagen on microfabricated PGS scaffolds, as evidenced by the increased collagen contents at 4 weeks versus 2 weeks. The increase of both collagen and DNA content from the scaffolds that were cultured for 2 weeks to the ones that were cultures for 4 weeks proves that the scaffolds support cell viability and expression of a differentiated phenotype (i.e., collagen synthesis is a hallmark of valvular interstitial cells). For both cell types the collagen content in the 4 week culture more than doubled from the content in the 2 week
culture. These results also provide support for the confocal images used in the F-actin orientation quantification. In particular, the substantial DNA content present within the scaffolds provides further evidence that the F-actin staining imaged in the confocal micrographs (Figures 5.4 and 5.6) and quantified by FFT image analysis indeed corresponded with cellular F-actin, not an artifact of the staining process. In comparing the collagen and DNA contents measured in the current study to previous studies, we find that the results are similar. For example, in the context of heart valve tissue engineering, Engelmayr et al. previously cultivated ovine vascular smooth muscle cells on 50:50 blend nonwoven poly(glycolic acid):poly(L-lactic acid) scaffolds for a period of 3 weeks under static and bioreactor-mediated cyclic flexure conditions [31]. By 3 weeks, collagen contents were reported as 546 ± 111 μg/g wet weight for the statically incubated samples and 893 ± 133 μg/g wet weight for the cyclically flexed samples. These values bracket the 4-week collagen results obtained for aortic and pulmonary valve interstitial cell seeded PGS scaffolds in the current study (i.e., 681.1 ± 153.9 μg/g wet weight and 615.1 ± 203.4 μg/g wet weight). In a more recent study, Engelmayr et al. reported collagen contents for nonwoven scaffolds seeded with ovine bone marrow-derived mesenchymal stem cells, with values ranging from 422 ± 98 μg/g wet weight (static culture), 530 ± 106 μg/g wet weight (cyclic flexure), 498 ± 95 μg/g wet weight (laminar flow), and 844 ± 278 μg/g wet weight (combined cyclic flexure and laminar flow) [35]. In the context of microfabricated PGS scaffolds similar to those used in the current study, Masoumi et al. recently reported collagen contents of 736 ± 193 μg/g wet weight for 2:1 aspect ratio diamond shaped scaffolds fabricated by laser microablation and cultivated with rat dermal fibroblasts for 3 weeks [23]. Regarding DNA content, values measured in the current study for 4 week samples (i.e., 26 ± 11.1 μg/g wet weight for aortic and 18.9 ± 9.3 μg/g
wet weight for pulmonary) were similar to those measured in the above-mentioned studies (e.g., 17.4 ± 4 μg/g wet weight in Masoumi et al. [23]).

5.3.3 Statically Stretched Scaffolds

It was hypothesized through that influence of static stretch, which would effectively elongate the diamond shaped pores in the scaffolds; the cells would become more aligned about the long axis. Unfortunately no quantification of the orientations of the cells was able to be completed since the cells fell of off the scaffold upon release of the 10% stretch. While the light microscope images seemed to show some orientation about the long axis, there is no way to tell if the alignment is superior to the original, non-stretched, values without this quantification. According to work done by Engelmayr et al., rectangular shaped pores with larger aspect ratios display better collagen alignment within the pores then those with a 1:1 aspect ratio do. It was also evaluated that diamond shaped pores are even more effective at guiding the collagen and cell alignment then those rectangular pores [32]. Thus it would be interesting to see how an increase in the aspect ratio of the diamond pores would affect cell alignment.
VI. CONCLUSION

The overall goals of the studies comprising this thesis were to develop a better understanding of native heart valve leaflet mechanical properties, as well as to compare two different scaffold materials for their ability to promote orientation of collagen and/or cells, thereby assessing, in part, their capacity to mimic native heart valve leaflet structures.

Although some studies were found to be inconclusive to date, some foundational facts were established through this work. In the case of the naturally-derived collagen gel scaffolds, a distinct difference in collagen and F-actin orientations was found between biaxially- and uniaxially-constrained gels. Specifically, a degree of orientation was seen in the uniaxial case, whereas a more random distribution of orientations was seen in the biaxial case. As for the synthetic, microfabricated PGS scaffolds, it was found that cells would preferentially align along the long-axis of the microfabricated scaffold pores. Collectively these results provide a foundation for future studies in heart valve tissue engineering.
REFERENCES


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The Pennsylvania State University
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University Park, PA
Schreyer Honors College
Expected Graduation: May 2012

Honors
The Pennsylvania State University College of Engineering Dean's list
4 Semesters: Spring 2010- Present

Academic Excellence Scholarship
1 Semester: Summer 2011
Total Amount Granted: $1,000.00
8 Semesters: Fall 2008- Spring 2012
Total Amount Granted: $14,000.00

International Thesis Research Grant
1 Semester: Summer 2011
Total Amount Granted: $2,000.00
1 Semester: Fall 2008
Total Amount Granted: $3,000.00

Research Experience

Lydia’s Design Team
BIOE 450W Senior Design student with Prof. Margaret Slattery at The Pennsylvania State University
Spring 2012- Present

- Leading a team in designing an assistive toileting device for a 7 year old girl who was born with no arms but has two fused finger like appendages off of each shoulder
- Managing the correspondences between design team and team sponsors
- Developing designs and testing prototypes

Heart Valve Tissue Engineering Research Laboratory
Honors undergraduate research assistant for Prof. George C. Engelmayr, Jr. at The Pennsylvania State University
Fall 2009-Present

- Comparing mechanical and cellular characteristics between native heart valves and poly(glycerol sebacate), PGS, polymer scaffolds
- Isolating and culturing of porcine aortic and pulmonary interstitial cells
- Casting of polymers, seeding of cells onto polymer, and analysis of cell attachment and proliferation
- Mechanical testing of native leaflets and PGS polymer and designing of bioreactor for polymers
- Staining by F-actin and visualization through reflectance Confocal Microscopy
- Drafting and preparing to defend Honors Thesis

Eindhoven University of Technology Biomedical Engineering Research Laboratory
Research Intern under Prof. Anita Dressien-Mol at Eindhoven, Netherlands
Summer 2011

- Studied the remodeling effects of extracellular matrix and cell interactions in 3D constructs subjected to uniaxial stress
- Cultured human vena saphena cells, constructed and seeded 3D collagen matrigel gels
- Used collagen and cellular probes to visualize cellular structures with Two-Photon Confocal Microscopy
- Quantified collagen and f-actin orientations through fiber tracking software created on Matlab
Mashavu Design Team  
BIOE 401 student with Prof. Peter Butler at The Pennsylvania State University

- Developed teamwork while designing, developing and testing a low cost thermometer for underdeveloped countries
- Led the experimental testing aspect of the project
- Produced prototypes and optimized the design
- Participated in weekly project updates and presentations

Pittsburgh Tissue Engineering Initiative Summer Internship  
Research Intern under Prof. Thomas W. Gilbert at The University of Pittsburgh

- Evaluated possibility of using decellularized lung tissue as a biological scaffold
- Decellularized rat lungs and trachea to make acellular scaffolds
- Cultured A549 cells and seeded cells on to decellularized scaffolds
- Created extracellular matrix powder and isolated and quantified DNA
- Stained using DAPI, H&E and Immunofluorescence

Activities

Penn State Dance Marathon  
Volunteered in the largest student run philanthropy in the world
 Raised money and enhanced awareness for pediatric cancer
 Helped set up events that supported the families of children with cancer

Relay for Life Participant  
Volunteered in the local community’s Relay for Life events
 Raised money and enhanced awareness for cancer patients and survivors

Intramural Sports  
Participated in intramural soccer and flag football teams

Publications


