Platform Technologies to Advance Clinically Relevant Tissue Engineered Heart Valve Products

Leslie Neil Sierad
Clemson University

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PLATFORM TECHNOLOGIES TO ADVANCE CLINICALLY RELEVANT TISSUE ENGINEERED HEART VALVE PRODUCTS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Bioengineering

by
Leslie Neil Sierad
August 2014

Accepted by:
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ABSTRACT

Diseased heart valves are commonly replaced by mechanical, bioprosthetic, or allograft heart valves. These replacements provide major improvements in cardiac function and quality of life, but have significant limitations and eventually require surgical replacement within 15-20 years. These risks are particularly prominent in pediatric patients and young adults. The field of tissue engineering and regenerative medicine, which combines scaffolds and cells, holds great promise in developing living replacement heart valves that would self-repair and grow in size along with the growing children.

The long-term goal of this project is to generate living, tissue-engineered heart valves from biological scaffolds and autologous stem cells – a goal that hinges on our ability to create tissue devices that withstand mechanical stresses immediately upon implantation without posing risks of immunological rejection. We hypothesized that these valves can be generated by optimal integration of three main factors: acellular heart valve root scaffolds, autologous stem cells, and construct preconditioning in a bioreactor. Furthermore, we hypothesized that the valves would not be generated without advanced bioreactor systems for the development, conditioning, and translation to clinical practice.

To reach this goal, we developed integrated platform technologies for complete aortic valve root (AVR) decellularization, stem cell seeding, and dynamic conditioning before implantation. Unique features include universal “no touch” valve-mounting devices, decellularization in a purpose-designed pulsatile perfusion system, and techniques for in vitro re-vitalization with adipose tissue-derived stem cells (hADSCs) followed by progressive conditioning in our heart valve bioreactors. Acellular porcine AVRs seeded
with autologous (sheep) ADSCs were implanted in 10 sheep as right ventricle to pulmonary artery shunts with complete clamping of the pulmonary aorta.

Results showed perfect decellularization of the entire porcine AVR and almost complete seeding with ADSCs. Bioreactor studies revealed stem cell pre-differentiation into cells resembling valvular interstitial cells as a response to dynamic stimulation. Animal studies with follow-ups to 12 months are ongoing.

Novel customized devices and bioreactor systems are vital to the successful development of tissue engineered heart valve products, especially in preparation for clinical translation. Herein is described some of the basic equipment and expertise necessary for the successful development of tissue-engineered cardiovascular products.
DEDICATION

I would like to dedicate this dissertation to my Lord and Savior, Jesus Christ. He is the Creator and God over all things, including my life and this research, and it is only by His grace that I have been able to complete my studies at Clemson.

Through the Bible came the knowledge of my sin and the realization that I needed to be saved from the penalty of those sins. Were it not for Jesus Christ’s perfect life given as an example, His death as a substitutionary payment for my sins, His resurrection as victory over death, and His offer of redemption as salvation for my soul, I would have no hope for my future. But by His grace, I have agreed with Him about the circumstances of my life and have accepted His gift of salvation. I place my faith entirely in Christ and His works, rather than any of my own, to save me. Therefore, I have a living hope for my future and a purpose for my life that is far greater than any other: to bring glory to God.

It is for this purpose that I live; it is for this purpose that I have performed research at Clemson University. As such, I dedicate this work to the Lord Jesus Christ, without whom I would have had neither the strength nor the skills to complete it.

And there is no other God besides Me,
A righteous God and a Savior;
There is none except Me.
Turn to Me and be saved, all the ends of the earth;
For I am God, and there is no other.
~Isaiah 45:21b-22~
ACKNOWLEDGEMENTS

I have learned more while pursuing my PhD than I ever could have imagined, and most of it was regarding far more than the research at hand. The experiences and opportunities provided to me by various organizations, individuals, and trials while at Clemson has contributed greatly to my life. To every one of them: Thank You.

Specifically, I would first like to extend deep gratitude and admiration to my advisor, Dr. Dan Simionescu as well as Dr. Aggie Simionescu. Your drive to advance the projects was second only to your concern for those working on them with you. Your endless support and guidance were as helpful as your granting of liberties to pursue the additional aspects of life and research that have shaped and molded each one of your students for the betterment of each of us, and therefore, the entire lab.

To all my colleagues in the BTRL of Clemson and TERM Lab of Romania: thank you for your various expertise on this project and for your friendship over the years. Each one of you have added something to the lab that is vital for its health. To the undergrads who have worked with me over the years: you have contributed greatly to this project and my life. I can only hope that you benefited from working with me. I have never seen an undergraduate work as hard as Laine Shaw or with such skill as Allison Kennamer. You both amaze me.

In addition to the University and the Department, many individuals within the University have contributed greatly to this project and my time here. Thank you to my other committee members, Dr. Ken Webb and Dr. Chris Wright for your expertise and guidance that has helped direct the outcome of this project. Thank you to Lisa Perpall in
the CURF office for your ability to remind and encourage me of the more widespread applicability to my projects. None of this work would have been possible without the assistance of Glenn Rankin and the entire Machining and Technical Services crew. MTS truly is a hidden gem at the heart of this university. Bob Teague was also instrumental in providing electronics and LabView capabilities for our lab.

Outside sources have also made this work possible. Thank you to Judy and the Snow Creek Meat Processing plant. Not only has your generosity fueled our entire department for years, but you have also made me a lover of great sausage. To each collaborator and supporter at the University of Pharmacy and Medicine of Targu Mures: thank you for your hard work on this project and for an unforgettable experience on the other side of the world. I would also like to thank the lab members of Dr. Jun Liao for their assistance in evaluating the mechanical properties of various tissues. Additionally, none of this would be possible without various funding from the NIH RO1 HL093399 and a grant of the Romanian National Authority for Scientific Research, CNCS – UEFISCDI, project number PNII-ID-PCCE-2011-2-0036.

My gratitude would not be complete without mentioning the wonderful people at University Baptist Church who have come alongside me in so many ways and at so many times. You have become my family and I am privileged to serve alongside you.

Most of all, thank you to my wonderful wife, Carli Sierad, as well as her entire family. Your love and support have carried me through many tough experiences. Thank you for believing in me during difficulties and being there to celebrate in the joyous ones.
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PART 1: INTRODUCTION AND BACKGROUND
CHAPTER 1: BACKGROUND INFORMATION

1.1 Anatomy and Physiology

1.1.1 Cardiac Anatomy and Physiology

The heart has two double-chambered pumps pictured in Figure 1-1, which demonstrates the pressures and flow through the chambers. The right pump pushes blood through the lungs, where waste gases are exchanged for nutritional gasses while the left pump propels blood through the peripheral organs. Within each pump, blood first enters the superior chamber, the atrium, which moves the blood through an atrioventricular valve into the ventricle, the inferior chamber. When these ventricles contract, blood propels through the pulmonary (right side) valve or ventricular (left side) valve and through the rest of the body. Following contraction, the pulmonary and aortic valves close to prevent blood flow back through the heart.[1]

The pumping heart is a dynamic feedback system that changes its physical and chemical outputs according to the body’s needs. Under average normal resting conditions, the heart beats about 70 times per minute (bpm), ejecting 70 mL (stroke volume) of its left ventricular volume of 120

Figure 1-1: Blood flow through the heart
Normotensive blood pressures are given in the green boxes for each chamber or area of the heart. However, these values can vary greatly and still be considered normal.[1,180]
mL (end-diastolic volume) each beat. This results in a total of about 4.9 liters of blood flow per minute. The ventricles’ cyclic contractions create pressures that drive the blood through the circulatory system. Upon contraction, the pressure in the left ventricle increases to approximately 120 mmHg. Almost simultaneously, the aortic valve snaps shut and blood flows away from the heart along the pressure gradient. As the pressure in the aorta drops toward 80 mmHg, the left ventricle has begun its contraction to pump another bolus of blood and return the pressure to 120 mmHg. Figure 1-2 shows the left ventricular volumes and pressures as described above.

Figure 1-2: The cardiac cycle for the left heart
By studying the pressure and volume correlations with time, we can understand the function and performance features of the heart that allow it to perform incredibly over a person’s lifetime.
1.1.2 Valvular and Cellular Anatomy

Aortic Valves, Cusps, and Extracellular Matrix

The heart valves are excellent examples of durability, design, and adaptability not found in man-made materials. The physiological composition of a human aortic heart valve is optimized to withstand rigorous physical stresses and to respond to small pressure changes during the diastolic and systolic stages of the cardiac cycle. It is constructed with very strong, yet very pliable fibrous tissue with three heterogeneous cusps attached to an annular ring and wall of the aorta (Figure 1-3a).[4] With every beat of the heart, the aortic valve, which acts as a one-way check valve, is opened to progressively move blood through the pulmonary and systemic circulation of the body and closed to prevent retrograde flow during diastole.[1,5] The continuous performance at 850 milliseconds per cycle leads to a lifetime average of more than three billion cycles.[6,7] Further, the trans-valvular pressures across the aortic valve change from roughly zero to 120 mmHg in the course of only 17-20 milliseconds that is required for the valves to close. This leads to a 13%
circumferential and a 24% radial cusp stretch. As a result, the heart valves are unequivocally the most mechanically stressed tissues in the body.

The cusps themselves have almost no blood vessels, but are thin enough to be nourished predominately by diffusion from blood. They also have no active part in opening and closing, but act solely in a passive manner in response to flow and pressure differentials. When they open during systole, they press against the aortic root wall and when they snap close at the start of diastole, they perfectly align to create a seal that can prevent backflow even with the 100 mmHg transvalvular pressure difference. The free edge of each cusp attaches to the portion of the aortic root surrounding one of the three aortic sinuses. The indentations of these sinuses change the fluid dynamics of the valve and likely cause the formation of vortices that aid valve closure. Two of the sinuses lead to coronary arteries and are therefore named the left coronary, right coronary, and noncoronary sinuses.

The aortic valve cusps are comprised of three distinct layers: the ventricularis, spongiosa, and fibrosa. These layers contain valvular endothelial cells (VECs), valvular interstitial cells (VICs), collagen, elastin, and glycosaminoglycans (GAGs) in a formation that provide the necessary biomechanical properties to withstand the 40 million rigorous cycles of loading each year. Figure 1-3b shows a view of the aortic valve and a cross-sectional view of a cusp. Error! Reference source not found. displays each component’s locations and major functions. Collagen gives the valve strength to withstand the pressures when closed while maintaining coaptation. Elastin extends and recoils in every valve cycle as the cusp moves. GAGs absorb the shear forces between the ventricularis and fibrosa layers.
Valvular Endothelial Cells

Although VECs are very similar to other endothelial cells at their basic structural and functional levels, they are phenotypically distinct from endothelial cells in various locations in the circulatory system, even in locations in close proximity, such as the adjacent aortic wall. Their differences possibly allow VECs to interact with VICs to mediate disease and maintain valve tissue integrity. An example of differences between VECs and other endothelial cells is their alignment perpendicular to flow while most endothelial cells align parallel to flow and their transcriptional gene expression profiles differ under the same mechanical environment. Differences even go so far as to distinguish between transcriptional profiles of endothelial cells on the aortic side versus the ventricular side of cusps.\[10,11\]

Table 1-1: Key cellular and extracellular matrix components of the aortic valve
Although not isolated in regions, many components of the aortic valve have primary locations and functions.\[10,183,184\]

<table>
<thead>
<tr>
<th>Component</th>
<th>Location</th>
<th>Putative Function</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelial cells (VECs)</td>
<td>Lining inflow and outflow valve surfaces</td>
<td>Provide thromboresistance, mediation of inflammation</td>
<td>Role in transducing shear and modulating VIC function; Functional differences from vascular wall EC</td>
</tr>
<tr>
<td>Interstitial cells (VICs)</td>
<td>Deep to surface, throughout all layers</td>
<td>Synthesize and remodel matrix elements</td>
<td>Currently considered the major modulator of long-term valve durability and a key mediator of disease</td>
</tr>
<tr>
<td>Collagen</td>
<td>All layers; Dense and circumferentially aligned in fibrosa layer.</td>
<td>Provides strength and stiffness to maintain coaptation against the pressure gradient during diastole</td>
<td>Likely the most important structural element; Crimp and alignment provide directional anisotropy of properties and accommodate cyclical cuspal shape changes</td>
</tr>
<tr>
<td>Glycosaminoglycans (GAGs)</td>
<td>Concentrated in spongiosa layer</td>
<td>Absorb shear and cushion shock between ventricularis and fibrosa during cyclical valve motion</td>
<td>Distribution of forces aid in longevity of valve components</td>
</tr>
<tr>
<td>Elastin</td>
<td>Concentrated and radially aligned in ventricularis layer</td>
<td>Maintains valve resilience and allows stretch in response to pressure changes</td>
<td>Extend in diastole, recoil in systole</td>
</tr>
</tbody>
</table>
Valvular Interstitial Cells

VICs are the most abundant type of cell in the heart valve. Under normal conditions, they synthesize extracellular matrix (ECM), and express matrix-degrading enzymes such as matrix metalloproteinases (MMPs) and MMP tissue inhibitors (TIMPs). These syntheses explain how VICs mediate the remodeling of the ECM structural components, thereby continuously repairing functional damage to collagen and other ECM components in the valve. In all, five distinct phenotypes of VICs have been classified, providing a diverse and dynamic population of cells in the valve. They include embryonic progenitor endothelial/mesenchymal cells (eVICs), quiescent VICs (qVICs), activated VICs (aVICs), postdevelopmental/adult progenitor VICs (pVICs), and osteoblastic VICs (obVICs). Error! Reference source not found. gives a brief overview of the characteristics of all five phenotypes of VICs.

A vast majority of adult VICs in situ are qVICs, having characteristics of fibroblasts. qVICs phenotypically change into myofibroblast-like aVICs upon external Table 1-2: Characteristics of VIC phenotypes
CD34 and CD133 are stem cell markers; S100 is an intracellular calcium-binding protein.[11,12]
Macroscopic stimuli such as alterations in the mechanical environment, certain chemical signals, or in response to injury. aVICs are characterized by an increase in stiffness, expression of alpha smooth muscle actin (α-SMA), and increased ECM biosynthesis.\[13\] This phenotypic change is healthy at a small scale for repair, but is widespread during development, after abrupt changes in the mechanical stress state of valves, and in disease states.

In conditions that promote valve calcification, qVICs and possibly pVICs can differentiate into obVICs, which actively participate in the valve calcification process. There is currently no evidence that obVICs are a separate cell population from aVICs, but the osteoblastic differentiation and calcification promotion of VICs significantly differs from normal aVIC activity, thereby warranting its own category.\[12\]

**Summary of VIC Phenotype Markers**

eVICs are present in development. Similar processes are thought to be present in the initial stages of disease, but a distinct cell type is not anticipated in these mature sources.

qVICs show two types of intercellular junctions: gap junctions and adhesion junctions, as seen by the expression of transmembrane gap junction proteins Connexin-26 and -45. Cell-cell adhesion junctions are especially present between the long processes of adjacent cells as detected by small amounts of N-Cadherin and desmoglein.\[12\]

pVICs are found as other cell types in the body such as endothelial progenitor cells (EPC) and dendritic cells (DC). EPCs can be identified by stem cell markers such as
CD133 and CD34, a high proliferative capacity, and the ability to form blood vessels. DCs are identified by an intracellular calcium binding protein, S100.\textsuperscript{12}

aVICs are most often characterized by expression of $\alpha$-SMA, which is normally not found in qVICs. They also have the features of myofibroblasts: increased contraction, prominent stress fibers, and other contractile proteins. Activated VICs also increase the secretion of cytokines, such as TGF-$\beta$.\textsuperscript{12}

obVICs express markers that characterize osteoblasts in bone such as alkaline phosphatase, osteocalcin, and osteopontin. There is no clearly defined method of identifying a VIC in the osteoblastic state.\textsuperscript{11}

1.2 Aortic Valvular Disease

1.2.1 Worldwide Prevalence

Although most people’s heart valves will last their entire lifetime and cycle about 3 billion times, about 2% of the American population will be affected by heart valve disease.\textsuperscript{14} By 2011, it was estimated that roughly four million people in the United States were diagnosed annually with a heart valve disorder.\textsuperscript{15} Worldwide, over 300,000 heart valves are replaced every year.\textsuperscript{15–17}

However, this number is not representative of the actual number patients in need of valve replacements. Statistically, 80% of all cardiovascular disease occurs in developing countries,\textsuperscript{18–20} but 85% of all open-heart procedures are performed in developed countries representing 11% of the world population.\textsuperscript{21,22} In developing countries, economies cannot support the vast number of open heart surgeries or valve replacements that would actually help the population. Furthermore, in those developing countries, we
see that it is children and young adults[20,21] that are the demographic most affected, not the population over 65 as is seen in the United States.[23]

While the majority of causes for heart valve diseases in industrialized nations is degenerative pathology, inhabitants of developing nations also often suffer from the persistent burden of rheumatic fever.[15,24] However, as the economies of these developing countries grow, so will healthcare. The cause of valve degeneration will shift and cardiac surgery will become increasingly more accessible to potentially millions more who are in need of valve replacements.[21]

1.2.2 Causes for Valve Disease

In a normal heart valve, the cyclical stresses (which are often exacerbated in cases of genetically malformed valves) and other systemic conditions[20,25] damage the valve in the form of micro tears and calcific deposits that must be continuously repaired by the specialized valvular interstitial cells (VICs).[7,12,26–29] Mechanical cues play an important role in this delicate regulation.[30–33] When an imbalance occurs in this damage-repair cycle,[20,34,35] the valve can become diseased.[35–37]

Valve disease most often occurs in the aortic valve and is commonly classified into two main types of abnormalities that disrupt blood flow: regurgitation and stenosis. In aortic stenosis, the left ventricle fails to empty adequately following contraction due to the aortic valve not opening properly. This creates a reduction in the valvular orifice. In aortic regurgitation, blood flows backward into the left ventricle after contraction due to the aortic valve failing to close properly. This can be caused by stenosis as well as other factors.[1]
The most frequent cause for aortic regurgitation is stenosis, which occurs when the cusps thicken, stiffen, or fuse together, most often due to calcific nodule formation.[38] This is most often a result of many risk factors that increase with age, but can also be seen in younger patients, especially those with congenital defects such as improper valve size, malformed leaflets, or an irregularity in the way the leaflets are attached.[24,39,40] In fact, evidence of a high percentage of valve replacement surgery patients having congenital abnormalities[41] suggests that patients with a congenital valve defect are predisposed to valve disease.[14] Stenosis may also be caused by damage acquired after birth as described above such as calcific deposits, changes in the structure of the valve, or infections like infective endocarditis and rheumatic fever.

While there are various causes for each of these problems, the final outcome is the same. Both stenosis and regurgitation lead to a reduction in the net stroke volume output of the heart, causing it to work harder to pump the same amount of blood through the body. Patients suffering from valvular heart disease will eventually need surgical intervention to repair or, more frequently, replace the damaged heart valve.[1]

### 1.3 Valvular Interstitial Cell Contributions to Stenosis

#### 1.3.1 Curing Valve Stenosis

The ability to stop progression, if not completely reverse calcification would be the optimal cure for stenotic valvular heart disease. This cure would help the roughly 30% of patients[42] previously classified as untreatable because they were unable to undergo the necessary surgery due to their age, poor health, or immune compromised status. The option of a pharmaceutical approach instead of surgical treatment would
reduce the factors that often accompany surgery such as hospital recovery, infection, and anxiety of open heart surgery.

This relatively new idea of medicinal treatment has developed in the last 10-15 years as researchers have found evidence that valvular stenosis is not a passive deposition of calcium with degeneration, but is actually an active biological process involving endothelial dysfunction, lipid accumulation, inflammation, ostegenic processes, and multiple ubiquitous cellular pathways. Some research in human aortic valves and in vivo animal models even suggest that degenerative valvular aortic stenosis is the result of active bone formation in the aortic valve that may be mediated by osteoblast-like differentiation processes.

As the evidence grows for an active process of calcification rather than a passive occurrence, novel areas are being investigated such as how to identify and classify the types of valvular interstitial cells (VICs), determining which exogenic factors contribute to calcium formation, and determining which endogenic pathways are involved in the pathology of this disease.

1.3.2 Factors Contributing to VIC Phenotype Shifts and Calcification

In normal valve function, the macroscopic mechanical stimuli like shear and solid stresses are translated into microscopic forces that impact VIC and VEC function. Sensing the local tissue microenvironment in response to the external factors, they transduce forces through cell-cell and cell-ECM interactions that mediate normal valve function and pathobiology. They also respond by transdifferentiating to perform phenotype-specific duties of maintaining homeostasis, adapting to altered stress states, and repairing
This phenotypical shift in VICs is a normal occurrence under many circumstances. However, certain factors have been identified that excessively promote this effect and even cause the cells to start producing calcium, as in obVICs. The pathways of this transdifferentiation are shown in Figure 1-4, showing known and possible transitions. While there is not a complete knowledge base on every transition, some factors have been identified that contribute to certain changes in phenotype.

**Embryonic Progenitor VICs (eVICs)**

eVICs are present during the developmental stages of life. Studies have shown that transforming growth factor beta (TGF-β), bone morphogenetic proteins (BMPs), Notch, and vascular endothelial growth factor (VEGF) have significant regulatory effects on eVICs becoming normal qVICs through the course of valve development. Correct levels of each are necessary to properly guide the differentiation.

**Quiescent VICs (qVICs)**

Evidence shows that over several passages in cell culture, qVICs are activated and become aVICs, but these culturing conditions have not been deeply investigated in an isolated manner. Isolated factors that have been found to initiate this phenotypic shift in vitro include cyclic mechanical stretch and TGF-β.

*Figure 1-4: Known and probable pathways of VIC transdifferentiation*

Hatched arrows depict possible transitions for which there is currently no solid evidence.
Post-developmental Progenitor VICs (pVICs)

In response to injury, the number of VICs present increases quickly. This is now thought to be a result of pVICs from bone marrow-derived cells, circulating cells, and resident valvular progenitor cells being activated to become aVICs. It is not known whether pVICs go through the qVIC stage before becoming aVICs. Two specific types of pVICs are the endothelial progenitor cell (EPC) and dendritic cell (DC). It is possible that these cells share a common circulating progenitor cell: the CD34+ hematopoietic stem cell. They have been shown to express mRNA for procollagen α1 and differentiate with morphological similarities to native VICs when engrafted into recipient heart valves. Progenitor cells from the pulmonary valve have been isolated and shown to transition to VIC-like cells in response to TGF-β2. Interestingly, VEGF causes these same cells to become endothelium rather than mesenchymal VIC-like cells.

Activated VICs (aVICs)

aVICs are the master cells of valves. Under conditions of pathological injury or unusual hemodynamic or mechanical stress, qVICs become aVICs and take on the features of myofibroblasts. The characteristic marker for aVICs is α-SMA, which is normally not found in qVICs. In culture, many VICs may express α-SMA, while exhibiting a number of different morphologies. It is believed that under the exogenic factors of injury or unusual stress, VECs and macrophages/foam cells are activated, releasing chemokines and growth factors that stimulate qVIC activation. The process surrounding aVIC activation and leading to valvular heart disease can be seen in Figure 1-5.
The normal adult heart valve is well adapted to its physiological environment, able to withstand the unique hemodynamic/mechanical stresses under standard conditions. Under conditions of pathological injury or abnormal hemodynamic/mechanical stresses, VICs become activated through activation of VECs and by inflammation and associated cytokine and chemokine signals. Macrophages will also be activated. aVICs increase matrix synthesis, up-regulate expression of matrix-remodeling enzymes, migrate, proliferate and undergo apoptosis, and undergo osteoblast transformation. These processes are regulated by a variety of factors, several secreted by

![Pathways of clinical valve disease](image)

**Figure 1-5: Pathways of clinical valve disease**
Pathological injury or abnormal hemodynamic/mechanical stresses can facilitate VICs activation through activation of VECs and by inflammation and associated cytokin and chemokine signals, which will also lead to macrophage activation. As a result, aVICs increase matrix synthesis, up-regulate expression of matrix-remodeling enzymes, migrate, proliferate and undergo apoptosis, and undergo osteoblast transformation. These processes are regulated by a variety of factors including those secreted by the aVIC. Progressive clinical valve disease can then result from these cellular processes, angiogenesis, chronic inflammation, fibrosis, and calcification.[12]
the aVIC. If the aVICs continue to promote these cellular processes, angiogenesis, chronic inflammation, fibrosis, and calcification result, leading to progressive clinical valve disease.[12]

**Osteoblastic VICS (obVICs)**

Currently, there is no evidence that obVICs are a separate cell population from aVICs, but they can be classified as VICS that undergo osteoblastic differentiation and promote calcification as seen when VICS are cultured in osteogenic and chondrogenic culture medium. This classification is useful when considering the evidence of cartilaginous nodule and mature lamellar bone presence in explanted valves. Associated proteins such as osteopontin, bone sialoprotein, alkaline phosphatase, and BMP-2 and -4 have also been found.

Numerous factors have been identified that promote the active calcifying action of VICS: TFG-β, 25-hydroxycholesterol, and BMP-2 increased the rate of nodule formation when cultured in growth medium supplemented with organic phosphate; Oxidized cholesterol stimulates calcified nodule formation; BMPs and TGF-β caused osteoblastic differentiation of VICS; VIC calcification and ECM degradation is induced by MMPs and may be promoted by Tenascin and early growth response-1 (Egr-1); Oxidized lipids, tumor necrosis factor-α (TNF-α), and hyperglycemia may all mediate osteoblast differentiation and thus, valvular calcification through pathways activated by BMP2; and VECs may transduce external signals to VICS, regulating the aVIC or obVIC function.[12]
1.3.3 Pathology and Pathways

Along with factors that affect the individual VIC phenotype and actions, there are many larger-scaled factors that contribute to VIC phenotype shifts and calcification. General risk factors include age, male gender, hypertension, diabetes mellitus, smoking, renal disease, hypercholesterolemia, hyperparathyroidism, and decreased bone mineral density. Some literature suggests that the molecular mechanism resembles that of atherosclerosis with hypercholesterolemia playing a role in this calcification, but statin treatments have had mixed results. Therefore, the overall valvular tissue response to disease, which further exacerbates the problem, can be characterized by four major processes: 1) accumulation of VICs and inflammatory cells, 2) neovascularization or angiogenesis, 3) increased matrix production, and eventually 4) fibrosis and calcification. Figure 1-6 shows some of the factors and mechanisms that will be discussed.

Cells

In response to injury, the number of VICs present increases quickly. This process is discussed above. Under normal conditions, when valve repair is complete, excess cells undergo apoptosis to restore the balance of cells in the valve. However, in α-actin-positive cells, mRNA and protein levels of the G1 and G2 cell cycle checkpoint genes p21WAF1/CIP1 and 14-3-3 sigma are down regulated several-fold, possibly giving rise to the observed increase in cellular density.
**Angiogenesis**

Normal valves are avascular, but a dramatic increase of angiogenesis in diseased conditions has been reported in a close association with valvular stenosis and inflammation. Endothelial cells in stenotic valves exhibit CEACAM1, a cell adhesion molecule involved in angiogenesis expressed in angiogenic but not quiescent endothelial cells. Angiogenic factors, VEGF and MMPs, have also been shown to play a role in valve pathology.\(^{[45]}\) While the presence of these factors contribute to angiongenesis, down regulation of the angioinhibitory factor, chondromodulin-I (chm-I) has been seen to permit pathologic angiogenesis in coronary artery endothelial cells. The work partly described in Figure 1-6 is the basis for a newly proposed mechanism for the onset of aortic valve degeneration where angiogenesis is the main contributing factor.\(^{[48]}\)

**ECM**

Certain proteins have been found in the matrix of cardiovascular calcifications that are not present in normal cardiovascular tissue. These include osteocalcin, osteopontin, osteonectin, TNF-α, BMPs, and many MMPs. Some components, such as the extracellular proteins tenascin C and cystatin C, alkaline phosphatase, Egr-1 (induces tenascin-C), are present in low levels in normal tissues, but have been found in higher levels in calcific valves.\(^{[45]}\)
The putative degenerative mechanisms for superficial endothelial cells, valvular interstitial cells, and extracellular matrices are shown (A–E). The table is a summary of several factors, including the function, mechanism (corresponding to A–E above), and effect on degeneration of the cardiac valve.[48]

<table>
<thead>
<tr>
<th>factor</th>
<th>function</th>
<th>mechanism</th>
<th>effect on degeneration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tenascin-C</td>
<td>MMP-2 expression/activity ↑</td>
<td>D</td>
<td>degenerative</td>
</tr>
<tr>
<td>Fibulin-4</td>
<td>maintain TGF-β signal, ECM integrity</td>
<td>D</td>
<td>protective</td>
</tr>
<tr>
<td>Filamin-A</td>
<td>maintain cytoskeletal architecture</td>
<td>D</td>
<td>protective</td>
</tr>
<tr>
<td>MMPs (-1, -2, -3, -9)</td>
<td>collasin degradation, collagen synthesis ↑</td>
<td>D</td>
<td>degenerative</td>
</tr>
<tr>
<td>TIMPs</td>
<td>MMP activity ↓</td>
<td>D</td>
<td>protective</td>
</tr>
<tr>
<td>Wnt3/β-catenin</td>
<td></td>
<td>E</td>
<td>degenerative</td>
</tr>
<tr>
<td>TGFβ1</td>
<td></td>
<td>A, C, D, E</td>
<td>degenerative</td>
</tr>
<tr>
<td>BMP2</td>
<td></td>
<td>C, E</td>
<td>degenerative</td>
</tr>
<tr>
<td>Notch1</td>
<td>Runx2 activity ↓</td>
<td>E</td>
<td>protective</td>
</tr>
<tr>
<td>RANKL</td>
<td>MMP-1/2, cathepsin K, Runx2 activity ↑</td>
<td>D, E</td>
<td>degenerative</td>
</tr>
<tr>
<td>osteoprotegerina</td>
<td></td>
<td>E</td>
<td>protective</td>
</tr>
<tr>
<td>β2 AR agonist</td>
<td>Runx2 expression in VIC↓</td>
<td>E</td>
<td>protective</td>
</tr>
<tr>
<td>P21 Waf1/Cip1,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-3-3 sigma</td>
<td>cell cycle ↓</td>
<td>C</td>
<td>protective</td>
</tr>
<tr>
<td>Chondroadminulin-1</td>
<td></td>
<td>A</td>
<td>protective</td>
</tr>
</tbody>
</table>
**obVIC Differentiation**

Some work has been done to identify which genes are expressed in obVICs. Characteristic gene expression includes those encoding osteocalcin, Sox9, Cbfa-1, Runx2, Osterix, Msx2 osteopontin, and bone sialoprotein. Additional genes that have been studied are receptor activator of nuclear factor kB ligand (RANKL) and osteoprotegerin (OPG). RANKL promotes matrix calcification and induces the expression of osteoblast-associated genes, transitioning VICs toward an osteogenic phenotype. OPG is present in normal valves, but without it, calcification occurs.\[14,45\]

Many signals present for cardiac valvulogenesis during development are also reported to be present in valve degradation. These include Wnt, TGF-β1, BMP, and Notch. In general, the overexpression of these signals results in valve disease and calcification.\[48\]

### 1.3.4 Current State

New observations in human aortic valves support the hypothesis that degenerative valvular aortic stenosis is the result of active bone formation in the aortic valve, which may be mediated through a process of osteoblast-like differentiation in these tissues.\[45\]

Some underlying mechanisms have been demonstrated including TGF-β1, tenascin-C, VEGF, angiogenesis, inflammation, and MMPs. However, a defining molecular target for heart valve disease has not been defined and significant gaps in this knowledge still exist. At this time, suppressing inflammation and preventing obVIC differentiation appear to be the best ways to prevent calcific progression in valves.
Progress needs to be made in the areas of VIC activation, valve stem cell contributions, initiation of an osteogenetic gene expression profile, aggravators and inhibitors of valve degeneration, what steps precede end-stage stenosis and calcification, and how valve calcification can be lessened or reversed. Measures to identify AoV disease earlier and treat AoV disease pharmacologically or with less invasive approaches would be a significant improvement over the current standard of care. These advances will only be possible with a better understanding of the molecular mechanisms underlying valve development and disease.\[14,45,48\]

1.4 Current Treatments

1.4.1 Pharmacological

Once diagnosed with aortic stenosis, there is currently little one can do to reverse the damage done to their valve. In the early stages, drugs can be used to ease the pain and treat the symptoms, thereby attempting to stop progression of the disease. Examples include diuretics to decrease the heart’s workload, beta-blockers to control the heart rate and decrease blood pressure, and anticoagulants to decrease the risk of clots forming on the diseased heart valve.\[38,49\] Studies have investigated using statin treatment to treat stenotic aortic valves because many of the same processes are characteristic of atherosclerosis.\[44,45,48\] However, there have been mixed results with some showing that the use of a statin stopped progression of aortic stenosis\[50\] while others have shown no effect on stenotic progression.\[51,52\]
1.4.2 Replacement Heart Valves

Ultimately, the only current treatment available for aortic valve stenosis is to repair\textsuperscript{[53,54]} or replace the valve, leading to the second most common type of open heart surgery in the western world. Without this surgery to replace the valve, 50% of patients with aortic stenosis die within two or three years of diagnosis.\textsuperscript{[42]} Repair may involve removing calcium deposits, reinforcing a valve that does not close properly, or mechanically altering a congenitally defected valve.\textsuperscript{[38]}

Most diseased valves cannot be repaired. Instead, they are replaced by mechanical heart valves (general practice for younger patients), bioprosthetic heart valves (general practice for older patients), or allograft heart valves (special cases when available). However, these treatments each have major drawbacks (see Figure 1-7). Thrombogenicity of the artificial surfaces and induced turbulent flow regimes necessitate lifelong antithrombotic therapy for patients with mechanical valves.\textsuperscript{[55,56]} The reliance upon an expensive drug therapy and the necessity of close patient monitoring is undesirable and the main factor that has kept mechanical valve replacement out of developing countries. Additionally, pregnancy and surgical procedures may require temporary suspension of this antithrombotic treatment. A

\textbf{Figure 1-7: Complications with current valve replacements}

Mechanical valves have a very low risk of reoperation, but carry a high risk of a major bleeding event that increases with implant age. Bioprosthetic valves last a limited amount of time, so are the choice for older patients who will likely die of other causes before the valve implant fails.\textsuperscript{[62]}

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solution to the thrombogenicity of mechanical valves was the advent of bioprosthetic valves (BHVs) that are made from either porcine aortic valves or bovine pericardium that has been chemically fixed to cross-link the tissue and mask the antigens present in the xenogeneic materials. These devices do not require the anticoagulation drug regimen, but are predicted to last only about 15-20 years due to calcific degeneration[^37,58] and even less in younger patients[^11]. Allograft or homograft valves are good choices for valve replacements, but they also suffer from degradation, chance of rejection, and general lack of availability[^59].

With these severe limitations, the current treatments have high risk of complications (Figure 1-8 Figure 1-7) and are not ideal procedures[^60–64]. Furthermore, they are sub-par solutions for young adults and children needing valve replacements because of infections or birth defects[^24,39,40]. In this demographic, multiple revision surgeries will be necessary to accommodate the increased degradation rate seen in younger patients and lack of device growth along with a growing child[^14,39,40,65], which is still a very frequent need in less developed nations[^66,67].
1.4.3 Valve Replacement in Children

The general drawbacks of current heart valve replacements are accentuated when considering that, in contrast to the United States, children and young adults in developing nations are the most affected demographic.[20,21,23]

Current methods of non-replacement treatment for children include repair of the valve, reconstruction of the aortic valve with autologous pericardium, or the Ross procedure, whereby the patient’s pulmonary valve is moved to the aortic position and replaced with a pulmonary homograft.[39,40] This is usually a sufficient short-term solution; the pulmonary valve does not have the robustness of an aortic valve, and as such, will eventually need replacing with a more traditional option. In recent trials, decellularized xenograft valves have even been used in the pulmonary position, demonstrating a preliminary shift toward a tissue engineering approach. However, none of these solutions are ideal.

Alternatively, the field of tissue engineering and regenerative medicine holds great promise[15,20,68] in developing replacement heart valves that would function just as native, healthy valves: not requiring antithrombotic or other pharmacological therapy, repairing themselves as micro-damages are incurred, and growing in size along with a growing child. As has been eloquently said,[40] “future advances with tissue-engineered heart valves […] may change the landscape for valve repair in the paediatric population.”
1.5 Heart Valve Tissue Engineering

1.5.1 Long-Term Goal

Tissue engineering approaches are being developed that seek to make curative solutions for patients in need of long-term treatment of disease and tissue degeneration. The constructs that are being researched and tested will not simply compensate for the damaged tissue, but rather create living tissue that can be implanted into a human that would function just as a native, healthy valve: not requiring antithrombotic or other pharmacological therapy, repairing itself as micro-damages are incurred, and growing in size along with a growing child.

Ideally, a tissue engineered heart valve will resemble both the size and shape of the native valve, be durable and fully functioning with good hemodynamics, be non-immunogenic, non-inflammatory, non-thrombogenic, and non-obstructive, respond to mechanical and biological cues appropriately, grow in size with the recipient, and adapt to changing conditions throughout the life of the recipient and valve. A valve such as this would dramatically change the way we treat heart valve disease in children and young adults all over the world.

1.5.2 Valve materials & various approaches

Multiple methods of creating the above valves are foreseeable and many are being thoroughly investigated and developed, such as decellularized animal valves,[69–73] polymers,[15,74,75] layered composites,[76–78] and 3D printing.[79,80] Among the most researched and advanced methods are those that utilize a decellularized xenographic aortic valve as a scaffold.[20,68,81] Combining those easily accessible scaffolds with the...
patient’s own stem cells before conditioning in a bioreactor and implanting would provide a replacement that is fully functional from initial implantation while able to maintain matrix homeostasis and reduce valve thrombogenicity for the lifetime of the patient.

Current progress of xenographic valve preparation has been widely successful in decellularizing pulmonary and aortic valve cusps.\[82–87\] However, when reported, investigators note that the aortic wall and sinus wall have not been completely decellularized\[84\] or they do not give definitive proof of complete decellularization.\[88\]

### 1.6 Valve Seeding and Animal Implantations

The key to achieving an implant capable of repairing itself in response to micro tears is the presence of cells to remodel the matrix as necessary.\[35,37\] In cases where the valve implant does not have the adequate mechanical properties to function properly upon implantation, in vitro cellular remodeling must prepare the valve scaffold prior to implanting.\[89–91\] Multiple groups have demonstrated cell seeding on the exterior surface of valve cusps and some have even achieved some interstitial seeding, though full revitalization has not been realized.\[92–94\] The surfaces of some valves have even been repopulated after implantation in animal models,\[95,96\] but the same is not guaranteed in the human system. Even if the valve can be initially covered with cells,\[73,97,98\] it is likely that these cells will need some sort of progressive conditioning to remain attached after implantation since application of sudden shear forces can detach cells. Overall, more progress is needed to achieve full and consistent external and internal recellularization of the cusps and to determine what methods are needed to allow the cells to remain, but autologous adult stem cells provide a promising source for this application.\[99,100\]
1.7 Heart Valve Bioreactors

Another major obstacle to the creation of such tissue engineered heart valves seen in many experimental approaches of biodegradable polymer scaffolding[4] is inadequate mechanical properties to withstand in vivo forces after implantation.[69] Conversely, many stabilized tissues have more than adequate mechanical properties, but are unable to degrade appropriately to facilitate the formation of a natural valve or have chemical properties that do not facilitate cellular in-growth.[101] As a result, research is being focused on decellularized valves that will allow the recipient patient’s cells to infiltrate the extra-cellular matrix, repopulate the valve, and eventually replace the slowly degrading donor scaffold with newly fabricated extra-cellular matrix.[11,13,57,102,103]

Bioreactors can be generally defined “as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions” such as pH, temperature, pressure, nutrient supply, and waste removal.”[104] Bioreactors for heart valves can use these monitored and controlled conditions to help develop well-functioning valves.[105] Requirements for such bioreactors and examples of previous aortic valve bioreactors are discussed below.

1.7.1 Bioreactor Requirements

Freed[106] says that a bioreactor must be able to perform at least one of the following five functions:

(1) Establish a uniform distribution of cells on a three-dimensional scaffold

(2) Maintain the desired concentration of gases and nutrients in the culture medium

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(3) Provide efficient mass transfer to the growing tissue

(4) Expose developing tissue to physical stimuli

(5) Provide information regarding the formation process of 3D tissues, which originate from the isolated cells.

One of the most important controls of a bioreactor is to supply an adequate amount of oxygen to a 3D tissue construct. Other biochemical factors, such as carbon dioxide and wastes also require adequate transport. In conventional cell culture, this is most often done by creating a large liquid to sterile air interface to facilitate the diffusive transport of oxygen. In a bioreactor, where the volume of culture medium present is much larger than in standard cell culture, additional methods must be used. These methods range from a sterile filter open to the external environment in combination with flow of the culture medium[107] to coiling gas-permeable tubing inside a culture medium reservoir.[108]

Physical stimuli such as tension, compression, shear stresses, pressure, temperature, and pulsatile flow of culture medium improve the structure and mechanical properties of engineered tissues.[109,110] These mechanical forces have an integral part in regulation of cell phenotype and growth and the repair or degradation of tissues.[109] Control of trans-valvular pressure, pulsatile forces, flow rate, frequency, stroke rate, and stroke volume are all important design parameters of bioreactor to ensure that the necessary physical stimuli are integrated into the design.[110]
1.7.2 Current Bioreactors

Many bioreactor systems have been developed\cite{94,107,111–118} to test and precondition valvular constructs prior to implantation. Although they vary in design, footprint, capabilities, and valve-holding mechanisms, they each share common attributes to be functional.

Current heart valve bioreactors are designed to test a single valve \emph{in vitro} at different levels of pulsatile flow under controlled conditions in a standard humidified incubator at 37°C and 5% CO2. Figure 1-9 shows the common main components of aortic valve bioreactors, which include the fundamental components of a driving force to move the fluid, a reservoir, a valve holder, a capacitance chamber, a resistive element following the capacitance chamber, and a method of gas exchange.\cite{118}

This schematic gives one representative layout of these components, but many bioreactors in use today have variations on placement, type, and extent of use of these components. Most bioreactors have control over parameters such as flow, pH, and stroke volume and can change those parameters as desired to create varying environments for the valves. One use of the controllable parameters is progressive adaptation, where increasing amounts of mechanical stimuli are applied to aid strengthening of the tissue construct and prevent immediate failure upon implantation.

There are currently numerous individual laboratories across the country using heart valve bioreactors. Many reviews\cite{8,104,106,109,110,119,120} contain extensive comparison and assessment of the variations between them. The bioreactors range in design, complexity, and function, performing at various levels and accuracy. Valve mounting methods and modes of assembly vary, often with little options or ability to
accommodate abnormal valve shapes. While many bioreactors are very powerful and can achieve high pressurization, few are able to simultaneously subject valves to actual physiological conditions of flow and pressure. Those that can are very cumbersome to set up and operate for the duration of an experiment, especially while maintaining sterile conditions. Furthermore, independent control of those physiological conditions is elusive. Overall, much progress has been made in the area of heart valve bioreactors. However, there is still room for improvement.

Figure 1-9: Schematic diagram of a model pulsatile flow bioreactor
Pulsatile flow loops for heart valve bioreactors all contain these minimum components. However, the form and means of use they take may vary from system to system.
CHAPTER 2: PROJECT RATIONALE

2.1 Introduction to Project

Most diseased heart valves cannot be repaired. Instead, they are replaced by mechanical, bioprosthetic, or allograft heart valves. These treatments each have major drawbacks and none are ideal for a growing child or young adult. The field of tissue engineering & regenerative medicine holds great promise in developing replacement heart valves that would function just as native, healthy valves: not requiring antithrombotic or other pharmacological therapy, repairing themselves as micro-damages are incurred, and growing in size along with a growing child. We hypothesized that implantable tissue engineered heart valves can be generated by optimal integration of three main factors: acellular heart valve root scaffolds, autologous stem cells, and construct preconditioning in a bioreactor.

In our preliminary studies we have developed a heart valve bioreactor and tested it with a variety of valves under physiological pressures, but flow rates and shear stresses were suboptimal. We have successfully decellularized cusps by simple immersion, but not the aortic and sinus walls. We have experimented with interstitial and external stem cell seeding followed by dynamic preconditioning, but the surface cells were not well retained under flow. Planning for large animal studies has revealed multiple translational challenges.

To address these shortcomings, we proposed to optimize the bioreactor performance through engineering design improvements, to achieve full decellularization of valve roots by novel, perfusion, pressure, and decellularization methods and devices,
and to mechanically precondition stem cell seeded roots under optimal adaptation conditions before implantation in large animals. Furthermore, we aimed to demonstrate that translational regenerative medicine is feasible with autologous stem cells for tissue engineering replacement therapy – a vital stage in the ability to remove barriers in commercializing tissue engineered products.\[68,123–125\]

2.2 Specific Aims

2.2.1 Aim 1: Heart Valve Bioreactor Optimization

Goal

To modify the heart valve bioreactor system to achieve physiological levels of \textit{flow}, \textit{pressure}, and \textit{shear stresses}.

Approach

Alternative components (compliance chambers, reservoirs, restrictor valves, etc) were developed to allow for physiological levels of \textit{flow} at \textit{pulmonic pressure} conditions. \textit{Shear stresses} were increased by adding dextran to the culture media. The controlling hardware and software was upgraded to allow simultaneous and independent operation of multiple bioreactors and accurate monitoring of the flow and pressure conditions.

Novelty & Innovation

Multiple heart valve bioreactors were simultaneously run under physiologic conditions with independent controls.
2.2.2 Aim 2: Aortic Heart Valve Root Scaffold Preparation

Goal

To decellularize all areas of the aortic valve roots (cusps, sinuses, ascending aorta, and muscular tissue near cusps).

Approach

Cusps were subjected to immersion conditions. The sinuses and ascending aortas were subjected to a transmural pressure gradient and cyclical mechanical stretching. Muscular areas were subjected to a pressure gradient and large flow through their tissues. This variety of techniques was simultaneously applied to the individual areas of the valve using a custom-built decellularization device. Histology and DNA analysis was used to evaluate the removal of cellular components. Mechanical tests were used to compare the valve scaffold with native tissue.

Novelty & Innovation

A novel device was designed, manufactured, and used to apply individualized conditions to multiple areas of the valve roots. We revealed, for the first time, fully acellular aortic valve root scaffolds.
2.2.3  **Aim 3: Dynamic Conditioning of Cell-seeded Heart Valve Scaffolds**

**Hypothesis**

Progressively pre-conditioning stem cell seeded acellular valve scaffolds in the improved bioreactor will allow internally seeded cells to *migrate within the cusps* and externally seeded cells to *maintain confluent coverage*.

**Approach**

Adipose-derived stem cells were manually injected directly and after pre-inflation into multiple areas of each cusp. Valves were rotated in a cell suspension for external coverage. Valves were placed in the bioreactors and flow, pressure, and viscosity conditions slowly increased until physiologic levels were reached. *Recellularization, cellular migration, and cell retention* were evaluated using histological and surface microscopy techniques.

**Novelty & Innovation**

A novel rotational device was used for external cell seeding of all valve surfaces. For the first time, heart valves revitalized with cells adapted to physiologic conditions were prepared for implantation.
2.2.4 **Aim 4: Translation to Pre-Clinical Large Animal Testing**

**Goal**

To overcome hurdles encountered while translating tissue engineered heart valves into *clinically relevant products*.

**Approach**

Acellular valve scaffolds were prepared, seeded with autologous adipose-derived stem cells and pre-conditioned using the bioreactor. Valves were implanted in young adult sheep in a right ventricle to pulmonary artery shunt position. A *valve mounting system* was modified to allow for *integrated* exchanges between decellularization, sterilization, cell seeding, and valve conditioning steps before implantation with minimal handling of the valve itself. Methods of aims 2-3 were continuously adjusted as necessary and re-verified to accommodate the clinical situation.

**Novelty & Innovation**

Stem cell seeded heart valves were implanted in an animal model. Products created in labs were effectively translated from bench to bedside, demonstrating translational feasibility with autologous stem cells.
PART 2: PLATFORM TECHNOLOGIES AND DEVICES
CHAPTER 3: A SELF-ADJUSTING, NO-TOUCH DEVICE FOR INTEGRATED PROCESSING AND HANDLING OF HEART VALVES

3.1 Introduction to Need

Advances in replacement heart valves available on the market have been relatively stagnant since the introduction of bioprosthetic heart valves in the 1970s. Although mechanical valves typically last a patient’s lifetime and bioprosthetic valves do very well for a limited time in patients who cannot be on anticoagulants, there is still a need for a tissue-engineered valve that can repair itself to last the lifetime of a young adult and grow along with a growing child.

Whether for study or implantation, natural and synthetic heart valve tissue is generally subjected to multiple treatment regimes. For instance, xenograft valve tissue must be decellularized to remove the native cells prior to either testing or implantation and it is common for some of the tissues to become thinner and drastically reduce in strength during these processes. In addition, mechanical testing by use of a conditioning system can be carried out to examine and alter tissue strength or to ensure suitable strength prior to implant.

Some researchers believe the key to a lasting tissue-engineered heart valve replacement lies not only in a mechanically durable scaffold that will function immediately upon implantation, but also requires the presence of cells. While some researchers are leaving recellularization until after implantation in large animals, others are revitalizing
natural or synthetic scaffolds with various cell types in vitro. These cells must not be removed through the normal handling of the valve tissue.

When these products reach the market or even clinical trials, they will also undergo the scrutiny of physicians needing a product that will hold up to the conditions required for implantation. Whether transcatheter deployment or open-heart surgery is the route of implantation, clinicians often desire a “no-touch” product. These products are held by external objects up-to and sometimes during implantation.

It has been common to secure valve tissue during the various treatment regimens by temporarily suturing or clipping the tissue to mounting rings (Figure 3-1). Unfortunately, physically attaching the tissue to the holding device can damage the tissue and cause mechanical weakening or physical change of the tissue. Moreover, securement systems generally do not provide a method for securing the tissue with a tight seal and fluid leakage around the tissue during the

*Figure 3-1: Valve holder requiring intense suturing*

Diagram (A) and picture (B) of an example valve holder requiring many sutures in standard locations to hold a heart valve during testing.[113] (C) A second example of a common valve tissue holder.[185]
treatment protocols can prevent effective conditioning and/or testing. For instance, decellularization can lead to a loss of tissue volume, and presently known systems do not account for the physical changes of the tissue associated with a volume loss. Thus, with present systems, the tissue can become loose in/on the holder and/or leaks can form between the tissue and the holder as decellularization takes place.

Regardless of the stage of the valve replacement technology, researchers and clinicians are in need of a device that can effectively grip a heart valve root through the entire process of valve preparation, testing, and implantation to prevent damage and facilitate easier product development and use. We have developed a device that provides for a totally no-touch procedure after initial mounting, secure retention during multiple treatment regimens, and self-adjustment to accommodate natural changes in tissue volumes. This device is of great benefit for natural or synthetic heart valve tissues alike.

### 3.2 Device Materials and Resulting Benefits

Initially, multiple valve bracing ring designs (Figure 3-2) were used for each of various valve preparation devices. Although the initial bracing rings share common sizes and some design features, they require constant user intervention when installing or transferring valves between devices. Furthermore, the purpose of certain devices required some bracing rings to have features that are incompatible with use in other devices. For instance, during recellularization, it is necessary to allow flow across the rings outside of the valve, but during bioreactor testing, this allows flow to bypass the lumen of the aortic root, thereby nullifying the purpose of the bioreactor.
3.2.1 Initial Bracing Rings

Figure 3-2A shows the initial bracing rings with offset ridges to clasp the tissue. These are held in place by the initial bioreactor pieces with six bolts (Figure 3-3) and require delicate maneuvering to install in place. These are used after tissue decellularization and subsequent recellularization and require significant manipulation of the tissue to function, thereby risking the sloughing of cells from the tissue surfaces. Furthermore, the tissue is at risk of drying out during the valve mounting and assembly of the bioreactor that follows. With the development of a larger bioreactor for potential use with cow heart valves, larger bracing rings (Figure 3-2C) were created that allowed for valves up to 60 mm in outer diameter to be tested, but these pose the same problems as the smaller rings.
3.2.2 Bracing Rings for Cell Seeding

When a device was developed to aid external cell seeding of heart valves\(^\text{[126]}\) by a master’s student in the lab (see section 5.2.3), additional bracing rings were designed. These bracing rings contain holes around the outside of the center hole to allow a cell suspension to flow around the external surfaces of the valve roots and through the valves during rotation of the seeding chamber (Figure 3-2C). After cell seeding, the bracing rings are replaced by new bracing rings not containing additional holes before placing the valve into the bioreactor as above. While the transfer of the valve from one set of rings to another is easier because of the indentations made in the tissue, it still requires handling of the valve that contributes to the removal of cells from the external tissue surfaces.

**Figure 3-3: Valve mounting procedure with initial bracing rings**
Details of mounting procedures for stented valves, mechanical valves and un-stented valves are shown. e) The basic valve holder, f) holder with one o-ring placed in the machined groove (arrow), g) bottom mounting ring placed on top of the o-ring (arrow). h) A stented valve was placed on top of the bottom mounting ring (arrow), i) a second mounting ring placed and o-ring over it and j) pushed slowly. k) Shows a mechanical valve (arrow) after mounting between the two rings. l) Porcine aortic valve root ready for mounting in bioreactor; m-o) valve mounted into a silicone root using 6-10 bottom sutures and 3-6 upper sutures (arrows). p) The valve was placed on the holder prepared as shown in (g); q) a second ring slid onto the root (arrow), r) a second o-ring added on top and s) the entire mount inserted into the aortic chamber (arrow).\(^\text{[117]}\)
3.2.3 *Heart Valve Quick-Tester*

Even with these slight improvements, the process of tightly clamping a heart valve root between the bracing rings and mounting them in the bioreactor for testing is complicated, difficult, and time-consuming. While it was necessary for work with sterile valves, a faster and simpler method was desired for testing iterations of synthetic and alternative valve designs under non-sterile conditions.

A novel tester was designed and built in cooperation with an undergraduate student for quick assembly and testing to assess integrity and function of valves. As shown in Figure 3-4, the primary components in the design are a ventricular chamber, bracing rings, an aortic chamber, and a cap. The tester is made from clear acrylic, allowing a clear view from all angles of the valve being tested. To manipulate the valve, the user operates two bellows so that when the ventricular bellow is squeezed (collapsed), fluid moves through the valve into the aortic chamber and the valve opens. The user then squeezes the aortic bellow to shut the valve and the fluid travels through the external tubing to return to the ventricular chamber. The aortic chamber is attached to the ventricular chamber via three adjustable draw latches that can quickly secure the two chambers while allowing for adjustments in height for different valve designs.

![Figure 3-4: Manual heart valve tester](image)

This tester can be very quickly assembled and is used for quick valve testing. Valves are placed between the bracing rings and the metal latched hold all other components together with a quick latch. The two rubber bellows are used to pump fluid through the valve to evaluate opening and closing.
Three screws are used to ensure the cap stays in the correct position and a seal is maintained. The tester utilizes the same set of bracing rings as previously described, which are placed between the two chambers and held in place by o-ring tension instead of bolts. Thus, this heart valve quick-tester results in faster and fewer steps to assemble than the bioreactor and thereby provides a faster alternative than previously possible when evaluating new valve designs.

3.2.4 **Self-Adjusting, No-Touch Valve-Mounting Device**

While the quick-tester decreased the time and work necessary to test iterations of non-sterile valves, there was still a need for a device that would clasp a valve for mounting without damaging it, adjust to maintain a constant holding force on the valve tissue, and allow for no-touch manipulation and transfer of the valve between devices. A valve-holding device was designed (Figure 3-5) from the ground up that meets these desires. While the bracing rings operate under the same clamping principal as the above bracing rings, their clasping mechanism was redesigned to improve hold during assembly, eliminate slip during use, and provide a better seal around the tissue in all processes.

The rings for this quick-mount (Figure 3-2D) can be

**Figure 3-5: Self-adjusting, no-touch valve mounting device**
Photograph (A), isometric (B), and blow-out (C) views of the new device to quickly mount heart valves and provide no-touch handling through the preparation process.
created in multiple sizes and shapes to hold various heart valve sizes and even types, such as the mitral valve (Figure 3-2E&F). In the current configuration, an angled series of ledges match together with a precise gap between the two bracing rings to clasp the final thickness of the tissue that remains after decellularization. The series of ledges create a clapping force that adequately seals around the tissue base. Grooves on the lower bracing ring are engaged by co-radial bumps on the upper bracing ring. Those bumps help prevent the tissue from slipping during processing as well as during mounting of the valve in the mounting device. They puncture the surface of the tissue and prevent slippage even when the lower bracing ring is not engaged, as seen in Figure 3-7G, which demonstrates the stepwise assembly of an aortic valve into the mounting device.

After clapping of the tissue, the bracing rings are held in place between two interacting threaded casing rings and a spring. The threaded casing rings can be adjusted to apply the desired initial amount of force on the clamped tissue. The spring allows that force to remain relatively constant during decellularization and the resulting dramatic thinning of the clamped tissue. The threaded casing rings contain holes around the outside of the center hole. The external ring of holes are used to engage with the tightening tools during assembly. The tightening tools have teeth with the appropriate heights to rotate the tissue and both bracing rings while not interfering with the tightening of the casing rings. In addition to facilitating the tightening of the casing rings, the holes can be aligned with one another. In this configuration, the holes allow a cell suspension to flow around the external surfaces of the valve roots and through the valves during rotation of the seeding chamber, just as with the bracing rings for cell seeding. If the external flow is not desired, an o-ring can be placed under the lower external casing
ring to prevent passage of fluid from the internal hole to the external series of holes. This is beneficial during decellularization to create a pressure differential between the inside and outside of the aortic root and during conditioning to cause the culturing medium to flow through the valve instead of around it.

### 3.2.5 Distal Aortic Root Constrictor and Stabilizer

When the pressure differential between the inside and outside of the aortic root is desired, a plug with a controlled outflow (Figure 3-6A) can be inserted into the distal portion of the aortic root and secured with cable ties. This outflow plug has a groove near its base in which the tissue and cable ties are seated to prevent tissue slippage. The outflow portion of the plug has a barbed fitting that is connected to tubing and can be constricted as desired by using external clamps.

While culturing the aortic root in a bioreactor setting, this plug is replaced by a two-piece stand (Figure 3-6B) to keep the valve root from collapsing under the backpressure applied during the bioreactor functioning. The first part of the stand consists of a ring with holes or grooves that is placed around the exterior of the aorta. It is attached to the most distal portion of the aorta via suturing or an internal retaining ring spring. The second part of the stand is the supporting ring with feet that surround the aortic root, yet do not interfere with the coronary arteries.

![Figure 3-6: Distal aortic root constrictor and stand](image)

The aortic flow constrictor (A) has a groove around which the aorta and cable ties can be tightened to hold the aorta in place for internal pressurization of the aortic root. The support stand (B) attaches an upper ring to the distal aortic root and uses a support stand to prevent the root from collapsing inward from the back pressure in the bioreactor.
The ledge on the first part of the stand rests on the top of the second part and by turning the first part “upside down,” it can accommodate longer or shorter aortic roots without the need for a larger or an alternative piece.

3.3 Method of Device Assembly and Mounting of Fresh Valves

The method of mounting an aortic heart valve into the self-adjusting, no-touch valve-mounting device is described below. While the description uses the most up to date bracing rings, it should be noted that the small initial bracing rings, large initial bracing rings, and cell seeding rings could all be used in their place. The upper casing ring contains counterbore cutouts corresponding to each size. It should also be noted that in certain designs, the upper and lower bracing rings may be combined with the upper and lower casing rings.

3.3.1 Tissue Cleaning and Preparation

Fresh porcine aortic valve roots with ascending aorta up to the branching of the brachiocephalic artery were collected from adult pigs at the local abattoir (Snow Creek Meat Processing Center; Seneca, SC) and transported to the lab in ddH₂O over ice. Aortic roots were further dissected to include 6-8 cm of intact aorta, 5-10 mm long coronaries, and about 3 cm of mitral valve leaflet and ventricular endocardium tissue with an intact mitral-myocardial junction. The valve roots were cleaned of fat and other extraneous tissues while maintaining a thin muscular shelf under the muscular cusp and a thin muscular layer all around the valve 360° with a thickness matching that of the mitral valve leaflet (about 1-3 mm). The coronaries were sutured shut and the valve roots were rinsed with ddH₂O.
3.3.2 Valve Mounting

The process of assembling the valve-mounting device can be seen in Figure 3-7. First, the tightening tool with short teeth is placed on the counter and the upper casing ring, spring, and upper bracing ring are placed, upside down, onto the tightening tool.
The height of the tightening tool allows the valve root to rest on the upper bracing ring without touching the countertop below. A prepared valve root is then placed, upside down, through the center hole of the upper bracing ring and the mitral valve leaflet and thinned endocardium are spread around the surface of the upper bracing ring. The appropriate vertical location is achieved by ensuring that the base of each cusp is about 2 mm beyond the reach of the upper bracing ring. Any excess muscle or mitral valve that proceeds radially to cover the external holes can be trimmed and the tissue pressed into the bumps on the upper bracing ring, providing a temporary hold until the lower bracing ring is placed onto the assembly. Following the lower bracing ring, the lower casing ring is placed, the external holes are aligned, and a second tightening tool with long teeth is placed onto the assembly, all while maintaining an exertion of force onto the assembly to prevent slippage of the tissue. Following placement of all pieces, the tools can be picked up or kept on the counter and used to twist the casing rings and engage the threading. The threading should be tightened until the top of the upper casing ring is flush or below the top of the lower casing ring. In cases where the tissue is extremely thick or alternative bracing rings/devices are used, the upper casing ring can extend above the lower casing ring, but should always have at least two full threads engaged. After this initial tightening, the spring will provide a relatively constant force on the tissue during any subsequent decellularization and processing.

3.3.3 Distal Aortic Root Stabilization

In preparation for decellularization, the distal ascending aorta is trimmed flat and the outflow plug is inserted into the aorta and secured with cable ties. This plug has a
groove near its base and when cable ties are secured around the external diameter of the aorta, the tissue and cable ties are held in place within this groove (Figure 3-6A). This plug is used to create a pressure differential between the interior and exterior of the valve root during further processing.

When the valve is fully decellularized and any subsequently desired sterilization and crosslinking is completed, the aorta can be cut just below the plug to remove any unwanted tissue that was clamped. Alternatively, the plug can remain in place to allow for more directed cell seeding to the interior of the heart valve during further processing.

Prior to bioreactor conditioning of the valve root, the two-piece aortic stand (Figure 3-6B) is placed around the exterior of the aorta and secured to the tissue via suturing or an internal retaining ring. This stand does not interfere with the valve and will prevent the root from collapsing during the backpressure created from bioreactor functioning.

### 3.4 Discussion of Integration into Valve Preparation Process

With the self-adjusting mounting device, a researcher can mount a fresh valve and transfer the valve between processing stages all the way to implantation without removing the valve from the mounting device or even touching the valve directly. One benefit that is highly desired by clinicians is the “no-touch” aspect. In the operating room and when dealing with living tissue, direct handling of the tissue can be damaging. By using external tools and manipulating only the portions of the aortic root that are not vital, inherent tissue integrity is not altered.

This device contains many pieces and performs multiple functions. However, during any given stage of processing, it is possible to remove or disable the aspects that
are either undesired or unneeded. At the same time, the valve is securely housed in one piece of equipment that can be transferred between processing devices. Furthermore, the features not currently in use do not interfere with the purpose or function of the immediate device.

Use of this device has truly streamlined the development of tissue engineered heart valves, especially in the context of transferring the valves between systems having a distinct purpose and design. It is the key to fast transfers between systems and as well as appropriate care of the living tissue. Although currently fabricated in stainless steel and ABS-like material, it could easily be made of ceramics or other plastics to facilitate magnetic imaging techniques, cost-effectiveness, or other desired properties to further expand its uses in additional systems.
CHAPTER 4: A CYCLICAL PERFUSION DEVICE FOR DECELLULARIZING WHOLE AORTIC VALVE ROOTS

4.1 Introduction to Need

Multiple methods of creating tissue engineered heart valves are foreseeable and many are being thoroughly investigated and developed. Among the most researched and advanced methods are those that utilize a decellularized xenographic aortic valve as a scaffold.\[20,68,81\] Combining those easily accessible scaffolds with the patient’s own stem cells before conditioning in a bioreactor and implanting would provide a replacement that is fully functional from initial implantation while able to maintain matrix homeostasis and reduce valve thrombogenicity for the lifetime of the patient.

While alternative ground up approaches have also been researched, a native scaffold gives the best mechanical properties and structural functionality to withstand the rigorous conditions of a replacement valve in the aortic position. Current progress of xenographic valve preparation has been widely successful in decellularizing pulmonary and aortic valve cusps.\[82–87,117\] Even attempts at decellularizing whole pulmonary artery roots has been adequately demonstrated.\[82\] Our own investigations with decellularizing aortic valve cusps has also been successful.\[117\]

However, if an entire aortic root is to be used as a tissue engineered heart valve replacement, it is vital that the entire tissue be free of native cells to reduce or eliminate chances of immune rejection and subsequent failure. The most severe example, wherein all native porcine cells were not removed from implanted tissue, resulted in the death of
three children due to severe inflammation, degeneration, and structural failure.[127] The removal of all foreign cells from the cusps, sinuses, ascending aorta, and muscular regions of an aortic root must be clearly demonstrated.

In some applications immersion decellularization techniques are frequently replaced with perfusion techniques for tissues that are difficult to decellularize.[86,128] When examining the properties of the aortic root tissues, it can be inferred that procedures that are more rigorous will be necessary to decellularize those portions than the procedures used to decellularize the cusps. However, the balance must be made to retain the structural integrity of the cusps while providing conditions harsh enough to decellularize the aortic wall. In this way, the entire aortic root would be prepared for recellularization with patient cells without the fear of an immune response caused by remnant xenogenic cells.

During perfusion decellularization, tissue is generally subjected to multiple treatment regimens including various chemicals, detergents, enzymes, and pressure profiles. What is needed in the art of decellularizing whole aortic heart valve roots is a system and method for decellularizing tissue that can provide independent conditions of immersion, pressure, and stretch to the cusp, sinus, aorta, and muscular portions of the aortic root. These areas are mapped visually in Figure 4-1.

**Figure 4-1: Mapping of tissues in the aortic root**
Main anatomical coordinates of the aortic root are displayed. Each component has different physical characteristics and function.[105]
4.2 Materials and Methods

Inadequate decellularization of entire aortic roots necessitated the creation of a perfusion decellularization system for aortic valve roots. Decellularized tissue samples from the cusp, sinus, aorta, and muscle were compared to fresh regions for DNA presence, matrix stability, and mechanical properties.

4.2.1 Perfusion Device Components and Assembly

Cleaned and prepared aortic valve roots were mounted in custom mounting rings as previously described (sections 3.3.1 and 3.3.2) and the distal portion stabilized with an outflow plug as previously described (section 3.3.3). If less than five valve roots were available for decellularization, acrylic cylinders having the outer dimensions of the lower casing rings were used as space holders. Figure 4-2D shows the assembled perfusion decellularization system. A brief overview of the process of assembly follows. All custom components were manufactured from stainless steel, acrylic, or polycarbonate materials by Clemson University Machining and Technical Services. Materials were chosen to provide strength in thin areas or transparency for visualization of aortas and cusps.

Phase 1: Attaching Through-Wall Fittings

Custom through-wall fittings (Figure 4-2A) were designed and manufactured that would protrude through the wall thickness while maintaining a seal, attaching to tubes, and not slip from their placement. Depending on need fittings either act as plugs or have barbed attachments on one or both ends. O-rings on either side of the fittings provide stabilization and a seal across the wall. A shoulder on one side of the fittings prevents the fitting from advancing through the wall in one direction while an external retaining ring
and groove prevents the fitting from reversing back through the wall. All fittings are pushed from the interior areas of the device through the wall and the retaining ring is attached on the portion of the fitting advancing on the outer portion of the wall. Fittings are attached to the lids for inflow and outflow and to appropriate areas in each other layer to provide the necessary circulation of fluid through the system.

Figure 4-2: Perfusion decellularization system for heart valves
The flow diagram (C) illustrates the main system components comprising a valve (v), peristaltic pump, pulse dampener (d), valves (c) and pressure transducers (p). The working setup is shown in (D). Direction of flow, indicated by arrows is as follows: from the pump through the dampener (D) enters the bottom of the system and pushes fluid through the valves. Fluid then collects via a manifold (white arrows) into a single line which feeds the system again and bathes the valves from the outside.
**Phase 2: Valve Clamping**

The next step of device assembly is to clamp the self-adjusting, no-touch valve holders in place between the three layers of the device that create a common solution chamber (between layers 1 and 2) and do the holding of the valves in the holders (between layers 2 and 3). The valves are placed in the circular grooves of plate 3 before plate 2 is lowered around them and plate 1 above that. The three layers are bolted together to create a seal that forces fluid from the solution chamber into the inflow side of the valve lumens.

**Phase 3: Chamber Assembly**

After the valve holders are clamped between the three plates, the next layer is placed onto the root side of the plate assembly to create the main chamber. The outflow plugs of each valve root are connected to the through-wall fittings of the main chamber and the final layer, the outflow lid, is placed on top of the main chamber. Layers, 3, 4, and 5 are then bolted together to create a sealed chamber around the valves.

**Phase 4: Flow Loop Assembly**

A five-port luer manifold with on/off valves is used to direct flow between the interior of the valves and the main chamber, where the solution bathes the tissues before exiting the system. This manifold and main chamber contain pressure transducers and a constriction valve between them to measure and control the pressure differential between the interior and exterior of the aortic roots. 500 mL bottles with glass-blown barbed attachments are used as reservoirs and pulse dampeners between the system and a peristaltic pump to create pressures and flow through the system.
Phase 5: Device Filling, Description of Flow, and Emptying

Four liters of solution are used to fill the system. The pressures during filling should never exceed the running pressures of the desired protocol (described below) and after filling, any air bubbles are removed component by component in the direction of fluid travel. Once full, the system can be subjected to the desired protocol. Pressures are monitored with the transducers and a custom LabView program (Figure 4-3) the proper flow protocol is set in the menu of the peristaltic pump. Pressure and flow are controlled by adjusting the speed of the peristaltic pump and the constricting valve coming from the outflow of the manifold.

4.2.2 Decellularization by Immersion

Aortic roots were cleaned and prepared as previously described (section 3.3.1), then decellularized in containers on an orbital shaker (immersion technique) at a ratio of 500 mL solution for 5 aortic roots. Decellularization steps consisted of hypotonic shock (ddH₂O, 24 h, 4°C), loosening of extracellular matrix and initialization of cell removal (0.1 M NaOH, 2 h, 22°C), decellularization solution (1% Sodium Dodecyl Sulfate, 1% Triton X-100, 1% Na-Deoxycholate, and 0.2% EDTA).

Figure 4-3: Monitoring of decellularization system
(A) The main decellularization system is comprised of 5 layers which help direct flow through, then around the aortic roots. (B) The LabView software reads direct pressure inside and outside of the aortic roots, conditions it to achieve steady values, then gives a direct output of the differential of pressure.
acid in 50 mM TRIS, pH 7.5, 8 days with fresh solution every 2 days, 22°C), and removal of nucleic acid remnants (720 mU/mL deoxyribonuclease & 720 mU/mL ribonuclease in 5mM Magnesium Chloride in 1xDPBS, 24 hours, 37°C). To reduce bio-burden, each extraction step was preceded by a 15-minute 70% ethanol treatment and appropriate rinsing. The roots were incubated in 70% ethanol (24 hours, 22°C) and sterilized[129] in 0.1% peracetic acid (2 hours, 22°C).

4.2.3 Decellularization by Perfusion

Aortic roots were assembled into the perfusion system as described above for decellularization. Perfusion decellularization steps were performed with a cyclic transmural pressure gradient of about 52±2 mmHg and mechanical stretching for 3 minutes on and 30 seconds off. Cyclical pressure steps were followed by rinsing with water, ethanol, or 1xDPBS as appropriate and consisted of hypotonic shock (ddH₂O, 24 hours, 22°C), loosening of the extracellular matrix and initialization of cell removal (0.1M NaOH, 2 hours, 22°C), detergent decellularization (1% sodium dodecyl sulfate, 1% Triton X-100, 1% sodium deoxycholate, and 0.2% EDTA in 50mM TRIS, pH 7.5, 16 days with fresh solution every 4 days, 22°C), enzymatic removal of nucleic acids (720 mU/mL deoxyribonuclease & 720 mU/mL ribonuclease in 5mM Magnesium Chloride in 1xDPBS, 4 days with fresh solution every 2 days, 37°C), and initial sterilization (0.1% peracetic acid, 1 hour, 22°C). Immediately following, the distal root outflow constrictors were removed with sterile tools and the mounted valve roots were transferred to individual 300 mL wide mouth glass containers with silicone membrane sealing lids on an orbital
shaker instead of the perfusion system for final sterilization (0.1% peracetic acid, 1 hour, 22°C).

Additionally, one group of aortic roots underwent the above protocol, but for 8 days instead of 16 days in the detergent decellularization step. Another group underwent 16 days of detergent decellularization, but was stopped immediately prior to enzymatic removal of nucleic acids.

4.2.4 **PGG Crosslinking Treatment**

High purity 1,2,3,4,6-Penta-O-galloyl-beta-D-glucose (penta-galloyl glucose, PGG) was a generous gift from N.V. Ajinomoto OmniChem S.A., Wetteren, Belgium (www.omnichem.be). Acellular valve scaffolds were rinsed in sterile PBS and then treated with sterile 0.15% PGG in 50 mM dibasic sodium phosphate buffer in saline containing 20% isopropanol, pH 5.5. Treatment occurred at 22°C on an orbital shaker for 21±1 hours. At the onset of fixation, cusps were lightly stuffed with sterile cotton balls pre-soaked in PGG solution to preserve the valve conformation in “closed” position. After treatment, the cotton balls were removed and the scaffolds were rinsed then stored in sterile 1xDPBS solution at 4°C.

4.2.5 **RapidGlut Crosslinking Treatment**

Valves were treated with 0.7% Glutaraldehyde (Polysciences, Inc) at 22°C on an orbital shaker for 15 minutes. Valves were rinsed with sterile 1xDPBS for 15 minutes then incubated in two rounds of 1% Glycine (Fisher Scientific) solution for 15 minutes
each. After overnight rinsing in 1xDPBS, valve scaffolds were stored in sterile 1xDPBS solution at 4°C.

4.2.6 DNA Analysis

DNA was extracted from aorta, sinus, cusp and muscle tissue samples (n=4) and purified with the DNeasy Blood & Tissue Kit (Qiagen), then analyzed by Ethidium Bromide agarose gel electrophoresis. Samples were also quantified by reading absorbance at 260nm on a NanoDrop 2000c (Thermo Scientific). Quantities of DNA were normalized to dry tissue weight and expressed as ng/mg dry tissue.

4.2.7 Histology and Immunohistochemistry

For histology studies, samples collected from the aortic wall, sinus, cusp and muscle were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm (3 µm for the aorta), and stained with DAPI for nuclei, Hematoxylin & Eosin (H&E), and Movat’s Pentachrome.

Immunohistochemistry (IHC) was performed to detect remaining components after decellularization. Biotinylated Griffonia simplicifolia (GS) lectin was used to detect Gala1–3Gal (α-Gal), the main porcine antigen responsible for acute rejection of xenotransplants. IHC for laminin, and type IV collagen was also performed. Tissue samples were rinsed with 1X PBS fixed at room temperature in 4% formaldehyde (BDH Chemicals). Following rinsing samples were blocked using 5% Bovine Serum Albumin (Rockland Immunochemicals, Gilbertsville, PA) with 0.05% Triton (BDH Chemicals) in 1X PBS for 2 hours at room temperature. The blocking solution was removed and 250
μL primary CD-31/PECAM1 Antibody (VM64) (NBP1-42152; Novus Biologicals, Littleton, CO) in blocking solution (1:2 dilution) was added for 1.5 hours at room temperature. The primary antibody was removed before rinsing 4 times with 1X PBS. The secondary antibody, Alexa Fluor® 594 Donkey Anti-Mouse IgG (Invitrogen, Grand Island, NY) diluted in blocking solution (1:2 dilution) was added for 1 hour at room temperature in the dark followed by 4 rinses with 1X PBS. Finally, 500 μL DAPI stain (Sigma-Aldrich) was added to each slide for 5 minutes at room temperature in the dark before fluorescent imaging. Primary antibody was omitted for staining negative controls.

4.2.8 Biaxial Mechanical Testing

Samples were prepared from native (fresh) aortic valve leaflets and the PGG treated decellularized aortic valve leaflets (N = 6 for each group) using the methodology previously reported. Square-shaped samples (~12 mm × 12 mm) were dissected from the belly region of the aortic valve leaflet, with one edge aligned with the leaflet circumferential direction and the other edge aligned with the leaflet radial direction. Thickness of each sample was measured in triplicate using digital calipers. Four dark markers were placed in the center region of the square sample (pasted on the ventricularis side of the leaflet). Samples were mounted onto the biaxial testing system via stainless steel hooks attached to 8 loops of 000 polyester suture of equal length (2 suture loops per sample edge). Membrane tensions (force/unit length) were applied to the circumferential direction and radial direction of the leaflet sample. A pre-load of 0.5 N/m was used during the biaxial mechanical testing. After 10 cycle preconditioning, the leaflet sample was loaded to an equibiaxial tension of 60 N/m. The leaflet extensibility was characterized by
the maximum stretch ratio along the circumferential direction ($\lambda_{\text{circ}}$) and the maximum stretch ratio along the radial direction ($\lambda_{\text{rad}}$). Biaxial testing was carried out with the samples submerged in PBS bath (pH 7.4) at 37°C.

### 4.2.9 Bending Mechanical Testing

Bending tests were performed by following a previously reported protocol.[87] The bending tests were carried out in a bath chamber with PBS. Aortic valve samples, both native and PGG treated decellularized valve groups, were dissected out of the belly region of the valve leaflet (N = 5 each group). These samples were further trimmed to tissue strips (~8 mm long by ~4 mm wide) in both the circumferential and radial directions. Two hollow posts (~4 mm) were attached to each end of the tissue strip for mounting purpose. One end of the tissue strips were attached to a post that was fixed on the inside wall of the bath chamber and the other end was mounted onto the bending bar. Each strip was mounted and subjected to simple bending testing with the ventricularis side up and the fibrosa side down. Five dark contrast markers used for tracking the leaflet strip curvature, i.e., marker 1 was pasted on the fixed post, marker 2 to 4 pasted along the edge of the tissue strip, and marker 5 pasted on the end of the bending bar (Figure 4–8D). The bending movement was tracked using a Firewire camera (DMK21AF04 model, The Imaging Source).

Due to the previously reported variation in material stiffness between the native and PGG treated samples,[130] two different sizes of Titanium bending bars (grade 23, Small Parts Inc.), one with a diameter of 0.38 mm and the other with a diameter of 0.71 mm, were used for testing the native leaflets and the PGG treated leaflets, respectively.
Both bending bars a length of 14 cm. The bending movement in the tissue strip was produced by moving the bath chamber toward the bending bar by a linear positioner controlled by a Velmex stepper motor (Velmex Inc., Bloomfield, NY). The corresponding change in bending bar deflection was recorded by tracking marker pasted on the end of the bending bar, and the force was calculated by reference to the bending bar calibration curve. Both the Velmex motor and Firewire camera were controlled by a custom written Labview program (version 2000, National Instruments).

In addition to circumferential and radial directions, each leaflet strip was tested by flexing the strip with the natural curvature (WC) and against the natural curvature (AC). The WC tests result in the ventricularis layer being in tension and the fibrosa layer being in compression, while the AC tests result in the ventricularis layer being in compression and the fibrosa layer being in tension.

4.2.10 Statistical Analysis

Results are represented as means ± standard deviation. Statistical analysis was performed with one way analysis of variances (ANOVA) and results were considered significantly different at p<0.05.

4.3 Results

4.3.1 Cyclical Perfusion Decellularization Device

The novel device for decellularizing a whole aortic root can fully decellularize up to five aortic roots by circulating 4 liters of solutions through the system under a cyclical pressure regime that creates a transmural pressure gradient of about 52 mmHg. Utilizing
a peristaltic pump to create flow, the system (Figure 4–2D) incorporates a pulse dampener, reservoir, constrictor valve to build pressure, and a series of plates and chambers to appropriately direct flow through and around an aortic root. A simplified cross section of the fluid flow path is shown in Figure 4–2C. Fluid flows from the pump and through the dampener into the lower level of the chambers. It is directed into each of the valves mounted in the system where it can build pressure due to the downstream constriction created by the manifold and outflow constricting valve. From the manifold, it circulates back into the system to bathe the outside of the tissue in solution at a low pressure. From there, the fluid freely flows out of the chamber and into a reservoir for recirculation. Pressure is measured in lines communicating with the outside as well as the inside of the aortic roots by transducers controlled by custom LabView software, which displays inner, outer, and differential pressures (Figure 4–3B).

4.3.2 DNA and Cellular Content

Figure 4–1 and Figure 4–5 show a mapping of each portion of tissue in the aortic root. While it is clear that the aorta, muscle, and cusp are different tissues, it should also be noted that the sinus is a different tissue than the aorta, with a different structure and function.

H&E and DAPI analysis for the 8-day immersion-decellularized valves revealed complete cellular removal from valve cusps (Figure 4–4A&B), which was confirmed with Nanodrop data (not shown). However, the sinus, aorta, and muscle were not completely decellularized using this immersion technique (Figure 4–4D).
While 8 days of perfusion treatment was sufficient to remove cells from the muscle and sinus as well as the cusp (Figure 4–5), a longer time was needed to decellularize the full thickness of the aorta. In unsuccessful attempts, cells remained in the central layers of the aorta, but were removed near the media and adventitia. Agarose gel imaging (Figure 4–5A), Nanodrop (Figure 4–5B), H&E (Figure 4–7), and DAPI (Figure 4–5) show that 16 days of decellularization treatment was adequate to remove sufficient amounts of DNA from all aortic root areas. Notably, the use of DNase and RNase enzymes were vitally necessary to remove cellular content from the aorta after 16 days as well as from the sinus at the 8 day time point as seen in the agarose gel images (Figure 4–6). Nanodrop analysis (Figure 4–6) confirmed the drastic reduction in DNA content after the full 16 day perfusion decellularization protocol.

4.3.3 Extracellular Matrix Components

Examination of the H&E and Movat’s Pentachrome staining, as in Figure 4–7, revealed an intact matrix primarily comprised of collagen and elastin. As expected, glycosaminoglycans were not seen after any decellularization steps.

Figure 4-4: Immersion decellularization is incomplete for all valve root areas
Fresh (left) and immersion decellularized (right) H&E staining of the cusp (A&B) and H&E and DAPI staining of the aorta (C&D) with elastin autofluorescence.
### Immersion decellularization

<table>
<thead>
<tr>
<th>Cusp</th>
<th>Muscle</th>
<th>Sinus</th>
<th>Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
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<tr>
<td>Decell</td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
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</tbody>
</table>

### Perfusion decellularization

<table>
<thead>
<tr>
<th>Cusp</th>
<th>Muscle</th>
<th>Sinus</th>
<th>Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 Days</td>
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<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>16 Days</td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
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### Macroscopic images

1. ![Image](image13)
2. ![Image](image14)
3. ![Image](image15)
4. ![Image](image16)

**Figure 4-5: Decellularization results of immersion and perfusion methods**

(A) Representative histology images of fresh aortic root sections stained for nuclei with DAPI (blue) before (Fresh) and after immersion decellularization (Decell). Arrow points to DAPI stained cell remnants after immersion decellularization. 

(B) Representative DAPI stained sections of aortic valve root areas after 8 and 16 days of perfusion decellularization. Arrow points to DAPI stained cell remnants.

(C) Macroscopic images showing a fresh intact aortic root (1) and a root cut open with one cusp removed to reveal the main anatomical coordinates (2). A fully decellularized aortic root is shown in top view (3) and cut open (4).
4.3.4 Cusp Mechanical Testing

Biaxial

The biaxial data obtained (Figure 4-8A) for both groups (native leaflets and PGG-treated leaflets) showed that the PGG-treated leaflets preserved the anisotropy of aortic valve leaflet tissue, i.e., a stiffer circumferential direction and more extensible radial direction. Moreover, the current PGG-treatment protocol generated leaflet tissue with extensibility very close to the native leaflets. We found that $\lambda_{\text{circ}}$ and $\lambda_{\text{rad}}$ of the native leaflets were $1.0193 \pm 0.0108$ and $1.2110 \pm 0.0504$, respectively; and $\lambda_{\text{circ}}$ and $\lambda_{\text{rad}}$ of the PGG-treated leaflets were $1.0245 \pm 0.01507$ and $1.2191 \pm 0.0509$, respectively. However, we noticed that the tension-stretch curve of

Figure 4-6: DNA analysis by agarose gel electrophoresis and nanodrop UV spectrophotometry
(A) Ethidium bromide agarose gel electrophoresis of genomic DNA extracted from fresh aortic root tissues (F) and tissues collected after 8 days (D8) and 16 days (D16) of perfusion decellularization. Samples were also collected before the nuclease treatment step from the 16 days groups (D16 -). S = DNA standards consisting of whole genomic DNA (*) and DNA ladder (#). (B) Nanodrop UV analysis of DNA extracted from fresh aortic root tissues and tissues collected after 16 days of perfusion decellularization (Decell).
the PGG-treated leaflets showed a much stiffer toe region in the radial direction when compared with the native leaflets.

**Bending**

The native aortic valve leaflets followed a similar trend as we observed in our previous study.[87] The moment-curvature curves (Figure 4–8B) showed that AC bending was stiffer than the WC bending, and the circumferential direction bending is stiffer than radial direction. After PGG treatment, the aortic valve leaflets showed a large increase in overall bending stiffness. In PGG-treated leaflets, we still observed that AC bending is stiffer than WC bending, and circumferential is stiffer than radial. Moreover, the degree of nonlinearity increases after PGG treatment, exhibiting a very stiff response when the bending curvature is small, but the increasing trend of the moment-curvature curve greatly slowed down after the bending curvature passed ~0.04 mm⁻¹.

**4.4 Discussion**

**4.4.1 Perfusion Decellularization Device**

Although widely used because of its simplicity to operate, immersion decellularization techniques are inadequate for decellularizing whole aortic roots. We see their successful utilization for tissues that are thin or relatively more porous than the aorta, which contains hundreds of layers of elastin that are difficult for solutions to penetrate. When considering the function of the aorta, to contain the pressurized blood within, it becomes apparent that simple immersion will not be adequate for solutions to reach all layers of this remarkable structure. Even the pulmonary artery is thinner and more porous than the aorta, resulting it its relative ease to decellularize.
Figure 4-7: Histology of decellularized root tissues
Representative images of H&E stained (A) and Movat’s pentachrome stained (B) sections showing fresh aortic root tissues before (Fresh) and after 8 and 16 days of perfusion decellularization. Arrows point to stained cell remnants. H&E stain shows nuclei in blue and cytoplasm and matrix in pink. Movat’s pentachrome stain reveals collagen (yellow), elastin (dark brown) and muscle cells (red).
A device and method of use was created for decellularizing the thick portions of the aortic root as well as the more fragile cusp portions. The cusps were subjected only to immersion conditions, the aorta and sinus were subjected to a cyclical transmural pressure gradient and resultant stretch, and the muscular regions were subjected to a transmural pressure gradient and increased perfusion. This combination of isolated conditions resulted in an aortic root that was overall, decellularized, yet maintained structural integrity in the most fragile and vital areas.

4.4.2 Decellularized Aortic Root

Upon initial evaluation of an immersion-decellularized aortic root, it was quickly apparent that the aorta regions of the root were not fully decellularized using our detergent and immersion method of decellularizing valve cusps. Further examination of the literature revealed a lack of evidence supporting full decellularization of the aortic roots. If an entire aortic root is to be used as a tissue engineered heart valve replacement, it is vital that the entire tissue be free of native cells to reduce or eliminate chances of immune rejection and subsequent failure.\cite{127} The removal of all foreign cells from the cusps, sinuses, ascending aorta, and even muscular regions of an aortic root must be clearly and satisfactorily demonstrated and the aforementioned reports of decellularized aortic roots are inadequate in their descriptions.

Our own experience indicates that cell removal must be evaluated by a variety of methods rather than one sole method of analysis. It can sometimes be difficult to classify an object as a cell or not in histological images. NanoDrop analysis is not as accurate at near-zero levels of dna, as evidenced by numerous negative readings, despite routine and
normal reads of our blanking solution. Perhaps the most identifying single analysis is the agarose gel. However, even that analysis is subject to human interpretation. Our demonstration of the necessity to utilize a perfusion system for decellularizing aortic roots reveals that many previously published methods should verify the cellular content of all areas of tissue to be used for implantation. Furthermore, the innermost portions of the aortic tissues should be verified to be free of cells and shown in publications. For the reasons stated above, multiple methods of analysis should be shown to demonstrate complete removal of cellular content.

4.4.3 Mechanical Analysis

The current protocol includes the step of leaflet decellularization followed by PGG treatment. It is well known that for most decellularization protocols, the aortic valve leaflets experience an increase in overall tissue extensibility due to the microstructural disruptions (e.g., disruption of collagen network and elastin fibers). On the other hand, PGG treatment generates crosslinking at molecular level, which has a stiffening effects on collagenous tissues. The treatment of PGG on the decellularized leaflets causes tissue crosslinking and stiffening, and hence the extensibility of the decellularized leaflets could be reduced accordingly. Interestingly, we found that the current protocol generated PGG-treated leaflets with an overall tissue extensibility comparable to the native leaflets. However, as we pointed out, the biaxial behavior of the PGG treated leaflets was not exactly the same as the native leaflets. The tension-stretch curves showed that the toe region of the radial direction is much stiffer than that of the native leaflets. The loss of a relatively flat toe region after PGG-treatment demonstrated
that the PGG-treated leaflet picked up mechanical load much quicker in the deformation initiation region.

We found that the PGG treated aortic valve leaflets had a much stiffer bending moment-curvature relationship than the native aortic leaflets. This reflected the fact that the crosslinking took place at the molecular level after PGG treatment. Tissue bending stiffness seemed to be more sensitive to this molecular level crosslinking, and the order of change we observed is very high (Figure 4-8). Interestingly, the subtle variations of leaflet flexure, in terms of AC vs. WC bending and circumferential vs. radial bending, were all preserved after PGG treatment. This observation implied that the PGG treatment still preserved the ultrastructural level subtlety while in overall increasing the tissue bending stiffness. The increase of the degree of nonlinearity after PGG treatment is another interesting finding. As we notice, the stiffening effect was more in the range of small bending curvature, and this observation echoed with the biaxial testing, which showed the toe region of the radial direction curve turned into much stiffer after PGG treatment. All the biomechanical observations seemly hinted that the mechanical effect of PGG treatment on tissue behavior was more noticeable in the deformation initiation range.
Figure 4-8: Mechanical properties of decellularized cusps

(A) Biaxial stress-strain analysis of fresh cusps and cusps which underwent decellularization and PGG stabilization, tested in both circumferential and radial directions. (B) Bending test results for fresh and PGG-treated decellularized cusps tested with curvature and against curvature.
CHAPTER 5: TECHNIQUES AND DEVICES FOR CELL SEEDING OF HEART VALVES

5.1 Introduction to Need

Cell seeding plays an integral role in the production of a tissue-engineered implant. Regardless of the target tissue, the idea of adding cells to a scaffold is a fundamental part of tissue engineering. Literature demonstrated numerous methods of adding cells to a scaffold, but no method has been deemed to work far better than any other does. Optimization of this process is considered to be an important aspect in the paradigm of tissue engineering.\[65\] Overall, seeding techniques can be categorized into three areas: static, dynamic, and perfusion.\[65,132\]

Static seeding methods (Figure 5-1A) usually involve manually pipetting a cellular suspension directly on the scaffold and re-pipetting the solution back onto the scaffold as necessary. After a specified amount of time of pipetting, the scaffolds are kept static to allow for strong attachment of cells. This is by far the simplest and most widely used method of seeding, but complex geometries of scaffolds often make uniform and repeatable results impossible.\[133\] Additionally, this method often results in low seeding efficiencies.

Dynamic seeding methods (Figure 5-1B) utilize fluid motion to maintain cells in a state of constant suspension around a tissue so they can contact many portions of the tissue evenly as they move. An example of this type of seeding is a commonly used spinner flask whereby the scaffold is placed inside the flask and the stir bar agitates the liquid to maintain cellular suspension. Although this constant mixing will provide cell contact to
many areas of the tissue, it can also cause the cells to detach and even cause cell death if shear forces are too high.\textsuperscript{[132,134]} Another approach to dynamic seeding involves creating random fluid motions by randomly rotating a sealed container with the scaffold housed within.\textsuperscript{[65,135,136]} This random cell movement increases the probability of cells attaching to multiple surfaces, but can sometimes require a more difficult method of mounting the scaffold. However, these methods can combine gravitational force to cause cells to fall and attach to specific portions of tissue when rotations are paused for a limited amount of time.

Perfusion seeding methods (Figure 5-1C) rely on hydrostatic pressure, magnetism, or electrostatic forces to move cells onto or into scaffolds.\textsuperscript{[132,137–139]} Hydrostatic pressure utilizes a pressure gradient to force seeding solutions into a scaffold by increasing internal pressures or pulling an external vacuum. Magnetic nanoparticles bound to cell surfaces can

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{figure51.png}
\caption{General methods of cell seeding}
\end{figure}

Static (A), dynamic (B), and perfusion (C) methods of seeding cells on a scaffold. Static methods rely on repipeting the cell suspension over the tissue. Dynamic methods use fluid movement to keep cells in suspension so they can contact and attach to many areas of the tissue. Perfusion methods use forces to drive cells deep into the tissue rather than relying on cellular migration alone.\textsuperscript{[65]}
allow the cells to be manipulated through precise use of magnets to direct cells onto specific surfaces of a scaffold. Electrostatic force methods involve inducing a positive surface charge on a scaffold to attract the negatively charged cells onto the scaffold. These methods are usually utilized in flat or tubular structures and could be difficult to apply to the complex geometry of a heart valve root. Additionally, they often involve complex equipment that makes maintaining sterility difficult.

The methods developed here serve to combine the advantages of multiple methods of seeding to achieve optimal results for heart valve and aortic root scaffolds. They utilize other devices created in the lab that eliminate some of the difficulties previously mentioned. They also seek to take into consideration the complex geometry of the scaffolds and do not rely on cellular migration, which has been shown to be extremely slow and not far enough into the tissue in our previous work.

5.2 Materials & Methods

Cell seeding methods were developed in close collaboration with other students and improved or modified as needed. Notably, internal cellular seeding protocols were initially developed by Jordan Maivelett and improved in collaboration with Allison Kennamer [140]. External seeding devices and protocols were developed by Richard Pascal [126] and underwent design improvements described herein.

5.2.1 Valve Scaffold Preparation

Fresh porcine aortic valve roots were collected, cleaned, and prepared as described in section 3.3.1. Aortic roots were decellularized by either the immersion (section 4.2.2) then mounted in the valve mounting rings as in section 3.3.1 or decellularized by 16-day
perfusion (section 4.2.3) techniques. Following decellularization, roots were crosslinked with PGG as in section 4.2.4 and neutralized for 18-24 hours in DMEM with 50% FBS and 1% Antibiotics/Antimycotics at 37°C.

5.2.2 Stem Cell Culturing

Human adipose-derived stem cells (hADSC; Invitrogen, Eugene, Oregon) were cultured on cell-culture flasks at initial densities of 5000 cells per cm². Cell culturing media consisted of Complete MesenPRO RS™ Medium (Invitrogen, Eugene, Oregon) with 1% Antibiotic/Antimycotic (Mediatech, Manassas, VA), and 0.36 g/L of L-Glutamine (Fisher Scientific). Passaging occurred at 75-85% confluence and, unless otherwise noted, hADSCs were not used beyond passage 6.

5.2.3 Rotational Seeding Device Design, Components, and Assembly

A LabView-controlled dynamic seeding device (Figure 5-2) was developed using SolidWorks software and engineering design techniques and manufactured out of acrylic and aluminum. The device was designed around the aortic root characteristics and with the intention of easily integrating into the overall valve preparation process. A combination of static and rotational seeding methods were deemed the best fit for this tissue and integration into the valve preparation process.

Initial methods of holding the aortic root involved previously used bracing rings described in section 3.2.2. These bracing rings contain holes around the outside of the center hole to allow a cell suspension to flow around the external surfaces of the valve roots and through the valves during rotation of the seeding chamber.
developments led to the improved method of mounting the aortic roots described in section 3.2.4. Utilizing these self-adjusting, no-touch valve mounting devices allowed an easier transition between devices and improved the retention of structural integrity of the tissue since no further handling was necessary. The improved valve mounting devices contain the same holes to allow fluid movement around the valve and fit well into the rest of the seeding device (Figure 5-2C) and methods.

Figure 5-2: External seeding device
Six chambers (B) can be held in the frame connected to the shaker plate (A) for cell seeding with agitation and pause cycles. The valve quick mount fits inside the chamber and allows flow to pass around the valve (C). The chambers are assembled and set into the chamber holder plates according to specific protocols that maintain consistency and ability to track valves (D). During rotation, cells are resuspended and during pausing at each angle, cells are allowed to fall to a valve surface and attach (E). During chamber assembly and placement into the chamber holder plates, the thumbscrews, degree marks, and ports must be aligned with specific characteristics in relation to each other and the set line or axial chamber holder plate.
The chambers (Figure 5-2B&C) to hold the valves were designed to use as little seeding solution as possible and reduce violent flow patterns that could cause cell removal from the scaffold or cell death. Rounded edges that fit closely to the outline of the aortic root were designed with dimensions that would cause the fluid to flow smoothly through the slits in the mounting device as well as the valve itself. The lid of the chamber contains two ports (Figure 5-2D) to allow pressure equalization and media or gas exchange as necessary. During end-over-end rotation, needleless ports can be added to the luer ports, but during axial rotation, luer plugs must be used for three chambers to fit into one chamber holder plate. It utilizes an o-ring seal to maintain sterility and slides into the outer chamber, thereby clamping the mounting device and holding it in place. The pressure of the o-ring seal provides temporary closure until the seeding chamber is mounted to the chamber holders described below. The chamber and lid was manufactured from acrylic material to allow visualization of flow and sterilization via ethylene oxide-gas treatment.

After or during attachment of the lids, each set of three lids should be lined up in relation to the chamber body so that each degree mark (0, 120, and 240) is each 1) aligned with both luer ports and 2) between two bolts. For example, on the first chamber, the 0° mark should be in line with both luer ports and also between two bolts; on the second chamber, the 120° mark should be in line with both luer ports and also between two bolts; and on the third chamber, the 240° mark should be in line with both luer ports and also between two bolts. This can be achieved by rotating the lid in relation to the chamber body and will be important when using the axially rotating chamber holder plate.
Up to three seeding chambers are mounted into aluminum chamber-holder plates by hand-tightened bolts between the lid and threaded portions of the chamber holder. These holders are mounted onto a frame containing a stepper-motor controlled by custom LabView software that provides rotation of the holders. The upgraded frame can hold two chamber-holder plates, resulting in the processing of up to six heart valves at one time. The frame can be mounted onto an orbital shaker that provides and additional direction of motion to help maintain suspension of the cells and create random fluid movement within the chamber (Figure 5-2A). The entire assembly of chambers, holders, frames, motors, and orbital shaker can fit into a standard cell culture incubator or heating oven to provide physiological temperature during seeding.

Two versions of chamber holder plates were designed that can be exchanged with by loosening set screws on either end of the plates. One chamber holder holds the seeding chambers so they rotate in an end-over-end fashion around the point directly in the center of each heart valve (Figure 5-2 – upper plate) while the second chamber holder holds the seeding chambers so they rotate around their natural axis (Figure 5-2A – lower plate). The end-over-end rotation and pauses at specific orientations will ensure that cells flow through the valve scaffold and contact multiple areas of the valve when they fall due to gravitational forces during the static phases. The axially rotating chamber holder will ensure cell contact with all interior surfaces of the valve root and may provide additional coverage over the alternative chamber holder.

Several marking systems (Figure 5-2D) were used to ensure uniform conditions between valve root samples and track areas already seeded with cells when performing multiple seeding steps. Each seeding chamber and lid set was labeled with a unique
number from 1 to 6 to identify and track each valve. The seeding chambers were also marked between each bolt with the degree marks of 0, 120, and 240. With three bolts, three degree marks, and by ensuring the non-coronary sinus was aligned with the 0° mark, we could ensure all three cusps and sinuses were treated equally as long as we performed three repetitions with $1/3$ rotation between each repetition. A simple dash mark was made on the end-over-end rotating chamber holder that would align with one of the three degree markings on the seeding chamber. When using the axially rotating chamber holder, the degree markings would be referenced to the location between the two bolts that hold the chamber to the chamber holder.

The LabView software allows the chamber holders to be programmed and rotated automatically. The hardware interfaces included the National Instruments cDAQ-9171 chassis and cDAQ-9401 module. Controllable parameters include rotation direction, rotation time, rotation pause time, orbital shaking speed, orbital shaking time, orbital shaking pause time, and number of repetitions. By using a finely-controlled stepper motor and calculating rotations per minute by time rotated, the final resting degree of rotation can also be controlled.

5.2.4 Changing Media and Gas Exchange in the Seeding Chambers

The needleless ports attached to the lid of the seeding chambers can be used for changing media, adding cells, cycling the gas content, or any other access necessary. To change media, the seeding chamber is removed from the A-frame and transferred to the cell culture hood. The chamber is drained by vacuum through a needleless port with an
empty 50 mL syringe attached to
the other port to allow sterile air
into the system (Figure 5-3).
Following removal of all liquid,
media is added to the chamber
through the 50 mL syringe until
the media just covered the stainless
steel mounting rings (about 120
mL). The attached vacuum will pull
the media from the syringe into the
chamber, but is not necessary for the chamber to fill. If additional gas exchange is
necessary, sterile filters can be attached to each needleless port and vacuum applied to
one port. This will draw CO₂ rich air into the seeding chamber. It should be noted that
compressed CO₂-rich air can be used, but only when chambers are securely latched to the
holding plate. If the lid is not attached, the pressurized air will force the lid out of the
system.

5.2.5 Internal Cellular Seeding via Injection

Valve scaffolds were prepared as above and seeded with cells as in Figure 5-4.[140] Following neutralization, the base and free edge of all cusps in the valve were inflated with sterile compressed air (15-20 psi) attached to a sterile 23G x 1.25 inch needle. A sterile pipet was used to stabilize the tissue and the needle was inserted into the middle layer of the cusp, causing the fibrosa and ventricularis layers of the cusps to delaminate.
Immediately after mixing, 4 million hADSCs in 1 mL were loaded into a 1 mL syringe and manually injected into the free edges of the cusps in multiple areas and in the base of the cusp through a 33G x 1.25 inch needle. Injection was repeated for each cusp for a total of 12 million cells into the interstitial cusp area of each valve.

Valves were then placed into a seeding chamber with the non-coronary sinus aligning with the 0° mark on the chamber. The chamber was filled with enough cell culture media (DMEM with 10% FBS, 1% Antibiotic/Antimycotic) to cover the stainless steel mounting rings (about 120 mL). The chamber was closed as described above and

Figure 5-4: Internal cell seeding of valve cusps
(A) Internal cell seeding was performed after mounting into the valve quick mount to assist in stabilization. (B) The bulb of a sterile pipet was inserted into the valve root to (C) hold the cusp in place. Compressed air (15-20 psi) was (D) pushed through a filter to sterilized the air before (E) injecting the base and free edges of the cusps with air. (F) After air injection, cusp layers delaminated and usually maintained pocket of air after needle removal. (G) Cell culture media was drawn into a syringe prior to (H) attaching a needle to the syringe. (I) Cells were injected into the previously delaminated cusps at the base and free edge.[140]
placed into the end-over-end chamber holder on the A-frame with the 0° chamber mark aligning to the corresponding mark on the end-over-end chamber holder.

After placing all the valves into the chamber holder, the shaker was set to speed 20%. The LabView program was set with the rotator at 5.0 RPM with a rotating time of 300 seconds and pause time of 300 seconds. Rotations alternated direction each time and the seeding chambers were rotated for 18-24 hours at 37°C at those conditions to improve cell attachment to the valve scaffolds.

5.2.6 External Cell Seeding via Rotational Device

One day prior to internal seeding (the same night as the neutralization step), the seeding chambers were assembled and filled with sterile 5% BSA in 1xDPBS to block attachment to the container and localize the future fibronectin/pronectin attachment to the valve. The next night, the freshly internally injected valve scaffolds were coated with 4 µg/cm² (approximately 4 µg/mL in this application) fibronectin (Sigma) or pronectin (Sigma) by adding the correct volume of fibronectin/pronectin to the culture media. Following interstitial cell seeding and coating with 4µg/cm² fibronectin or pronectin, the exterior surfaces of the valves were seeded by one or a combination of the methods described below.

External Seeding Method 1

In the first method, 16-24 million hADSCs were seeded in one night using end-over-end rotation of the valves. For this method, the seeding chamber was removed from the A-frame and drained as described in section 5.2.4. 16-24 million hADSCs (as
determined by the experiment trial) were resuspended in 35 mL cell culture medium and added to the seeding chamber through the syringe, followed by about 90 mL of additional cell culture media to fill the chamber.

The chamber was placed into the end-over-end holder on the A-frame with the 0° chamber mark aligning to the corresponding mark on the end-over-end chamber holder (position B in Table 5-1) and the rotational regimen in Table 5-1 was followed overnight.

**External Seeding Method 2**

For the second method, 30 million hADSCs were seeded over the course of three nights using end-over-end rotation of the valves. For this method, the seeding chamber was removed from the A-frame and drained as described in section 5.2.4. 10 million hADSCs were resuspended in 35 mL cell culture medium and added to the seeding chamber through the syringe, followed by about 90 mL of additional cell culture media to fill the chamber.

The chamber was placed into the end-over-end holder on the A-frame with the 0° chamber mark aligning to the corresponding mark on the end-over-end chamber holder.
The chamber holder was positioned with the root up (position A in Table 5-2) and the rotational regimen in Table 5-2 was followed overnight. On the second day of seeding, the media was aspirated and another 10 million hADSCs were added to the chamber in 120 mL media through the needleless ports as described above. The seeding chamber was then placed back into the end-over-end holder on the A-frame with the 120° chamber mark aligning to the corresponding mark on the end-over-end chamber holder (position B). The chamber holder was positioned with the root up (position A) and the rotational regimen in Table 5-2 was followed overnight with the only change being position B was at 120° on the set mark.

On the third day of seeding, the media was aspirated and another 10 million hADSCs were added to the chamber in 120 mL media through the needleless ports as described above. The seeding chamber was then placed back into the end-over-end holder on the A-frame with the 240° chamber mark aligning to the corresponding mark on the end-over-end chamber holder (position B). The chamber holder was positioned with the root up (position A) and the rotational regimen in Table 5-2 was followed overnight with the only change being position B was at 120° on the set mark.

### Table 5-2: External seeding method 2 dynamic regimen

<table>
<thead>
<tr>
<th>Position A (holder orientation)</th>
<th>Position B (rotation in holder)</th>
<th>Time (min)</th>
<th>Shaker Speed</th>
<th>Rotator Speed</th>
<th>Rotating Time (seconds)</th>
<th>Pause Time (seconds)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Setting: Root Up</td>
<td>0° on set mark</td>
<td>00.13</td>
<td>Off</td>
<td>5.00</td>
<td>11</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>Run for 12-15 seconds, then hit circular stop button during the first pause time.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotating</td>
<td>0° on set mark</td>
<td>30.05</td>
<td>20%</td>
<td>2.00</td>
<td>1805</td>
<td>5400</td>
<td>1</td>
</tr>
<tr>
<td>Root Up + 30 degrees</td>
<td>0° on set mark</td>
<td>90.00</td>
<td>Off</td>
<td>30.05</td>
<td>20%</td>
<td>1805</td>
<td>5400</td>
</tr>
<tr>
<td>Root Up + 330 degrees</td>
<td>0° on set mark</td>
<td>90.00</td>
<td>Off</td>
<td>30.05</td>
<td>20%</td>
<td>1805</td>
<td>5400</td>
</tr>
<tr>
<td>Run the above for 1 cycle = 4 hours. Be sure to turn shaker off/on as appropriate.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second pre-Setting</td>
<td>0° on set mark</td>
<td>00.03</td>
<td>Off</td>
<td>5.00</td>
<td>1</td>
<td>5400</td>
<td>1</td>
</tr>
<tr>
<td>Run for 2-5 seconds, then hit circular stop button during the first pause time.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotating</td>
<td>0° on set mark</td>
<td>15.30</td>
<td>20%</td>
<td>1.00</td>
<td>930</td>
<td>6300</td>
<td>100</td>
</tr>
<tr>
<td>Root Up + 180 degrees</td>
<td>0° on set mark</td>
<td>105.00</td>
<td>Off</td>
<td>15.30</td>
<td>20%</td>
<td>930</td>
<td>6300</td>
</tr>
<tr>
<td>Root Up</td>
<td>0° on set mark</td>
<td>105.00</td>
<td>Off</td>
<td>15.30</td>
<td>20%</td>
<td>930</td>
<td>6300</td>
</tr>
<tr>
<td>Run the above until next seeding day. Can leave shaker off if needed.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
end-over-end chamber holder (position B). The chamber holder was positioned with the root up (position A) and the rotational regimen in Table 5-2 was followed overnight with the only change being position B was at 240° on the set mark.

**External Seeding Method 3**

For the third method, 30 million hADSCs were seeded over the course of three nights using axial rotation of the valves. After removing the seeding chambers from the A-frame, the end-over-end chamber holder was replaced with the axial chamber holder on the A-frame. For this method, needleless ports were replaced with luer plugs before the first media aspiration to allow three chambers to fit into the axial chamber holder. The seeding chamber was drained as described in section 5.2.4. 10 million hADSCs were resuspended in 35 mL cell culture medium and added to the seeding chamber through the syringe, followed by about 90 mL of additional cell culture media to fill the chamber. Following the additional media, luer plug caps were place onto the luer fittings.

The chamber was placed into the axial holder on the A-frame with the 0° chamber mark being set between the bolts holding the seeding chamber to the chamber holder (position B in Table 5-3). The chamber holder was positioned with the bolts up (position A in Table 5-3) and the rotational regimen in Table 5-3 was followed overnight.

On the second day of seeding, the media was aspirated and another 10 million hADSCs were added to the chamber in 120 mL media through the luer ports as described above. The seeding chamber was then placed back into the axial holder on the A-frame with the 120° chamber being set between the bolts holding the seeding chamber to the chamber holder (position B). The chamber holder was positioned with the bolts up
(position A) and the rotational regimen in Table 5-3 was followed overnight with the only change being position B was at 120° between the bolts.

On the third day of seeding, the media was aspirated and another 10 million hADSCs were added to the chamber in 120 mL media through the luer ports as described above. The seeding chamber was then placed back into the axial holder on the A-frame with the 240° chamber being set between the bolts holding the seeding chamber to the chamber holder (position B). The chamber holder was positioned with the bolts up (position A) and the rotational regimen in Table 5-3 was followed overnight with the only change being position B was at 240° between the bolts.

### 5.2.7 Live/DEAD® Imaging

Presence of live cells on tissue surfaces was analyzed using Live/DEAD® stain (Invitrogen, Eugene, Oregon) according to manufacturers’ directions, using 20 µL of EthD-1 and 5 µL of Calcein-AM in 10 mL of 1X PBS. The Live/DEAD® solution was
added to cover each sample and covered with foil at 37°C for 20 minutes before fluorescent imaging with an inverted microscope.

5.2.8 SEM Analysis

Scanning electron microscopy (SEM) was used to visualize the 3-D morphology of cells on the cusp surfaces. After rinsing in 1xDPBS, the cusps were fixed in Karnovsky’s fixative (2.5% glutaraldehyde, 2% formaldehyde in 0.1 M cacodylate buffer, pH 7.4) for at least 24 h. Samples were dehydrated in increasing ethanol concentrations until absolute alcohol was reached, then stored in 100% ethanol for up to 12 hours. Following immersion in hexamethyldisilazane (Polysciences, Inc., Warrington, PA) for 15 min, samples were left to air dry. Samples were then coated with platinum for 2 min (Hummer 6.2, Anatech LTD, Union City, CA) before imaging with a Hitachi S4800 or TM3000 Tabletop scanning electron microscope (Clemson University Electron Microscope Facility, Anderson, SC).

5.2.9 Histology

For histology studies, samples collected from the cusp were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm and stained with DAPI for nuclei and Hematoxylin & Eosin (H&E).

5.3 Results and Discussion

5.3.1 Injection Seeding

Several trials of injecting cells into the cusps were used to determine the best sizes of needles and other tools to use during inflation and injection. Initial trials using carbon
particles were helpful in tracking where the injected media traveled, but did not travel in the same manner as the cell suspension. Figure 5-5B shows the route of needle injection into the central layer of the cusp where a bolus of localized carbon particles can be seen in black.

Initial placement of cells by injection yielded similar boluses of cells in the free edges of the cusps (Figure 5-5D). The base of the cusp (Figure 5-5C) yielded the most complete air inflation. This allowed the injected cells to spread through the tissue more than in the free edges of the cusps. Overall, our method of injecting cells into the central layers of aortic cusps yields an initial distribution of cells that is concentrated in boluses in various locations of the cusp. We will rely on cell migration and dynamic forces to cause these cells to spread throughout the tissue during culture.

Other trials using static seeding and relying on cellular migration to repopulate the scaffolds are clearly inadequate up to six weeks when using hADSCs (Figure 5-6). Briefly, hADSCs were seeded onto 1 cm² scaffolds with dropwise methods in 12-well plates and kept in static conditions for 6 weeks with media changes every 2-3 days. We saw complete cell coverage by the 2-week time point with apparent proliferation beyond
that, but extremely few cells penetrated the surface of the scaffolds. Although not ideal, the injection method of recellularizing the internal layers of the valve cusps holds promise. While other methods of achieving a uniform repopulation of the cusp layers are being developed, this method is substantially faster and more effective than current methods available.

5.3.2 Rotational Seeding

Following rotational seeding with method 1, valves were analyzed immediately for cellular attachment, spreading, and alignment in comparison to fresh valve cusps (Figure 5-7). Analysis using Live/DEAD® imaging and scanning electron microscopy for the cusps revealed substantial recellularization. Many surfaces appeared to have as many cells present as the native cusps. The exception to this is seen in Figure 5-7K, where fewer cells are covering the ventricularis side of the cusp. However, during this trial of cell seeding, the rotational regimen was varied and the valve rested in the “root down” position for 5 minutes instead of 90. Since this is the position in which the ventricularis would have been seeded, this is likely the cause for less coverage here.
Figure 5-6: hADSC migration into valve root scaffolds

hADSCs seeded onto the surfaces (Live/DEAD images) of each type of scaffold proliferated, but did not penetrate the surface except in a few instances (C,G,H). The brightness/contrast settings in the DAPI images have been artificially increased to enable visualization.
Overall, initial cell repopulation was very successful with this method. However, the aortic wall and sinus are not as well covered as the cusp (data not shown). Data is not available for initial seeding using methods 2 and 3, but analysis of the surfaces after further conditioning suggests that these methods are equally or more effective at recellularizing the sinus and aorta portions of the valve root than method 1 (see Chapter 8: for details). One drawback to these methods is the large number of cells to be used at one time. For example, a study recellularizing five heart valves by injection and a combination of methods 2 and 3 for full surface revitalization would use over 360 million cells. As demonstrated in Figure 5-7, fewer cells can achieve excellent results, but modifications to the rotational protocol and alternative chamber holders may be necessary to accomplish similar seeding densities on the sinus and aorta luminal surfaces.

![Figure 5-7: Initial cell coverage of valve cusps](image)

Fresh cusp surfaces (A-D) are compared with recellularized surfaces (E-L). External seeding method 1 was used for these valves. I-L show external seeding method 1 with very limited time resting in the root down position, resulting in fewer cells attached to the ventricularis surface.
5.3.3 Rotational Device

Improvements to the previously developed seeding device allow for a semi-automated process. Minimal user intervention is necessary, but observation during active phases of seeding is recommended to prevent unintended scenarios of rotation until the software and hardware can be further developed. By calculating the speed and time in relation to the physical parameters of the stepper motor, a stopping angle can be determined and set by the software and timing. Software and hardware is being developed that can control the speed of the shaker plate.

The additional seeding chamber holding plate to provide an axis of rotation along the axis of the valve root was important in achieving full recellularization of all surfaces of the valve root. Furthermore, capabilities to seed up to six valves on the same system will improve future experiments by allowing for higher sample size. Overall, the rotational seeder works very well for creating an initially recellularized valve surface.
CHAPTER 6: A PLATFORM HEART VALVE BIOREACTOR

6.1  Introduction and Background

Bioreactors have many applications from the bioreactors used for microbial expansion to those intended to prepare a tissue for implantation. In the realm of tissue engineered heart valves, they have two main purposes: 1) to elucidate the mechanisms that influence interactions between cell types or cells and scaffolds and 2) to prepare a tissue engineered construct for implantation into a living animal. However, even within the application of preparing or testing valve constructs, many various functions are performed by bioreactors. From seeding cells, to testing cell retention, to developing new extracellular matrix, bioreactors fulfill many purposes that are otherwise unavailable with conventional 2D or even some 3D methods.

6.1.1  Previous Bioreactor Systems

Many bioreactors focus on subjecting valves, cusps, or cells to stretch, flexure, flow, or a combination thereof. Commercial systems such as Flexcell (Flexcell International, Hillsborough, NC) and other noncommercial systems can subject cells or small pieces of tissue to various physiological levels of stretch. Flexure bioreactors have been created to test the effects of bending on isolated segments of scaffolds with or without cells. Flow systems have frequently been used in many applications to explore the effects of sheer stress on cellular function. Furthermore, many systems have been created to combine the effects of various conditions for the purpose of studying independent vs synergistic effects of those conditions.
Despite the wide array of bioreactors present, relatively few are capable of preparing a tissue engineered construct for implantation. However, it is in this final area, that the most complicated of systems are necessary. They must be capable of subjecting the replacement to a gradually increasing set of physiological conditions until the final conditions that represent or exceed those seen in the body are reached. Furthermore, these types of bioreactor systems must keep the environment sterile and allow for other complications seen in the laboratory environment such as limited space, assembly techniques, and general lack of ready to use equipment for such purposes.

The requirements for a heart valve bioreactor system have been discussed in previous sections. Although not the most sophisticated system in the literature, understanding the function of our previously published version will provide the foundational exposure necessary to see the further advances described herein.

6.1.2 Previous Clemson Heart Valve Bioreactor

The first edition Clemson heart valve bioreactor was developed to test and condition all types of tissue engineered, replacement, and native aortic and pulmonary heart valves under adjustable physical stimuli similar to those experienced in vivo.[117,149] It was designed with the following 10 important considerations:

1) To allow mounting of various sizes and shapes of free-standing (“stentless”) and stented valve designs into the bioreactor.

2) To ensure that the valve opens and closes cyclically due to measurable changes in trans-valvular pressure.
3) To maintain the desired concentration of gases and nutrients and waste removal in/from the culture medium.

4) To expose cell-seeded tissue engineered scaffolds to physical stimuli similar to in vivo, including trans-valvular pressures, pulsatile forces, flow rate, frequency, stroke rate, stroke volume and sheer stresses on the valve surfaces.

5) To allow full control over parameters and to allow for implementation of progressive adaptation protocols.

6) To ensure maximum visibility and the ability to continuously and remotely monitor and record valve function.

7) To use materials which are non-toxic, non-degradable, and easy to sterilize.

8) To ensure reasonable durability, easy setup, and compact size to fit in standard cell culture incubators.

8) To maintain the system at 37°C, 5% CO₂, and 95% humidity in sterile conditions.

9) To yield repeatable results while fulfilling all of the above conditions.

The valve bioreactor is based on a functional principle proposed by Hoerstrup et al[111] with numerous modifications. The pneumatic-driven conditioning system (Figure 6-1C) consists of a three-chambered heart valve bioreactor (1), an optional pressurized compliance tank (2), a reservoir tank (3) with sterile filter (4) for gas exchange, one-way valves (5), resistance valves (6), pressure transducers, a webcam (7), and a ventilator pump routinely used in clinics for intensive care and anesthesia (Siemens 900E, Soma Technology, Bloomfield, CT). The entire system (except ventilator) can be
sterilized using conventional methods (ethylene oxide gas for the acrylic and high-density polyethylene components and autoclave for the silicone, PVDF, and stainless steel components) and accommodates all clinically relevant sizes of stented or stentless biological, mechanical, or tissue engineered valve substitutes. The valve bioreactor is made of acrylic, composed of three compartments and measures 6 inches in diameter and 8.5 inches in height and is completely transparent (Figure 6-1A). The three parts of the bioreactor are held together by stainless steel screws.

The air chamber is connected to the external pump and is the only chamber not filled with culture medium. It is separated from the ventricular chamber by a clear silicone rubber membrane. During the inspiration phase of the ventilator (ventilator causing the patient to breath air in), air is pushed from the ventilator into the air chamber, the membrane bulges into the ventricular chamber, and the residing media is pushed through the heart valve into the aortic chamber, opening the valve. Once completed, the exhalation phase of the ventilator begins (ventilator relaxes pressure to allow the natural patient exhalation) and the ventilator releases pressure in the air chamber, allowing the hydrostatic pressure of the remaining fluid in the ventricular chamber to push down on the silicone membrane, creating vacuum pressure inside the ventricular chamber. The resultant pressure gradient between the ventricular chamber and the reservoir causes culture medium to flow from the reservoir tank, through the one-way valves, and into the ventricular chamber in preparation for the next cycle (Figure 6-1B).
Figure 6-1: First edition heart valve bioreactor and conditioning system

a) CAD designs (left) and manufactured heart valve bioreactor (right) showing the four main components. A transparent silicone membrane diaphragm is mounted between the air chamber and the ventricular chamber. b) Air and media flow through the system during systole (left) and diastole (right). Color coding aids identification of the components and black arrows indicate direction of air and media flow. c) Schematic overview of the entire conditioning system: a three-chambered heart valve bioreactor (1), an optional pressurized compliance chamber (2), a reservoir tank (3) with sterile filter (4) for gas exchange, one-way valve (5), pressure-retaining valve (6), a webcam (7), and a ventilator pump (air pump). The entire setup fits within a standard cell culture incubator. d) Two identical bioreactor systems (Br 1 and Br 2) with endothelial cell-seeded, functioning valves inside an incubator; the bioreactors are in the front row while their corresponding reservoirs are in the back row. The webcams normally mounted onto the top viewing windows of the aortic chamber have been removed to reveal bioreactor details. [117]
During the pumping phase, the curvature of the ventricular chamber and angle of media inlets contributes to the circulation of culture medium throughout the chamber. Once through the valve, the medium enters the aortic chamber then flows through a compliance chamber and into the reservoir. Both the aortic and ventricular chambers have multiple ports for easy access of pressure transducers, as inlets and outlets for media change, or for other probes.

Figure 6-1D shows two bioreactors set up in one standard size incubator. The clear, flat top of the aortic chamber facilitates the viewing of the functioning of the heart valve via the webcam. A permanent fluorescent lamp mounted inside the incubator provides lighting. Using websites such as www.livestream.com and others, continuous broadcast of the bioreactor and valve performance is possible over the internet, allowing the investigators remote viewing of valve performance from any computer and sharing of information with collaborators.

6.1.3 System Limitations and the Need for Further Development

The manufactured and tested first edition bioreactor system fulfills most of the original conditions, including mounting of various types of valves and excellent valve opening and closing characteristics. The system ensured good cell and valve viability under sterile conditions for extended periods of time and excellent reproducibility. It also allowed for implementation of progressive adaptation protocols, ensured perfect visibility and the ability to continuously and remotely monitor and record valve function. Many successful experiments have been conducted using that edition including cell seeding of valves and testing under flow conditions as highlighted in Figure 6-2.
Most heart valve bioreactors in the literature are designed for use with only the valve type investigated in that laboratory. Many facilitate testing of decellularized valves\cite{84,115} or laboratory-manufactured polymer valves.\cite{150} However, the Clemson heart valve bioreactor is able to test all clinically relevant sizes and variations of stented or stentless biological, mechanical, or tissue engineered valve substitutes with multiple mounting methods allowing for variations in tissue thickness, engineered material, valve diameter, amount of excess tissue, and presence or absence of aortic sinuses. Our bioreactor’s mounting design easily facilitates many different valves in a variety of non-restrictive options.

Figure 6-2: Cell analysis of endothelial cell-seeded valvular scaffolds after functioning in the first edition bioreactor
Representative SEM images showing native cusp surfaces (a, b), at time zero (T=0d), i.e. immediately after seeding (c, d) and after 17 days (T=17d) in the conditioning system (e-h).
Incremental stroke volumes from 4.7 to 22.3 mL were achieved. For the lowest stroke volume, the valve was only slightly opening. Under no added pressurization, systolic and diastolic pressures had an average value of 40/15 mmHg, respectively. After pressurization, these values increased to a stable 80/70 mmHg. Higher pressures could be attained, but this halted circulatory flow and the pressures were not stable. The pressure limitations were the result of an inability of the system to circulate fluid effectively. A very high level of pressure could be reached if the pumping mechanism could match and overcome that pressure. By using an alternative pumping mechanism that would preserve energy by reducing air compression, modifications could be made to increase the flow to match and surpass even high physiological values. The stroke volume and pressure of our first edition bioreactor do not yet meet aortic physiological levels and as the tissue engineering technology improves and valves get closer to implantation stages, we will need to be able to reach those higher values.

Our heart valve bioreactor system’s primary function is to prepare tissue engineered heart valves for animal implantation. Thus, representing the whole spectrum of physiological conditions as well as those leading to those conditions and sometimes exceeding them is necessary. Although the current system is capable of meeting some of those needs independently, improvements were necessary to reach the desired outputs and capabilities of the system. Thus, one goal of this research was to modify the heart valve bioreactor system to achieve simultaneous physiological levels of flow, pressure, and shear stresses while maintaining the independent adjustment of each.


6.2 Materials, Methods, and Description

Incremental improvements have been made to the bioreactor system to reach the current capabilities. Each increment (Figure 6-4) added specific value that should not be veiled by the consummated design.

6.2.1 Second Edition Heart Valve Bioreactor

Aside from the technical capabilities, the largest pitfall of the first edition bioreactor was its susceptibility for contamination and cracking of the acrylic material around the threaded ports. Not only did this consume a tremendous amount of resources to repeat contaminated experiments, it was also very time consuming to assemble in a sterile hood. A method of connecting the multiple chambers that did not utilize threaded fittings was investigated. A quick connect fitting similar to those available from Colder Products Company (Figure 6-3A) was desired, with the only difference being that the male end plugged into a bulkhead (the side wall of the acrylic chamber) instead of another fitting. If the second fitting was used, the same problem of connecting that fitting to the acrylic chamber would occur.

After exhausting all known distributors and suppliers as well as inquiring about custom fittings, a novel quick-connect fitting (Figure 6-3B) was developed and patented[151] that would...
maintain a sterile seal and be quick to install without utilizing Teflon tape, threading, or difficult to sterilize materials. This quick connect fitting is a male fitting with an actuating member that engages with a groove in the wall of the bioreactor to lock it in place. It utilizes a reliable o-ring seal to maintain sterility and allow for a quick-connection. Additionally, it can be manufactured out of materials that can be cleaned and sterilized multiple times or 3-D printed and autoclaved for single uses.

Although the hospital ventilator was able to fulfill the role of pumping media, the controls of this ventilator did not provide adequate tuning of the conditions desired and are often difficult and expensive to acquire. A custom LabView program was written to control and monitor custom equipment (Figure 6-4B) for setting the stroke volume, stroke rate, duty cycle, trans-valvular pressures, and overall flow rate. Figure 6-5B demonstrates the second edition bioreactor’s

![Figure 6-4: Editions of the heart valve bioreactor](image)

The conditioning system (A) consists of a three-chambered heart valve bioreactor (1), an optional pressurized compliance tank (2), a reservoir tank (3) with sterile filter (4) for gas exchange, one-way valves (5), resistance valves (6), pressure transducers (7), a flow meter, a webcam (8), and an air supply. External LabView software and custom hardware (B) monitor and control the system. The bioreactor has come through three iterations (C) with the current design shown here (D).
attainable pulmonic and aortic levels of pressure at 75 beats per minute, which is adjustable for all relevant values. The new system for providing the pumping mechanism was powered by a standard air compressor instead of a hospital ventilator. Bioreactor pressures are easily adjusted by controlling input air pressure and can currently reach any desired level. Maximum stroke volume levels achieved under sterile conditions with the second edition bioreactor are roughly 30 mL (70 mL is normal average).

An additional upgrade in this edition was utilizing custom glass-blown bottles as compliance chambers and reservoirs. By using standard media bottles with barbed attachments, we were able to replace multiple assembly steps. Together, the new bottles and fittings were able to drastically reduce system setup time and connection points and therefore reduce the opportunity for contamination of the system during initial setup. While the first edition took 4–6 hours to set up depending on user skill, the second edition took less than 2 hours. More improvements in the third edition bioreactor system would further reduce that time to about 30–45 minutes for an experienced user.

User-friendliness was drastically improved with the second edition bioreactor. As a result, it was able to support more valve testing experiments than previously possible,
but at sub-physiological flow rates. Shear stress on the tissue was also low because the viscosity of the fluid in the system was much lower than that of blood. As the main objective of developing a tissue engineered heart valve progressed, additional improvements were desired to reach full physiological conditions, decrease complexity of the system, and allow for seamless integration into the entire valve preparation process.

### 6.2.2 Third Edition Heart Valve Bioreactor

The next edition of the heart valve bioreactor had slight modifications to its main chambers. It eliminated unnecessary ports; added custom threaded nuts to reduce the number of bolts used and eliminate any threading in the acrylic components; and utilized a re-designed method of holding the heart valve tissue. This tissue holder (described in Chapter 3:) is installed around a fresh heart valve and is used to hold, transport, and seal the valve in the various treatments of decellularization, fixation, cell seeding, condition, and final preparations for implantation. It provides a no-touch approach to handling the valves and fully integrates the entire valve preparation process. Utilizing this valve holder significantly reduced the time to install a valve into the bioreactor, which not only decreased overall assembly time, but also increased cell viability because of the decreased amount of time the cell-seeded valves were outside of culturing media.

3-D printing technology provided by Clemson University Machining and Technical Services (both Printer companies, places) was utilized to reduce the footprint and number of bioreactor components and subsequently, those connections. It allowed the quick connect fittings to be directly attached to custom-shaped reservoirs and compliance chambers. By printing the external modules of the bioreactor in a material
that could be autoclaved once, we were able to pre-assemble a majority of the components outside of the hood, saving time and decreasing the difficulty of assembling a majority of the system in a laminar flow hood.

The new components and custom shapes also allowed for reduced overall footprint, lower media volumes used, and an alternative air filter for gas exchange. Very few filters were found that could provide the volume transfer over the low pressure drop provided by the bioreactor system. Although two options remained, the large flow filter was utilized.

One of the requirements of this edition of the bioreactor was to reduce complexity by utilizing disposable or autoclavable components that could be pre-assembled. Recently developed turbine flowmeters (EquFlow, Netherlands) were acquired that could be autoclaved, but were also relatively inexpensive and could be discarded if necessary. They also acted as a partial constriction valve by their inherent functional properties. Alternative disposable pressure transducers were utilized that would connect directly to ports on the 3-D printed fittings. Normally utilized for invasive blood pressure monitoring, these transducers are very precise.

Utilization of the new flowmeters and pressure transducers also required an upgrade to the hardware and software of our controls devices. A chassis system with interchangeable modules (National Instruments) that could support the new systems components was utilized. This chassis system allowed for numerous counters for the flowmeters and data conditioning required by the pressure transducers. This chassis system was also beneficial for use in controlling the other components and devices utilized in the valve preparation process.
6.2.3 Achieving Physiological Pressure, Flow, and Viscosity with the Third Edition Heart Valve Bioreactor (Fourth Edition Bioreactor)

Although the changes made for the third edition bioreactor enable normal and pathological conditions of pressure, flow, and shear stress, further modifications were necessary to provide those conditions in a sterile environment and control them independently. To modify fluid viscosity, 2.5% dextran (Sigma Aldrich) was added to the media to thicken the media.

As previously described, all normal and pathological levels of pressure can be achieved by adjusting the input air pressure pumping the system. By increasing the pressure, the flow will also increase. However, the physical components of the previous versions have limited the flow to about 30 mL per stroke (normal stroke volume is 70 mL per stroke) at physiological pressures and 75 beats per minute (bpm). As a result, larger flow paths and different compliance chambers were developed with the goal of achieving up to 100 mL per stroke at 75 bpm with physiological pulmonic and aortic pressures.

After increasing all fluid lines to at least 0.9 inches in diameter, we determined that initial air volumes of 8 and 1.5 liters would be necessary for pulmonic and aortic conditions, respectively to provide adequate flow. This can be calculated from the compression ratio of air from 10 to 25 mmHg and 80 to 120 mmHg for pulmonic and aortic pressures, respectively. However, it is also dependent upon having enough time and low enough resistance to move the fluid by that amount under those relatively small pressure changes.

Since 10 liters is too large of a volume to fit multiple systems into one incubator, we investigated alternative methods of creating such a compliance chamber.
approach of using a liquid head of pressure for pulmonic conditions was investigated. Since 30 mmHg is equivalent to roughly 40 cm H₂O, we determined that a cylinder that extended at least 40 cm above the valve would be adequate to create pulmonic pressures. Additionally, this configuration would fit inside a standard sized incubator. The final approach was to attach a 3-D printed cylinder with specific variances in diameter to the fluid circulatory line of the system (Figure 6-6A) to create the appropriate pressure head on the valve. During diastole, as the fluid continued to flow out of the compliance chamber cylinder and into the reservoir, the fluid level would lower and the pressure would decrease. By changing the diameter of the cylinder and varying it along the length, we could adjust how fast the fluid dropped, which affected the stroke volume and the diastolic pressure. This rapid change in air volume in the system was only possible by allowing airflow into and out of the compliance chamber. To accommodate, an air filter was
attached to this chamber as well as the reservoir that had a large enough surface area to accommodate the rapid change in air over the relatively low pressure changes.

A two-liter custom glass-blown bottle (Figure 6-6B) was used as a compliance chamber for the aortic conditions. This size bottle allowed all ranges of stroke volumes, depending on how much air was initially in the system at 80 mmHg. By altering the amount of fluid in the bottle (and subsequently, how much air) we could control stroke volume of the system independently from pressures. Designs have been made to adjust the volume via an external source of fluid while maintaining system sterility. This would require the least amount of culture media inside the flow loop to reduce supplies. In addition, this bottle has a port through which an initial air pressure can be supplied to ensure low media usage.

Incremental improvements in the bioreactor design have allowed us to control the compliance tank size, compliance tank shape, pumping air pressure, media viscosity, pumping rate, resistance, and duty cycle. Variations of certain combinations of these factors allow for the independent control flow, pressure, and shear stresses applied to the valves. Furthermore, each can be controlled while maintaining sterile testing conditions. While the current system meets all the research needs, further improvements are recommended to aid during assembly by novice users. These can be found in Error! Reference source not found.: Error! Reference source not found..

6.2.4 Validation of Function Testing

St. Jude mechanical heart valves used for validation of functions. Viscosity adjustments were made in accordance with published results. Performance testing

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was performed on the benchtop to aid in fluid visualization, but closed systems as those seen in sterile experiments were maintained. A custom LabView program was used to control pumping and monitor flow and pressure during operation. This software utilized a National Instruments cDAQ-9178 chassis and appropriate modules. Bioreactor pumping utilized module 9472.

Flow between the compliance chamber and reservoir was measured by an EquFlo turbine flowmeter. In this position, continuous flow occurred with cyclical increases in flow rate during systole. All fluid passes through this one constriction point, but it should be noted that if any regurgitation occurred, this volume of fluid would not be accounted for in the flow calculations. Flow is measured in a turbine by counting the pulses sensed from an infrared sensor placed in line with the turbine spokes. As the spokes pass, the sensor is blocked and a pulse is generated. A National Instruments cDAQ-9401 module was used to count pulses and calculations were made dependent upon each individual flowmeter’s pulse/liter rating.

Pressure was measured by DTX Plus pressure transducers. A National Instruments cDAQ-9237 module was used to measure and condition the incoming signals. Transducers were located at the level of the valve or the baseline adjusted appropriately. Each transducer was zeroed to atmospheric pressure in the software before initial use.

### 6.3 Results and Validation of Function

Overall, the bioreactor system has come through four iterations of design. Alternative components (compliance chambers, reservoirs, restrictor valves, etc) were developed or acquired to allow for physiological levels of flow at pulmonic and aortic pressure conditions while maintaining sterility. The controlling hardware and software
was upgraded to allow simultaneous and independent operation of multiple bioreactors and accurate monitoring of the flow and pressure conditions.

Shear stresses were increased by adding dextran to the culture media. Other researchers had determined that a concentration of 2.5% Dextran (2x10^6 MW) in DMEM with 10% FBS would result in a fluid viscosity to match that of blood. Their previous experimentation to measure fluid viscosity was deemed unnecessary to repeat.

### 6.3.1 Pulmonary and Aortic Conditions

Although stroke rate and duty cycle can be adjusted to all reasonable levels, testing was performed at 75 bpm with a systole time of 0.3 seconds and a diastole time of 0.5 seconds. Dextran can be easily added to thicken the media to the appropriate viscosity. Full physiological conditions were met for pulmonic position testing. These included flow rates exceeding 75 mL per stroke, and pressures up to 35/20 including the normotensive pressure of 25/10 in that position. Figure 6-7 demonstrates a valve in the open and closed position being tested in the bioreactor at those pulmonic conditions. Full physiological conditions have been met for aortic position testing using non-sterile conditions only. The newest updates will allow those conditions to be met while maintaining sterility, but the final details to accommodate are not yet in place. However, using the aortic module on the benchtop, flow rates can reach nearly 100 mL per

![Figure 6-7: Valve in the functioning bioreactor at pulmonic conditions](image-url) Valves open and close well at both pulmonic and aortic conditions of pressure and flow.
stroke and pressures can rise well beyond 300 mmHg. Normotensive pressures of 120/80 mmHg and average flow rates of 70 mL per stroke are also reachable in this configuration. However, until final device details are in place, increased amounts of media are necessary to reach those conditions.

6.4 Discussion of Further Potential Uses

The most recent platform bioreactor system developed has numerous applications for tissue engineering, mechanical testing, and pharmacological development. The resulting small footprint, ease of assembly, modularity, and means of sterile media exchange have led to a truly platform system that can be utilized in many fields. While many systems described in the literature or that are commercially available can perform a single function well, this system performs multiple functions well with a simple change of module when necessary. For instance, it can:

1) Provide a variety of physiological pulmonary and aortic conditions.
2) Provide a flow loop condition for the mitral valve.
3) Maintain a sterile environment or be used on the benchtop.
4) Precondition a tissue engineered valve.
5) Mechanically test the opening of a valve.
6) Allow visualization and capture of cusp movement.
7) Test the placement and perivalvular leakage of transcatheter-deployment.
8) Assist in the training of transcather deployment of replacement valves.
9) Create normal or pathological conditions for use in disease modeling.
10) Independently control up to four systems inside one cell culture incubator.
In all, nearly 100 experiments have been performed with these systems and improvements have been made with each through the knowledge learned about the system. With the knowledge gained from these experiments, we see an expansive field of use for this and future editions of the Clemson heart valve bioreactor.
PART 3: RESEARCH AND CLINICAL APPLICATIONS
CHAPTER 7: BIOREACTOR VALIDATION CASE STUDIES

7.1 Introduction to Multiple Uses of the Heart Valve Bioreactor

Bioreactors have many applications from the bioreactors used for microbial expansion to those intended to prepare a tissue for implantation. In the realm of tissue engineered heart valves, they have two main purposes: 1) to elucidate the mechanisms that influence interactions between cell types or cells and scaffolds and 2) to prepare a tissue engineered construct for implantation into a living animal.\[118\] However, even within the application of preparing or testing valve constructs, many various functions are performed by bioreactors. From seeding cells, to testing cell retention, to developing new extracellular matrix, bioreactors fulfill many purposes that are otherwise unavailable with conventional 2D or even some 3D methods.

Our heart valve bioreactor system’s primary function is to prepare tissue engineered heart valves for animal implantation. Thus, representing the whole spectrum of physiological conditions as well as those leading to those conditions and sometime exceeding them is necessary. However, at other times, our system has been utilized for other purposes. Projects from undergraduates, other subprojects, and even other main graduate student projects have, at various times, made use of the bioreactor systems herein described. Disclosed here are other applications of our platform bioreactor system that serve to demonstrate its versatility of uses including testing valve designs, creating a diseased condition to evaluate drug treatment regimens, and even differentiating stem cells into valvular interstitial cells\[78\]. It should be noted that with a simple switch of
modules, the aortic/pulmonic bioreactor has been adjusted to become a mitral valve bioreactor. As this is the focus of another student’s ongoing project, details are withheld.

7.2 Gelatin-Coated Resorbable Polymer Mesh as a Novel Scaffold for Heart Valve Tissue Engineering

7.2.1 Abstract

A gelatin coated resorbable polymer scaffold was used to create tissue engineered heart valves (TEHV) in order to address the issues associated with the longevity of current artificial heart valves. It was hypothesized that the combination of specific elements is essential to development of a functional TEHV: biomimetic geometry, flexible and durable scaffolds, proper cell seeding, and mechanical stimulation. Testing of this hypothesis included iterations of aortic valve scaffold designs to mimic native heart valve architecture using stainless steel and bioabsorbable polymer meshes. The functionality of the valve was tested with a pulsatile flow bioreactor. Preliminary scaffolds were too rigid and displayed only a minimal effective orifice area (EOA) for blood flow through the valve. The bioabsorbable polymer mesh provided adequate structural support and greater flexibility for the gelatin heart valve when compared to the stainless steel mesh. A new suturing technique was then incorporated to increase the EOA. Initial studies demonstrated that the new suturing technique allowed for natural movement of the root and closure of the cusps. However, improvements are still needed to further increase the EOA during valve opening. Future research plans include modification of suturing techniques as well as the acquisition of more compliant polymer meshes. The gelatin-coated scaffolds were also tested for cytotoxicity by seeding with cells. Preliminary
results displayed cell attachment, but ingrowth was limited. Long-term goals include endothelial cell seeding on the most effective polymer scaffold, which will then be cultured in a bioreactor where it will be subjected to mechanostimulation.

**7.2.2 Introduction**

The human heart performs approximately forty million cycles a year – an estimated three billion cycles for the lifespan of the average human.\textsuperscript{[11]} However, increased applied working loads as a result of patient-dependent factors such as stress, high blood pressure, and stenosis can lead to degeneration and premature failure of the native valve.\textsuperscript{[153]} Malfunction of heart valves can also be caused by non-patient-dependent factors such as congenital deformation, inadequate leaflet strength, calcification of leaflets causing stenosis, or inflammation of the valve.\textsuperscript{[11]} The aforementioned pathologies can cause inadequate blood flow through the valve when the leaflets do not fully open, as well as regurgitation when the leaflets do not properly seal the pathway upon closure\textsuperscript{[154,155]} as shown in Figure 7-1. In modern times, an increasing lifespan has proportionally increased the occurrence of degradation in heart

![Figure 7-1: Normal vs stenotic valves](image)

Comparison in orifice area of aortic valve with normal function and stenotic aortic valve.
valves. This failure has led to an increased demand for heart valve replacement surgeries. The aortic and mitral valves are more commonly replaced than the tricuspid and pulmonary, with more than 100,000 patients requiring replacement of a diseased or dysfunctional valve every year in the United States.

There are currently two categories of replacement heart valves: mechanical heart valves constructed from pyrolitic carbon and bioprosthetic heart valves composed of biological tissue. These heart valves ensure an improved quality of life, but are hindered by a decrease in their viability over time. Mechanical heart valves are more durable, but patients who receive them are administered anticoagulants as long as they have the implant. Bioprosthetic valves are made from animal tissues and materials that are compatible with the body and therefore do not require the administration of anticoagulants post implantation. However, the valves are more likely to degenerate leading to patients requiring revision surgeries. To overcome these issues, researchers have sought to create an entirely tissue-engineered valve capable of integration into the body and remodeling to become living tissue. As a deviation from the ideology of mere disease mitigation, tissue-engineered heart valves promise to be the ultimate fix, serving as a living replacement for a diseased component of the human anatomy. Although several groups have demonstrated the feasibility of this new technology using animal studies, there are a number of unmet design problems. Therefore, it is pertinent for a new aortic valve design to be fabricated.

The focus of the present study was to create living, tissue engineered heart valves which can surpass the longevity of current artificial heart valves and optimize structural integrity. It was hypothesized that the combination of specific elements are essential in
development of a functional tissue engineered heart valve: biomimetic geometry, flexible and durable scaffolds, proper cell seeding, and mechanical stimulation. In this study, gelatin-coated polymer mesh sutured to mimic porcine valve geometry was used as a flexible scaffold and mechanically stimulated in a pulsatile flow bioreactor. Effective orifice area and movement of the root and cusps without cells seeded were assessed. Fibroblasts were seeded onto the valve materials and were assessed for reaction and ingrowth into the materials. This paper describes the design process in an effort to report both successful and unsuccessful methodologies to assist future tissue engineering heart valve researchers. The final version of the valve, presented herein, is a result of an iterative design process, whereby many fabrication techniques were tested to develop the best method for mimicking the functionality of a native heart valve while also providing optimal durability and biocompatibility.

7.2.3 Methods and Materials

Valve Design Process

The valve was designed by selecting materials, patterns, and techniques based on effectiveness and ease of assembly. In general, the desired cusp and wall shapes were cut from a mesh, assembled in the form of a tri-leaflet valve, coated with gelatin, and tested to assess valve functionality compared to native valves. The first generation design began with the use of a stainless steel mesh (Small Parts, Inc., Miramar, Florida, USA) that was cut using the contiguous scalloped leaflet template (Figure 7-2a) and sutured (Ethicon, Inc., Somerville, New Jersey, USA) to the valve wall (Figure 7-2b). The sutured mesh was then fit onto the silicon mold and coated using a gelatin injection technique (described
below). Initial testing in a heart valve bioreactor showed very little cusp movement and an opening in the center of the valve where the cusp edges were not large enough to touch each other upon closing.

The second generation valve was constructed using a high-density biodegradable polymer mesh sutured together to create the valve scaffold. We used the contiguous peaked leaflet template (Figure 7-2c) to allow the cusps to touch in the center, and finalized it with the gelatin injection technique. This valve was tested in the heart valve bioreactor (see description below) and showed adequate closure. However, cusp movement was not ideal due to an accumulation of gelatin at the base of each leaflet. For this reason, a gelatin submersion technique (see description below) was developed and replaced the injection technique to provide a thinner layer of gelatin that would better facilitate cusp movement.

This new gelatin submersion technique was used in combination with a more pliable mesh of the same material that had a lower density (AM6-A) and a new design: the individual peaked leaflet template (Figure 7-2d). This new leaflet template was designed to create more freely flowing movement of the valve with the use of individual leaflets. After testing, these selections were seen to provide optimal valve function and served as the third generation valve.

To further improve function and ease of assembly, a number of different meshes and attachment methods were tested. These included a 19 courses per inch (cpi) low tension reverse locknit, a 28 cpi low tension reverse locknit, and a 40 cpi low tension tricot (generously donated by Poly-Med, Inc., Anderson, SC). Mesh materials and architecture were analyzed for valve functionality as well as cellular compatibility. In
addition, the suturing method of assembly was compared with a cauterizing method and a combined suturing/cauterizing method.

**Gelation Injection Technique**

For non-sterile studies, Knox gelatin (Kraft Foods, Tarrytown, New York, USA) was dissolved in boiling water on a hot plate at a concentration of 0.1 g/mL and 2\% antibiotic-antimycotic solution (Mediatech, Inc., Manassas, Virginia, USA) was added after cooling. Once the mixture thickened, it was injected into a silicone mold containing the mesh framework and wrapped in plastic piping to prevent gelatin from seeping out. The mold was then stored at 4°C in a refrigerator for 24 hours to allow thermal gelation to occur.

**Gelation Submersion Technique**

Gelatin was prepared in the same method as the gelation injection technique. Using tweezers to hold the feet of the valve wall, the valve was submerged in the

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**Figure 7-2: Leaflet templates**

(2a, b) Contiguous scalloped leaflet design. (2c) Valve root design as used with original continuous scalloped leaflet design. (2d) Individual peaked leaflet design with valve root and valve wall.
gelatin (Figure 7-3). After each submersion, a paintbrush was used to evenly distribute gelatin on the valve and avoid undesirable gelatin accumulation at the base of the leaflets. Submersion and painting was repeated four times and the valve was allowed to set for three minutes. After setting, the valve was submerged and painted three more times to allow for sufficient coverage. The valve was stored in a humidifier box at 4°C for 24 hours in an inverted position to encourage the leaflets to remain in the closed position post thermal gelation.

**Bioreactor Studies**

To effectively reproduce and measure the effects of the human body on the proposed tissue-engineered heart valve, a pulsatile flow bioreactor designed for heart valves[117] was used. The entire assembly has been tested thoroughly and has performed consistently in more than 30 experiments with various valve designs.[78] It allowed for testing the opening and closing of leaflets in order to qualitatively assess similarities to native leaflet movement.

The bioreactor was designed to allow for mounting valves of various sizes and shapes, as well as allow proper exposure to mechanical stimuli for preconditioning of valves. To mount a valve in this bioreactor, the valve base must be clamped between two
o-rings. For these experiments, the valve design consists of a valve wall with attachment feet (Figure 7-4) that allow for easy installation into this bioreactor.

**Manual Heart Valve Tester**

This novel tester is intended for quick assembly and testing of valves to assess integrity and function. By requiring fewer steps during its assembly than the bioreactor (described above), it provides a faster alternative when evaluating new valve designs. As shown in Figure 7-5, the primary components in the design are: a ventricular chamber, bracing rings, an aortic chamber, and a cap. The aortic chamber is attached to the ventricular chamber via three adjustable draw latches that can quickly secure the two chambers while allowing for adjustments in height for different valve designs. For convenience, the tester was designed to utilize the same set of bracing rings as used in the bioreactor described by Sierad, et al., which are placed between the two chambers. Three screws are used to ensure the cap stays in the correct position and a seal is maintained. To manipulate the valve, the user operates two bellows so that when the
ventricular bellow is squeezed (collapsed), fluid moves through the valve into the aortic chamber and the valve opens. The user then collapses the aortic bellow to shut the valve and the fluid travels through the external tubing to return to the ventricular chamber. The components of the tester in contact with fluid are made entirely from clear acrylic and are easy to sterilize. This allows the valve being tested to be clearly viewed from all angles.

**Analysis of Effective Orifice Area**

One important measure of valve functionality is the effective orifice area (EOA). EOA is the area between the leaflets when a valve is in the open position. EOA relates to
the amount of work it takes for the heart to push blood through the valve. The larger the orifice, the more blood can go through with one pump of the heart and the less work required when compared to a smaller opening.

Effective orifice area was calculated using ImageJ software (National Institutes of Health) for valves consisting of high-density bioabsorbable polymer mesh sutured using the contiguous scalloped leaflet template (second generation valves) and the thin mesh sutured using the individual peaked leaflet template (third generation valves). The EOA for an aortic porcine valve was calculated as a control comparison. Images were taken of the valves during testing in the pulsatile flow bioreactor. From a series of images taken, images of the valve when open were collected and analyzed using ImageJ. The images were made binary and inverted so that the valve opening was black. A freehand selection drawing was made around the opening and the ‘analyze particles’ command was used to calculate the amount of black space in the freehand selection. The particle size was set to 50-infinity pixels² and the circularity was 0-1.00. The area reading was recorded. The oval selection command was used to evaluate the maximum effective orifice area possible if the leaflets were fully open (the inner diameter of the valve). This evaluation was performed using the ‘measure’ command. The effective orifice area was divided by the total area possible and multiplied by 100. In this study, EOA is represented as a percentage of the total possible opening of the valve.

Figure 7-6: Mesh valve study design
12- well plate used for cell study showing rows for Live/Dead Assay, H&E staining, and the control.
Cell Study

To test cytotoxicity and cell integration of the low-density bioabsorbable polymer mesh coated with gelatin (used in the third generation valves), a 3-week cell study was performed. 12 samples of the mesh were cut into 1 cm² pieces, sterilized with ethylene oxide, and coated with gelatin using the gelatin submersion technique. Once coated, the samples were placed in a 12-well plate, one sample per well (Figure 7-6). After thermal gelation, the gelatin coated mesh samples were crosslinked using 0.075% glutaraldehyde in 0.1 M Hepes buffered saline at pH 7.4 for 24 hours. The glutaraldehyde was then removed and replaced with a 1:1 ratio of Fetal Bovine Serum (FBS) to Dulbecco’s Modified Eagle’s Medium (DMEM) for 24 hours for neutralization of glutaraldehyde residues. After the allotted time, the FBS/DMEM was removed and the mesh was coated in glycine for 2 hours. 3T3 fibroblasts were seeded at a seeding density of 100,000 cells/well in DMEM/10%FBS media. After 3, 7, 14, and 21 days, samples were examined using the Live/Dead assay (Invitrogen) and Hematoxylin and Eosin staining. Samples without cells served as a control.

7.2.4 Results

Effective Orifice Area

Figure 7-7 shows one of the initial valves mounted in the bioreactor. Looking closely, you can see spaces between the cusps, showing the inability of the initial valves to completely coapt. For the low-density bioabsorbable polymer mesh valve made using the individual peaked leaflet template (third generation valve), the average percent opening was 16.6% for the images from the first day and 15.7% for the second day. A
student’s T test determined that there was no significant difference in EOA between the first and second day. This shows consistency in the valve over the course of the time spent in the bioreactor. The average percent opening for the high-density bioabsorbable polymer mesh valve made using the contiguous scalloped leaflet template (second generation valve) was 6.6%. The average percent opening for the decellularized porcine valve was 15.4%. There was no significant difference in average percent opening between the low-density bioabsorbable polymer mesh valve (third generation valve) and the porcine valve. These results reveal that the EOA of the low-density bioabsorbable polymer mesh valve (third generation valve) is comparable to that of the porcine valve and there is a significant difference between the high-density bioabsorbable polymer mesh valve (second generation valve) and the porcine valve showing a large increase in valve functionality in the transition to a thinner mesh and a different scaffold shape.

Figure 7-7: Bioreactor testing of mesh valves
Apical view of the second and third generation valves in the bioreactor, showing the inability of the initial valves to fully close and open.
Cell Study

The results of the LIVE/DEAD Viability study are shown in Figure 7-8. The images are overlays of the green and red fluorescent images created using ImageJ software. The results of Day 3 and Day 7 showed only a few dead cells and a large amount of live cells surrounding the low-density bioabsorbable polymer mesh. Day 14 and Day 21 had an increased amount of dead cells, but still showed an abundance of live cells surrounding and incorporating within the mesh fibers.

The results of the hematoxylin and eosin stains are shown in Figure 7-9. The controls and samples were stained and observed under bright field microscopy. The results of cellular integration with the mesh were inconclusive, but the images depict the gelatin in purple integrating throughout the circular polymer mesh fibers.

7.2.5 Discussion

Analysis of Valve Efficacy

The first iteration of the valve, constructed with a stainless steel scaffold, was tested in a pulsatile flow bioreactor for 3 days. This valve exhibited a small effective orifice
area. Bioreactor testing showed that motion was hindered by an accumulation of gelatin at the base of each leaflet. Further, complete coaptation was not evident and the leaflets did not display biomimetic motion. These problems can be attributed to the choice of material and the template used to make the valve. Understanding that this valve exhibited poor compliance to flow lead to the development of a new valve design that more accurately mimics the native valve.

In the second iteration, the high-density bioabsorbable polymer scaffold used a contiguous peaked architecture that attempted to better represent native leaflets. This valve, however, showed poor movement due to an accumulation of gelatin at the base of each leaflet, preventing proper motion in vitro. It was determined that the gelatin injection technique applied more gelatin than desirable for effective leaflet motion.

In comparison with the stainless steel scaffold, the second iteration using the high-density bioabsorbable polymer scaffold appeared to allow sufficient motion, but valve architecture was still a hindrance to valve movement and coaptation. It was determined that a contiguous design was preventing valve responsiveness to flow and leading to fabrication of a stenotic valve. With these results in mind, alternatives for gelatin coating, as well as polymer mesh density and leaflet architecture were considered. A gelatin submersion technique, as well as a single peaked leaflet design and use of more porous polymers were developed for the third iteration of the valve.

Figure 7-9: Gelatin integration around scaffold
Hematoxylin & Eosin sample showing gelatin integration at 100x (left) and 200x (right).
In the third iteration valve using low-density bioabsorbable polymer scaffold, each leaflet was constructed with a mesh of different courses per inch (cpi), allowing for monitoring of individual leaflet motion. Bioreactor analysis suggested that improvements were made by switching from the stainless steel to a polymer mesh, but more improvements are necessary to reach a fully functional tissue-engineered heart valve.

The third valve, a low-density bioabsorbable polymer scaffold, addressed the issues discussed above and the tests were more successful. The results of testing in the bioreactor showed an increase in the EOA of the valve during opening. The low-density bioabsorbable polymer scaffold leaflets presented mechanical properties similar to native heart valve leaflets, as a result of their compliance to flow. Tight closure of the leaflets was observed with little or no backflow.

7.2.6 Conclusions

Our data suggests that by using the individual peaked leaflet design, the geometry and movement of our valve more closely mimicked that of a native human aortic valve. Combining a low-density, bioabsorbable mesh with a gelatin submersion technique allowed for an increased EOA as well as more natural and flexible physiological motion of the scaffold. Further advancements should include modification of suturing techniques and improved gelation techniques to produce a more compliant scaffold while maintaining structure integrity, thus increasing EOA.

Growth of fibroblast cells on the gelatin and mesh scaffold demonstrated that the chosen materials could foster the growth of tissue. The growth of new tissue in parallel with scaffold degradation will allow for preservation of the original valve architecture.
and ensure normal blood flow. Future work includes the growth of endothelial cells, the native blood contacting surface, on the low-density bioabsorbable polymer scaffold and exposing this construct to mechanical stimulation in the bioreactor. Mechanostimulation is known to promote the growth of cells, and would lead to an improved design.

### 7.2.7 Acknowledgements

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7.3 Toward an Endothelial-Cell Covered Mechanical Valve; Surface Re-Engineering and Bioreactor Testing of Mechanical Heart Valves

7.3.1 Abstract

Mechanical valve replacements for diseased heart valves have excellent long-term durability, but necessitate a lifetime anticoagulation regimen. We aimed to create a more hemocompatible device by modifying the leaflet surfaces to promote adherence and retention of endothelial cells under physiological shear forces.

St. Jude Medical mechanical valves were autoclaved, plasma treated, coated with a collagen/fibronectin solution, and seeded with porcine aortic endothelial cells. The next day, the valves were placed in a custom-made bioreactor where pressures were gradually increased until pulmonary or aortic pressures were reached. Conditioning continued for 7 days. Cellular retention, viability, and morphology were investigated using Live/DEAD® staining, immunofluorescence for CD-31, and scanning electron microscopy.

Results demonstrated successful adhesion of the collagen/fibronectin substrate to the pyrolytic carbon surface. Complete endothelial coverage of the leaflet surface in the static control group indicates that our surface modification approach created a suitable environment for the cells to attach, proliferate, and remain viable. Moreover, after 7 days of dynamic conditioning at pulmonary pressures, a significant portion of the endothelial cells remained adherent to valve surfaces, improved cell coverage over static controls, remained viable, increased cell-cell interactions, and maintained expression of CD31. Similar results were seen at aortic pressures but with increased cell removal due to higher
shear stresses. Further work is needed to improve cell retention in areas of high shear stress, but surface modification and endothelial cell coating may ultimately aid in limiting coagulation and reduce the need for anticoagulation medication in patients receiving mechanical heart valve implants.

7.3.2 Introduction

The overall prevalence of heart valve disease, adjusted to the US 2000 population, was 2.5\%\cite{160} with about 99,000 heart valve operations yearly.\cite{157} Most of these procedures were directed at repairing or replacing the mitral or aortic valve with either a bioprosthetic or mechanical valve. Whereas the bioprosthetic valves function well with usually no medication needed, many fail within 15 years of implantation.\cite{56} The mechanical valves have several positive attributes to their structure including strength, durability, and ease of operation. However, they initiate thrombosis within the blood stream because of the foreign surface as well as high shear forces.\cite{161–163} To prevent this process, the patient must be medicated with anticoagulants and blood-thinning medication for their remaining lifespan. Even with the prescription of anticoagulants, the patient is still at high risk for blood clots, which can cause stroke, myocardial infarction, or pulmonary embolism.\cite{164,165} Thrombosis can also cause the heart valve to malfunction, requiring the patient to reenter surgery for repairs. Even if the anticoagulant regimen successfully prevents clotting, there is still the increased risk of hemorrhages associated with blood thinning medication.\cite{166}

Endothelialization of blood-contacting surfaces with autologous cells to reduce the implants thrombogenicity is a common procedure in the literature. For example,
vascular grafts have been seeded with endothelial cells to improve hemocompatibility, showing successful attachment of cells to vascular prostheses where the surface had been modified using ePTFE and a collagen matrix.\textsuperscript{[167–169]} Studies have also been conducted to analyze the morphology of endothelial cells attached to vascular prosthesis\textsuperscript{[114,170]} as well as heart valves.\textsuperscript{[15,93,171]}

Previous work has been performed with the idea of modifying a mechanical valve surface to reduce or eliminate the amount of anticoagulants patients would need to take.\textsuperscript{[169]} In that experiment, researchers modified mechanical valves by seeding a confluent layer of cells on the surfaces. The valves were then implanted into the mitral position in pigs for one hour. Although cells initially attached to these leaflets, no cells remained attached after explantation, possibly due to the shear forces of the blood on the cells and their lack of initial attachment to the surfaces. A drastic improvement in cellular retention under physiological shear forces is needed for this method of valve improvements to be successful.

The long-term goal of the present study is to prevent thrombosis in patients with mechanical heart valves by improving previous methods\textsuperscript{[169]} of reducing the amount of coagulation-causing factors and more specifically by generating endothelial cell-covered mechanical valves. For this study, we hypothesized that surface modification will enhance initial endothelial cell coverage and further retention under dynamic flow conditions. To test this hypothesis, mechanical heart valves were treated with plasma for surface activation, coated with a type I collagen/fibronectin matrix, seeded with endothelial cells and maintained in static cell culture. Endothelial cell-seeded mechanical valves were then subjected to flow and simulated pulmonary and aortic pressure conditions using our
custom-made heart valve bioreactor. Viability tests, scanning electron microscopy and immunofluorescence of dynamically challenged cell seeded valves showed that surface-bound endothelial cells remained attached to the modified valves, proliferated and maintained their endothelial phenotype.

### 7.3.3 Materials and Methods

Overall, we first modified the surfaces of isolated leaflets from St. Jude Masters Series mechanical heart valve via plasma treatment and matrix attachment. The modified surfaces were analyzed with Coomassie Blue staining and scanning electron microscopy to confirm matrix attachment and uniformity. Initial cell viability studies were performed with fibroblasts. Endothelial cells were then used to further investigate the most effective surface modification combination for this application. Cellular analyses were performed by Live/DEAD® staining.

After obtaining the desired surface modification method and confirming its cellular compatibility, we cultured and attached porcine aortic endothelial cells on the outflow surface of intact St. Jude Master Series mechanical heart valves. These cell-seeded valves were then dynamically tested under pulmonic and aortic pressures for seven days using a previously described heart valve bioreactor. Valves kept under static conditions overnight and for 7 days were used as controls. We analyzed the cells via Live/DEAD® imaging, scanning electron microscopy, and immunofluorescence for morphology, coverage, and expression of the endothelial cell marker, CD-31 (PECAM), respectively.

**Surface Modification**
**Sterilization, Surface Modification, & Matrix Attachment**

Whole valves or individual leaflets removed from St. Jude Medical (St. Paul, MN) Masters Series (AJ25) mechanical heart valves (1A) were sterilized via steam autoclaving. After allowing the valves/leaflets to completely cool and dry, they were plasma treated for 5 minutes using an Expanded Plasma Cleaner (PDC-001) from Harrick Plasma (Ithaca, NY) at 50 mTorr to modify the valve surfaces and promote protein attachment. Immediately after plasma treatment, the valves were placed in a sterile 6-well plate and immersed in a collagen solution. The collagen solution was prepared by mixing (on ice) 4 parts 3.1 mg/mL PurCol type I collagen solution (Advanced Biomatrix, San Diego, CA), 4 parts ddH$_2$O, 1 part 10X phosphate buffered saline (PBS) (Mediatech, Manassas, VA) and 1 part 0.1M NaOH (Fisher Scientific). After matrix deposition was confirmed, 100 µL of 1 mg/mL fibronectin (Sigma-Aldrich) were added to 7.5 mL of each collagen batch to complete the collagen/fibronectin solution. The whole valves or individual leaflets were then placed in a cell culture incubator (37°C, 5% CO$_2$) overnight.

**Quantification of Protein Adhesion**

Individual leaflets (n=6) were autoclaved and distributed evenly into the following treatment groups: Group 1 – with plasma, with collagen; Group 2 – no plasma, with collagen; Group 3 – with plasma, no collagen. Leaflets were modified as above and rinsed three times with 1X PBS. Following rinsing, 200 µL of Coomassie Blue stain solution (1% Coomassie Blue R-250 in 10% methanol, 80% ddH$_2$O, 10% acetic acid) was placed on the leaflets for five minutes at room temperature. Leaflets were then rinsed with ddH$_2$O and three times with Coomassie Blue wash solution (38% methanol, 60% ddH$_2$O, 2% acetic acid) before adding 250 µL eluent solution (50% ethanol, 50% 50mM NaOH). After 30
minutes, 200 µL of the eluent solution was transferred to a 96 well plate and absorbance was measured in a Biotek plate reader at 620 nm with a reference of 450 nm. Eluent solution was used as a blank.

**SEM Analysis of Modified Surface**

Scanning electron microscopy (SEM) was used to visualize the level of homogeneity of the collagen solution on the leaflet surface. After sterilization, two leaflets were plasma treated, coated with collagen solution as described above, and allowed to dry. One additional leaflet was used as a control with no further modification after sterilization. For SEM analysis, the leaflets were fixed in Karnovsky’s fixative (2.5% glutaraldehyde, 2% formaldehyde in 0.1 M cacodylate buffer, pH 7.4) for at least 24 h. Samples were dehydrated in increasing ethanol concentrations until absolute alcohol was reached, then stored in 100% ethanol for up to 12 hours. Following immersion in hexamethyldisilazane (Polysciences, Inc., Warrington, PA) for 15 min, samples were left to air dry. Samples were then coated with platinum for 2 min (Hummer 6.2, Anatech LTD, Union City, CA) before imaging with a Hitachi S4800 or TM3000 Tabletop scanning electron microscope (Clemson University Electron Microscope Facility, Anderson, SC).

**Cellular Attachment**

*Initial Cell Viability Static Test using 3T3 Fibroblasts*

Three leaflets were sterilized and plasma treated before applying the collagen solution as described above. 3T3 fibroblasts (Cell Applications Inc, San Diego, CA) were expanded in DMEM (Mediatech, Manassas, VA) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and 1% Antibiotic-Antimycotic (Ab/Am) Solution (Mediatech,
Manassas, VA) and 80,000 cells were seeded onto each leaflet for 20 minutes before adding additional media to each well to minimally cover the leaflet. Media was changed every 3–4 days for 21 days before analyzing with Live/DEAD® stain for viability as described below.

**Endothelial Cell Culturing**

Porcine aortic endothelial cells (pAEC; Cell Applications Inc, San Diego, CA) were cultured on cell-culture flasks coated with 50 µL/cm² fibronectin solution (1 mg/mL; Sigma-Aldrich) in a 20 µg FN/mL dilution. Cell culturing media consisted of MCDB-131 (Mediatech, Manassas, VA) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 1% Antibiotic/Antimycotic (Mediatech, Manassas, VA), and 0.36 g/L of L-Glutamine (Fisher Scientific) and passaging occurred at 80-90% confluence.

**Live/DEAD® Imaging**

Presence of live cells on valves and leaflets was analyzed using Live/DEAD® stain (Invitrogen, Eugene, Oregon) according to manufacturers’ directions, using 20 µL of EthD-1 and 5 µL of Calcein-AM in 10 mL of 1X PBS. The Live/DEAD® solution was added to cover each sample and covered with foil at room temperature for 30 minutes before fluorescent imaging with an inverted microscope.

**Dynamic Bioreactor Conditioning**

**Mechanical Valve Seeding**

The aortic surfaces of sterilized, plasma-treated, and matrix-coated valves were aseptically seeded with porcine aortic endothelial cells (1,250,000 per leaflet) in 500 µL
media in a 6-well plate. If any suspension flowed through, it was re-pipetted over the valve. This process was repeated for 20 minutes. Valves and leaflets were submerged in culture media (about 10 mL for valves and about 3 mL for individual leaflets) before placing them in an incubator for overnight attachment. The next day, the valves were processed according to dynamic and static conditions below. Media was prepared using MCDB-131 with 10% FBS, 2% Ab/Am, 0.36 g/L L-Glutamine, and 0.2% Gentamycin (50mg/mL, Sigma-Aldrich).

**Dynamic Conditioning**

The previously described Clemson Heart Valve Bioreactor\[117] was used to simulate the dynamic conditions placed on the valve. The entire assembly has been tested thoroughly and has performed consistently in more than 35 experiments with various reported\[78\] and unreported valve designs. The bioreactor was designed to allow for mounting valves of various sizes and shapes, as well as allow proper exposure to mechanical stimuli for preconditioning of valves.

Three valves tested under dynamic conditions were split between high (aortic pressures of 120/80 mmHg, one valve) and low (pulmonary pressures of 40/25 mmHg, two valves) pressure conditions. The valves were inserted into the bioreactor where 1.2 L of media was circulated through the system at 60 beats per minute, and roughly 20 mL (1200 mL/minute) or roughly 10 mL (600 mL/minute) stroke volume for high and low pressures, respectively. Both low and high pressure valves began at a pressure of 7 mmHg. For progressive conditioning, systolic pressure was increased to 20 mmHg after 3.5 hours, to 30 mmHg after 6.5 hours, and to 40/25 mmHg after 15.75 hours. The valve at low pressure condition was left at this pressure until 7 days passed. The valve at high pressure...
continued to undergo pressure increases to 64/43 mmHg after 19.83 hours, 85/60 mmHg after 24.72 hours, 100/75 mmHg after 27.12 hours, and 120/80 mmHg after 47.17 hours, where it remained until seven days had passed. Media was changed after three days in all bioreactor studies. On the seventh day, all the valves were removed and prepped for Live/DEAD® imaging (one low pressure valve) or immunofluorescence (one low pressure valve and the high pressure valve). After Live/DEAD® imaging, valves were fixed for SEM as indicated below.

**Static Controls**

One valve and leaflet were processed after an overnight incubation under static conditions. After overnight cell attachment, the valve was prepped for Live/DEAD® imaging and SEM and the leaflet was prepped for immunofluorescence imaging. Another valve and leaflet were placed in a 6-well plate with 10 mL and 3 mL of media, respectively. The media was changed every 24 hours for seven days. The valve was then prepped for Live/DEAD® imaging and SEM and the leaflet was prepped for immunofluorescence imaging.

**Immunofluorescence**

Valves or leaflets for immunofluorescence imaging were rinsed with 10 mL of warm 1X PBS 2 to 3 times and fixed for 30 minutes at room temperature in 4% formaldehyde (BDH Chemicals). Following rinsing with 1X PBS, the bottom of the valves was sealed with parafilm and placed into a custom made, pre-formed, silicone mold (CopyFlex Liquid Silicone, Culinart, Inc.) to minimize volume needed for staining (Figure 7-10B). Samples were then blocked using 5% Bovine Serum Albumin (Rockland...
Immunochemicals, Gilbertsville, PA) with 0.05% Triton (BDH Chemicals) in 1X PBS for 2 hours at room temperature. The blocking solution was removed and 1000 µL (250 µL for leaflets) primary CD-31/PECAM1 Antibody (VM64) (NBP1-42152; Novus Biologicals, Littleton, CO) in blocking solution (1:2 dilution) was added for 1.5 hours at room temperature. The primary antibody was removed before rinsing 4 times with 1X PBS. The secondary antibody, Alexa Fluor® 594 Donkey Anti-Mouse IgG (Invitrogen, Grand Island, NY) diluted in blocking solution (1:2 dilution) was added for 1 hour at room temperature in the dark followed by 4 rinses with 1X PBS. Finally, 500 µL DAPI stain (Sigma-Aldrich) was added to each leaflet for 5 minutes at room temperature in the dark before fluorescent imaging. Primary antibody was omitted for staining negative controls.

7.3.4 Results

Surface Modification

![Image](image-url)
The combination of autoclave sterilization, plasma treatment, and submersion in the collagen/fibronectin solution proved to be an effective method to attach a cell-friendly matrix to the inert surface of a mechanical heart valve. Coomassie Blue elution analysis showed that collagen indeed attached to the surface while confirming the necessity to use the combination of both plasma treatment and matrix submersion rather than either one alone (Figure 7-10C). Plasma alone and ECM coating alone resulted in negligible readings, whereas the combination of the two gave an expected reading for the presence of proteins, demonstrating that significant levels of collagen had strongly bonded to the pyrolytic carbon surface and were not rinsed away during the coomassie blue procedure. SEM analysis (Figure 7-11) provided further visual evidence that a collagen-based matrix formed on the leaflet surfaces in a fairly homogeneous distribution after plasma treatment and submersion in the collagen solution whereas unmodified leaflets were still smooth.

**Cellular Attachment**

Initial cell attachment and viability tests using fibroblasts cultured on the leaflets for three weeks demonstrated that the matrix-coated leaflet was well accepted by the cells and did not appear to detach from the leaflet surface. Cells had proliferated to become nearly 100% confluent in most areas (Figure 7-12). Under these static culture conditions,
over 99% of the cells remained viable, demonstrating excellent cellular compatibility of the re-engineered mechanical valve surfaces.

**Dynamic Bioreactor Conditioning**

In order to evaluate the ability of the re-engineered, endothelial cell seeded mechanical valve surfaces to withstand dynamic conditions, we subjected valves to conditioning in a valve bioreactor, with valves and leaflets incubated in static conditions serving as controls. The bioreactor setup seen in Figure 7-13 served to supply and simulate physiologic pressures and shear stresses on the mechanical heart valves, causing them to fully open and close (Figure 7-13).

**Static Controls**

The valve processed after overnight attachment provides a control from which to gauge cellular changes after seven days on the surface of the valve in the flow and non-flow conditions. Live/DEAD® (Figure 7-14A-C), SEM (Figure 7-15A-C), and immunofluorescence (Figure 7-16A, B) imaging of the valve under static conditions revealed the successful attachment of endothelial cells onto the re-engineered surfaces. The Live/DEAD® stain showed the majority of the cells fluorescing green rather than red, showing excellent initial cell viability. Endothelial cell attachment was successful on all areas.

**Figure 7-12: Fibroblast coverage of leaflets**

Live/DEAD® staining (live=green, dead=red) of fibroblasts seeded on the surface of a mechanical heart valve leaflet after 21 days in static culture conditions. A) shows cells on the rounded edge of the leaflet, B) in a central region and C) on the straight edge.
of the valves, with cells appearing as spindle to triangular shaped and exhibiting no uniformity in cellular alignment. A nearly confluent layer of cells was present on the static valves after overnight incubation. However, small void spots were still present, which apparently left room for cell proliferation to provide almost complete coverage of the valve, which was seen after seven days (Figure 7-14D-F, Figure 7-15D-F, and Figure 7-16B). Most of the cells retained their spindle shape and exhibited tendency to align in swirl patterns, but noticeable distinctions are seen between cellular alignment and morphology between time points, especially in the higher magnifications. These results indicate that the extracellular matrix created through our resurfacing protocol is a suitable environment for the cells to proliferate and survive. The positive CD-31 staining (Figure 7-16A, B) demonstrates the ability of our matrix to facilitate a monolayer coverage of anti-thrombotic endothelial cells, which is a key component to our objective of reducing thrombogenicity of mechanical heart valves.
**Dynamic Testing**

The imaging of the dynamic seven day valves under pulmonic pressures revealed three distinct zones of cell coverage (Figure 7-14G-I). The first region is the edge of the valves, where most cells were no longer present after seven days of dynamic conditioning. This region composed roughly only 10% of the total leaflet area. Moving inward from the edges, region two is described as the area of slightly less dense cell coverage than the central regions (region three). In these slightly less dense regions, the cells apparently

![Figure 7-14: Cell coverage of leaflets after testing by Live/Dead](image)

Live/DEAD® staining (live=green, dead=red) of aortic endothelial cells on the surface of modified mechanical heart valves in static overnight group (A, B, C), static seven day group (D, E, F) and dynamic pulmonary seven day group (G, H, I). The upper row (A, D, G) shows the middle of the valve where the two leaflets join together. The middle row (B, E, H) shows the hinge or edge of the valve where the leaflet inserts into the outer ring. White lines in G and H show the edges of the leaflet. H shows the central region (right) as well as the edge region of the leaflet (left).
exhibit a larger surface area with a more rectangular morphology than the spindle shape seen in the central regions. In this third region (about 85% of the leaflet area), the spindle shaped cells do not exhibit a complete uniformity of alignment, but long bands of 10-30 cells in width are conforming to a single alignment in swirling patterns across the leaflet.

Compared to the static valves, the pulmonic pressure valves had more complete cell coverage of the pyrolytic carbon. The small gaps that existed after static conditions were not present in the dynamic valves. This could be seen in the Live/DEAD® images in Figure 7-14F and Figure 7-15I, but was more clearly noticed in the SEM images in Figure 7-15:

**Figure 7-15: Cell coverage of leaflets after testing by SEM**

Scanning electron microscopy images of aortic endothelial cells on the surface of modified mechanical heart valves in static overnight group (A, B, C), static seven day group (D, E, F), and dynamic pulmonary seven day group (G, H, I). Note that under dynamic pulmonary conditions endothelial cells are forming a flat sheet with numerous cell-cell junctions that makes it difficult to distinguish the individual cellular boundaries (I).
Figure 7-15F and Figure 7-16I where the cells formed more complete junctions almost appearing as a cell “sheet” with fewer spaces in the dynamic conditions than in the static conditions. The SEM images in Figure 7-15 also revealed that in both static and dynamic conditions, the cells appear to be quite flattened on the pyrolytic carbon surface.

Bioreactor conditioning under pulmonic pressures apparently resulted in stronger, more consistent CD-31 expression than under static conditions (Figure 7-16B, D). Under aortic pressures, cells remained attached to the valve and continued to express CD-31 (Figure 7-16, F).

Conditioning under aortic pressure conditions had similar results to that of pulmonary conditions, but fewer cells apparently remained attached to the mechanical valve surfaces. Lower cell retention was expected for the dynamic valve under high

Figure 7-16: Cell coverage of leaflets after testing by immunofluorescence
Immunofluorescence images for CD-31 marker (red) and dapi stain for nuclei (blue) of aortic endothelial cells on the surface of modified mechanical heart valves in static overnight group (A), static seven day group (B), dynamic pulmonary pressures seven day group (C, D), and dynamic aortic pressures seven day group (E, F). Inserts in A, and B are immunofluorescence negative controls.
pressure because of the greater shear force and stress. Overall, we noted good cell viability, retention, and CD-31 expression (Figure 7-16E, F) for the cells under aortic conditions.

7.3.5 Discussion

Surface Modification & Cellular Attachment

The objective of the present study is to modify the surface to allow for complete endothelial cell coverage of the pyrolytic carbon mechanical heart valve leaflets and enhanced retention under shear forces. A collagen/fibronectin matrix was used for leaflet re-engineering because of their biocompatibility and documented facilitation of cell attachment[167] as well as cellular integrin’s ability to strongly bind to the RGD sequences found on the fibronectin strand.[173,174] These features allow strong initial attachment of cells and subsequent extracellular matrix formation.

We successfully attached our collagen/fibronectin matrix to the autoclaved leaflet surfaces after modifying the pyrolytic carbon surface through plasma treatment, which proved to be necessary for the matrix to attach. Plasma treatment uses free radical chemistry to clean, micro-etch, crosslink, and surface activate the material. This treatment can result in a 2 to 10 fold increase in protein adhesion.[172] From our initial data, it is apparent that the collagen matrix completely and evenly bonded to the surface of the pyrolytic carbon through functional groups. Whereas Bengtsson and associates have previously demonstrated complete endothelial cell coverage on pyrolytic carbon through manual seeding with no surface modification,[169] our studies show that not only do both fibroblasts and endothelial cells attach to the collagen/fibronectin coated valves, but that the matrix actually promotes a more complete cell coverage of the valve
over non-altered valves at these seeding densities (data not shown). Although not the aim of this particular study, we speculate that fibroblast pre-seeding, as a preamble to endothelial cell seeding, might further improve the stability of the ECM bound to the pyrolytic carbon surface.

Successful seeding of endothelial cells on to the modified mechanical valve was demonstrated by the results of immunofluorescent and SEM imaging. Images of the overnight time point under static conditions suggest that the seeding method used was sufficient in creating an even layer of cells on the entire leaflet. After seven days of growth in static conditions, a nearly confluent endothelial monolayer was achieved on the mechanical valve, but small gaps still appeared between cells, possibly signifying a lack of cell-cell interactions that was present in the dynamic conditions. A more spindle shaped than triangular shape was also seen after seven days in static conditions. This shape reduces cell area, and since cell coverage appeared to be similar or greater after seven days, suggesting that cell spreading, rearrangement, and ultimately proliferation is taking place. Thus, imaging of the static valves supports the hypothesis that the collagen/fibronectin matrix bound to plasma-activated pyrolytic carbon surfaces is not cytotoxic.

**Bioreactor Conditioning**

Subjecting the cells to mechanical conditions in the heart valve bioreactor was necessary to demonstrate the ability of the matrix modified leaflet to retain cells under native conditions before further animal testing. It was also important to test their ability to maintain their phenotype in dynamic conditions to ensure a non-thrombogenic surface. The heart valve bioreactor previously developed by our lab,[@117] which creates nutrient...
transfer and pressures similar to that of the heart, was utilized to simulate dynamic conditions. Valves conditioned using the bioreactor showed a difference in cell coverage, cell morphology and alignment, cell-cell interactions, and CD-31 expression compared to valves in static conditioning.

The valve under pulmonic pressures of 40/25 mmHg exhibited better cell coverage in the central 90% of the leaflet compared to valves under static conditions overnight, as the cells closed the gaps between cells that were present in the static valves. The outer 10% of leaflet area, especially around the hinges, contained very few cells possibly due to the shear forces on the cells.[175–177] A further increase of shear forces on the cells under aortic pressures of 120/80 was correlated to a decrease in final cell coverage. The large numbers of cells present on the surfaces after 7 days of exposure to aortic pressures indicate that our approach could yield non-thrombogenic mechanical valves. However, since mechanical heart valves are already known to cause hemolysis due to high shear forces around the leaflet edges and hinges, these areas vacant of cell coverage pose an obstacle to reducing hemolysis. Ideally, full coverage of the leaflets is desired, especially around the areas with higher shear stress to mitigate the risk of hemolysis and the resulting cascade of health issues.

Cell morphology, orientation, and alignment under dynamic conditions was similar to that in static conditions after seven days, but the attached cells appeared longer, more flattened, and had a larger surface area under the dynamic conditions. The complete coverage in dynamic conditions also leads us to believe that the cells are proliferating, as in the static conditions.
SEM imaging suggested that the cells are not only flattening out, but also interacting with one another more under dynamic conditions than static conditions. This might be promising for the creation of a continuous monolayer of cells to reduce thrombogenic effects of the foreign surface. The expression of the endothelial cell marker, CD-31, under both static and dynamic conditions demonstrates that the cells appear to retain their endothelial phenotype and therefore their anti-thrombotic function while attached to the surface of the mechanical heart valve. Clinically, this means that an autologous endothelial-cell coated mechanical heart valve would create a non-thrombogenic surface, thus possibly limiting coagulation occurring in patients receiving mechanical heart valve implants.

**Challenges in Heart Valve Recellularization**

Providing a confluent endothelial cell surface on any blood contact surface is a common objective among regenerative medicine cardiovascular implants. Achieving such is not simple. We have faced many obstacles and questions in the search for a method to do this with a mechanical heart valve. Bonding a matrix to the pyrolytic carbon for improved cell attachment may be one breakthrough in this challenging task. However, this study has several limitations and opens avenues for further studies with every question we face.

First, what treatment to the valve surface would facilitate a more complete cell coverage and stronger bond for the cells upon subjection to shear stresses, especially on the inflow side where larger forces are seen than on the outflow side of the valve? Alternative matrix components or concentrations could yield results that are more favorable.
Second, what progressive mechanical conditioning regime would allow more cells to remain attached? Further studies should examine incubating the cell-seeded valves under static conditions for several days before subjecting the valves to dynamic conditions and/or slowing the rate of increase in progressive adaptation to reach physiologic pressures. Our studies progressed to aortic conditions over two days whereas recent experiments indicate that progression lasting up to three weeks may be necessary.

Third, if cells can be retained to reach physiological conditions, how would they phenotypically and morphologically respond to appropriate conditions of shear and valve opening/closing speeds? Studying cellular response with appropriate flow rates and after adding a thickening agent such as dextran to the media may help answer these questions.

Finally, will the layer of endothelial cells on a mechanical valve leaflets functionally reduce thrombogenicity of mechanical heart valves? It is well documented that high shear forces around the valve – not contact with the pyrolytic carbon surface – is responsible for hemolysis leading to clotting. Functional thrombogenicity in *in vitro* testing with whole blood or purified platelets, as well as *in vivo* large animal implantation as valve substitutes are required for validation of this mechanical heart valve re-surfacing and re-engineering approach.

### 7.3.6 Conclusions

Surface modification of mechanical heart valves via plasma treatment facilitates attachment of a cell-friendly surface layer of matrix proteins, which supports endothelial cell attachment, spreading, and proliferation in static conditions. In short-term dynamic conditioning under physiologic pressures, the new endothelial monolayer maintains
viability, cell-cell interactions, and endothelial phenotype. Although the cell retention in these studies is promising, improvements are greatly needed when subjecting the cells to physiologic shear stress, especially in areas around the edges and hinges of the leaflets. This is intensified when considering that the preservation of the endothelial coating on the leaflet in areas subjected to high shear forces will determine the success of reducing thrombogenic effects caused by mechanical heart valves. Work is needed to develop a progressive pre-conditioning regime that maintains cell attachment before steady subjection to pulmonic or aortic pressures and flow. Additionally, further *in vitro* and *in vivo* testing is necessary to demonstrate the decreased thrombogenicity of endothelium-covered mechanical heart valves. Success of such advances could ultimately aid in reducing the need for anticoagulation medication in patients receiving mechanical heart valve implants.

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A portion of this work was performed as part of the “Tissue Engineering Heart Valves” Creative Inquiry Class at Clemson University. Undergraduate, Eliza Laine Shaw contributed equally to this work. Additional students and instructors involved in this class include Ryan Launius, Shannon McBride, Cassie Storholt, Ryan Poole, Daniel Spence, Katie Miller, Lauren Sosdian, Kaity Allen, Lauren Burton, Anita Iari, Dr. Jiro Nagatomi, and Dr. Dan T. Simionescu. Gratitude is extended to St. Jude Medical for generously providing the mechanical heart valves, Clemson University Machining & Technical Services for bioreactor Manufacturing, Bob Teague for LabView programming,
7.4 Discussion of Bioreactor Applications

The bioreactor systems developed have numerous applications for tissue engineering, mechanical testing, and pharmacological development. In all, dozens of experiments have been performed with these systems and improvements have been made with each through the knowledge learned about the system. The resulting small footprint, ease of assembly, modularity, and means of sterile media exchange have led to a truly platform system that can be utilized in many fields.

Beyond tissue engineering and testing heart valves, the platform bioreactor system has great potential for developing in vitro models for comparing drugs and screening potential treatments for diseases. Animal models do not fully reproduce human pathology and are difficult to combine multiple risk factors seen in dialysis-sustained ESRD, besides their restrictive cost considerations. A testing platform to would assist in the development of such a pharmacological treatment for aortic valve disease.

While many systems described in the literature or that are commercially available can perform a single function well, this system has been shown to perform multiple functions well with a simple change of module when necessary. It can provide a variety of normal and pathologic physiological conditions including those for aortic and mitral valves while maintaining a sterile environment to precondition a tissue-engineered valve. These conditions can also be made to mimic diseased states for evaluating drug treatment regimens. It can be used on the benchtop to test the opening of a valve, TAVR valve
placement, and perivalvular leakage. It can assist in the training of transcatheter deployment of replacement valves and allows visualization and video capture of cusp movement regardless of location. Furthermore, it is compact enough to fit four independently controlled systems inside a standard cell culture incubator. The described experiments have demonstrated the utility of this system and are but a sampling of its capabilities.
CHAPTER 8: TISSUE ENGINEERED HEART VALVE CONDITIONING

8.1 Introduction

The key to achieving an implant capable of repairing itself in response to micro tears is the presence of cells to remodel the matrix as necessary.\cite{35,37} In cases where the valve implant does not have the adequate mechanical properties to function properly upon implantation, \textit{in vitro} cellular remodeling must prepare the valve scaffold prior to implanting.\cite{89–91} Multiple groups have demonstrated cell seeding on the exterior surface of valve cusps and some have even achieved some interstitial seeding, though full revitalization has not been realized.\cite{92–94} The surfaces of some valves have even been repopulated after implantation in animal models,\cite{95,96} but the same is not guaranteed in the human system. Even if the valve can be initially covered with cells,\cite{73,97,98} it is likely that these cells will need some sort of progressive conditioning to remain attached after implantation since application of sudden shear forces can detach cells. Overall, more progress is needed to achieve full and consistent external and internal recellularization of the cusps and to determine what methods are needed to allow the cells to remain, but autologous adult stem cells provide a promising source for this application.\cite{99,100}

8.2 Materials and Methods

8.2.1 Study Overview

Fresh porcine aortic valve roots were collected, cleaned, and prepared as described in section 3.3.1. Aortic roots were decellularized by 16-day perfusion as in section 4.2.3
or in the case of one valve, by 8-day immersion as in section 4.2.2. All valves were then crosslinked with PGG as in section 4.2.4. Prior to seeding, valves were neutralized for 18-24 hours in DMEM with 50% FBS and 1% Antibiotics/Antimycotics at 37°C. Stem cells were cultured as in 5.2.2. Many cell-seeded valves were tested for this compilation study, but due to device failures, data for only 13 valves will be reported. Additionally, valves evaluated for initial cell placement (internal and external) have been reported in Chapter 5: and details will not be repeated here.

One valve was externally seeded with 16 million cells by method 1 of section 5.2.6 before being placed in the bioreactor for progressive conditioning and testing. Results from this trial indicated that additional preconditioning was necessary. Following the first experiment, 10 more valves were internally seeded with 4 million cells at passage 6 per cusp as in section 5.2.5. Six of those valves were also externally seeded with 30 million cells at passage 6 by method 2 of section 5.2.6. Furthermore, three of the latter group were seeded as above and successively seeded with an additional 30 million cells at passage 7 by method 3 of section 5.2.6. A summary of the cell-seeded valves can be seen in Table 8-1.

Initially seeded valves were described in Chapter 5: . Internally and externally seeded valves were investigated for each group, but are often contained in the same valve, leading to a seemingly high total sample size. Final test groups can be seen in Table 8-2 and include:

1) **Static controls** (ST): Valves kept in the incubator with media changes every two days, daily, or 2 hours as necessary.
2) **Rotationally conditioned** (RC): Valves conditioned by the seeding chamber frame described in section 5.2.3.

3) **Rotationally and bioreactor conditioned** (RCBR): Valves rotationally conditioned then bioreactor conditioned with the third (section 6.2.2) or fourth (section 6.2.3) edition bioreactor.

4) **Bioreactor conditioned** (BR): Valves conditioned in the third (section 6.2.2) edition bioreactor immediately after cell seeding.

After appropriate conditioning, valves were analyzed for cellular attachment, spreading, retention, alignment, and viability using the Live/DEAD® assay, scanning electron microscopy (SEM), histology, or immunohistochemistry as appropriate for each group.

### 8.2.2 Static Controls

For the static valves, sterile filters were attached to the needleless ports in the lid of the chambers and the seeding chambers remained in the cell culture incubator for 13-15 days. Media was changed every two days, daily, or 12 hours as necessary based on pH colorimetric indicators in the media.

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**Table 8-1: Summary of cell-seeded valves**

<table>
<thead>
<tr>
<th>Number of Valves</th>
<th>Internal Seeding</th>
<th>Primary External Seeding</th>
<th>Secondary External Seeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>Method 1: 16e⁶ cells, p5</td>
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</tr>
<tr>
<td>6</td>
<td>4e⁶ cells/cusp, p6</td>
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<td>0</td>
</tr>
<tr>
<td>3</td>
<td>4e⁶ cells/cusp, p5/p6</td>
<td>Method 2: 30e⁶ cells, p5/p6</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>4e⁶ cells/cusp, p5/p6</td>
<td>Method 2: 30e⁶ cells, p6</td>
<td>Method 3: 26e⁶ cells, p7</td>
</tr>
</tbody>
</table>
8.2.3 **Pulmonic Bioreactor Conditioning and Testing without Rotational Conditioning**

After external seeding, distal aortic root stabilizers (section 3.2.5) were added to each valve root for support during bioreactor conditioning and testing. The valves were transferred to the pre-assembled third edition bioreactor, where pressure was increased every 12 hours until final pulmonic parameters were reached, as seen in Figure 8-1.

Upon reaching final pressures of 40/25 mmHg and flow of roughly 30 mL per stroke at 75 beats per minute (bpm), valves were tested for an additional 13 days before analysis for a total of 17 days in the bioreactor. Media was changed every 3.5 days (twice per week) throughout conditioning and testing.

8.2.4 **Rotational Conditioning**

After the desired internal and external seeding, valves were conditioned further in the rotating chambers. The end-over-end rotating chamber holding plate was attached to the frame and needleless ports were attached to each seeding chamber. The chambers were placed into the chamber holder plate. Rotating and orbital conditions progressively increased every 12 hours until a shaker speed of 40% and rotational speed of 4 rpms were achieved for short-term conditioning. Long-term conditioning followed the schedule seen in Table 8-3 with media changes occurring during every adjustment (roughly every 12

<table>
<thead>
<tr>
<th>Group</th>
<th>Number Internal Seeded Valves</th>
<th>Days</th>
<th>Number External Seeded Valves</th>
<th>Days</th>
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</thead>
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<td>0</td>
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<td>13</td>
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<tr>
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<td>1</td>
<td>13</td>
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<tr>
<td>Rotationally and Bioreactor Conditioned (RCBR)</td>
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<td>23-25</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>Bioreactor Conditioned (BR)</td>
<td>2</td>
<td>23</td>
<td>1</td>
<td>17</td>
</tr>
</tbody>
</table>
hours) after day 3. Position A (holder orientation) from previous rotation angles was not monitored from this point on. After 13-15 (short term) or 23-25 (long term) days of rotational conditioning, valves were analyzed or transferred to the bioreactors for adaptive conditioning and testing.

Media was changed every two days, daily, or every 12 hours as necessary based on pH colorimetric indicators in the media. For these changes, the chambers were removed from the frame and drained by vacuum through a needleless port with an empty 50 mL syringe attached to the other port to allow sterile air into the system. Following draining, about 130 mL of cell culture media was added to each chamber (until the media just covered the stainless steel mounting rings). The syringes were removed and the chambers placed back into the chamber holders with the 0° mark aligning with the set mark on the holding plate and rotated.

Figure 8-1: Bioreactor preconditioning without rotation conditioning
This trial reached pressures of 40/25 within 4 days.
Table 8-3: Conditioning regimen

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<th>Position B</th>
<th>Time Set</th>
<th>Day #</th>
<th>Shaker Speed</th>
<th>Rotator Speed</th>
<th>Rotating Time (seconds)</th>
<th>Pause Time (seconds)</th>
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</table>

8.2.5 Pulmonic Bioreactor Conditioning and Testing with Rotational Conditioning
After 5 or 25 days of the above rotational conditioning, distal aortic root stabilizers (section 3.2.5) were added to each valve root for support during bioreactor conditioning and testing. The valves were transferred to pre-assembled third (for 5 day conditioned valves: “short pre-conditioned”) or fourth (for 24 day conditioned valves: “long pre-conditioned”) edition bioreactors (see section 6.2.3 for details of this system).

The short pre-conditioned valve was increased to 25/15 mmHg at 75 bpm and a stroke volume of 35 mL over the course of eight days (Figure 8-2). Increases in pressure occurred every 12 hours. At each increase, systolic pressure was increased by 0.5 mmHg each time for the first five days and 4 mmHg each time for the final 3 days. Media was changed every 3.5 days (twice per week) throughout conditioning. Upon reaching final pressures of 25/15 mmHg and flow of 35 mL per stroke, valves were analyzed by Live/DEAD, histology, and immunohistochemistry.

8.2.6 Live/DEAD® Imaging

Presence of live cells on tissue surfaces was analyzed using Live/DEAD® stain (Invitrogen, Eugene, Oregon) according to manufacturers’ directions, using 20 µL of EthD-1 and 5 µL of Calcein-AM in 10 mL of 1X PBS. The Live/DEAD® solution was added to cover each sample.

Figure 8-2: Bioreactor preconditioning without rotation conditioning
This trial reached pressures of 25/15 within 7 days after rotation conditioning for 5 days.
and covered with foil at 37°C for 20 minutes before fluorescent imaging with an inverted microscope.

### 8.2.7 Histology and Immunohistochemistry

For histology studies, samples collected from the aortic wall, sinus, cusp and muscle were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm (3 µm for the aorta), and stained with DAPI for nuclei, Hematoxylin & Eosin (H&E), and Movat’s Pentachrome.

Immunohistochemistry (IHC) was performed to detect remaining components after decellularization. Biotinylated Griffonia simplicifolia (GS) lectin was used to detect Gala1–3Gal (α-Gal), the main porcine antigen responsible for acute rejection of xenotransplants. IHC for laminin, and type IV collagen was also performed. Tissue samples were rinsed with 1X PBS fixed at room temperature in 4% formaldehyde (BDH Chemicals). Following rinsing samples were blocked using 5% Bovine Serum Albumin (Rockland Immunochemicals, Gilbertsville, PA) with 0.05% Triton (BDH Chemicals) in 1X PBS for 2 hours at room temperature. The blocking solution was removed and 250 µL primary CD-31/PECAM1 Antibody (VM64) (NBP1-42152; Novus Biologicals, Littleton, CO) in blocking solution (1:2 dilution) was added for 1.5 hours at room temperature. The primary antibody was removed before rinsing 4 times with 1X PBS. The secondary antibody, Alexa Fluor® 594 Donkey Anti-Mouse IgG (Invitrogen, Grand Island, NY) diluted in blocking solution (1:2 dilution) was added for 1 hour at room temperature in the dark followed by 4 rinses with 1X PBS. Finally, 500 µL DAPI stain
(Sigma-Aldrich) was added to each slide for 5 minutes at room temperature in the dark before fluorescent imaging. Primary antibody was omitted for staining negative controls.

8.3 Results and Discussion

8.3.1 External Cell Response

As shown previously and repeated in Figure 8-3, initial cellular attachment to the cusp surfaces was very successful with method 1. However, the surfaces of the sinus and aortic wall required additional seeding. Utilizing methods 2 and 3 have resulted in excellent initial cell coverage of those areas as well.

Cell-seeded valves were placed directly into the bioreactor for 17 days. As a result some cells remained attached to the valve and were further spread and aligned than static controls and initial time points. However, many of the cells had been removed from the scaffolds, presumably because of the applied shear stresses. We believed that a gentler pre-conditioning regimen, including more time in the rotating seeding chambers, would increase cellular retention. Studies were performed to evaluate the effect of increasing the cell quantity, rotational conditioning time, and the time it took for the bioreactor to reach full pulmonic conditions. A summary of this data can be seen in Figure 8-3.

Valves conditioned in the rotating conditioner for 13 days are subjected to lower shear stresses than those in the bioreactor and thus, they retained full cell coverage on both sides of the cusp with similar, but less noted spread and alignment compared to bioreactor valves. This indicates that a substantial layer of cells was growing on the surface of the cusps prior to bioreactor conditioning.
Figure 8-3: External and Internal cell seeding and retention

Live/DEAD® and H&E imaging of fresh valve cusps (A-C) in comparison to valves initially seeded (D-E), static for 13 days (G-I), bioreactor conditioned for 13 days (J-L), rotating conditioned for 13 (M-N, R), 15 (O), and 24 (S) days, and rotating conditioned for 5 days then bioreactor conditioned for 8 days (P-Q). (R) is internally and externally seeded.
Valves were placed in rotational conditioning regimens for 5 and 25 days prior to being placed into the bioreactor. Once in the bioreactor conditions were slowly increased until 25/15 mmHg at 75 bpm was reached. The first set of valves achieved 35 mL per stroke and was cultured in the bioreactor for a total of 8 days. The second set of valves achieved 70 mL per stroke and 2.5% dextran and was cultured in the bioreactor for a total of 29 days. Surface imaging indicated that the 5-day rotational pre-conditioning was inadequate. Similar or fewer cells remained on the valve surfaces for this experiment than the previous bioreactor experiment having no rotational conditioning. The second set of valves took that into consideration and had an extensive rotational conditioning step before being placed into the bioreactor.

Unfortunately, during the rotational conditioning, it is likely that many of the cells died due to a pH imbalance. As the conditioning progressed, the pH in the media inside the seeding chambers was dropping, as indicated by the phenol red indicator turning orange faster and faster). At one point, an extended period elapsed between media changes (indicated by ** in Table 8-3). After this time point, the media did not change to orange nearly as fast. Initial evaluations of the valves indicated that most of the cells had been removed from the scaffold surfaces. Despite this, one valve was placed in the bioreactor in case external cells remained on that valve or the internally seeded cells were still viable.

Overall, additional rotational conditioning is necessary for cell retention once the valves are placed in the bioreactors. However, improved devices, methods of gas exchange, and protocols must be investigated to reduce the chances of the seeded cells dying during this phase.
8.3.2 Internal Cell Response

Internal seeding of hADSCs into porcine aortic valve cusps yielded promising results. Initial cell placement was concentrated in boluses at the injection sites with improved initial distribution near the base of the cusps. Static and short-term rotational conditioning yielded little to no cellular migration. However, after 24 days in rotational conditioning and bioreactor conditioning, cells were beginning to migrate into the surrounding tissue layers (see especially Figure 8-3O). Although slow and not ideal, this method of repopulating the internal areas of the aortic cusps has yielded promising results.

Immunohistochemistry (Figure 8-4) of the injected cells immediately after injection and after 24 days in the rotating conditioner or bioreactor conditioner.

Figure 8-4: Immunohistochemistry of conditioned internal cells
Slides stained for Vimentin, alphs-SMA, and P4HA3 immediately after injection and after 24 days in the bioreactor or rotating conditioner.[140]
demonstrated some changes in marker expression. Notably, alpha-smooth muscle actin had a higher expression after bioreactor conditioning than rotating conditioning. The expression of this marker indicates a myofibroblast-like phenotype typical of cells that are remodeling their matrix.

Further IHC analysis suggests that the cells undergoing rotational conditioning resemble q-VICs. There is strong expression of vimentin and little to no true staining for α-SMA and prolyl-4-hydroxylase. These reports are promising, as the goal of achieving a quiescent-VIC phenotype is ideal prior to further mechanical conditioning.
CHAPTER 9: ANIMAL IMPLANTS OF TISSUE ENGINEERED HEART VALVES

9.1 Introduction

Laboratory research is often disconnected from clinical practice. One day, we may be able to develop a functional tissue engineered valve, but if the technology cannot be utilized in a clinical setting, it may be necessary to revise the technology from a fundamental level. If we can adapt our laboratory practices to consider clinical requirements during the research and development stage, translation into clinical practice will be more easily realized. Initial animal trials must be performed to reveal hurdles we will need to overcome in our process of translating tissue engineered heart valves into clinically relevant products. We will then be able to adapt our laboratory practices to consider clinical requirements during the research and development stage.

Our initial investigations into animal implantation revealed a necessity for many of the specific features of the devices described in previous chapters. For example, it was vital to have a user-friendly valve mounting system that will be used to house the valve from initial cleaning to just before implantation with minimal to no handling of the valve itself. This chapter will describe the experiments made possible by the technologies and research previously described. Herein, we aim to generate implantable tissue engineered heart valves by optimal integration of three main factors: acellular heart valve root scaffolds, autologous stem cells, and construct preconditioning in a bioreactor. As the study continues, additional translational challenges will inevitably necessitate further
alterations to our laboratory protocols for valve preparation as we translate the laboratory research into a clinical setting

By implanting these tissue engineered heart valves, we will be implanting the first stem cell seeded heart valve in an animal model to eliminate the risk of immune response due to remaining cells. These studies will also help to demonstrate that translational regenerative medicine is feasible with autologous stem cells for tissue engineering replacement therapy – a vital stage in the ability to remove barriers in commercializing tissue engineered products.[68,123–125]

9.2 Materials and Methods

The research team in Targu Mures, Romania has prepared acellular porcine valve scaffolds using the devices and methods developed in chapters 3 and 4. They will isolate adipose derived stem cells from sheep, multiply them in culture, and use them to repopulate the acellular scaffolds as in chapter 5. They will then precondition the cell-seeded scaffolds using rotational conditioning methods developed in chapter 8. After preconditioning, the surgical team will implant the autologous cell-seeded tissue-engineered valves in a right ventricle to pulmonary artery shunt in the young adult sheep from which the cells were originally taken. Future studies will analyze these valves at 6- and 12-month time points at the macroscopic and microscopic level for calcification, necrosis, structural damage, cell infiltration, and cell retention.

9.2.1 Primary Stem Cell Isolation and Culture

Sheep adipose-derived stem cells (sADSCs) were isolated (Figure 9-1) from well-vascularized, soft subcutaneous fat samples in the rear subscapular region under sterile
conditions and anesthesia from individual animals. Established collagenase digestion protocols\[178\] were used to isolate the stem cells, which involved cutting the tissue fragments into small pieces and digesting with 20 mL/50 mL tube collagenase type I (Sigma) 1mg/mL 1% BSA, PBS at 37°C, 1 hour with agitation in the water bath. Tissue digest was filtered through 100 µm sieves into new 50 mL tubes and centrifuged at 1000 rpm for 5 minutes before aspirating the supernatant. The cell pellet was resuspended in 155 mM NH₄Cl with 0.1 mM EDTA in PBS and incubated 5-10 minutes at room temperature to lyse red blood cells. After another centrifugation at 1000 rpm for 10 minutes, the supernatant was aspirated and the pellet was resuspended in DMEM with 10% FBS and 2% Antibiotics/Antimycotics and plated. Media was changed every 3-4 days and passaged with trypsin at 70% confluence

9.2.2 Scaffold Preparation

Fresh porcine aortic valve roots were collected, cleaned, and prepared as described in section 3.3.1 with the restriction that the donor pigs weigh between 45-50 kg. Aortic roots were decellularized by 16-day perfusion (section 4.2.3) techniques, crosslinked with
PGG as in section 4.2.4 (later trials also utilized RapidGlut as in section 4.2.5), and neutralized for 18-24 hours in DMEM with 50% FBS and 1% Antibiotics/Antimycotics at 37°C.

9.2.3 Stem Cell Seeding

Stem cells were isolated from sheep and cultured as described above. Each primary source of cells was tracked and a single source of cells was used to seed valve scaffolds internally with 4–6 million cells per cusp as in section 5.2.5. The remaining cells from the culturing were in suspension, but the valve position was not controlled during rotation and pausing.

9.2.4 Valve Conditioning and Preparation for Implantation

After seeding, the valves were conditioned further in the rotating chambers by simple rotation in the incubator. Media was changed every two days until implantation. Each valve was tracked for final implantation into the same sheep from which the cells came for an autologous tissue engineered implant.

Before implantation, conduits of decellularized bovine pericardium were attached to the proximal and distal segments of the valve root by a continuous suture (Figure 9-2A & B). This created a pipeline for the valve that will be intercalated between implant locations for routing of the host circulation.

9.2.5 Surgical Procedure: Right Ventricle to Pulmonary Artery Conduit Implant

Juvenile male sheep (5–6 months, 35–40 kg) are chosen for these studies because of several benefits including growing in size, similar anatomy and hemodynamic...
parameters to young adults, and prior testing of heart valve bioprostheses degeneration and calcification. Animals were obtained from the local Animal Research Station and the sheep were housed under optimal conditions and monitored pre and post-operatory for general health, food intake, behavior, and blood tests.

Animal implantation protocols were approved by the Institutional Review Board of the University of Medicine and Pharmacy of Târgu Mureș. The shunting procedure (Figure 9-2C&D) involves attaching pericardial tubes to each side of the valve (Figure 9-2A&B) then attaching those tubes to their respective portions of the right ventricle or pulmonary artery. Animals were set under general anesthesia with controlled ventilation and continuous monitoring of ECG, arterial pressure, pulse-ox, and temperature. Surgical procedure generally followed the steps here:

1. Access via left thoracotomy at the level of the 4th intercostal space.
2. Incision of the parietal pleura and pericardium.

Figure 9-2: Tissue engineered valve preparation, implantation, and follow up
Pericardium tubes are attached to the valve (A&B) before implanting the conduit between the right ventricle and pulmonary artery (C-D). Postoperative housing is indoors (F) before longer term outdoor housing. (F) Echocardiogram and other methods are used for follow up.
3. Lateral clamping of the right ventricle followed by a 3 cm long
ventriculotomy.

4. The proximal end of the valved conduit was anastomosed to the ventricle
at this level using a continuous suture (Surjet with Prolene 5.0),
strengthened by Teflon pledgets where needed.

5. The pulmonary trunk was then clamped and the distal end of the valved
conduit was sutured to the pulmonary trunk with same suturing method.

6. After declamping, the pulmonary trunk was ligated shut at 1-2 cm above
the level of the pulmonary valve.

7. After hemostasis and checking sutures and conduit for leaks, the
pericardium and pleura were left open to prevent tamponade.

8. Finally, cavities were drained and the thoracic cavity was closed with
Dexon 2.0. Closing was in layers and isolated sutures at the tegument.

9.2.6 Postoperative Care

Following surgery, the sheep are temporarily placed in indoor monitoring
facilities (Figure 9-2E) before being released to their outdoor living spaces until explant
and analysis. Animals are cared for by standard techniques concerning monitoring,
supervision, pain killer medication, X-ray monitoring of the chest, laboratory tests and
food, with the consultation of a veterinarian, surgeon, anesthesiologist and cardiologist.
Animals will be monitored post-op for respiration (frequency, thoracic X-ray, blood
gases), cardiac frequency, hydration status, pH equilibrium, nutrition, hematology
(hemoglobin, hematocrit and electrolytes). Sheep will be sacrificed at 6 months (n=6) and
12 months (n=6). At explanation, valves will be analyzed for morphology and function. Those future studies will analyze these valves at the macroscopic and microscopic level for calcification, necrosis, structural damage, cell infiltration, and cell retention.

9.3 Results and Discussion

9.3.1 Scaffold Preparation

Aortic valves were harvested from pigs of various ages and weights to assess the anatomical details of the aortic valve (size, diameter, and position) that best corresponds to implantation in sheep hearts in the pulmonary position. The optimum weight was identified at 45-50 kg to obtain an aortic valve corresponding to the size of the sheep pulmonary tract. Additional modifications to the research protocol were necessary to accommodate the surgical setting. One such change has been the method of valve cleaning and mounting during decellularization to allow pericardial tubes to attach to the valve. The resultant conduit is necessary for the current placement of the valves and gives relevant information about the ability to suture the resulting valve root scaffold.

9.3.2 Preliminary Sheep Results

Progress has been made toward testing a revitalized valve root in an ovine right ventricle to pulmonary artery shunt model. Three surgeries have been performed with glutaraldehyde treated valves to increase familiarity with the surgical procedure before a tissue-engineered valve is utilized. The first resulted in animal death due to anesthesia difficulties. The animals survived both the second and third procedures and operating time decreased for each case.
In all, at least 25 surgeries have been performed and they are still ongoing. More recent trials utilizing tissue-engineered valves have had varied success. Surgical procedures are still being adjusted to meet the needs of the sheep. Initial trials with valves crosslinked only with PGG indicated that the valves were deteriorating more rapidly than expected. This has also led to the modification of our research protocols as seen in the addition of RapidGlut crosslinking treatment to later valve trials.

Overall, the implantation of tissue-engineered valve implants seeded with autologous stem cells is one of the first such trials being performed in large animals. As the teams become more familiar with the procedures and modifications are made to improve outcomes, the advancement of a clinically relevant product will take great strides. The knowledge gained by these studies will contribute important information for a commercial valve replacement product.

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PART 4: CONCLUSIONS AND REFLECTIONS
CHAPTER 10: DISCUSSION, CONCLUSIONS, AND FUTURE WORK

10.1 Conclusions of Research and Recommendations

The advance of heart valve replacements is moving in the direction of tissue-engineered devices. This research has shown how core devices are a vital part to developing and commercializing valve replacements. This research has also served to progress the field of tissue-engineered heart valves forward by means of the novel devices as well as scientific findings.

10.1.1 Valve Holding Device

A tool to handle a scaffold for a tissue-engineered product is integral to the many components and processes used for developing those products. Without appropriate handling, the scaffold could be damaged or even unable to transition between the various treatments involved in developing these products. The valve holder developed through this research provides an excellent hold on the tissue during assembly and use, provides a reliable seal around the tissue in all processes, and is easy to use with many versatile applications.

Recommendations

- The distal root stabilizer currently utilizes sutures to hold the root up during bioreactor functioning. This method is very versatile, but can also be time consuming, even with the relatively few sutures needed. A means of quickly attaching the distal aortic root to the stand and stabilizer would prevent the valve and cells from drying
out during valve preparation and would also greatly decrease the time necessary to prepare a valve for bioreactor functioning. Initial designs and prototypes have utilized an internal retaining spring that can be applied with plier tools. Concave grooves in the upper ring or stand itself would help seat the retaining spring and prevent dislocation during valve functioning.

- Nonmetal materials should be sourced for the potential use of the valve in an MRI device or for disposability. However, care should be taken to ensure chemical compatibility with all decellularization, crosslinking, and culturing solutions. An inert ceramic or plastic that would provide adequate strength would serve well.

### 10.1.2 Perfusion Decellularization Method and Device

Immersion decellularization techniques are inadequate for whole-root decellularization. The thick, elastin-rich areas require a more robust method of decellularization. The perfusion system designed for this research is the first of its kind for heart valve roots. Its capabilities to treat individual components of the valve root with independent conditions allows it to be rigorous enough to decellularize the aortic root while gentle enough not to destroy the cusps. Any scaffold to be implanted must be free of all animal cells and the valve root is no exception. Each area of cusp, sinus, aorta, and muscle must be free of native cells. However, we are currently unaware of the effects of this process on the mechanical durability of the valve root components.

**Recommendations**

- The current device uses nearly 4 liters of fluid for each media change. While an adequate fluid-to-tissue ratio is necessary, this system is excessive and potentially
wasteful. A main chamber that reduces the area around the valves should be built. This chamber would essentially be contoured to the shape of the valve roots while allowing adequate circulation. Alternatively, individual chambers or a reduced number of valves could be processed with the above in mind.

- Future versions of the system should be manufactured with slight variances to the layers and materials to ease assembly and reduce components. For instance, the central holding plate (layer 3) can be combined with the main chamber portion (layer 4) and the material should be a rigid plastic such as Delrin. Additionally, by thickening the other layer that holds the valves (layer 2), a dampening section can be dug out of that layer along one side. That dampening section would be full of air while not increasing the volume of solution used to eliminate the dampening bottle.

- Individual chambers could also allow the valves to be crosslinked inside the system without the need for packing the cusps with cotton balls. By reversing the flow, a slight pressure gradient can be created to crosslink the valves in the naturally position.

- Upgrades to the software should include connecting the peristaltic pump to the computer so it can be monitored and controlled via a feedback loop by the software. The pump includes that capability and software, but LabView programming and potentially another piece of hardware will be necessary to provide the interface.

- Mechanical evaluation should be performed on the valve roots. Compliance data can be obtained through the clear wall of the system by inserting a ruler into the main chamber of the decellularization system and diameters measured at varying pressures using the existing setup. Biaxial or uniaxial testing should be performed on the aortic
root to determine if excessive damage is being done to the elastic properties and strength of the native tissue.

- The decellularization protocol may also be optimized. A shorter time for various steps of the process could reduce the overall time required to achieve full decellularization.

10.1.3 Cell Seeding by Injection and Rotational Seeding and Conditioning Device

Cell seeding by injection is much more effective than relying on cellular migration, but it is not the optimal means of revitalizing a tissue because of its damaging and inconsistent aspects. It is also technically difficult and reproducibility is user-dependent. The rotational seeding device works well, but requires routine user input and observance. The optimal methods of achieving a confluent layer of cells on all surfaces of the valve root are also deficient. Current methods achieve the goals, but may be using too many cells and supplies.

Recommendations

- A new method of recellularizing the internal portions of valve cusps is necessary. This method should provide more uniform coverage and result in less tissue damage. Other methods currently being investigated in the lab are promising. Details are purposefully omitted to maintain confidentiality.

- External seeding could be optimized by clamping the distal aorta with existing equipment and sealing the inflow with o-rings as done in other places. This would localize the cell seeding solution within the valve lumen to allow a much higher concentration of cells to attach to the valve, resulting in higher seeding densities with fewer cells used. Care should be taken to ensure that the devices fit inside the chamber.
and that the external surfaces of the root maintain moisture, although concern for the second point is minimal given the small air volume in the system.

- Seeding protocols should also be optimized. A combination of method 2 and 3 should be compared with method 3 alone to determine the most efficient means of fully recellularizing all of the aortic root surfaces. Reducing the number of cells or repetitions could also yield results that are just as effective.

- Improvements to the frame and software should allow monitoring of rotation angles to enable pausing at specified angles. Automated control of the speed of the shaker plate should also be incorporated into the controls of the system. The seeding method and regimen should be entered into LabView in “spreadsheet style” instead of “on/off” style. These upgrades will allow complete customization of rotational scenarios. Bob Teague is currently working on automated control of the shaker plate and Charlie McDonald is currently working on spreadsheet style of entering data into LabView.

- Gas exchange inside the seeding chambers needs immense improvement. Securing the lids and flowing appropriately mixed gases through the ports and sterile filters by pressure or vacuum would improve this, but must be compatible with the rotation of the system. Preliminary designs have been discussed that involve routing a pressure/vacuum line through the axle of the chamber holder and incorporating a gas line inside the holding plate that would protrude next to each chamber position. Hydrophobic sterile filters could be attached to the system to allow gas movement and timing could be aligned appropriately with the placement of the gasses near the ports if necessary.
10.1.4 Fourth Edition Heart Valve Bioreactor Device

The fourth edition of the bioreactor is a sophisticated system that provides capabilities not seen in most other systems in the research or commercially available. In addition, its small footprint, relatively simple assembly, and ability to fine-tune make it a valuable component to the laboratory’s research. The current edition is nearing completion to provide pulmonic or aortic conditions of flow, pressure, and viscosity, but additional improvements and modifications are necessary to reach those conditions or improve ease of use.

Recommendations

- The first objective should be to finalize the size and shape of the bottle used as an aortic compliance chamber. The means of providing an internal balloon membrane should also be finalized. A component of this chamber will include an input pressure regulator and filter to control the internal air pressure of the chamber without opening it to non-sterile air or using excessive amounts of fluid.

- The quick-connect latches of the quick-connect bioreactor fittings should be adjusted to function with the 3-D printed materials. Although the o-rings provide a reliable seal, there is potential risk that the fittings could slide out of the main bioreactor chamber without the latches in place. External design services of the inventors can be sourced for this task.

- Additional tools should be developed to assist in the assembly of the bioreactor. These include a membrane cutting and placement tool, a camera holder, a pressure transducer holder, and any required supports for the modules.
• As with all future devices made of a clear plastic, future versions of the chambers should be machined out of polysulfone rather than acrylic. This material is semi-transparent and can be sterilized by autoclave.

• A manifold system should be developed inside the incubator that allows the small through-wall port of the incubator to provide enough real-estate to service four or more bioreactors within the same incubator.

• A more aseptic method of adding media to the bioreactor should be developed. Current methods work well, but have risks of contamination. If media could be delivered by attaching bagged media to the bioreactor through one of the needleless ports and applying a small vacuum force to the main chambers, the media could be added very quickly without drying out the valve sample.

• Studies should be performed to evaluate the cytotoxicity of all printed or other materials on cells. While no effects have been seen, validation of the appropriate choice of material should be performed.

• Additional modules should be developed to provide capabilities to test mitral valves and transcatheter implantations. The validation an in vitro system for creating diseased states and testing drug treatments is still a possible use for the bioreactor systems that should not be abandoned.

• Living aortic valves should be collected from the slaughterhouse and mounted into the bioreactor. The bioreactor should be run for at least four weeks to demonstrate its capabilities to provide a healthy, native environment to heart valves. This scientific
evidence will be the bedrock to support claims that this bioreactor system can adequately prepare a tissue-engineered valve for implantation.

- Additional case studies should be performed to widen the applicable uses of the heart valve bioreactor. From providing physiologically relevant flow to enabling another technology, the bioreactor system is truly a platform system that can be applied in many areas.

### 10.1.5 Tissue-Engineered Heart Valve Conditioning and Animal Implants

Progressive adaptation regimens to allow seeded cells to remain attached to scaffold surfaces are vital to a valve’s self-modulation of the repair of the cusps. Included studies have shown that this progressive adaptation is necessary for cell retention and a select few trials have been performed to develop the appropriate protocol. Factors of other systems (gas exchange in the seeding chambers, etc) have prevented further study herein.

Autologous stem cell-seeded scaffold being implanted into large animals is new in this field. Many hurdles have been faced in translating this research project into clinical applications, but the animal studies have revealed very important aspects of our research that must continuously be adapted to fit the need.

**Recommendations**

- Trials should be performed to determine the necessary pre-conditioning regimens for adequate cell retention after reaching pulmonary and aortic conditions. A period of gentle conditioning followed by more rigorous conditioning in the seeding chambers/rotating frame before transferring the valves to the bioreactor has shown great promise, but needs to be optimized and validated.
• Cell staining (such as for focal adhesion kinases, FAK) to observe the cell-cell interactions as well as the cell-matrix interactions should be performed to evaluate cellular attachment to the scaffolds.

• Overall, fewer cells should be used to revitalize the scaffold. Current methods employ more cells than reasonably collected from a patient without in vitro culturing.

• One ongoing question is if externally seeded cells are necessary for these implants. After pre-conditioning regimens are developed to retain cells at physiological conditions, trials should be performed using externally seeded valves.

10.2 Final Discussion of Progress

The field of heart valve devices has come far since the advent of mechanical valves in the middle of last century. There have been improved hinge designs, alternative bioprosthetic materials, minimally invasive implantation procedures, and even animal trials with decellularized or tissue-engineered valve replacements. As our understanding of this complex and astounding structure increases, we will continue to develop better replacement products that perform closer and closer to the way the valve was designed to work in a healthy person. There is much work to be done, but we stand on a hill of giants gone already deeply entrenched and dedicated to its progress. We look forward to where our future innovations, learning, worldwide need, and even funding will lead us. There is no doubt that a robust tissue-engineering replacement heart valve will soon be available to improve lives all around the world.
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