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Feasibility Studies on Using a Thermoplastic Polyurethane Scaffold for Hybrid Tissue-Engineered Heart Valves

THESIS

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MASTER OF SCIENCE

in Biomedical Engineering

by

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DEDICATION

To

my family and friends

in recognition of their love and support

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ABSTRACT OF THE THESIS

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By

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Master of Science in Biomedical Engineering

University of California, Irvine, 2017

Professor Arash Kheradvar, Chair

Heart valve replacement procedures currently use either mechanical or bioprosthetic valves made from animal tissue. This thesis examined the use of polymers as the scaffold material for novel hybrid tissue-engineered heart valves and as the leaflet material for polymer heart valves. The concept of a polymeric heart valve was also examined when testing material durability and functionality with accelerated wear testing and a pulsatile flow simulator. Human aortic smooth muscle cells (HASMC), normal human lung fibroblasts (NHLF), and umbilical vein endothelial cells (HUVEC) were examined on a thermoplastic polyurethane, Aromatic Carbothane. Each cell type was seeded on individual pieces of both fibronectin-treated and untreated Carbothane to test biocompatibility. Samples were stained using Cell Tracker Red CMTPX and imaged using fluorescence microscopy. Fluorescence imaging confirmed HASMC and NHLF adhesion and viability on the polyurethane substrate. HUVECs initially attached on both fibronectin-treated and untreated substrate, but they began to detach and clump together within 24 hours. Trilayered tissue samples were successfully grown on a lasercut Carbothane mesh scaffold using the three cell types. A Carbothane heart valve was sewn and placed in a heart flow simulator to examine geometric orifice area. The valve opened to about 49% of the

calculated orifice area, but it showed very symmetrical coaptation as it closed. The valve showed no signs of damage after 20 million cycles in the AWT. Carbothane exhibited excellent biocompatibility, and its durability, ease of manufacturing, and flexibility make it a promising candidate for future studies in the field of heart valve engineering.

CHAPTER 1

Introduction

1.1. Valvular Heart Disease

Cardiovascular diseases are among the world's leading causes of death.[1] In the United States alone, they are responsible for over 800,000 deaths each year.[1] Patients can be affected by many types of cardiovascular diseases ranging from arrhythmia, cardiomyopathy, valvular heart disease (VHD), and congenital heart defects. Common causes of many of these diseases include atherosclerosis, high blood pressure, smoking, and diabetes.[1,2] Atherosclerosis is a prominent cardiovascular disease that often leads to more serious conditions; it is defined as the buildup of plaque in the arteries that causes fibrosis of the inner walls.[3] While the cause of this disease is not well understood, it is often a major factor in the progression of VHD.[4,5]

VHD can affect any of the four valves in the heart (Figure 1.1).[6] Heart valve dysfunction can be congenital or an acquired disease. Acquired degenerative valve diseases are generally characterized as either valvular stenosis or regurgitation.[5,7] Valvular stenosis prevents sufficient blood flow through the heart because of thickened or calcified leaflets.[5] The thickening and calcification reduce the valve's ability to open. Valvular regurgitation, on the other hand, is due to improper closure of the valve leaflets and results in a leaky valve.

Valvular heart diseases comprise approximately 20 percent of all cardiac surgical procedures in the United States.[8] As patients grow older, the prevalence of acquired valve diseases increases along with the need for treatment.[9] Prevalence of VHD in 18- to 44-year-old patients was 0.7 percent and increased to 13.3 percent in patients age 75 and older (Figure 1.2).[9]

The risk of developing a degenerative valve disease is significantly higher in developing countries where larger groups of people are affected by rheumatic fever or rheumatic heart disease.[9,10] Bacterial infections associated with rheumatic fever cause inflammation of the heart and blood vessels, often leaving the heart valves permanently damaged.[10] An estimated 15 million people worldwide are affected by rheumatic heart disease, and many of the afflicted require frequent hospitalization.[10,11] Sadly, many of those with rheumatic heart disease are not able to receive regular treatments.[11] With an estimated 5 million Americans diagnosed with VHD each year and many more globally, the need for reliable and efficient treatment is substantial.[1]

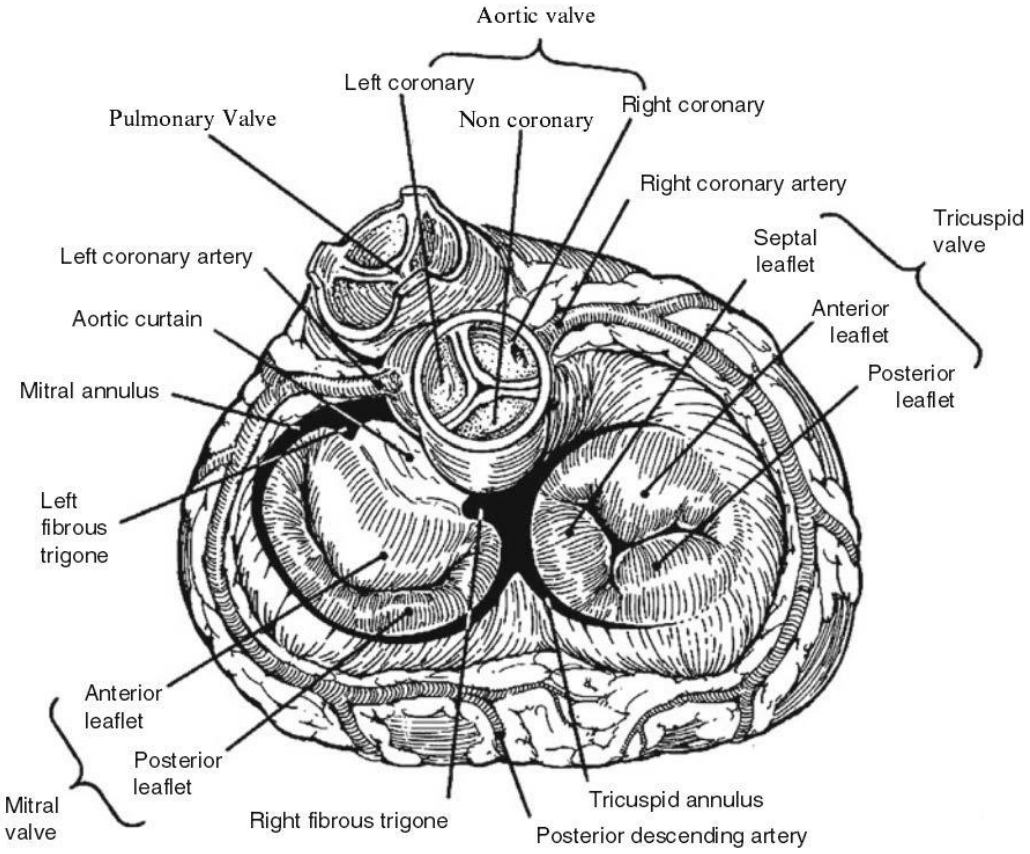

Figure 1.1 Cross section of the heart showing the four valves. The aortic valve, pulmonary valve, and tricuspid valve are tricuspid valves. The mitral valve is a bicuspid valve. Image from Iaizzo, 2009.[6]

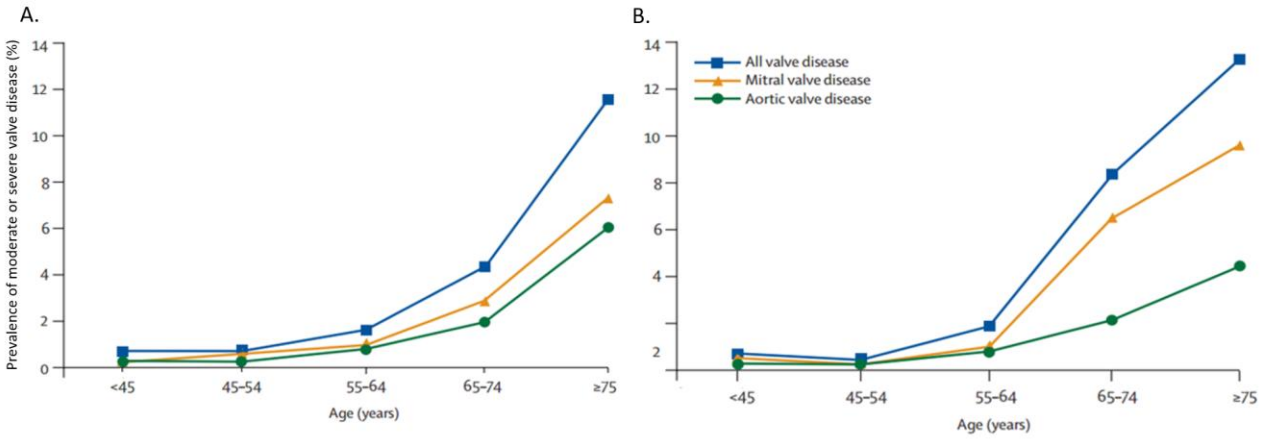

Figure 1.2. The prevalence of valvular heart disease significantly increases in older patients. Population studies at the national level are shown in A) while data collected for Olmsted County, MN is shown in B). Figure taken from Nkomo, 2006.[9]

1.2. Replacement Valve Treatment Options

There are currently no treatments to reverse or prevent VHD. Fortunately, there are many other surgical treatment options available. Valvular stenosis and regurgitation, the most prevalent acquired valve diseases, are commonly treated with a heart valve replacement using either a mechanical valve or a bioprosthetic heart valve (BHV) (Figure 1.3).[5,7,12] The mechanical valves are very durable and long lasting, but they tend to have higher risks of thromboembolism which may lead to a stroke or heart attack. Mechanical valves are generally implanted in younger patients due to their durability, but lifelong anticoagulation therapy is necessary to reduce the risk of stroke or heart attack.[12] Bioprosthetic heart valves are made from bovine or porcine tissue and have greatly improved hemodynamic and biocompatibility properties, so they do not require anticoagulation therapy. However, they have lower durability than mechanical valves, and, depending on the age of the patient, may eventually need to be replaced. Each type of replacement valves has its own set of advantages and disadvantages, and selecting the right option depends on each patient's specific conditions. Both the aortic and mitral valves can be

treated using a surgical valve replacement. However, this requires open heart surgery and long recovery times that increase the risk of post-operative complications. In fact, nearly 33 percent of patients at least 75 years of age diagnosed with severe aortic stenosis were denied treatment because of the higher risk of post-operative complications.[13]

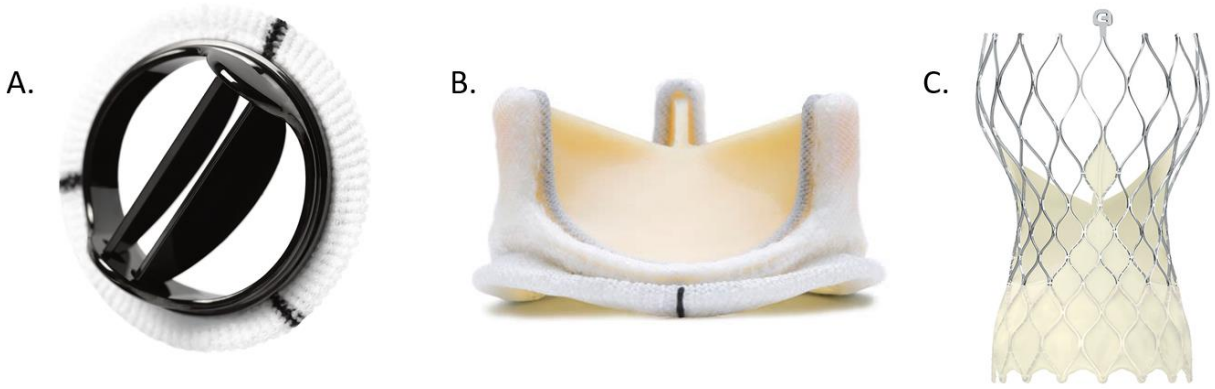

Figure 1.3. Three different types of commercial heart valve replacement devices. A) The St. Jude Medical Regent™ mechanical heart valve. B) The Edwards Perimount™ Bovine Pericardial Aortic Valve. C) The Medtronic CoreValve Evolut Pro™ Transcatheter aortic valve.

Paradoxically, the age group most commonly affected by acquired valve diseases is also the group most often denied surgical replacement. This gap in treatment options has led to the development of other forms of heart valve replacements. Transcatheter aortic valve implantation (TAVI) is a newer, alternative technique used to replace diseased heart valves (Figure 1.3).[14] Much less invasive than surgical replacements, TAVI uses a catheter system and a "crimped" bioprosthetic valve to replace the target valve without requiring extensive surgery.[14] This method is mainly used for high-risk patients, but as the technology improves, many more will become eligible for a transcatheter valve replacement. One of the obstacles that TAVI faces is the fact that there is little clinical data on the long-term durability of the replacement valves, although they do have excellent hemodynamic and anti-thrombogenic properties. As with any bioprosthetic valve, the exposure to the many cyclic mechanical stresses present in the heart can

lead to transcatheter valve calcification.[15] Thus, younger patients who can recover more easily from open heart surgery are often not eligible for TAVI due to their inferior durability compared to mechanical valves. As more data is gathered on the long-term durability of transcatheter replacement options, it is likely that younger patients will become eligible for this less invasive procedure.

Even with the extensive surgeries required, surgical heart valve replacements remain the most common type of treatment for VHDs. Of this group, bioprosthetic heart valves comprised the largest market share. As the technology improves, however, TAVI is predicted to dominate the market share in the near future. The global market in 2015 for all heart valve replacements was valued at 3.14 billion dollars, and it is expected to grow to 5.3 billion dollars by 2021.[16] There is also considerable economic value for improving patient health and longevity; the net value of the extended life years of aortic valve replacement patients in the U.S. was 11.3 billion dollars.[17] As new therapies are developed, these numbers will continue to increase, along with the patients' quality of life.

A new area of research with the potential to change the way VHD is treated is tissue-engineered heart valves. A living heart valve replacement would reduce or even eliminate many of the durability issues associated with bioprosthetic heart valves. Rather than tearing or calcifying because of exposure to the shear stress and other mechanical forces linked to dynamic blood flow, these living valves would be able to regenerate and replicate native cellular structure to compensate for the stresses. The cells used to grow the replacement valve are taken directly from the patient, so adverse immune responses to the implant are not likely to occur. Over the past decade there has been extensive research into this idea, but no successful treatments have been developed yet. The approaches that have been researched on this topic involve using both

synthetic and natural scaffolds as a base on which to seed the appropriate cardiovascular cell types.[18-20]

Synthetic scaffolds are generally composed of polymer complexes that simulate the biological extracellular matrix, and they are designed to break down over a short period of time.[18,19] Cells are seeded directly onto the scaffolds, and as they attach and proliferate, the cells begin to develop their own natural extracellular matrix (ECM) and collagen structures to replace the synthetic scaffold.[19,21] Depending on the environment in which the tissue-engineered heart valves are cultured in, the ECM and collagen architecture may remodel in an attempt to optimize cell exposure to nutrients and prevent excessive shear forces.[21,22] While this treatment sounds ideal, it is very difficult to grow the entire valve *in vitro*, and the complicated structure of native heart valve tissue is still not well understood. The biodegradable scaffolds also give rise to durability issues during the growth process.

Another approach to develop tissue-engineered heart valves is by using decellularized animal tissue valves.[20,23] Using a number of chemical treatments, the valves can be stripped of all viable tissue as the ECM maintains a high level of structural integrity.[24] Stem cells are seeded on the decellularized heart valve scaffolds and are cultured in dynamic environments to facilitate autologous recellularization of the valve *in vitro.*[23,24] This method has several drawbacks; depending on the method of decellularization, the collagen and ECM maybe be structurally weakened which can lead to scaffold shrinkage.[23-25] Using a decellularized xenograft scaffold also affects immunogenic and thrombogenic properties of the implant. Inflammation and increased thrombogenicity are common side effects when using decellularization.[24]

1.3. Polymer Heart Valves

The ideal treatment for Valvular heart diseases is a prosthesis which is minimally invasive, completely resistant to mechanical deterioration, and has excellent hemodynamic properties. Anticoagulation medication would not be necessary, either. Since the 1960s, the use of flexible polymers as the leaflet material has been extensively studied.[26] While many polymers boast the necessary flexibility to achieve ideal hemodynamic properties, few have been able to eliminate the need for anticoagulation therapy. Polycarbonate urethanes and polyurethanes were commonly used because of their excellent durability.[26-28] These valves often withstood more than 600 million cycles during accelerated wear tests. However, calcification of the polymer leaflets *in vivo* remained a debilitating issue. In fact, many animal and human clinical trials for various polymeric heart valves have been halted due to calcification and thrombosis.[26] However, recent advances in polymer research have resulted in new materials with excellent biocompatibility. A novel polystyrene showed significantly less thrombotic potential than glutaraldehyde-fixed porcine tissue, and it was not significantly different from another polymer that was previously approved for cardiovascular applications.[29] Many polymers are also now able to be modified at a microscopic level to meet specific mechanical needs, which will improve the lifespan of the material in a dynamic environment. Once successful, a polymeric heart valve would have a major impact on the treatment of valve diseases. Not only would they last a lifetime, polymeric heart valves would be significantly cheaper and easier to manufacture, which would enable millions more people living with valvular heart diseases to be treated.

1.4. Outline of the Thesis

The development of a new scaffold for tissue-engineered heart valves is the main objective of this thesis. A scaffold that promotes successful recellularization while minimizing immunogenicity and thrombogenicity is of utmost importance. A strong, biocompatible base on which to grow a patient's tissue will promote proper cellular function and regeneration. The development of such a heart valve would greatly improve patients' quality of life. In this research, previous experiments and various materials were first studied to develop an understanding of the type of scaffold needed for such a device. The mechanical properties of several polymers and other nondegradable materials were compared to the properties of native heart valves and bioprosthetic valves. Chapter 2 describes this process and the results found from the scaffold material investigations.

Once an ideal scaffold material was chosen, biocompatibility and cell viability were examined with native valvular interstitial cell types. These experiments are discussed in detail in Chapter 3. Layers of each cell type were grown on the chosen scaffold material, and the cells' responses were monitored using both light microscopy and fluorescence microscopy. After confirming the scaffold biocompatibility, tri-layered tissue constructs were grown as a method to study the scaffold at a tissue level.

Production of the leaflets for the hybrid tissue-engineered heart valve (TEHV), as well as full valve functional testing is outlined in Chapter 4. The design of the leaflets was completed in CAD software, and multiple paths of scaffold production were considered. A slightly modified version of the scaffold was created in order to perform accelerated wear testing and functional testing of the scaffold material. Because the leaflets are perforated to create a cohesive tissue-polymer hybrid for the actual valve, fluid dynamics could not be studied. Leaflets were made

without the perforations to examine the scaffold's reaction to various heart flow simulations as well as to test its long-term durability in an accelerated wear tester. Finally, a hybrid tissue-engineered heart valve prototype will be grown.

Future research on this topic include new frame and leaflet designs for the valve. These may be optimized to improve the valve's ability to regulate blood flow and increase durability. *In vivo* studies will also be performed using a sheep model for the valve. The future topics of research and further discussion are outlined in Chapter 5.

CHAPTER 2

Hybrid Tissue-Engineered Heart Valve Scaffold Material Selection

2.1. Background

Growing a viable tissue-engineered heart valve presents many challenges. Along with overcoming the standard challenges such as durability and thrombogenicity, a tissue-engineered heart valve needs to promote and sustain specialized tissue growth. Scaffold structures attempt to mimic the extracellular matrix's strength and flexibility but degrade over time, allowing the seeded cells to deposit their own ECM. In natural ECM, collagen fibers give the tissue strength and rigidity, elastin allows the heart tissue to expand and contract without damage, and glycosaminoglycans protect the tissue from impact forces and shear during the valve cycle.[19] Synthetic scaffolds generally use polymer mixtures to capture these properties. Common polymers are polylactic acid (PLA) and polyglycolic acid (PGA); they are synthetic, biodegradable, and biocompatible.[30,31] PGA is a hydrophilic substance and degrades quickly in the body; therefore, it loses its mechanical strength very quickly. PLA, however, is hydrophobic, maintains its mechanical properties, and thus does not degrade as quickly.[19] Scaffolds used for heart valve engineering need to degrade quickly, but they also must maintain their mechanical stability long enough to support tissue growth and ECM production. By using hydrogel matrices comprised of both polymers, an ideal ratio of mechanical stability and degradability can be achieved.

This method of using PLA-PGA hydrogels has shown promise in the field of tissue engineering. That said, there are drawbacks to these copolymers including difficulties with proper cell adhesion and varying mechanical properties of the gels. Because they do not have any of the ECM proteins natural tissue contains, cells react differently to the polymers. Another issue with using synthetic scaffolds is the potential for inflammation. As the scaffold breaks down and degrades, seeded cells proliferate and secrete ECM into the empty space. Unfortunately, oxygen diffusion through the tissue may not be sufficient due to the lack of tissue vascularization, which is a major hurdle toward successful engineering of thick, complex tissues.[19,32]

Rather than creating a hydrogel scaffold from synthetic materials, the existing ECM protein scaffold in porcine tissue can be obtained through decellularization. This method employs a series of chemical detergents to remove cellular tissue and leave this protein scaffold relatively untouched.[33,34] Decellularization takes advantage of the ECM structure formed when tissue grows in a natural, dynamic environment instead of using a statically developed synthetic hydrogel scaffold. The decellularized scaffold can be recellularized via two methods: *in vitro* cell seeding, or *in vivo* growth of autologous cells.[35,36] Before recellularization in either method can be completed, the scaffold must undergo ample washing because the detergents are highly toxic to the cells.[35] Once the scaffold is sufficiently washed, valve interstitial cells are seeded on the decellularized scaffold and allowed to attach. To add more complexity to an already intricate procedure, the valves must be incubated in specialized bioreactors that improve nutrient and oxygen supply to the growing tissue.[36,37] The bioreactors mimic the dynamic environment of the heart and may lead to a more natural engineered tissue. I*n vivo* growth of autologous cells greatly simplifies this process by eliminating the dynamic incubation needed to recellularized the scaffold *in vitro*. The decellularized scaffold can be directly implanted into the animal or patient,

and the recellularization process occurs inside the body. This method also has significant drawbacks. The scaffolds can degenerate causing structural failures of the implant as well as inflammation of the valve.[38] Cell repopulation *in vivo* is a slow process which can allow bacteria to infiltrate the scaffold and cause negative immunogenic responses.[38] While both methods show promise as a future treatment of VHD, neither are currently viable options.

2.2. Materials Investigated

The goal of this research was to develop a nondegradable scaffold for tissue-engineered heart valves. By having a biocompatible, permanent scaffold to facilitate tissue growth, many of the mechanical and immunogenic problems could be addressed. To be successful, the material would need to be flexible, nontoxic, and nonhemolytic. In previous studies, nitinol was investigated as the scaffold material.[39,40] Nitinol was used because of its excellent shape memory characteristics and its proven biocompatibility. Although it is highly durable, it is significantly stiffer than a native valve which may cause long term durability problems. To solve this problem, the use of polymers such as various polyurethanes were investigated. The ideal polymer needed to be biocompatible, nonhemolytic, nondegradable, and highly elastic. To maintain structural integrity, the material also needs to have negligent water absorption levels. The combination of these properties would enable the scaffold material to be successful as a long-term implantation.

Two important values investigated for both new materials and native heart valves were the ultimate tensile strength, or the maximum stress a material can withstand before failing (e.g. breaking or tearing), and the elastic or flex modulus of the material.[41] The elastic modulus measures an object or material's resistance to deformation along an axis caused by an opposing

force.[41] Both properties are static mechanical properties, and since a heart valve is located in a highly dynamic environment, they do not fully capture every mechanical characteristic of a native heart valve. The fluid forces that act on the heart valve leaflet surfaces are not uniform, so various areas of the heart valve may be exposed to higher than normal stress[42]. Due to the anisotropic nature of heart valve tissue, additional layers of complexity are added when characterizing the tissue's mechanical properties[43]. It is extremely difficult to generate a model that accurately portrays the tissue structure and function of a heart valve, and it often requires custom biaxial stress testing equipment[44]. Developing an accurate model of the mechanical properties of a native heart valve was not the goal of this study, and the elastic modulus and ultimate tensile strength of the materials were used because these properties still provide valuable information about the polymers' mechanical properties. As a reference for polymer comparisons, the commissures of a human aortic valve have an elastic modulus of 13.80 +/- 3.16 MPa and an ultimate tensile strength of roughly 2.6 MPa.[45,46] Nitinol has drastically different properties; its elastic modulus ranges from 41 to 75 GPa and an ultimate tensile strength of greater than 1070 MPA, both of which are several orders of magnitude larger than a human aortic valve.[47]

Three groups of medical-grade polyurethanes were compared to native heart valves and nitinol: Biospan SPU (DSM Medical), Tecothane TPU (Lubrizol, Inc), and Aromatic Carbothane TPU (Lubrizol, Inc). Each polymer is available in multiple hardness levels, so the mechanical properties of each hardness were compared (Figure 2.1). The weakest material was Tecothane Soft Polyester, which had a maximum ultimate tensile strength of 30.4 MPa. While it did have the closest tensile strength to native aortic valves, Tecothane Soft Polyester 62A also had a flex modulus value lower than aortic valves that reduced the likelihood of it being an acceptable

scaffold material. Both Biospan and Carbothane had ultimate tensile strengths higher than an aortic valve, but the flex modulus of Biospan was almost an exact match to the aortic valve (14.55 MPa and 15 MPa, respectively).[45] In addition to their ideal elastic moduli and tensile strengths, both Biospan and Carbothane exhibit no cytotoxic effects, are hydrolytically stable with negligent water absorption, and are nonhemolytic. To further qualify these polyurethanes as excellent candidates for hybrid tissue-engineered heart valve scaffold material, both have been determined to be suitable for long-term implantation in humans. One characteristic of Carbothane that differentiated it from Biospan is that it is a thermoplastic polyurethane— that is, Carbothane can be repeatedly heated and cooled without impairing its mechanical properties. Because highly dynamic forces are continuously acting upon heart valve leaflets, a material with slightly stronger mechanical properties may enhance long term durability of the prosthetic valve. Thus, Carbothane TPU 95A was selected as the substrate material for further biocompatibility and functional testing.

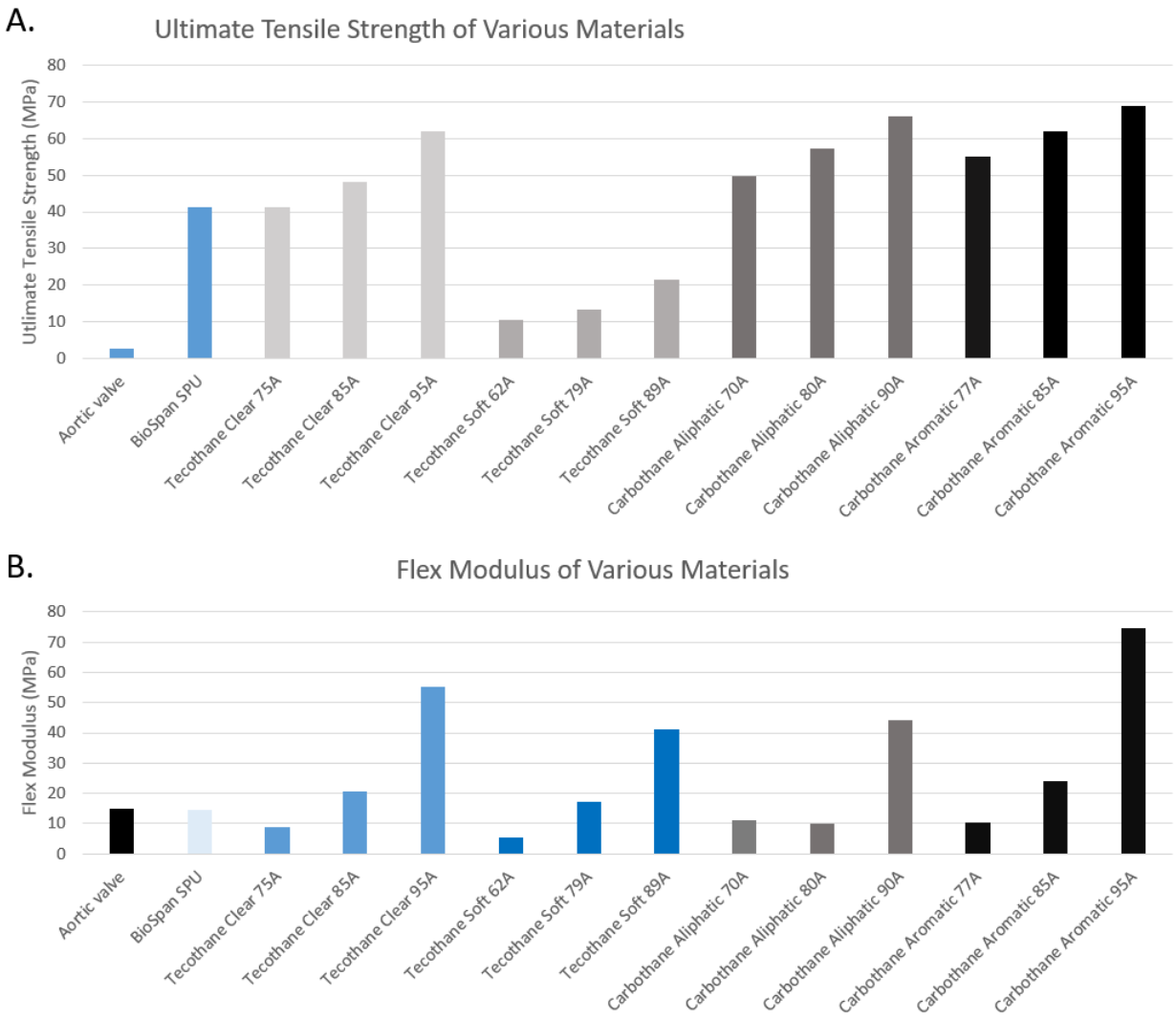

Figure 2.1. A) The ultimate tensile strengths and B) flex modulus values of each polymer were compared to that of native human aortic valves. All product information was obtained from DSM and Lubrizol product information pages.

CHAPTER 3

Biocompatibility and Cell Viability on a Carbothane Scaffold

3.1. Background

The field of tissue engineering has long been idealized as the answer to many biomedical problems ranging from heart valve replacements to entire organ growth *in vitro*. It has proven to be an extremely complicated subject to research, and progress toward achieving these lofty goals has been slow. To grow a tissue-engineered heart valve successfully, the heart's structure and function at the cellular level needs to be better understood. From previous studies, valve interstitial (VIC) and endothelial cells (VEC) have been found to play an important role in regulating molecular pathogenesis in heart valves.[48-51] Vital to the proper functioning of native heart valves, healthy VICs maintain proper proliferation and the secretion of critical matrix compounds and ECM molecules.[49] Without appropriate regulation, VICs may begin to express myofibroblastic phenotypes that cause the valve tissue to stiffen.[48,49] A cascade effect of tissue inflammation and stiffening follows; the myofibroblastic VICs continue to release large amounts of collagen and begin osteogenesis which leads to valve stenosis and calcification.[49] In healthy heart valves, VECs secrete nitric oxide (NO) which inhibits osteogenic differentiation and calcification of VICs and allows them to express normal phenotypes.[49] Any attempts toward successfully growing a healthy tissue-engineered heart valve must provide a similar cellular environment that promotes proper phenotypic expression.

VICs exhibit morphological similarities to both fibroblasts and smooth muscle cells, so these cell types are commonly used to study VIC characteristics *in* vitro.[51] In this portion of the study, cell adhesion, viability, and tissue growth of human aortic smooth muscle cells (HASMC), normal human lung fibroblasts (NHLF), and umbilical vein endothelial cells (HUVEC) were examined on Carbothane scaffold material. These tests determined whether the new scaffold material was biocompatible and could be a suitable material for hybrid-TEHVs.

3.2. Materials and Methods

To test cell adhesion and viability, single layers of each cell type were grown on $1cm^2$ squares of Carbothane. Before seeding on the substrate material, HASMC, NHLF, and HUVEC (Lonza Group) cell lines needed to undergo maturation. In early generations, the young cells express different characteristics and proliferate much more quickly. Each cell type was cultured in appropriate cell culture media (Lonza Group), which contained all necessary growth factors, cytokines, and supplements (Table 3.1). Penicillin-streptomycin antibiotics were used to prevent any bacterial growth during cell culturing. Cell lines were cultured at 37ºC and 5% CO_2 until the fifth generation was reached in order to prepare adult populations for the study. Samples from each generation were cryogenically stored in liquid nitrogen to build a cell bank for later use.

FGM-2 Fibroblast Growth Medium		SmGM-2 Smooth Muscle Growth Medium		EGM-2 Endothelial Cell Growth Medium	
Ingredient	Quantity (mL)	Ingredient	Quantity (mL)	Ingredient	Quantity (mL)
Fibroblast Basal Medium	500	Smooth Muscle Basal Medium	500	Endothelial Basal Medium	500
Penicillin-Streptomycin	10	Penicillin-Streptomycin	10	Penicillin-Streptomycin	10
Fetal Bovine Serum (FBS)	10	FBS	25	FBS	10
Insulin	0.5	Insulin	0.5	Hydrocortisone	0.2
rhFGF-B	0.5	hFGF-B	1.0	hFGF-B	2.0
GA-1000	0.5	GA-1000	0.5	VEGF	0.5
		hEGF	0.5	R3-IGF-1	0.5
				Ascorbic Acid	0.5
				hEGF	0.5
				GA-1000	0.5
				Heparin	0.5

Table 3.1 The contents of each growth medium contained several growth factors and supplements to promote cell viability and proliferation *in vitro*. Every 500 mL of media also contained 2% penicillin-streptomycin antibiotic.

Flat strips of Aromatic Carbothane 95A were obtained from Lubrizol prior to beginning the study. Approximately $1cm^2$ squares were cut from the strip and placed in a 50 mL centrifuge tube with 70% isopropyl alcohol (IPA) for sterilization. The tube was placed in an ultrasonic bath for fifteen minutes. Fresh IPA was added to the tube under a cell culture hood, and the process was repeated three times. After sterilization, the Carbothane squares were transferred into individual wells on a six-well culture plate. To examine cell adhesion and viability properly, both fibronectin-treated and untreated Carbothane samples were used. Three Carbothane samples were submerged in a fibronectin bath for 1 hour prior to cell seeding. To facilitate cell growth only on the Carbothane and not the surrounding well surface, roughly 100,000 cells of each cell type were pipetted directly onto the Carbothane squares without spilling over. 200 uL of cell media was also pipetted onto each sample, and the six-well plate was placed in the incubator for

1 hour. Samples were then examined under a microscope for signs of cell attachment. When suspended in media, all three cell types appear circular and float when the suspension is disturbed, and as cells attach to a surface they begin to elongate. Roughly 2 mL cell media was gently pipetted into each well, and the cell culture plate was returned to the incubator where it remained overnight. To provide enough nutrients for the cells to survive, cell media was replaced every 24 hours. Images were captured under a light microscope every 24 hours for 7 days, and cell adhesion and viability were observed.

Using the samples at the end of the 7-day incubation period, cell proliferation was studied. Carbothane squares with single layers of each cell type were transferred to fresh cell culture plates. Cells were dissociated using trypsin, followed by centrifugation and resuspension in appropriate cell culture media. An automated cell counter was used to determine the final population count of each cell type.

In parallel to the 7-day cell adhesion, viability, and proliferation study, these characteristics were examined further using fluorescence microscopy. A nearly identical experiment was prepared with the exception that following the initial 1 hour incubation, samples were submerged in Cell Tracker Red CMTPX (Thermo Scientific) and incubated for an additional 45 minutes. Cell Tracker Red dye was used because it is well retained by the cells once ingested, and its signal can be viewed for up to 72 hours. The dye was then replaced with appropriate cell culture media, and initial images of each sample were taken using the fluorescence microscope (Figure 3.1).

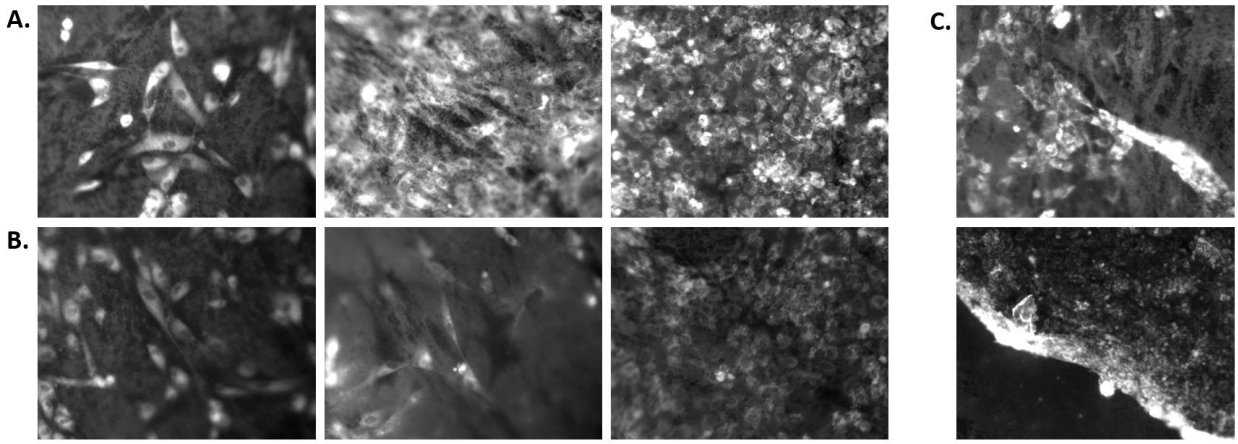

Figure 3.1 Fluorescence microscopy images of HASMCs, NHLFs, and HUVECs were captured on Carbothane samples. (A) HASMCs (left), NHLFs (middle), and HUVECs (right) show attachment on fibronectin coated AC-4095A. (B) Attachment is seen for cells on non-fibronectin coated AC-4095A. (C) HUVECs begin to clump together and die on fibronectin coated (top) and non-fibronectin coated (bottom) AC-4095A within 24 hours.

3.3. Results

Carbothane showed positive adhesive properties with HASMCs and NHLFs. The cells' level of elongation was used as a factor to determine their ability to adhere to and survive on the Carbothane material. Both cell types attached and survived when cultured on fibronectin coated and untreated AC-4095A. Cells survived on the substrate for the entire 7-day period of the experiment. Fluorescence imaging confirmed HASMC and NHLF viability on the polyurethane substrate (Figure 3.1). HUVECs initially attached to the fibronectin coated substrate, but they began to clump together within 24 hours. Similarly, for untreated AC-4095A, HUVECs showed initial attachment but clumped together and died within 24 hours of seeding. The proliferation tests confirmed the results that HASMCs and NHLFs exhibit normal growth properties, while HUVECs do not. A significant increase in population for both HASMCs and NHLFs was observed (215,000 $\pm$ 55,000 and 490,000 $\pm$ 100,000, respectively) (Figure 3.2). HUVEC

populations decreased significantly (60,000 ± 20,000), likely due to weak adhesive properties of these cells on the Carbothane surface.

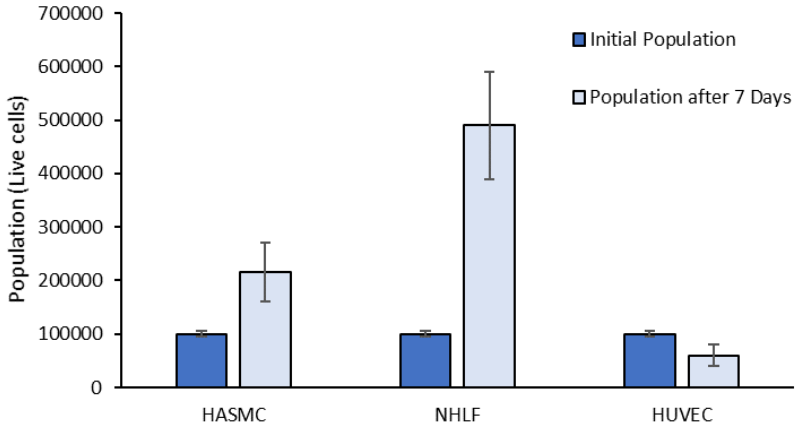

Figure 3.2 Cell proliferation on Carbothane was successful with HASMCs and NHLFs. HASMC populations rose by a factor of 2, and NHLFs increased nearly five-fold. HUVEC populations, however, declined over the 7 day period.

The biocompatibility properties of Carbothane AC-4095A were tested using HASMCs, NHLFs, and HUVECs. Each cell type was seeded on fibronectin coated and untreated substrates, and cell attachment and viability were observed using fluorescence microscopy. HASMCs and NHLFs exhibited good attachment and proliferation on both fibronectin coated and untreated substrates, while HUVECs did not attach as well to either substrate. HUVECs began to clump together and die within 24 hours of seeding. Fortunately, HUVECs will not contact Carbothane when used for hybrid tissue-engineered heart valves. Because of the order of tissue layers, Carbothane AC-4095A is an ideal candidate for hybrid-TEHV scaffold material due to its excellent durability and biocompatibility.

CHAPTER 4

Design of a Carbothane Scaffold for Tissue Growth and a Carbothane Heart Valve for Functional Testing

4.1. Background

Because of the trilayered structure of the proposed hybrid-TEHV, it is not necessary for all three layers to show adhesive ability to the Carbothane surface. HASMCs were the base layer, followed by NHLFs, and HUVECs created the outer layer. The two innermost layers consist of cells that showed excellent adhesive properties on Carbothane. Additionally, it was previously confirmed that a trilayered tissue construct of HASMCs, NHLFs, and HUVECs is possible in culture.[40] Carbothane has significantly better manufacturability than other polymers because it is a thermoplastic polyurethane. It also has excellent biocompatibility, and because it is nondegradable, it does not lose its mechanical stability over time. The use of polymers for mechanical heart valves has been studied for years. However, research has stagnated because of the difficulty in finding a polymer that exhibits good biocompatibility, hemocompatibility, and an excellent long term flex life. Many attempts at a polymer valve did not eliminate the need for anticoagulation therapy nor did it address concerns of leaflet calcification. As polymer technology has improved, the potential to find a suitable material has risen.

4.2. Materials and Methods

Following confirmation of cell survival and proliferation on the Carbothane squares, tri-layered tissue growth on Carbothane mesh was examined. The ability of the cells to create even layers of viable tissue is a top priority of this study. Uneven layers may create areas of high shear force when placed in a dynamic bioreactor or when used for *in vivo* studies. Carbothane sheets were lasercut to create a mesh to allow for the growth of tissue through the scaffold to enhance the strength of the leaflets. A Versa Laser Systems VLS2.30 laser equipped with a high-power density focusing optics (HPDFO) lens was used to cut the mesh from a 0.5mm thick strip of Carbothane. The hole diameter for the mesh was 1 mm. Optimized settings were required to prevent excessive melting or burning of the material (Table 4.1). The mesh was examined under a light microscope for signs of burned or extremely melted edges.

Versa VLS2.30 Laser System	
Power	5%
Speed	5%
Points per Inch (PPI)	500

Table 4.1 Optimal settings for a laser are highly dependent on the substrate material. Because Carbothane was a thin thermoplastic polyurethane, very low power and speed were necessary to eliminate excessive melting.

Once the Carbothane mesh was created, it was cut into 1 cm^2 squares and sterilized by the same process as before. The cell seeding technique for trilayered tissue growth is very different from the previous biocompatibility tests; cells will be suspended in a collagen matrix prior to seeding on the scaffold which allows them to grow in a three-dimensional structure instead of a flat layer of cells. Collagen polymerization can be confirmed visually when the mixture becomes clouded. This process begins almost immediately following the addition of

sodium hydroxide (NaOH), so the cells and scaffold must be ready beforehand. Once the cell-collagen mixture was seeded, it was placed in the incubator to accelerate the polymerization process.

Timing of the polymerization process must be carefully regulated because if it is left in the incubator for too long before adding cell media, the cells may starve. Incomplete polymerization, on the other hand, may cause the cells to migrate off the Carbothane scaffold once the cell media is added.

HASMCs were suspended in 5mg/ml collagen type I, and 250,000 cells were pipetted directly on the mesh. The cell-collagen mixture underwent polymerization in an incubator for 45 minutes before being submerged in cell media and returned to the incubator. Every 24 hours, the mesh samples were flipped, and another layer of cells was seeded onto the scaffold. Cell media was also changed every 24 hours. This process was completed two times for each cell type until the tri-layered tissue was fully seeded, and the cell media contained equal ratios of all three media types. Samples were then incubated for seven days, with the cell media mixture changed every 24 hours. Cell viability and proliferation were monitored by visual inspection under a microscope. Following completion of the tissue growth process, samples were fixed using 4% formaldehyde. The samples were sent out for histology imaging and cross-sectioning to determine the growth success of the tri-layered tissue.

The lasercutting process for the initial mesh development was adapted to produce the heart valve leaflets. Although the thickness of the Carbothane sheet used for the leaflets was 0.01 inches (250 μm), or half the thickness used for initial testing, the laser system's settings were the same. By using a thinner scaffold, the overall thickness of the final leaflet would be reduced and be a better representation of a native leaflet. A thinner leaflet may also give the polymer heart

valve better hydrodynamic function. Next, two separate leaflet designs were created: a perforated mesh leaflet with suture guide holes along the margin of attachment (MOA), and a solid leaflet containing only the suture guide holes along the MOA (Figure 4.1). Each leaflet was designed with the goal of obtaining even stress distribution throughout while optimizing leaflet coaptation.

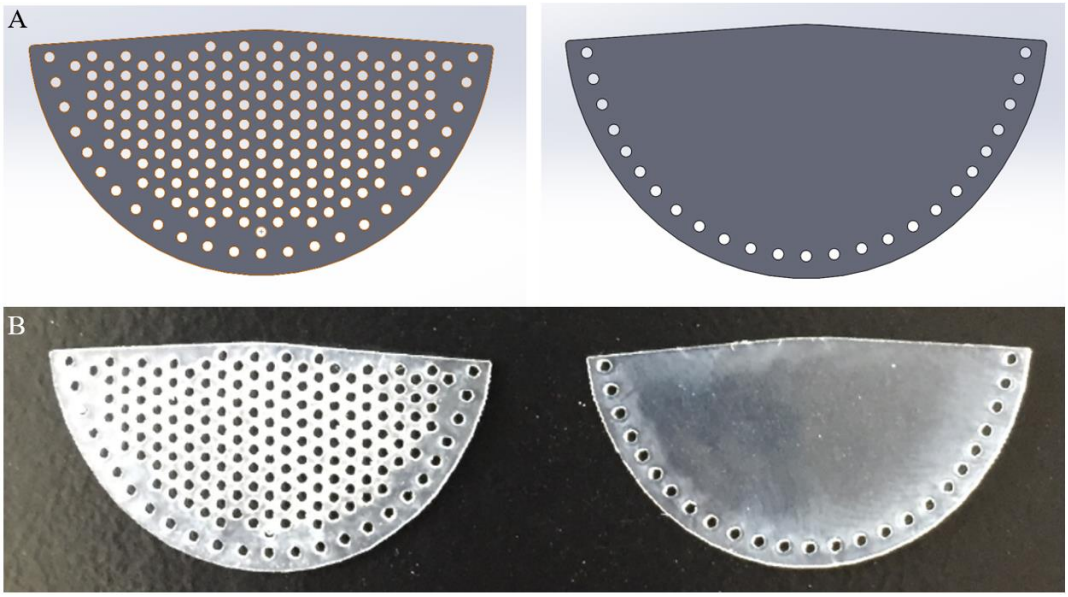

Figure 4.1 Two different leaflet designs were used during durability and functional testing. A) Solidworks models of a mesh leaflet (left) and a solid leaflet (right). Perforations on both leaflets were 0.5 mm. B) Each leaflet was successfully lasercut without any burning or excessive melting.

Using the mesh leaflets during hydrodynamic testing would not give any usable data because it would simulate a valve with extreme levels of regurgitation. Thus, the solid template was used to cut leaflets for the accelerated wear testing (AWT) and pulsatile flow simulator tests. The leaflets were then sent out to be sewn into functional valves (Figure 4.2). The leaflets were sewn into nitinol frames, and separate commissure posts were used in both designs. By using separate commissure posts, the forces exerted on the leaflets could be transferred more easily to the sturdier nitinol frame.

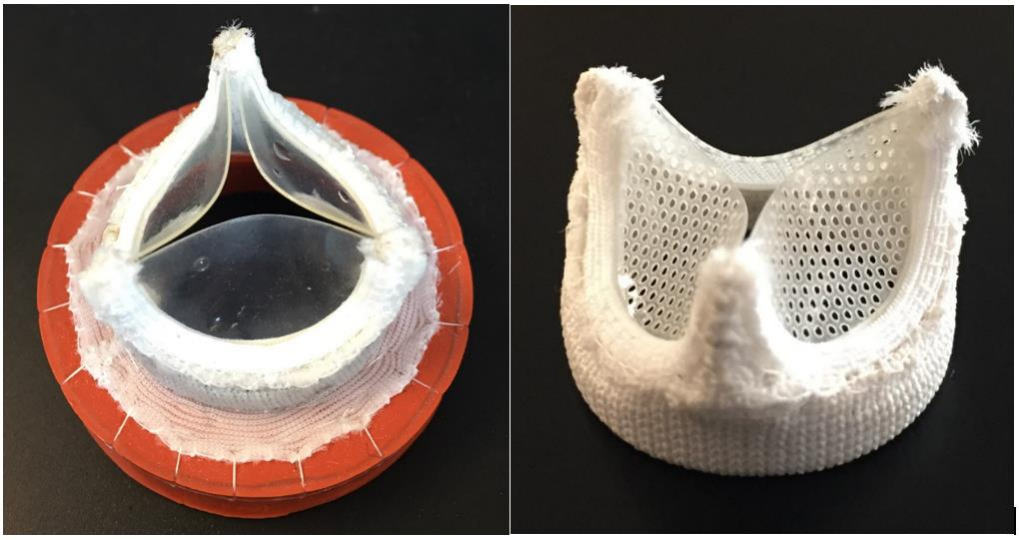

Figure 4.2 The solid leaflet valve (left) was sewn to a gasket for use in the AWT and pulsatile flow simulator. Layers of tissue will be grown on the mesh leaflet valve (right) prior to use. In both valves, an additional elastic band is sewn between the leaflets and the nitinol frame.

Accelerated wear testing is often described as the gold standard of heart valve testing. The purpose of studying a Carbothane valve in the AWT was to determine the durability of the leaflet material and expose any mechanical design flaws. By simulating physiological pressures at a very high cycle rate, projections for a valve's lifespan can be obtained. In this study, a Dynatek Labs M6 Heart Valve Durability Tester was used to perform the accelerated wear testing. A "heart rate" of 800 cycles per minute (cpm) and a pressure of 120 mmHg, which is considered as normal systolic pressure in the left ventricle, were used for the duration of the test. Calibrated pressure transducers were placed near the inflow and outflow regions of the heart to measure transvalvular pressure gradients during the test.

The pulsatile flow simulator is another very useful test when studying heart valve design. The machine simulates left ventricular pressures, flow rates, and fluid mechanics across a prosthetic heart valve. A silicone model of the left ventricle is used to create flow properties very close to those found in a human heart, and the cardiac output of the simulator is also generally

set to normal levels between 3-5 L/min. The valve set up for this test is nearly identical to that in the AWT; the heart valve is sewn to a rubber gasket that is placed in a stationary position in the machine. Cardiac output is monitored on a separate controller connected to sensors before and after the heart valve. A camera was placed directly above the heart valve chamber to record videos of the cardiac cycle that were used for valve orifice area analysis using ImageJ analysis software.

4.3. Results

Carbothane turned out to be highly responsive to the lasercutting process. Initially, the power settings on the laser were too high and caused excessive melting and even blackening around the edges. Once the settings were optimized, the material was cut very cleanly by the laser. The diameter of the holes was reduced from 1mm to 0.5mm when cutting the leaflet patterns. Even after long periods of continuous laser exposure, the material heat did not cause any unwanted melting. The trilayered tissue consisting of HASMCs, NHLFs, and HUVECs was successfully grown on a Carbothane mesh scaffold. The entire growth process, including one final week of incubation, took 30 days. Prior to tissue fixation in 4% formaldehyde, the samples were examined under a microscope for viability. Throughout the entire tissue, cells showed elongation comparable to the levels found under two-dimensional cell culture conditions (Figure 4.3). Microscope imaging was difficult due to the thickness of the tissue, but healthy cells could be seen at every layer. Unfortunately, it was not possible to perform histological cross-sectioning and staining because of the scaffold at the center of the tissue. It was very difficult to cut through the Carbothane without severely damaging the surrounding tissue. Perhaps a thinner sheet of Carbothane would mitigate the cross-sectioning issues without compromising any mechanical properties. Other methods of cross-sectioning the samples will be investigated in the future, but

even without histological analysis these results were enough to validate Carbothane as a nondegradable and biocompatible scaffold material.

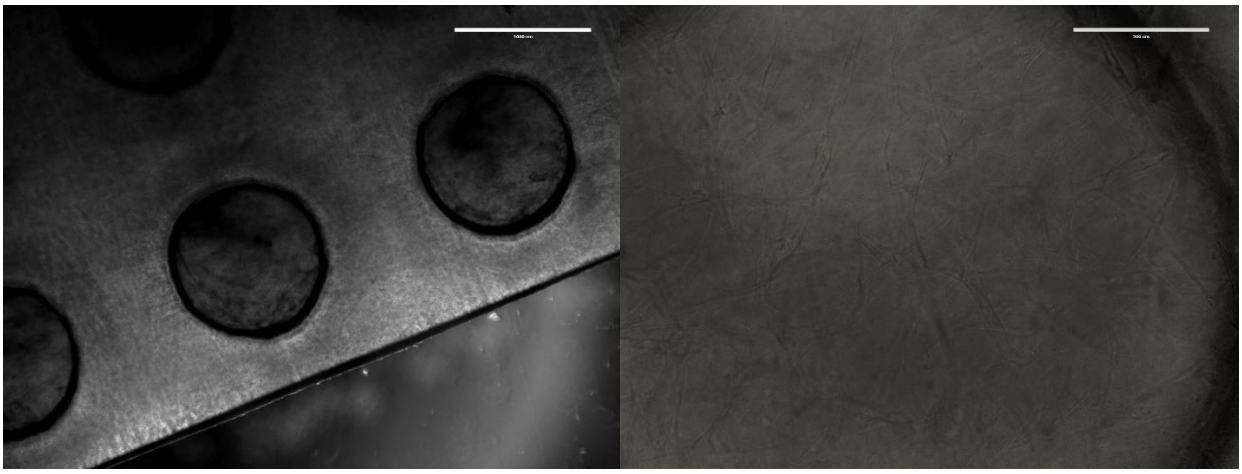

Figure 4.3 The trilayered tissue was successfully grown on a Carbothane scaffold. At 4X (left), dense areas of cells can be seen inside the mesh holes and as the distance increases from the edge of the square. Individual cells can be seen fully elongated at 20X (right).

Pulsatile flow simulations showed good ability of the solid Carbothane leaflets to close completely without any prolapse. They also showed good ability to open under normal conditions, although the valve was opening only in a triangle (Figure 4.4A). The geometric orifice area (GOA) was measured at cardiac outputs of 3 L/min and 5 L/min, and there was no significant difference between the GOA at either rate. The calculated orifice area (COA), or the maximum orifice area the valve can achieve, was estimated to be 309.84 +/- 5.62mm. When compared to the COA, the GOA was roughly 49 percent of the potential area (Table 4.2).

Attempts were made to improve the GOA of the Carbothane valve. A second valve design was made which had leaflets that were narrower in the radial direction. When placed in the pulsatile flow simulator, the new valve showed similar results as the first design (Figure 4.4B and C). The new design closed in a completely symmetrical pattern with no regurgitation, but it still did not open to its full extent. At 3 L/min, the valve only opened to approximately 37

percent of the COA, and it opened to 54 percent of the COA when the cardiac output was 5 L/min. As a reference, both Carbothane valves were compared to a transcatheter porcine pericardial bioprosthetic valve. The pericardial valve's GOA was significantly closer to its COA (246.65 +/- 1.71 mm, and 286.08 +/- 3.52 mm, respectively). It opened to 86 percent of the theoretical orifice area (Figure 4.4D).

There are several likely reasons that the Carbothane valve did not compare as well to the pericardial tissue valve. Although Carbothane is several orders of magnitude more flexible than nitinol, it is still significantly stiffer than native aortic valve tissue. The higher stiffness is a compromise for obtaining a higher durability in a Carbothane valve or leaflet scaffold. However, the triangular valve opening and less than optimal GOA were likely caused by the valve design rather than the material. For example, the separate posts may be too flexible and cause them to absorb too much of the stress. The leaflets may also be too small, which may increase lateral tension through the leaflets between posts. When the leaflet's radial dimensions were reduced in the second design, the valve closed more symmetrically, but it did not improve the valve's GOA. While the results from the pulsatile flow simulations were not perfect, they were able to prove that Carbothane is responsive to normal flow rates, and only minor design adjustments may be necessary to improve functionality dramatically.

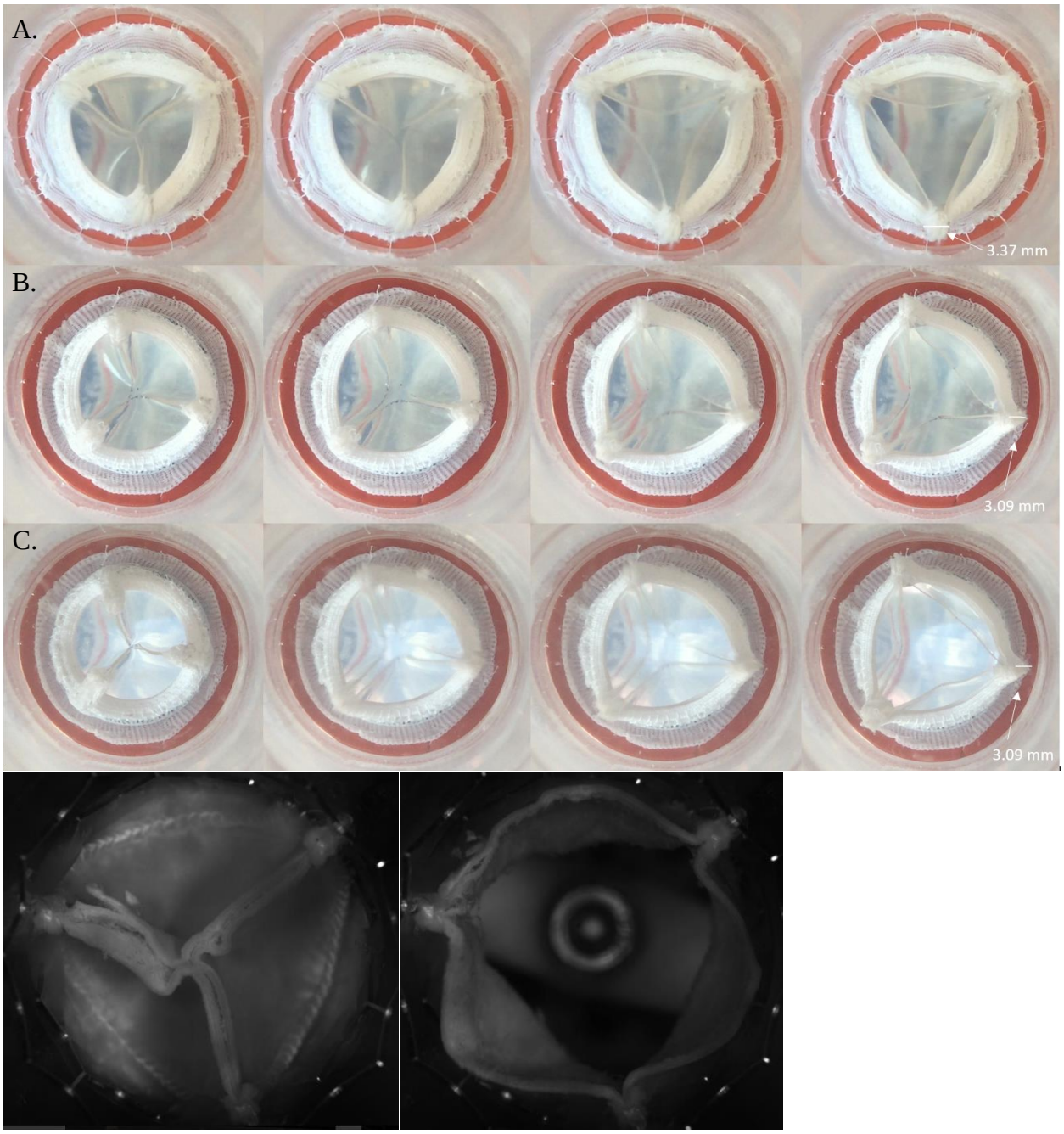

Figure 4.4 A) The Carbothane valve was photographed at several stages of the cardiac cycle. The valve fully closed at normal pressures (left), and all three leaflets opened evenly until its maximum triangular opening (right). B) and C) At 3 L/min and 5 L/min, respectively, the second valve design showed symmetrical closing with a low GOA. D) A porcine pericardial bioprosthetic valve opened and closed more efficiently than the Carbothane valve.

Carbothane Heart Valve: Design 1			
Cardiac Output	GOA (mm^2)	Standard Deviation (mm^2)	GOA:COA
3 L/min	151.87	2.30	0.49
5 L/min	154.69	1.32	0.50
Carbothane Heart Valve: Design 2			
Cardiac Output	GOA (mm^2)	Standard Deviation (mm^2)	GOA:COA
3 L/min	148.62	2.26	0.37
5 L/min	190.92	5.32	0.54

Table 4.2 The geometric orifice area in the Carbothane valve was not significantly different at either flow rate. However, when compared to the calculated orifice area, the valve only opened to about 49% of its theoretical capability at both flow rates.

Following completion of the pulsatile flow testing, the valve was placed in the AWT. The machine was set to run until valve failure at 800 cpm and a pressure of 120mmHg. The inflow and outflow pressures were monitored throughout the test. It is important to note that while the pressure transducers were calibrated correctly, there was a difference of 25mmHg between the transducers and the AWT controller's software. Taking this difference into account, the valve consistently opened between 115 mmHg and 120 mmHg, and it closed between 55 mmHg and 60 mmHg (Figure 4.6). Further inspection of the outflow pressure showed a consistent drop in pressure while the valve closed. A stroboscope was used to view the valve's opening and closing mechanics. The stroboscope revealed minor regurgitation that may be caused by the same potential design issues suggested following the pulsatile flow testing. It was also discovered that one of the leaflets was sewn about 1 mm higher than the others that caused the valve to close in a spiral. This asymmetry was likely the main cause of the valve regurgitation. The valve was removed at 10 million cycles and examined for damage. While no damage to any part of the valve was found, the stationary valve retained some of the spiral closing shape (Figure 4.6). Since the valve was still acceptable, it was returned to the AWT to continue running until failure. The second version of the polymer heart valve was placed in the AWT at this time as well. The

first design of the valve is currently approaching 25 million cycles with no signs of damage, and the second design is approaching 10 million cycles without any damage.

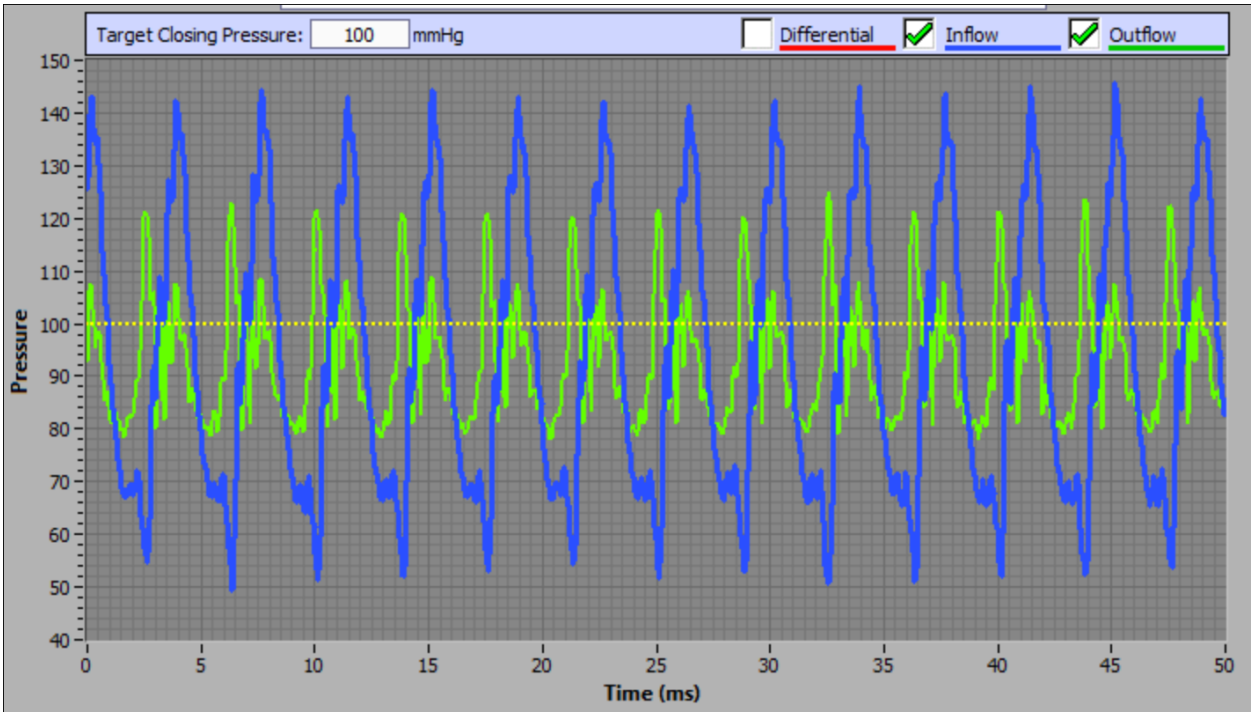

Figure 4.5 The Inflow pressure (blue) shows the valve opening between 115-120 mmHg. The outflow pressure (green) shows a consistent drop in pressure while the valve is closed, which shows that there is significant regurgitation. Note that the graph above does not account for the difference of 25 mmHg between both pressure transducers and the AWT controller software.

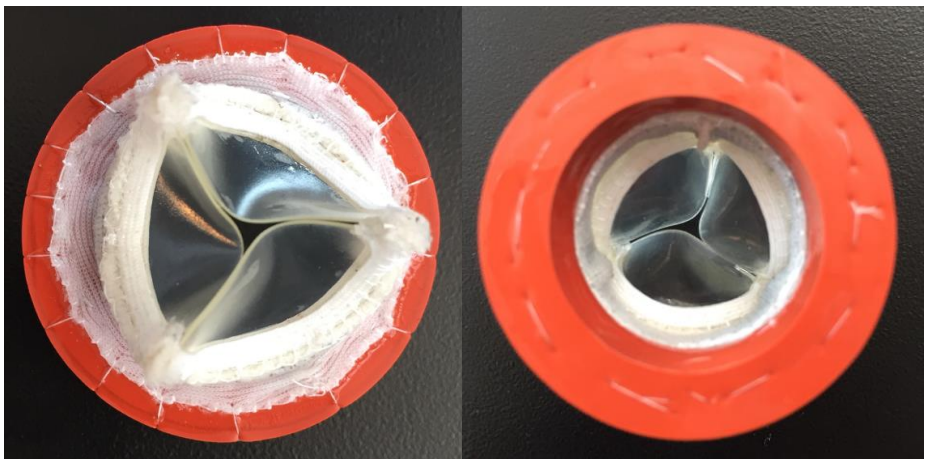

Figure 4.6 After 10 million cycles, the valve showed an uneven, spiral-like coaptation. This is likely due to asymmetrical sewing of the leaflets to the posts and frame.

CHAPTER 5

Conclusions

5.1. Conclusions

This collection of experiments confirmed that Carbothane exhibited many of the necessary properties and characteristics needed to be a successful scaffold in hybrid tissue-engineered heart valves. Its excellent biocompatibility with cell types representative of valve interstitial cells and its strong, yet flexible mechanical structure make it a great candidate. Carbothane was used successfully as the scaffold to grow a trilayered tissue construct consisting of HASMCs, NHLFs, and HUVECs. The cells continued to proliferate and elongate similarly to cells grown in a standard cell culture flask. Its thermoplastic properties made it astoundingly simple to manufacture mesh scaffolding and valve leaflets without compromising its other mechanical properties. Once sewn into complete valves, the solid leaflet version was studied in a pulsatile flow simulator and an accelerated wear tester. In both tests, the valve showed good ability to open and close; however, its geometric orifice area was only 49 percent of its maximum calculated orifice area. The small GOA was likely due to the design of the heart valve rather than the leaflet material used. Slightly stiffer commissure posts would allow the leaflets to absorb more force and potentially open in a more circular geometry. The design of the leaflets may also have contributed to the smaller than ideal orifice area. A longer leaflet in the circumferential direction may also give it additional flexibility, while a narrower leaflet in the radial direction may help improve symmetrical leaflet coaptation. So far, the AWT has shown excellent durability with no signs of damage. With more precise valve assembly, regurgitation

could be minimized or eliminated altogether. As more data was collected and the biocompatibility was researched further, it also became apparent that this material may be used as a fully synthetic heart valve that would not require anticoagulation therapies. Carbothane has been shown to be nonhemolytic with negligible thrombogenicity.[53] These properties would eliminate the need for a lifetime of anticoagulation therapy that is currently needed for mechanical valve recipients. The material can also be manufactured in sheets as thin as 0.001", which could make the leaflets more responsive to the pressures found in the heart. Leaflet thickness would need to be optimized to prevent the loss of durability. If successful, prosthetic heart valve costs would plummet while the quality of life of the patients could greatly increase. The material's durability would allow them to be implanted in younger patients without the fear of requiring another operation, even one as minor as a transcatheter valve implantation procedure. This research has shown many benefits for the use of Carbothane as both a scaffold for hybrid-TEHVs as well as on its own as a synthetic polymer heart valve, and it has helped lay the groundwork for future research in the field of heart valve replacement options.

5.2. Future Work

The compilation of data collected from initial material selection, biocompatibility, functional, and durability testing on Carbothane as both a polymer heart valve and as a scaffold for hybrid-TEHVs shows great promise for success in future experiments. Already planned future projects include accelerated wear testing to failure and pulsatile flow simulations for a newly designed leaflet pattern as well as a single-piece frame with fixed posts. Biaxial stress testing of Carbothane, pericardial tissue, and native heart valve tissue would provide a more accurate comparison between the materials. This may lead to additional changes to the

mechanical design of the prosthetic heart valve. Changes to the design may positively affect the geometric and effective orifice areas of the heart and can eliminate the presence of regurgitation during the cardiac cycle. Using a more flexible material for the frame is also an opportunity for major design improvements. A more flexible, yet very durable and biocompatible material such as polyether ether ketone (PEEK) would make an ideal single-piece frame that still allows for distribution of mechanical forces from the leaflets through the posts without compromising leaflet coaptation at closure. A benefit of using PEEK is that it already has a vast library of biocompatibility studies that show its suitably for long term implantation.[52]

Another experiment planned for the near future is an *in vivo* animal study of a hybrid-TEHV using Carbothane as the scaffold. The valve's function and biocompatibility will be monitored following implantation in an adult sheep. A biopsy of the sheep's jugular vein will be taken, and the cells will be cultured until a sufficient population is ready for seeding on the Carbothane scaffold. A sample of the cultured cells will also be sent out for identification. A customized three-dimensional cell culture mold will be used to seed and grow the tissue on the mesh valve. The mold consists of two pieces— a base with a pocket that the valve is placed in upside down, and a top piece that fits snugly on the inside of the valve. Once set up, there is a narrow gap on either side of the leaflets where the cell-collagen mixture will be seeded. A higher concentration of collagen will also be used to increase the mechanical strength of the tissue while the cells grow. Within 24 hours of successful cell seeding, the valve will be implanted in the sheep, and the tissue will grow in the implanted location. This way the tissue benefits from growing in a dynamic environment and can constantly absorb the necessary nutrients.

In the case that the sheep expires prior to the conclusion of the animal study, the cause of death will be determined, and the valve will be explanted for examination. Otherwise, the study

will continue until the study has concluded, at which point the valve will be explanted and examined for signs of thrombus formation, calcification, or any other type of damage. The surrounding heart tissue will also be examined for signs of inflammation, which would point to an immune reaction to the valve. Data collected during the animal study will be used to improve the valve's mechanical design, if necessary. Several rounds of animal studies are planned to improve the device design and streamline the methods used to produce hybrid tissue-engineered heart valves. Future research may eventually provide enough positive data to prove that this technology is a viable and rewarding path toward improving cardiovascular treatments and extending patient lives.

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