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Fabrication and Characterization of an Extracellular Matrix Hydrogel for Aortic Valve Applications

Brady Culbreth
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FABRICATION AND CHARACTERIZATION OF AN EXTRACELLULAR MATRIX HYDROGEL FOR AORTIC VALVE APPLICATIONS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Brady Culbreth
May 2016

Accepted by:
Dr. Dan Simionescu, Committee Chair
Dr. Aggie Simionescu
Dr. Martine LaBerge
Dr. Ken Webb
ABSTRACT

With an estimated 5 million people suffering from valve disease in the United States, valve disease is currently the leading cause of cardiovascular disease. Each year, between 80,000 and 85,000 aortic valve replacements are performed in order to treat the stenotic heart valves. Despite this being a worldwide epidemic, the current valve replacement options that are on the market have distinct limitations. Furthermore, a viable alternative does not exist for the patients that are not candidates for the current treatment methods. Our proposed solution to this epidemic is to create a highly viable injectable scaffold that would allow for the minimally invasive delivery of human adipose-derived stem cells (hADSCs), as well as to provide necessary biological cues for growth and remodeling to the scaffold.

A process was created to create a hydrogel derived from decellularized porcine aortic cusp tissue. The aortic cusp was solubilized using an acid-pepsin solution, neutralized and reformed as a hydrogel structure. This processing was analyzed for effectiveness of decellularization, retention of the extracellular matrix components, scaffold architecture, and cell interaction and viability.

Histology showed proper decellularization while maintaining the components of the extracellular matrix throughout the fabrication process, collagen content analysis provided further evidence of this. Quantitative analysis of H&E sections revealed a highly porous scaffold, conducive to cell migration. Rheological
studies revealed shear thinning properties that is advantageous for the ability of the scaffold to be injected. A Live/Dead assay of the scaffold showed an extremely viable scaffold in static conditions, as well as a tendency of the cells to contract and remodel the hydrogel.

Present studies have optimized the technique for creation of the hydrogel, characterized the biological and physical properties of the scaffold, and determined the viability of the scaffold for seeding of hADSCs. These aortic cusp-derived scaffolds provide an environment that mimics the aortic cusp ECM. This research will advance cardiovascular tissue engineering and further aid in the search for the ideal tissue engineered heart valve.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Dan Simionescu, and committee members, Dr. Aggie Simionescu, Dr. Ken Webb, and Dr. Martine LaBerge. I would also like to thank the Biocompatibility and Tissue Regeneration Lab and Cardiovascular Tissue-Engineering and Regenerative Medicine Lab partners, and Dr. Lee Sierad for all their help throughout the project. I would also like to acknowledge my peers within the Clemson University Bioengineering Graduate School, the Clemson University Bioengineering Department. Lastly, I would like to thank Snow Creek Meat Processing facility for donating the porcine hearts used for the research.
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CHAPTER 1: INTRODUCTION
AND BACKGROUND
1.1 **Structure and components of the cardiovascular system**

The cardiovascular system serves as a closed system that circulates blood throughout the body in order to deliver nutrients and oxygen to the body. This system consists of two circuits, the pulmonary circuit and the systemic system. The pulmonary circuit is responsible for oxygenating the blood and eliminating the carbon dioxide from the blood. The systemic circuit is responsible for delivering the oxygenated blood and nutrients to the body, as well as to remove the waste from those cells. These circuits work together to keep the cells from becoming deprived of the necessary nutrients to survive. At the center of this system is the muscular pump in charge of pushing the blood throughout the system, known as the heart. The heart works through continuous contractions and relaxations, known as the cardiac cycle. The heart is a very hard working organ that is responsible for pumping 7,000 liters of blood through the system, every single day.2

1.1.1 **Pericardium and the tissue layers of the wall**

The protection of the heart is charged to the pericardial sac, which covers the heart with three layers of pericardium: the fibrous pericardium, parietal pericardium, and the visceral pericardium. The outermost layer of the pericardial sac, the fibrous pericardium, is responsible for providing a tough and protective layer of dense connective tissue. This layer is attached to the diaphragm, the vertebral column, and the large blood vessels that emerge from the heart2. The other two layers, the visceral and parietal pericardium, form a double layered serous membrane. Between these two layers is a space called the pericardial cavity,
containing serous fluid. This fluid is secreted by the membranes of the pericardial cavity in order to reduce the amount of friction between the pericardium and the heart. The innermost layer, the visceral pericardium, corresponds to the epicardium, which is the outer layer to the wall of the heart.³

The wall of heart is comprised of three different layers of tissue: the epicardium, myocardium, and endocardium. The epicardium is the outermost layer of the wall, which is connected to the visceral pericardium. This layer of tissue reduces the amount of friction that the heart is subjected to, which helps contribute to protecting of the heart. This layer consists of connective tissue that includes capillaries for both blood and lymph, as well as nerve fibers. The next layer of the wall is the myocardium, which is made up of cardiac muscle tissue that contracts to pump blood out of the chambers. This layer consists both of the muscle fibers used to contract the chambers and the connective tissue that allows blood and lymph capillaries to run through the muscle fibers. The innermost layer of the wall is the endocardium, which is comprised of connective tissue, epithelial tissue, blood vessels and Purkinje fibers. This layer lines all the chambers and covers the heart valves and other structures of the heart. The anatomy of the wall of the heart, as well as the pericardial sac, is pictured below in Figure 1.²
1.1.2 Valves and chambers of the heart

There are four internal cavities that divide the heart into sections, known as chambers. There are two chambers on the right and two chambers on the left, each of which lead to different parts of the cardiovascular system. The upper chambers of the heart are known as the atria, which are thin walled and accept the blood that is returning to the heart through the cardiovascular system. The lower chambers, known as the ventricles, are responsible for forcing the blood inside the chambers of the heart back out into the arteries. The right atrium is responsible for receiving blood from the superior and inferior vena cava, which returns blood from the body that is low in oxygen. The left atrium is responsible for receiving oxygenated blood from the pulmonary veins, which returns the blood from the lungs. The left ventricle is specifically responsible for receiving the blood from the left atrium and forcing the blood to all other parts of the body. The right ventricle is...
responsible for receiving the blood from the right atrium and pumping it out to the right ventricle. The walls of the left ventricle are therefore much more muscular in order to be capable of moving the blood the required distance. The layout of the chambers of the heart, as well as how they interact with the rest of the cardiovascular system, is shown in Figure 2 below.³

The chambers of the heart are divided by four valves, which function as gateways for the blood to be pumped through. These valves allow the blood to move from one side to the other without backflow occurring. Similar to there being two types of chambers, there are also two types of valves. One of the types of valve is called the atrioventricular valve and as its name suggests, these two valves separate their respective atria and ventricle. These valves open when the blood pressure is greater on the side of the atria and the valve will close when the blood pressure is greater on the side of the ventricle. The tricuspid valve covers the large
orifice between the right atria and right ventricle with its three leaflets structure and the mitral valve covers the large orifice between the left atrium and left ventricle with its bi-leaflet structure. These valves also have chordae tendineae, or fibrous tissue strings, that prevent the valves from swinging backwards into the atrium. The second type of valves are the semilunar valves, the name of which comes from the half-moon shape of the cusps of the valve. The two valves that make up this category are the pulmonary valve, which covers the entrance to the pulmonary trunk, and the aortic valve, which covers the entrance to the aorta. These valves open upon contraction of the ventricular wall to allow blood to rush out of the heart and into the rest of the system. These valves close upon the relaxation of the ventricular wall to prevent backflow into the ventricles. The anatomy of these four valves are shown in Figure 3 below.²
1.1.3 **Blood vessels and blood flow**

To understand the need for the complexity of the cardiovascular system, the route, purpose, and interactions of the blood with the rest of the body must be fully understood. Deoxygenated blood travels enters the heart, leaving the venae cava and coronary sinus and enters the right atrium. The blood then passes through the tricuspid valve into the right ventricle, which then enters the pulmonary circuit upon contraction of the right ventricle and opening of the pulmonary valve. The blood travels to lungs through the pulmonary arteries in which the oxygen exchange occurs in the capillaries of the alveoli. Once the gas exchange occurs, the oxygenated blood returns to the heart through the pulmonary veins, enters the left atrium, then passes into the left ventricle through the mitral valve. The blood is then pushed through the aortic valve upon ventricular contraction and into the systemic circuit. This circuit is responsible for delivering the nutrients and gas that the newly oxygenated blood into the tissue cells through the thinly walled capillaries. The deoxygenated blood then returns through the systemic veins and into right atrium, completing the entire circuit. The entirety of this process can be seen in Figure 4.²
Due to the blood traveling through the systemic circuit having a much larger distance to travel, the aortic and mitral valves is subjected to a larger amount of shear stress than the pulmonary and tricuspid valves. This increase in shear stress contributes to the higher incidence of disease in the aortic and mitral valves.

1.2 **Anatomy and physiology of the aortic valve**

In order to design an effective replacement for a diseased aortic valve, the entirety of the aortic valvar complex should be completely understood. This complex consists of all the components of the aortic root and the ascending aorta. The aortic root is the lower part of the aortic valvar complex that contains the leaflets of the valve, the sinotubular junction, the annulus, the inter-leaflet triangles, and the Sinus of Valsalva. The anatomy of the aortic valvar complex is shown below in Figure 5.
The normal aortic valve has a tri-leaflet structure and the interaction between these leaflets is responsible for whether or not the valve is functioning properly. These leaflets work together to form a boundary between the ascending aorta and the left ventricle and acts as a hemodynamic junction. This tri-leaflet design is optimized for a low resistance opening and high level of efficiency in the cardiovascular system. This tri-leaflet structure is very important to the functionality of the valve, which can be seen with the pathology of the bicuspid defect in those with congenital defects. Recreating the functionality of the tri-leaflet stricture when trying to create a working valve replacement is imperative creating a permanent solution to a diseased valve.

Every one of these components works in harmony in order to create a functioning complex that pumps oxygenated blood throughout the body. When an
aortic valve becomes diseased it can be a result of the valve not functioning properly, but can also be caused by dysfunction in the other parts of the complex.

1.2.1 Tissue layers of the aortic valve

While the structure of the aortic root complex, the structure of the leaflets of the aortic valve are also extremely important to the functionality of the valve. The importance of the structure is at both a macro- and a micro-level, as seen in Figure 6. The three layers are known as the fibrosa, spongiosa, and ventricularis.

![Figure 6: Cross section of an aortic cusp, showing the three layers: fibrosa, spongiosa, and ventricularis.](image)

These layers contain different components that ultimately decide the properties of the valve. The ventricularis is the layer located on the left ventricular side of the valve. This layer of the valve is made up of a layer of elastin fibers with are arranged radially to reduce the strain caused by the inflow of blood. The middle layer, known as the spongiosa, is made up of glycosaminoglycans (GAGs). These GAGs are long, unbranched polysaccharides which help to lubricate the ventricularis
and fibrosa layers, helping to cut down on the shear and deformation that the cardiac cycle causes. The layer on the aorta side of the valve is called the fibrosa. This layer is made of two types of fibrillar collagen (Type I and Type III), which serves as the load-bearing layer of the leaflet. This is done with a circumferential formation of these collagen fibers. Each of the layers also has layers of valve endothelial cells (VECs) lining the outside of the leaflets and valve in valve interstitial cells (VICs). The endothelial lining of the leaflets help to maintain homeostasis of the valves. This is done through a variety of mechanisms including regulating permeability, paracrine signaling, and inflammatory cell adhesion. The valve interstitial cells of each layer serve as the source of components for the extracellular matrix of the tissue. These components consist of collagen, elastin, and GAGs and they provide the necessary strength and elasticity for a functioning leaflet, as seen in Figure 7.

![Figure 7: Three layers of the aortic valve: the fibrosa, spongiosa, and ventricularis. Each layer is lined with valve interstitial cells.](image-url)
1.2.2 Importance of the Valve Interstitial Cells and Valve Endothelial Cells

As discussed in the previous section, VICs and VECs both play an enormous role in the biological function of the aortic valve. The interstitial cells are of particular interest because they are the most common cell present in the valve. This cell type is a mesenchymal cell type and therefore can be differentiated into the different phenotypes of VICs from the embryonic mesenchymal cell type. The five different phenotypes of VICs are the embryonic progenitor mesenchymal stem cells, quiescent VICs, activated VICs, progenitor VICs and osteoblastic VICs. Each of these different phenotypes serve a different function and all of them can be found in the leaflet of the heart valve. Because of the diversity of the functions of the different phenotypes, it is important that when trying to utilize these cells in tissue engineered aortic valves, the correct phenotype is used in order to utilize the correct function and give the most functional valve. The quiescent VICs are responsible for maintaining the valve’s structure and function, as well as to inhibit angiogenesis in the valve. The activated VICs function as the repairmen of the valve, working to repair and remodel diseased valves. These cells respond to the abnormal hemodynamic or mechanical conditions consistent with pathological valves and work to remodel the extracellular matrix of the leaflet. The progenitor VICs are a recently discovered phenotype of VICs, thought to be derived from hematopoietic cells during a time of injury, meaning they also respond to the degenerated and diseased tissue and help to remodel the cells. This pathway of differentiation from a separate cell line is particularly interesting, as these cells have
a similar function to that of the activated VICs, but only arise in the presence of pathogenic valves and have different markers than the activated VICs. The last phenotype of adult VICs is osteoblastic VICs. This last phenotype is differentiated from the quiescent VICs, but unlike those cells, the osteoblastic has a role in calcification of the heart valve and the degradation of the ECM. This particular phenotype gets its name from its function, which is very similar to that of the osteoblasts that remodel the bone matrix. This cell is undesirable in the leaflets because it can lead to a stenotic valve through the calcification of both a native valve and a tissue engineered valve, so steps must be taken to inhibit this differentiation process in a TEV. A number of methods to inhibit the calcification of the osteoblastic VICs such as the addition of an anti-apoptic agent ZVAD-FMK or the use of matrix metalloproteinases (MMPs) to regulate calcification and ECM degradation in the leaflets, but ultimately very little is known about the inhibition of these osteoblastic VICs. More research should be done with this phenotype in order to be able to effectively inhibit the differentiation and function that leads to stenotic heart valves.
1.3 **Pathology of the aortic valve**

1.3.1 **Prevalence of aortic valve disease**

Cardiovascular disease (CVD) is one of the most common causes of death in the world, killing 678,000 people in 2010 in the United States alone, 1.68 million worldwide. The most frequent subtype of CVD is an aortic valve disease (AVD), which can be diagnosed by a few different characteristics. One of the more common type of AVD is a congenital defect, or one that is developed at birth, known as the bicuspid aortic valve (BAV). BAV is a defect in which the affected party effectively only has two leaflets of the aortic valve, either by partial or total fusion of two leaflets. This defect is estimated to be present in three million people across the world and is also estimated to occur in 1.3% of new born infants. Because this defect of the aortic valve is congenital, the incidence is not affected by age, as it is acquired at birth. In addition to a congenital defect of the aortic valve, AVD also can
include aortic valve stenosis, which is present in about 54,000 people in the United States. This disease is found more frequently in older patients, as it is usually a result of calcium build up on the leaflets of the valve over time.

1.3.2 Pathology of common aortic valve diseases

There are many risk factors associated with the development of a valve disease, many of which are also associated with the myriad of cardiac diseases that can lead to cardiac events such as infarctions and cardiac failure. Advanced hypertensive, diabetic individuals, or individuals that have been exposed to certain infections, such as the bacteria associated with rheumatic fever, are some factors that put individuals at a higher risk for developing a heart valve disease. Once the valve begins to function abnormally, it is characterized by an irregular heartbeat, or a murmur, and can usually be heard before any other symptoms occur in patients. This murmur of the heart is usually a sign that the valve is becoming stenotic, or is unable to open fully and properly, obstructing blood flow.

In the pathogenesis of these diseased heart valves, there are a few pathways that the valve can take to reach a state of dysfunction. An advanced bacterial infection caused by either the staphylococcus bacteria or syphilis could cause an inflammation and thickening of the valve, but only in the event that the valve had already been previously inflamed by another event, such as a myocardial infarction (MI). The valve undergoes a thrombotic effect through the collagen of the valve attracting platelets to the surface, resulting in thrombus formation, and the resultant endothelial damage to the valve can be permanent. This condition is called
infective endocarditis, though the advent of penicillin and the decline of rheumatic fever have rendered this pathway of disease a thing of the past in the modern world.

1.3.3 Calcification of the aortic valve

Another pathway of valvular degeneration is by way of the calcification of the heart valves, which is limited to the left side of the heart’s valves, the aortic and the mitral valves. This calcification is one of the main causes for stenosis of the valves, due to the calcium deposits causing a narrowing and stiffness of the valve. This degenerative calcification develops in the cusp fibrosa tissue and results in calcific aortic valve disease (CAVD). The calcium deposits on the surface of the cusps of the valve, which forms masses of calcium build up and stiffens and thickens the valve. The valve will eventually accumulate enough calcium on the surface that it loses its mobility, becoming stenotic, as seen in Figure 9.

![Figure 9: A comparison of a healthy aortic heart valve and a stenotic valve caused by calcification.](image)

The calcium deposits can also become so large that they project into the aortic sinuses, which can result in further blocking of blood flow. Calcium build up
usually occurs over time, and the sites of the initial binding suggest that the repeated damage from flexing may be the initiating factor\textsuperscript{13}. Abnormal valves, such as congenital bicuspid aortic valves, are associated with a higher risk of calcification, due to its abnormal anatomy and function.

The other abnormal valve function that can result in complications, aside from stenosis, is valve regurgitation. Regurgitation is caused by incompetence in the valve in which the valve does not close fully, allowing back flow of blood into the ventricle. Regurgitation, like stenosis, can be caused by calcium build up or from a congenital defect in the valve. Patients who have regurgitation usually tolerate it well and may never need to seek treatment, but they may eventually experience ventricular hypertrophy\textsuperscript{13}. Patients who have diseased heart valves can relate their symptoms to that of the early signs of heart failure, which can also be an end result of a diseased heart valve. Thus, if left untreated, a diseased heart valve can be a fatal condition. Therefore, treatments should be sought to replace the diseased valve before late stage heart failure begin to occur.

1.4 **Current valve treatment options**

Surgical replacement of a diseased aortic valve Patients who have diseased heart valves can relate their symptoms to that of the early signs of heart failure, which can also be an end result of a diseased heart valve. Thus, if left untreated, a diseased heart valve can be a fatal condition. Therefore, treatments should be sought to replace the diseased valve before late stage heart failure begin to occur. There are two kinds of procedures that physicians use to repair or replace the
diseased valves. Physicians have the choice between using a minimally invasive procedure, such as a balloon valvuloplasty, to repair the valve and a surgical procedure to replace the valve entirely with a mechanical or bioprosthetic valve.

1.4.1 **Minimally invasive repair of diseased valves**

Stenosis of the valves can be diagnosed rather easily through the use of a stethoscope in the presence of a murmur of the valve. Once the diseased valve is discovered, it can be evaluated and monitored through the use of an echocardiogram (echo). Once the diseased valve reaches a point of concern for the physician, the valve can possibly be repaired in one of the few following ways, if the valve is not fully dysfunctional.

The cardiologist can use cardiac catheterization to perform balloon valvuloplasty in order to repair a diseased valve. The balloon valvuloplasty is a comparatively simple procedure to perform, seen in Figure 10. This method is appealing due to its minimal invasive nature and thus the minimal recovery time and stress it puts on the patients. Unfortunately, this balloon valvuloplasty is not very effective in the long term, often resulting in restenosis of the aortic valve within a year of the procedure\textsuperscript{15}, but has been shown to be more effective in the mitral valve stenosis. Thus, the balloon valvuloplasty should be seen as a method to relieve symptoms, but not as a permanent resolution to the underlying disease.
1.4.2 Surgical replacement of diseased valves

In the event the stenosis of the valve becomes advanced and symptoms become dangerous, the cardiologist may elect to proceed with a surgical replacement of the stenotic valve. There are two major types of heart valve replacements: mechanical valves and bioprosthetic valves. Each type of offers its own advantages and disadvantages, and thus it is not always clear which type is more suitable for the treatment of the patient with valve disease. This choice involves a balance of the inherent advantages and disadvantages that each valve replacement type has to offer.

Mechanical valves are well known to be extremely durable, due to their excellent mechanical properties, thus decreasing the need for a future surgery to replace due to wearing out. With that inherent advantage comes some major disadvantages though; the mechanical valves are thrombogenic, which means that
the patient will need to be on anticoagulants for the rest of their life and will be at an increased risk of hemorrhaging.

The bioprosthetic valve offers the patient a non-thrombogenic alternative to the mechanical valve, as it is more biocompatible. Another advantage that the bioprosthetic heart valve offers over the mechanical valve is its ability to be utilized in a newer surgical method, known as the transcatheter valve replacement (TVR). This procedure allows for the valve replacement to be surgically put into place through a catheter, and therefore is minimally invasive and offers all of the advantages of a minimally invasive procedure. This procedure is most commonly performed on aortic stenosis, and is known as TAVR in this case. This is procedure is performed fairly similar to the cardiac catheterization described prior, but on the end of the catheter there is a valve that has been engineered to push the old valve out and replace it, either by balloon or by spring, shown in Figure 11.

Figure 11: Transcatheter aortic valve replacement (TAVR) procedure
Again, with this advantage comes a distinct disadvantage, as the bioprosthetic valves only last about 10-15 years, due to the material undergoing structural deterioration\textsuperscript{16}. Part of this structural deterioration that comes with the bioprosthetic heart valves is its tendency to calcify, or re-calcify in the cases of CAVD patients, which leads to the restenosis of the valve and thus the need for another valve replacement. This calcification of the bioprosthetic heart valves is a main area of concern for current tissue engineering, in order to engineer a bioprosthetic replacement valve that is both durable and non-thrombogenic.

1.4.3 Limitations of the current valve replacements

Calcification of the implant is not a problem that only affects bioprosthetic heart valves, but one that has been affecting a variety of cardiovascular implants, such as the aortic homograft and tri-leaflet polymeric valve prosthesis.\textsuperscript{18} This pathological pathway has caused the dysfunction of these different medical devices prematurely through the buildup and mineralization of calcium phosphate molecules on the surface. This calcification can lead to the dysfunction of these replacements by either causing mechanical dysfunction (a failure in opening and closing), an obstruction of the vasculature, or the calcium deposits breaking off or causing an embolism.\textsuperscript{18} There are many steps in the pathology of the calcification of a bioprosthetic tissue replacement, which lead to the degenerative nature of the valve, shown in Figure 12.\textsuperscript{19} This process was initially thought to be unregulated, but it is now thought to be a regulated event through the use, and loss of function of, mineralization inhibitors. This physiological process is similar to that of the
mineralization of bone. This calcification deposition and degradation occurs in the replacements due to the calcium in the blood binding to the mechanically damaged cell fragments of the bioprosthetic tissue. This calcium deposition is especially problematic for the bioprosthetic tissue due to the chemical cross-linking with glutaraldehyde fixation. This fixation disables the calcium pump in the cells, which in healthy cells allows calcium ions to be pumped out of the ability of cells in the tissue to pump calcium out of the cells. The calcium in the membranes will then bind to the phosphate, which results in the buildup, and eventual enlargement of calcium crystal molecules. This is crystal growth ultimately stiffens and weakens the tissue until dysfunction or failure begins to occur.

Figure 12: The hypothetical model of how calcification occurs in bioprosthetic valves.¹⁹
Some further regulatory investigations suggest that this calcification may be regulated by noncollagenous matrix proteins, such as osteopontin, osteonectin, and osteocalcin. Osteopontin is an active calcium binding phosphoprotein that has a high affinity to hydroxyapatite, a key component in bone formation. This and high-density cholesterol (HDL) function as inhibitors for calcium binding. Other factors that have been demonstrated to have a regulatory role in calcification of bioprosthetic heart valves include TGF-α, and tenascin-C. Evidence suggests that hypercholesterolemia may play a role as a risk factor, due to the large amount of low-density cholesterol (LDL) in the blood causing inflammation. The pathway for vascular cell calcification was mimicked in genetic studies in transgenic mice using a metalloproteinases (MGP) gene knock out, or inactivation of the osteopontin gene, and it was observed that severe calcification of the blood vessels occurs. This confirms that the osteopontin gene is essential to the regulation of mineralization, and also that calcification can be inhibited through the inhibition of matrix remodeling metalloproteinases.
Another potential pathway of calcification that has been postulated is that of the inflammatory and immune processes upon valve replacement\textsuperscript{19}. This pathway has gained some ground due to antibodies specific to valve components being detected in some patients with valve dysfunction and failed tissue having mononuclear inflammation. Although these two components provide some a hint that they play a role in the calcification process, there have been studies in nude mice (T-cell function knocked out) that still exhibited calcification morphology, which points to the fact that the immune and inflammation responses may be a secondary response to the valve damage caused by calcification, rather than the causal factors of failure.\textsuperscript{19}

1.4.4 The ideal engineered valve replacement

Tissue engineering is a field that deals with the creation of tissue based "scaffolds" in order to replace or repair a diseased structure in the body. This
process is can be done in a number of different ways, but the ultimate goal is to create a scaffold that has the same or similar mechanical, chemical, and structural properties of the original organ or tissue. In order to create the ideal functional tissue-engineered scaffold the following objectives must be met, as proposed by the leaders in the musculoskeletal field:

1. Define the functional success of the tissue before implementing
2. Understand biomechanical properties of both the engineered and native tissue
3. Establish design requirements of the scaffold
4. Understand microenvironment of the cells within the scaffolds
5. Control cellular differentiation through biophysical stimuli
6. Provide a permanent solution as a treatment

These objectives must be met for the construct to be considered a viable option in implementation. One method of creating the ideal scaffold has been proposed to be achieved by using biological scaffolds, such as repurposing a piece of animal tissue that has been decellularized and then recellularized with a cell type that have no cytotoxicity and illicit no immunologic response. These scaffolds would allow for the proper biochemical and mechanical signaling for the culture of the cells that are seeded onto the scaffold because they are derived from the original tissue type. Other methods include creating a scaffold from a polymer, synthetic or natural, to mimic the structure of the original tissue.

The ideal tissue engineered valve would provide three components that would work together to replace or repair the diseased tissue. These three
components are the physical structure of the tissue, the cellular components, and the chemical properties and these components would be as similar as possible to that of the native tissue before disease. In addition to these three components, the ideal tissue engineered valve should not be cytotoxic, not illicit an immunologic response, have a small diffusion barrier to allow nutrients to diffuse into the scaffolds, and the control of the degradation time of the scaffold.21 This last characteristic is very important because it allows for the proper amount of time for integration of the scaffold at both the cellular and tissue levels.

1.4.5 Adipose stem cells in tissue engineering

Tissue engineering, or regenerative medicine, relies on many different variables in order to repair or replace a damaged organ. Despite the recent advances in material science, which have resulted in the ability to fabricate fully biocompatible scaffolds that allow for cell infiltration and angiogenesis, a challenging aspect of this multidisciplinary endeavor remains to be the availability and utilization of stem cells. In order to find the ideal stem cell, the desired stem cell should meet the following guidelines, described by Gimble et al. The stem cells must be found in abundance, acquired with a minimally invasive procedure, have multiple cell lines that the cell can differentiate into, is able to be transplanted safely into both autologous and allogenic hosts, and can be manufactured within the Good Manufacturing Practice guidelines23. These guidelines rules out most cell types, due to their inability to be harvested efficiently, but a particular cell type stands out as one that falls within the guidelines described above. This cell type is the adipose
derived stem cell and they are isolated through the tissue harvested from liposuction, meaning there is plenty of available tissue due to the increasing popularity of the liposuction procedure. These cells are also capable of being differentiated into many different cell lines, as seen in Figure 14 below.

![Figure 14: Possible lineages of adipose-derived stem cells.](image)

One of the major aims of tissue engineered scaffolds is to create a scaffold that is fully populated with cells prior to their implantation. The problem with the seeding of these scaffolds is that the cells like to adhere to the surface of the tissue, but usually cannot penetrate inside the extracellular matrix. There are a few different techniques that have been developed in tissue engineering to try and uniformly seed the scaffold with cells throughout its entire thickness.
1.4.6 **Limitations and challenges in valve tissue engineering**

Obviously, there are some strict guidelines and regulations that come with the design of a tissue engineered heart valve. But in today’s current prosthesis regime, there is no real “gold-standard” for the replacement valve, due to either the mechanical or the bioprosthetic valve coming at a significant cost, the increase of complications. Thus the ultimate challenge in any tissue-engineering field, and it holds true for the fabrication of bioprosthetic valves, is to create that “gold-standard” with the least amount of complications. There is a limitation, therefore, to the materials that are most likely to be durable and sustainable inside the body, while maintaining a non-thrombogenic effect.

One of the biggest challenges is presented not by the valve itself, but by the exposed aortic wall. The aortic wall is much harder to treat with anti-calcification methods, due to the differing effects of the pretreatment methods\textsuperscript{19}. Thus a material must be found that can be effective on both the aortic wall and the valve itself, or a combination therapy must be utilized to gain the beneficial effects necessary to make the new bioprosthetic valve a viable option to the alternative that is already on the market.

Another challenge that comes inherently with working with the heart valve is that, in order to gain valuable and meaningful data, a 15 to 20 year clinical study must be completed before any conclusions can be drawn, and therefore the bioprosthetic valves that are on the market are between 15 to 20 years behind the current research. There is recent literature that has shown a recent shift to the
study of hydrogels. This research is in its early stages, and therefore there are very little clinical results to draw conclusions from the hydrogel paradigm at this time, but they do offer a promising and exciting future for the of valve tissue engineering, specifically when it comes to the inhibition of calcification.

The future of current research in bioprosthetic heart valve replacements seems to be headed in the direction of modifications of the current bioprosthetic model. These modifications must first be tested in clinical settings, which takes a large amount of time to research. Once the calcification of the modifications have been sufficiently researched, there seems to be a shift towards the use a multi-material valve paradigm, possibly coupled with the use of another anti-calcification therapy in order to maintain the structural and mechanical properties necessary to avoid primary failure of the bioprosthetic valve replacement, while still eradicating the need to replace the valve in 10 to 15 years due to the degradation and restenosis from calcium deposition.

1.5 **Hydrogels in tissue engineering**

Hydrogels have been utilized in a variety of medical applications over the last 60 year. The first documented use of a synthetic hydrogel was in 1954, when Wichterle and Lim developed a soft contact lens. Sense then, hydrogels have been applied to plenty of different technologies across multiple disciplines, such as hygiene products, agriculture, food additives, and biomedical applications.

Hydrogels are becoming especially popular when it comes to designing scaffolds in tissue engineering and regenerative medicine for a variety of reasons.
These hydrogels are capable of replicating the structure of the extracellular matrix of the original tissue. The hydrogels are also appealing due to their ability to be synthesized in mild conditions and therefore they are relatively easily to fabricate. The cause for the shift focus to hydrogels as scaffolds in the tissue engineering field is due to the ease of fabrication and the minimally invasive nature of the application of these hydrogels. Some of the major uses for the hydrogels include drug delivery, growth factor delivery, and cell delivery to the site of injury. These scaffolds can either be created from natural or synthetic materials to try and mimic the native tissue.

The design and material selection for a tissue engineered scaffold is dependent on the desired application of that scaffold. The goal of most of these scaffold materials is to replicate a natural structure in the body, such as the extracellular matrix, in order to replicate the structural and mechanical properties of the native tissue that is being replaced. Unfortunately, the solid scaffold structures are usually synthesized in an environment that results in an acellular scaffold, either through the decellularization of a natural construct or the fabrication of a polymer structure in severe conditions. This factor makes the incorporation of viable cells onto the scaffold exceedingly difficult to achieve. In order to achieve the seeding of cells onto the desired scaffold, there has been a shift to the use of a highly hydrated scaffold, known as a hydrogel. These hydrogel scaffolds can be composed of either natural-derived materials, synthetically derived materials, or a composite hydrogel that is composed of both natural and synthetic materials. The hydrogels
have many advantages over the traditional scaffold due to the properties gained through their highly hydrated structure and mild conditions during their synthetization method, while potentially maintaining similar mechanical and structural properties to that of tissue and the extracellular matrix.

Hydrogels are three dimensional hydrophilic polymer constructs that have been imbibed with large amounts of fluids and crosslinked, which allow the hydrophilic hydrogel to be water-swellable without becoming water soluble. These hydrogels most closely resemble soft tissue and are usually made up of components that are either similar to or derived from native tissue components, such as the ECM.

1.5.1 Current hydrogels in tissue engineering

The strategies for creating a hydrogel for tissue engineering applications vary based on the both the desired application and the type of materials available. The desired material is dependent on the type of properties that the target tissue require. Materials that are commonly used for scaffolds, while FDA approved mechanically strong, can be hydrophobic and created in very harsh conditions making the incorporation of cells into the scaffold impossible. However, hydrogels can be synthesized in much milder conditions and therefore can serve as a great alternative to traditional scaffolds. These hydrogels, both natural and synthetic, can take the place of the commonly derived scaffold materials when the entrapment and incorporation of viable cells are required. Hydrogels are made up of overly hydrated polymers, resulting from the incorporation of hydrophilic polymer chains into the scaffold. This leads to hydrophilic scaffold materials and in turn the incorporation
of viable cells into the scaffold becomes possible. There are many different types of hydrophilic polymers that are used to form hydrogels and hydrogels can be created from either synthetic and naturally derived materials, or a composite material comprised of both.

1.5.2 Classification of hydrogels

Natural derived materials can be a desirable material to form hydrogels for tissue engineered scaffolds due to their naturally occurring prevalence in the human body. These naturally occurring polymers include agarose, alginate, collagen, chitosan, fibrin, gelatin, and hyaluronic acid, which are used based on the fact that they either naturally occur in the extracellular matrix or contain macromolecular components similar to the ECM.

Collagen specifically is a very attractive material for the use in these hydrogels, due to its abundance in mammals as the main protein occurring in the ECM. To date, there have been 28 different types of collagen discovered, and each type of Collagen is composed of three polypeptide chains, which wrap around each other, binding using hydrogen and covalent bonds, to form a triple-helix structure that is shown in Figure 15. Another reason collagen is a desirable scaffold material is the fibers have the ability to self-aggregate into the three stranded structure and form stable fibers. Collagen degradation is relatively easy to control through the use of collagenase, allowing its incorporation into an engineered hydrogel fairly easily and the degradation from the body in a locally controlled manner.
Other materials that have been investigated as possible hydrogel scaffold materials include hyaluronic acid and chitosan, due to their structural similarities to that of naturally occurring glycosaminoglycans (GAGs). These GAGs are found in every living mammalian tissue and are important to the wound healing process of diseased tissue. The degradation of these materials are also easily controlled through the use of enzymes, hyaluronidase and lysozyme respectively. While there are no FDA approved gels for the encapsulation and injection, currently many natural hydrogels on the market that have proven cell-viability.

These naturally derived materials are usually used to replicate the ECM and other cellular structures that have degraded through one of the various degradation methods, or to otherwise to act as biological carriers for cells into the body.
Due to the somewhat unpredictable nature of naturally derived materials, sometimes the hydrogel should be derived from a synthetic polymer. These synthetic polymers are much easier for scientists to reproduce with consistent mechanical and degradation properties. The gelation properties can also be controlled through the manipulation of the specific properties of the gelation properties.

Three examples of synthetic materials that are commonly used for hydrogel applications: PEO, PVA, P(PF-co-EG). PEO is formed using a photoinitiator and a subsequent exposure to UV radiation. These hydrogels are thermally reversibly, or able to be de-gelled, and are also able to be combined with the co-polymers PLA. PVA can be used in space filling and drug delivery applications, and can be crosslinked with different chemicals or other polymer solutions to form hydrogels. However, once it is cross-linked with other polymers it is no longer dissolvable in aqueous solutions.

These synthetic materials are generally used in space filling and drug delivery biomedical applications. This is due to the materials’ ability to be controlled using crosslinking or combining with other polymers. The major advantage of these synthetic materials is their ability to be controllably combined with other polymers for delivery into the body or otherwise change the properties of the formed hydrogel. However, these materials are not advantageous for cell delivery applications due to their harsh fabrication conditions.

In order to achieve the desired properties required for certain biomedical applications, some of these hydrogel materials need to be combined. The hydrogels
are labeled composite hydrogels and can be a mixture of synthetic and natural
derived material, synthetic with another synthetic material, or natural and another
natural material. One specific example of a composite hydrogel is the combination
of hyaluronic acid with collagen. This hydrogel is combined for the purpose of
combining the structural integrity of collagen with the wound healing properties of
GAGs.

While the material the hydrogels are derived, or the source material, from is
a very effective way of classifying and sorting the different types of hydrogels, this is
not the only way to sort them. Sometimes certain properties may be desired in a
hydrogel, meaning that it is very useful to sort the hydrogels based on their other
properties. These classifications can include the polymeric composition,
configuration, type of cross-linking, physical appearance, or network electrical
charge.27

1.5.3 General properties of hydrogels

When designing a hydrogel, there are specific design variables of the
hydrogel that need to be of concern. These design variables are contingent on the
type of application they are being applied to and the environment they are being
deployed in. The general properties of the ideal hydrogels are, as defined by
Ahmed: 27

1) Highest absorption capacity in saline

2) the desired rate of absorption for the application

3) Highest absorbency under load
4) The lowest soluble content (resilience in application) and lowest residual material post-gelation

5) Neutral pH of end product (post formation of hydrogel)

6) Photo-stability (resistant to chemical change)

7) Degradation without toxicity to surrounding environment

8) Durable in a saline or swelling environment and stable

9) Total non-toxic, odorless, and colorless

10) Re-wetting capability, or the ability to return the original solution if required

11) The lowest cost material possible

It is impossible to find a hydrogel that incorporates excellence in all of the characteristics described above, as some of the characteristics are contradictory to each other. This means that some sacrifices must be made in order to obtain the ideal hydrogel for specific applications. In order to choose the best possible hydrogel, one must take into consideration the importance of each characteristic to their specific application and then optimize the balance between contradictory properties.

1.5.3.1 Environmental stimuli sensitivity of hydrogels

Hydrogels precursors are typically made up of a polymer network that has been highly hydrate into a solution of the polymer. In order to have these soluble polymers form a solid structure, they must be cross-linked in one of a variety of different methods. The properties of a hydrogel rely heavily on the type of cross-
linking method. Using a physical stimuli response will yield a reversible bond in the polymer chains, yielding a very weak hydrogel composed of hydrogen bonds. As shown in Figure 16, the other type of cross-linking method is a chemical stimuli, which always results in a strong hydrogel via covalent bonds. Using these different types of cross-linking methods, different properties of the hydrogel can be achieved. As previously stated, if a strong hydrogel is desired then a chemical cross-linking method, such as pH or use of specific solvents. These hydrogels may be stronger than those the physical cross-linking method, but the chemical cross-linking can affect biocompatibility and biodegradability. Thus, sometimes it is desirable to use a physical stimuli, which requires no potentially toxic method to cross-link the hydrogel. This means it is probably desirable for cell encapsulation than that of the chemically cross-linked hydrogels. The softer hydrogels (formed by physical stimuli) also can minimize the irritation to the surrounding tissue caused by their introduction to a site of injured tissue. Ultimately, it is possible to change the chemical and physical properties of a hydrogel scaffold by manipulating the method of cross-linking of the polymer network.

These environmental stimuli don’t only affect the way that hydrogels initially cross-link and form. The various stimuli can affect the properties of the swollen hydrogel as well. A thermosetting hydrogel, when in a heated environment, can also be swollen to a greater extent by submerging it in the desired fluid to be imbibed in the hydrogel. This is especially important when proposing to inject a thermosetting hydrogel into physiological conditions, because this means the
hydrogel, if it has not reached equilibrium prior to injection, will uptake some bodily fluid and this will change the porosity of the hydrogel.\textsuperscript{30}

1.5.3.2 Porosity and microarchitecture

The porosity of a tissue engineered scaffold is very important for tissue regeneration. Due to their swollen structure hydrogels have the advantageous characteristic of having an increase in the porosity of their structure, while maintaining the structural components of the extracellular matrix.\textsuperscript{31} This increased porosity is beneficial to the biological performance of the tissue because it allows for a beneficial impact on their diffusion of nutrients and oxygen. This allows for a superior environment to the traditional tissue engineered scaffold, because this environment will allow for local angiogenesis to occur, which is required for vascularization.\textsuperscript{30} The porosity of these hydrogels are also controllable through the swelling or cross-linking of the hydrogel, as well as the addition of electrospun
fibers or freeze-drying the scaffold. This is very important because different porosities are useful for different applications in specific tissue regeneration. The optimal pore sizes for neovascularization and fibroblast growth is 5-15\(\mu\)m, adult skin tissue is 20-125\(\mu\)m, 100-350\(\mu\)m for bone regeneration, and 500\(\mu\)m for rapid vascularization.\(^{30}\) Thus, the larger pores of hydrogels can be used for different applications in biomedical engineering for designing scaffolds. The voids caused by the large pores can be filled with medication that can be used for drug delivery. Due to the hydrogels degradation, usually by hydrolysis, a drug can be controllably delivered over time using the encapsulation of the drug in the hydrogel.\(^ {27}\) This porosity property is also useful for cell-encapsulation.

1.5.4 Importance of rheological properties for cell encapsulation

When trying to inject a cell seeded solution through a small diameter needle, the shear stresses can have a large effect on the viability of the cells. This is due to the cells lack of protection to the shear stresses, which can induce apoptosis in cells.\(^ {32}\) The use of a hydrogel in cell encapsulation becomes much more important when injecting, because of the hydrogel's ability to protect the cells from the shear stress, due to their high viscosity.\(^ {33}\) Most naturally occurring hydrogels also exhibit a rheological characteristic known as stress-thinning. This property is important to the injection method because the viscosity of the hydrogel decreases as it is subjected to the shear stress from the wall of the needle, leading to the lubrication of the needle from the less viscous form of the hydrogel, allowing the rest of the hydrogel to flow through the needle with less resistance. This further protects the
cells encapsulate from the stresses of the needle, allowing for a high viability of injection.\textsuperscript{34}

1.5.5 \textbf{Mechanotransduction and cellular interactions with hydrogels}

Most hydrogels can be formed at mild, physiological condition when compared to traditional scaffolds developed in tissue engineering. For this reason, hydrogels are regarded as a very promising material for encapsulating cells in the regenerative medicine community. Some specific characteristics of hydrogels that are particularly crucial to cell survival on a scaffold are the following:

1. Ability to be remodeled by cells\textsuperscript{35}
2. Permeability to nutrients, oxygen, and metabolic waste\textsuperscript{36}
3. Ability to mimic the ECM structure of natural tissue\textsuperscript{35}
4. Viscoelastic tissue properties\textsuperscript{37}

One of the most important characteristics of the cell-hydrogel interaction is the ability of the cell to take the hydrogel scaffold and restructure it into a tissue-like structure. This behavior is imperative to the tissue growth and the repair of damaged tissue. The mechanical interactions with the hydrogel result in the embedding of the cells into the hydrogel is the mechanism by which these cells remodel, as can be seen in Figure 17.\textsuperscript{35} For this reason, hydrogels have been used as 3-dimensional constructs for the study of how the cell interacts with remodeling, repair, and regeneration of damaged tissue, based on the biochemical and mechanical stimulation of the environment surrounding the cells.
The ability of these scaffolds to be viable and remodel makes them potentially ideal scaffolds. The ability for the cells to remodel the hydrogel scaffold lies within the cell surface receptors called integrins. These integrins allow the cells to bind to ligands inside the hydrogel, those on the collagen strands, and initiate remodeling. These ligands are available on collagen and fibrin, though not on other hydrogels such as those made from agarose and alginate. The morphology of those cells that have attached is different than those that have not initiated attachment. In cells that have adhered to the ligands of the hydrogel, there is a distinct spread morphology. Those cells that have been encapsulated in hydrogels without available binding sites cannot adhere, and therefore do not
exhibit this spread morphology. Instead, they appear rounded and lack visual fiber attachment throughout.\textsuperscript{39}

The cellular adhesion throughout the scaffold is not the only type of stimulation that leads to proper cell activity for cellular regeneration. Mechanotransduction, or the effect of mechanical forces on cellular behavior, has proven to be very important in the correct cell activity.\textsuperscript{40} The application of these forces change the cytoskeleton structure, leading to activation of ion channels and phosphorylation. The opening of the correct ion channels leads to the signaling molecules being released, signaling correct pathways. These forces can be replicated \textit{in vitro} using a bioreactor, using a different type of bioreactor based on the types of forces desired to be replicated.\textsuperscript{41}

\subsection*{1.5.6 Applications for hydrogels in tissue engineering}

\subsubsection*{1.5.6.1 Hydrogels used for drug delivery and cell encapsulation}

The cell delivery therapy considers the importance of the presence of healthy, living cells at the target site but it does not solve the problem of cellular retention. Injectable hydrogels were proposed to be the solution to this problem, allowing for the cells to bind to the hydrogel.\textsuperscript{42} Some of these hydrogels are naturally derived from a decellularized version of the tissue that the hydrogel is being applied to, such as decellularized myocardium used as a scaffold for ischemic myocardium therapy. Other hydrogels are made from synthetic materials, such as Poly-acrylic acid hydrogels, and composite hydrogels that are a combination of two different types of materials.
The synthetic hydrogels give the distinct advantage of control of the components in order to control and optimize the properties of the gel. These gels can be manipulated to have stronger mechanical properties than naturally derived gels and therefore have been a strong interest in cardiovascular tissue engineering. While these gels show promise in multiple applications, such as drug delivery and mimicking the extracellular matrix, a large majority of these materials have drawbacks in their biodegradability or stimulation of the inflammatory response.

When using a material for cell delivery, the FDA requires at least 70% viability upon completion of the study. If this viability is not achieved, other cytotoxicity testing is required to test for potentials cytotoxins in the material causing cell death.

Figure 18: Strategy for encapsulating cells inside a hydrogel scaffold
1.5.6.2 Hydrogels used for tissue replacement or regeneration

In designing a hydrogel that can serve as a tissue replacement or to regenerate damaged tissue, there are a few design variables that are advantageous for those applications. The following characteristics of a hydrogel are particularly advantageous in the use of artificial muscles: the hydrogels must be biocompatible, have material properties similar to the tissue that they are simulating, non-biodegradable, able to have its properties manipulated chemically, have an easily adjusted shape, and be a low-cost to manufacture. Furthermore, these gels must have the ability to expand and contract under the pressures that the native biological tissue is subject to in physiological conditions.

1.5.7 Cardiac repair via injectable hydrogels

There has been a shift in recent research to the use of non-invasive treatments to facilitate the repair of damaged cardiac tissue. This shift has led to much research in the field of hydrogels. The need for these hydrogels arose from the lack of effective treatments, with the inability to treat ischemic cardiac tissue post myocardial infarction a large motivating factor. The damaged tissue has been treated via a combination of pharmaceutical agents, interventional therapies via medical devices, or heart transplantation. Heart transplantation was the only truly effective of these techniques in the treatment of these diseases, while the other two only work to optimize the function of the remaining living tissue.44 Due to heart donors being in very short supply, the need for an alternative treatment method is very apparent.
This alternative treatment needs to allow for growth and repair of the tissue without the need to remove or replace the damaged tissue. One of the proposed treatment methods to solve this need is cell delivery via injection of the appropriate cell type or stem cells at the site of the damage, thereby providing the proper cellular components to the ischemic tissue. Unfortunately, this procedure has a low yield of cell retention at the target site and therefore is not a highly effective procedure. However, this idea gave rise to the idea of cellular therapy via a different delivery method such as delivery via drugs or delivery via cell-encapsulated hydrogels.
CHAPTER 2: PROJECT AIMS AND RATIONALE
The valve replacements that are currently on the market have many drawbacks to their use, including the lifelong need for anticoagulants, or the wearing down of the replacement. There is also usually an invasive surgery that is required, which results in There are currently many endeavors to create the ideal heart valve replacement through the use of tissue engineered constructs, using various methods. However, there is a growing need for a non-invasive treatment method, designed for the high risk patients that are not strong candidates for a valve replacements. The motivation behind my project is to create a new material that will be injected non-invasively, in order to act as a delivery method for stem cells to the diseased or damaged site on an aortic valve.

The specific aims of my project to contribute to the landscape of tissue engineering efforts to create an effective and long-lasting treatment for heart valve disease include:

1. To fabricate and optimize a novel hydrogel scaffold derived from decellularized porcine aortic cusp tissue for aortic valve applications
2. To achieve and optimize the encapsulation of the hydrogel scaffold through seeding of hADSCs
3. Create a method to transition hydrogel into the bioreactor for dynamic conditioning
CHAPTER 3: MATERIALS AND METHODS
3.1 Study 1: Fabrication of hydrogel using decellularized aortic cusps

3.1.1 Collection and harvesting of porcine aortic cusps

Porcine hearts were received from Snow Creek Meat Processing immediately after death and put on ice. The hearts were then put in a bucket of ddH₂O on ice while setting up the tools for harvesting. The aortic root was isolated from the rest of the heart and the rest of the heart was disposed of in biohazard waste. The major vessels, atria, fat and ventricular muscle were removed from the heart so that only the aortic root and mitral flap (5-10 mm past the base of the cusp) remained. Figure 19 shows the appropriate size of the harvested aortic root, keeping the aortic root and cusps intact with no damage to the desired tissue.

Figure 19: Harvested aortic root for decellularization. (Left) Side view of the aortic root showing the mitral flap and aortic root. (Right) Top view, inside of aortic root and aortic cusps
3.1.2 **Decellularization of porcine aortic cusps by immersion**

Each root was decellularized in detergents already shown to be effective in decellularizing cusp tissue, while maintaining its extracellular matrix structure. The tissue was briefly washed in changes of distilled water and stirred in 0.02% sodium azide overnight. The tissue was then rinsed in ethanol and water, stirred again in 0.02% sodium azide overnight, and rinsed in phosphate buffered saline (PBS) for one day. The aortic roots are immersed in a DNase/RNase solution at 37°C for two days, and then rinsed with PBS for one day. The aortic roots were sterilized using 0.1% Peracetic acid and rinsed PBS for one day, then the cusps were dissected to remove the remained of unwanted tissue so only cusps remain, shown in Figure 20.

![Harvesting of Porcine Aortic Cusps](image)

**Figure 20: Aortic Cusp Harvesting**
One cusp was removed, processed, sectioned at 5 µm and stained with hematoxylin and eosin (H&E) to confirm the absence of cells. Fresh aortic cusps were also processed, sectioned at 5 µm and stained for H&E as a reference. Following the decellularization process, the aortic cusps were deconstructed and lyophilized using the following procedure to create a powder.

### 3.1.3 Decellularization verification of aortic valve tissue

Sufficient decellularization was first verified with agarose gel electrophoresis and histology using hematoxylin and eosin, DAPI fluorescent, and Movat's Pentachrome stains. Fresh and decellularized samples were fixed in 4% paraformaldehyde overnight (24 hours maximum) placed in a processing cassette, paraffin-embedded using the automatic tissue processor according to the following preset protocol.

- 10% buffered formalin (1 min) at 37°C
- 10% buffered formalin (1 min) at 37°C
- 70% ethanol (15 min) at 37°C
- 80% ethanol (15 min) at 37°C
- 95% ethanol (15 min) at 37°C
- 95% ethanol (15 min) at 37°C
- 100% ethanol (15 min) at 37°C
- 100% ethanol (15 min) at 37°C
- Xylene (5 min) at 37°C
- Xylene (10 min) at 37°C
- Paraffin (15 min) at 60°C
- Paraffin (15 min) at 60°C
- Paraffin (15 min) at 60°C
- Paraffin (15 min) at 60°C

Samples were then stored in a warming drawer and the tissue samples were embedded in blocks of hot paraffin on the back of the cassettes, cooled using a cold plate at -5°C and then transferred into a bucket of ice water for sectioning. The embedded samples were then sectioned at 10 µm using a microtome and the sectioned paraffin ribbons were transferred to a water bath at 44°C and carefully mounted on glass microscope slides by moving the slides underneath the ribbons without disturbing air bubbles that build up on the bottom of the water bath. The slides were then patted dry using a kimwipe, then put in a plastic slide holder and transferred into an oven at 60°C to bake the tissue sample onto the slide. The slides were then deparaffinized in xylene for 5 minutes, rehydrated in a succession of dilutions of ethanol and are now prepared for various stains, including: hematoxylin and eosin (H&E), DAPI fluorescent, Movat’s pentachrome, and Masson’s trichrome.

3.1.3.1 Histology: Hematoxylin & eosin staining and imaging

The following protocol for H&E staining was established and verified by the Clemson University Bioengineering Department. After the rehydration series from above, the slides are rehydrated in ddH₂O, stained in hematoxylin for 7 minutes, rinsed with ddH₂O and tap water, and stained for eosin for 45 seconds. The samples
are then dehydrated in a series of concentrations of ethanol and xylene.

Immediately put coverslips on the microscope slides using paramount medium to glue the coverslips on allow to dry. The samples were then imaged using a light microscope at 10X, 20X, and 40X magnifications.

3.1.3.2 Histology: DAPI nuclear staining and imaging

After the rehydration step, the slides are rinsed in ddH₂O and mounted using Vectashield mounting slips with DAPI fluorescent medium. The slides are place in a closed slide holder in order to keep slides in the dark, and placed in refrigerator (4°C) and allowed to dry. The slides are then imaged in the dark using a fluorescent bulb on a light microscope and a blue fluorescent filter.

3.1.3.3 Histology: Masson’s Trichrome and imaging

After the rehydration step, slides are rinsed in ddH₂O and fixed using Boulin’s fixative at 56°C for one hour. Samples were rinsed with running tap water for 10 minutes and then stained with Weigert’s iron hematoxylin working solution for 10 minutes. Samples were again rinsed with warming tap water for 10 minutes, washed in ddH₂O and stained with Biebrich scarlet-acis fuchsin solution for 10-15 minutes. After the Biebrich staining, the samples were washed in ddH₂O and differentiated in phosphomolydic-phosphotungstic acid solution for 10-15 minutes. Without rinsing, sections were transferred to aniline blue solution for 5-10 minutes and then rinsed with 1% acetic acid solution for 2-5 minutes. Distilled water was then used to wash the samples, dehydrated in ethanols, and cleared in xylene.
Coverslips were then mounted using paramount medium and the samples were then imaged using a light microscope at 10X, 20X, and 40X magnifications.

3.1.3.4 Histology: Movat’s Pentachrome and imaging

After the rehydration step, slides are rinsed in ddH$_2$O and placed in 1% Alcian Blue for 20 minutes. The samples are then washed using running tap water for 3 minutes and placed in alkaline alcohol (pH=8) for 2 hours. The samples are washed again with running tap water for 10 minutes, then rinsed in 70% alcohol, placed in Resorcin Fuchsin Working Solution for 16 hours. The samples were washed in running tap water for 10 minutes, rinsed in ddH$_2$O, and placed in Weirget’s Hematoxylin Working Solution for 15 minutes. Tap water and ddH$_2$O were used to rinse again and the nuclei are then differentiated using Woodstain Scarlet—Acid Fuchsin Solution for 5 minutes, rinsed using 0.5% Acetic Acid, and differentiated in 5% Phosphotungstic Acid for 10-20 minutes. Again, the samples were rinsed in 0.5% Acetic Acid and rinsed thoroughly with 100% EtOH. Saffron Du Gratinais was used for 15 minutes and then the samples were dehydrated in a series of dilutions of ethanol and xylene and coverslips were immediately mounted using paramount mounting medium. The samples were then imaged using a light microscope at 10X, 20X, and 40X magnifications.

3.1.3.5 Agarose gel electrophoresis for DNA loss verification

Both native and decellularized aortic cusps had their DNA purified using the Qiagen Kit, following the manufacturer’s instructions for tissue samples. The agarose gel was formed by placing 1% agarose gel in the microwave for 1.5 minutes,
cooled to 60°C and 10μl of ethidium bromide is added to the gel. The gel is then poured in the mold and the comb is placed based on the number of combs. The standard and samples were loaded into each well and were run at 100V for 60 minutes. The gel was imaged using the ethidium bromide setting on the ChemiDoc MP+ BioRad imaging system.

3.1.4 **Powdered aortic cusp preparation**

In order to prepare a sterile powdered aortic cusp tissue, each cusp was first shredded into small pieces using sterile scalpel blades. The tissue was then blended using the homogenizer at the maximum speed setting. The tissue was lyophilized and weighed in preparation for the next step, stored in freezer (-20°C) if not immediately preparing solubilized tissue solution.

3.1.5 **Preparation of solubilized aortic cusp for gelation**

To generate a gelation form of cusp extracellular matrix, the decellularized matrix was solubilized using a method adopted from a previously published protocol by K.L. Christman. Pepsin (Sigma P7125, 400 U/mg) and 0.01 M hydrochloric acid (HCl) solutions were first used to determine the optimal concentration of pepsin to digest the ground tissue matrix. The ideal pepsin:matrix ratio was found to be 1.5:1 (m/v), or 600 units of Pepsin per milligram of aortic cusp tissue, and a 10:1 concentration of 0.01M HCl:matrix (m/v) was used. The matrix was allowed to digest for 48 hours under constant shaking, via an orbital shaker, until completely solubilized. The solution was then neutralized to a pH of 7.2-7.6 by the addition of sodium hydroxide (0.1N NaOH) and diluted using a 1:10
concentration of chilled 10X PBS and 1X PBS to dilute to the desired concentration of 6 mg/ml. The final solubilized matrix was then incubated at 37°C for two hours to induce gelation process. In order to inhibit the gelation of the solutions, keep on ice for short term storage store at 4°C until use, for long term storage store in -80°C.

3.1.6 Preparation of PureCol® hydrogel

The protocol for preparation was adapted from the Advanced BioMatrix manual for PureCol®, Bovine Collagen Solution, Type I (3 mg/ml). While keeping all solutions on ice, add 1 part 10x PBS to 8 parts collagen solution. The pH was adjusted using 0.1 M NaOH to 7.2-7.6, monitored using a pH paper strip. The final volume was brought to 10 total parts with sterile 1xPBS. To initiate gelation, the gel was warmed in incubator (37°C) for approximately 1 hour.

3.2 Study 2: Characterization of aortic cusp-derived hydrogel

3.2.1 Thermal gelation of hydrogel

The thermal gelation was evaluated using a temperature controlled water bath, increasing the temperature every 5 minutes from room temperature (25°C) until the gelation point is reached. Gelation was tested using the inversion test, where the solutions were inverted inside a micro-centrifuge tube at each time point, until the solution was no longer fluid inside the tube. The time of the complete loss of fluidity, or full gelation, was recorded for each gel solution.

3.2.2 Swelling and solubility behavior of the hydrogel

In order to quantify the swelling behavior and equilibrium water content, a swelling behavior assay was performed, adapted from Sanders, 2014. Solutions of
the hydrogel immaterial were prepared at as described above at a volume of 100 µl samples and at three different concentrations of gel; 6mg/ml, 4.5mg/ml and 3mg/ml of the aortic cusp derived gel and a PureCol® gel. The hydrogels were allowed to form for 24 hours at 37°C, and weighed yielding the initial wet weight ($W_{w1}$) of each hydrogel. Each of these hydrogels were frozen at -80°C, lyophilized for 24 hours and weighed ($W_{d1}$). The hydrogels were then allowed to swell in an excess amount of 1X PBS (pH=7.4) at 37°C for 24 hours. The excess buffer on the hydrogels were then blotted off and the hydrogels were weighed ($W_{w2}$). The hydrogels were again frozen at -80°C, lyophilized for 24h and weighed ($W_{d2}$).

Where $\rho_g$ is the hydrogel density and $\rho_s$ is the density of 1X PBS (1 g/ml).

The equilibrium water content (%EWC) is calculated using the following equation:

$$\text{%EWC} = \frac{(W_{w2} - W_{d2})}{W_{w2}} \times 100$$

*Equation 1: Equilibrium Water Content Percentage of Hydrogel*

$$\text{Gel Fraction (hydrogel%) = } \left( \frac{W}{W_f} \right) \times 100$$

*Equation 2: Calculation of the solubility of a hydrogel*

### 3.2.3 Collagen content quantification of hydrogels

The samples were frozen (-80°C) overnight and lyophilized to obtain 10-15mg of dry weight. The samples are submerged in 4N NaOH and hydrolyzed at 120°C for two hours. Complete the hydrolysis inside a hydrolysis bottle and covered in tin foil in order to avoid evaporation of the sample. Using 1.4N citric acid, the solution should be neutralized to a pH between 7.2 and 7.6. The final
volume of the sample is measured and allow the precipitate to fall off before analyzing the solution. Add the 200-μl of the sample into a small borosilicate tube, adding one mL of Chloramine T to each sample and the standard, allow to incubate at room temperature for 20 minutes. Add 1-mL of the PDMAB solution (p-dimetilamino-benzaldehyde, n-propanol, 60% H₂SO₄) to the samples and standards to create the colorimetric binding of the sample. The samples should be vortexed and incubated at 65°C for 15 minutes, again covering with tin foil to avoid evaporation. The samples are each then pipetted in triplicate and the standards in duplicate into a 96-well plate are pipetted at a volume of 300-μl. The plate is read using a plate reader and the Gen5 software, at an optical density of 550nm. A standard curve is made using the standard readings, and a trend line is created to obtain the concentration of hydroxyproline that corresponds to the absorbance reading. The collagen percentage in the tissue is then calculated based on the equation below.

\[
\text{% Collagen in tissue} = \frac{100 \times \frac{\mu g \text{ Hyp}}{ml} \times \text{Vol sample after neutralization} \times 8.33}{\text{tissue weight (mg)}}
\]

*Equation 3: Calculating the percent collagen in a construct from a hydroxyproline assay*

### 3.2.4 Rheological studies for viscosity of hydrogel

The viscosity of the aortic cusp-derived hydrogel precursor was tested using the Brookfield DVIII Ultra Programmable Rheometer. One-mL of hydrogel precursor was injected onto the CP42 spindle and run using the parameters in Figure 21, each ramp being run for 3 minutes, for a total of 15 minutes per experiment. The viscosity of the precursor solution was then first calculated using
Equation 4, where $F/A=$ shear stress, $\eta$ is viscosity and $V/h=$ shear rate. Therefore, the apparent viscosity can be found as the slope of shear stress vs. shear rate. The fluid was then fit to a better model, the Power Law model, which is used for fluids that exhibit a non-Newtonian behavior. The viscosity was then compared to literature values of PureCol®.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ramp 1</strong></td>
<td>1 RPM</td>
<td>1.00 RPM</td>
</tr>
<tr>
<td><strong>Ramp 2</strong></td>
<td>2 RPM</td>
<td>3.25 RPM</td>
</tr>
<tr>
<td><strong>Ramp 3</strong></td>
<td>3 RPM</td>
<td>5.50 RPM</td>
</tr>
<tr>
<td><strong>Ramp 4</strong></td>
<td>4 RPM</td>
<td>7.75 RPM</td>
</tr>
<tr>
<td><strong>Ramp 5</strong></td>
<td>5 RPM</td>
<td>10.0 RPM</td>
</tr>
</tbody>
</table>

Figure 21: Protocol used for Rheology

\[
\frac{F}{A} = \eta \ast \frac{V}{h}
\]

Equation 4: Equation for viscosity of a Newtonian fluid\(^{46}\)

\[
\eta = k\gamma^{n-1}
\]

Equation 5: Power law model for pseudoplastic materials\(^{33}\)

3.2.5 **Porosity analysis of aortic cusp hydrogel, decellularized cusp, and PureCol®**

The H&E sections were analyzed using the ImageJ software for porosity size, number of pores, and overall area of the pores. In order to do this, the scale was set using the scale bar in the image, the threshold was set so the pores appear red (Figure 22: Porosity protocol for H&E sections (Figure 22, left). The feret’s diameter (Figure 22, right) was then analyzed of each pore using the software. The results
were transferred into an excel file and averaged across three photos (n=3) for each study group. A student’s t-test was used to analyze the difference in pore size, pore number and overall area of the pores across all samples.

Figure 22: Porosity protocol for H&E sections

3.3 Study 3: Encapsulation of hADSCs onto different hydrogel scaffolds

3.3.1 hADSCs culture and subculture

Human adipose stem cells were removed from cryopreservation liquid nitrogen and rapidly warmed using a water bath at 37°C. The cells were transferred into approximately 5-mL of fresh, warmed culture media (90% 1x DMEM, 9% Fetal Bovine Serum, 1% Antibiotic/Antimycotic) and centrifuged at 12,000 rpm in order to achieve a cell pellet. The culture media was aspirated, the cells were resuspended
in fresh, warm culture media and were plated onto a Corning T-175 flask at a density of about 5,000 cells/cm². Culture media was replaced every 3-4 days.

In order to subculture the cells, cell were rinsed with warmed PBS, aspirated, then trypsin EDTA was used to detach the cells from the flask for 5-7 minutes. The trypsin was deactivated using culture media and the cell suspension was transferred to a 50 mL conical tube. The cell suspension was centrifuge at 12,000 rpm for 5 minutes to achieve a pellet, the old media was aspirated off, and the cells were resuspended the cells in fresh culture media. The cells were then re-plated on T-175 flasks at the desired density.

3.3.2 Encapsulation of hADSCs onto hydrogel scaffolds

All steps for this study were performed in sterile conditions. The aortic cusp hydrogel and PureCol® precursor solutions were made as described previously (3.1.5) up until the addition of 1X PBS, which was replaced with DMEM culture media for this study. The hydrogel precursor was kept on ice until cells were ready to encapsulate. The cell culture of hADSCs was passaged as described previously (3.3.1) and the cells were counted using the cell scepter in order to determine the total number of cells. The cells in culture media were then transferred into the hydrogel precursor solutions in order to achieve a cell density of 800,000 cells/mL in both of the solutions. The precursor solutions were then brought to their desired concentration using DMEM culture media (6 mg/ml for the aortic cusp derived hydrogel and 3 mg/ml for the PureCol® hydrogel). The solutions are mixed via pipette mixing, note that the aortic cusp derived hydrogel is extremely viscous and
the pipette tip needed to be cut wider using a sterile razor blade in order to successfully pipette. The hydrogel precursors were plated on a 24-well place at a volume 0.5-mL per well using the schematic shown in Figure 23.

Once hydrogels were plated, the well plate was placed in incubator (37°C, 5% CO₂) for two hours to ensure full gelation. After full gelation has occurred, 1-mL of DMEM culture media was pipetted on top of the hydrogels without disturbing them. The culture media was changed every 3 or 4 days, being careful not to disturb the hydrogel on the plate. Images were taking every 24 hours using the ChemiDoc MP+ BioRad system for contraction analysis. This study was run for a total of 24 days.
3.3.3 **Contraction analysis of hydrogels**

The images taking daily from the ChemiDoc MP+ BioRad system, the images were imported into the ImageJ software. Using the drawing tool in ImageJ, each hydrogel was outlined every day and the surface area was taken using the measure function. The scale was based on the known dimensions of a 24-well plate. The surface areas of each test group was averaged and graphed as a function of time.

3.3.4 **LIVE/DEAD cytotoxicity assay**

The media from each well was aspirated in the plate as washed using pre-warmed 1XPBS. The Live/Dead assay from Molecular Probes was used to evaluate the viability of the cells on each hydrogel after 24 days. A mixture of 4mM Calcein AM, 2mM ethidium homodimer-1 (EthD-1) in 1XPBS was used to stain the wells. The well plate was incubated for 20 minutes at 37°C, and then imaged using a fluorescent microscope using a green filter for living cells and red filter for dying or dead cells.

3.4 **Study 4: Hydrogel encapsulation of hADSCs in varying concentrations**

3.4.1 **Encapsulation of various concentrations of hADSCs in aortic cusp hydrogel**

All steps for this study were performed in sterile conditions. The aortic cusp hydrogel precursor solution was made at a concentration of 6 mg/mL, as described previously (3.1.5) up until the addition of 1X PBS, which was replaced with DMEM culture media for this study. The hydrogel precursor was kept on ice until cells were ready to encapsulate. The cell culture of hADSCs was passaged as described
previously (3.3.1) and the cells were counted using the cell scepter in order to determine the total number of cells. Prior to seeding of the cells, $100\mu\text{L}$ of hydrogel precursor was coated onto each well in order to ensure that no cells would adhere to the well plate and would stay on inside the scaffold until full gelation took place. The hydrogel precursors were then seeded at the densities described in Figure 24: Cell seeding schematic for study 4 Pictures were taken every day using the ChemiDoc MP+ BioRad Imager in order to gather the images necessary to do the contractile analysis described in the prior study (3.3.3).

After 13 days, a Live/Dead assay was run on each sample in accordance with the protocol described earlier (3.3.4).

<table>
<thead>
<tr>
<th>Seeding Density</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>$3.2 \times 10^6$ cells/ml</td>
<td>$8.0 \times 10^5$ cells/ml</td>
<td>$2.0 \times 10^5$ cells/ml</td>
<td>0 cells/ml</td>
<td></td>
</tr>
</tbody>
</table>

*Figure 24: Cell seeding schematic for study 4*
3.4.2 **Contraction analysis of hydrogels**

The images taking daily from the Chemi Doc MP+ BioRad system, the images were imported into the ImageJ software. Using the drawing tool in ImageJ, each hydrogel was outlined every day and the surface area was taken using the measure function. The scale was based on the known dimensions of a 24-well plate. The surface areas of each test group was averaged and graphed as a function of time. A student’s t-test was used to analyze the difference in contraction rate across all samples at every time point.

3.4.3 **Quantification of glycosaminoglycan content using DMMB assay**

The samples were frozen (-80°C) overnight and lyophilized, dry weights of each sample were recorded for normalization. Sample are digested using 1-mL of 15mg/ml proteinase K in 30mM Tris Buffer (pH 8.0) for 24 hours at 50°C. In a 96 well plate, a 1:24 mixture of the sample and Tris Buffer is transferred in each well and was combined with 200 of DMMB reagent (40mM NaCl, 40mM Glycine, 46μM DMMB). A standard was made using a 0.025 μg/μl CS stock solution in Tris-buffer. The absorbance was immediately read at 525nm using a plate reader and the Gen5 software. Using the standard, the absorbance was related to glycosaminoglycan (GaGs) content and the GaGs content was calculated for each sample and normalized to their dry weight. A student’s t-test was used to analyze the difference in chondroitin sulfate content across all samples.
3.4.1 Viability analysis of LIVE/DEAD images

The protocol previously described in 3.3.4, live and dead images were obtained of each group of seeded hydrogel. The viability of the cell encapsulated was then calculated using the ImageJ program (NIH freeware) and the protocol described by Labono.47 The cells were isolated from the image by first turning the image to greyscale and then using the threshold feature to only visualize the cells. Note, if there was overlap of cells, then the watershed feature was used to isolate the cells further. The image was then turned into a binary image format (Figure 25) and analyzed using the analyze particles feature, excluding <10μm² to reduce the noise of the signal analysis. The number of analyzed particles is the number of cells counted. The percent viability is then calculated based on Equation 6: Viability analysis equation. A student’s t-test was used to analyze the difference in cell viability across all samples.

Figure 25: ImageJ isolation of cells
\[
\%\text{Viability} = 100 \times \frac{\#\text{Live}}{\#\text{Live} + \#\text{Dead}}
\]

*Equation 6: Viability analysis equation*

### 3.4.2 Sample preparation for scanning electron microscopy

Scanning electron microscopy (SEM) was used to image and characterize the surface morphology of the hydrogel samples. Each sample was fixed overnight at room temperature using Karnovsky’s fixative (a mixture of 2.5% Glutaraldehyde, 2.0% Formaldehyde buffered with 0.1M Cacodylic Acid in ddH\(_2\)O). The samples were washed with three changes of 1X PBS and then washed three times with ddH\(_2\)O. The sample is again fixed, this time incubating in a 0.1% Osmium Tetroxide in ddH\(_2\)O fixative for 30 minutes. The samples are again washed three times using ddH\(_2\)O. The samples were dehydrated using the succession of ethanol (electron microscopy grade) changes, as follows:

- 50% EtOH 10 minutes
- 70% EtOH 10 minutes
- 85% EtOH 10 minutes
- 95% EtOH 10 minutes
- 100% EtOH 15 minutes
- 100% EtOH 15 minutes

Upon completion of the dehydration of the sample, the sample is critical point dried (CPD) via incubation in excess of Hexamethyldisilazane (HMDS) for 20 minutes. The HMDS is aspirated off and air dried at room temperature until fully dry. The samples are stored in a desiccator overnight, in order to avoid rehydration.
of the sample. Mount the samples on aluminum stubs with double sided copper
tape and sputter coat the samples for 2 minutes with platinum using the Hummer
6.2 Sputter Coater, shown in Figure 27, at the Advanced Materials Research
Laboratory (Clemson University Electron Microscope Facility, Anderson, SC). Store
in a desiccator until imaging. The samples are imaged at the AMRL on the Hitachi S-
4800 scanning electron microscope at magnifications ranging from 2,000x to
50,000x.

Figure 26: Hitachi S-4800 Scanning Electron Microscope
3.4.3 **Collagen fiber diameter analysis of aortic cusp hydrogel**

Using 50,000x images that were obtained from scanning electron microscopy, then imported into the ImageJ software. Using the scale bar provided by the image from SEM, the scale is set in ImageJ and the diameter of the individual collagen fibers can be measured using the measure tool. Ten random fibers from each sample group were measured and averaged together for comparison.

3.4.4 **Sample preparation for transmission electron microscopy**

Scanning electron microscopy (SEM) was used to image and characterize the surface morphology of the hydrogel samples. Each sample was fixed overnight at room temperature using Karnovsky’s fixative (a mixture of 2.5% Glutaraldehyde, 2.0% Formaldehyde buffered with 0.1M Cacodylic Acid in ddH$_2$O). The samples were washed with three changes of 1X PBS and then washed three times with ddH$_2$O. The sample is again fixed, this time incubating in a 0.1% Osmium Tetroxide
in ddH₂O fixative for 30 minutes. The samples are again washed three times using ddH₂O. The samples were dehydrated using the succession of ethanol (electron microscopy grade) changes, as follows:

- 50% EtOH 10 minutes
- 70% EtOH 10 minutes
- 85% EtOH 10 minutes
- 95% EtOH 10 minutes
- 100% EtOH 15 minutes
- 100% EtOH 15 minutes

Upon completion of the ethanol dehydration, leave the samples in 100% EtOH overnight. The samples are then infiltrated using a 1:1 mixture of EPON resin and 100% EtOH for 4-6 hours in the refrigerator (4°C), then embedded in 100% EPON resin and placed in the drying oven (60-70°C) for 24 hours. The embedded samples are then sectioned using the microtome (Figure 28) and a glass the sections are captured for imaging using a small copper circle with a carbon mesh grid. The samples are stored overnight in a desiccator to dry and then further fixed for cells using 4% Uranylacetate for 30 minutes in the dark, in order to stain the nucleic acids, proteins and free amino groups. This is follow by a staining of cellular membranes using Reynold’s lead citrate, for one minute in the absence of CO₂. The samples were then dried for at least two hours prior to imaging, again in a desiccator. The samples were then imaged using the Hitachi H7600 Transmission
Electron Microscope (Figure 29) at the Advanced Materials Research Laboratory (Clemson University Electron Microscope Facility, Anderson, SC).

3.4.5 **Immunohistochemistry for analysis of vimentin, α-smooth muscle actin, and laminin presence**

The immunohistochemistry (IHC) protocol was adapted using the general IHC protocol that was developed by a former lab member, Dr. James Chow. Samples were sectioned onto glass slides as described in 3.1.3.1. The slides were deparaffinized in xylene and rehydrated to water using succession of decreasing ethanol concentrations. For antigen retrieval, the sections were circled using the wax Vector Immedge Pen and 2N hydrochloric acid (HCl) was dropped onto each individual section and incubated for 20 minutes. The slides were then rinsed using a tris buffer saline (TBS) two times for 5 minutes. The slides were rinsed in 0.025% triton for 5 minutes in order to permeabilize the sections and were again rinsed in TBS for 5 minutes. A 1.5% normal blocking serum made from normal horse serum
in TBS was used to block non-specific binding for 45 minutes. The normal blocking serum was wicked from the slides and the primary antibody in TNB buffer was added to the sections, Vimentin (Abcam, ab92547) at a concentration of 4ug/ml, α-smooth muscle actin (Abcam, ab5694) at a concentration of 5ug/ml, and laminin at a concentration of 40ug/ml (Abcam, ab11575) and was incubated at room temperature for 1 hours. Following the primary antibody incubation, the antibodies were extracted and saved, rinsed twice with TBS. The endogenous peroxidase was blocked using a 0.3% H2O2 and 0.3% normal horse serum in TBS for 30 minutes, rinsed with TBS for 5 minutes, and the secondary biotinylated antibody was applied, made of 30μl normal horse serum and 10 μL anti-mouse in 2 mL TBS for 30 minutes at room temperature. The slides were again washed using TBS two times for 5 minutes and then were incubated in the Avidin Biotin Complex for 30 minutes. Again, the slides were rinsed with TBS and then were developed using the DAB solution, made of 5mL H2O, 2 drops of Buffer Stock solution and 4 drops of DAB solution from the Vector kit, and 2 drops of H2O2. Once the brown tint of the sections was observed the slides were then quenched using ddH2O. The slides were then imaged using a light microscope at the 10X magnification.

3.4.6 Western blot for α-smooth muscle actin and laminin

Using a protocol previously established by Dr. Jeremy Mercuri in the BTRL, the protein of various samples was extracted using RIPA buffer for 20 minutes on ice. The protein concentration was determined using a Bicinchoninic acid (BCA) colorimetric assay. The samples were prepared so there were 20 μg of protein per
well using reducing buffer and 6-mercaptoethanol. SDS-PAGE was run for 90 minutes at 100V prior to western blotting. The gels were transferred to PVDF membranes and run for 60 minutes at 100 V. The membranes were then blocked in 2% Non-fat dried milk (NFDM) for 2 hours and then incubated in the primary antibody in 2% NFDM. Then they were washed in Tris-Tween 3x and in 0.5% NFDM 2x, then in the secondary primary for 90 minutes. The membranes were then washed in Tris-buffer, prepared in detection solution (Thermo Fischer) and imaged using the ChemiDoc MP+ (BioRad).

3.5 Study 5: Injection of cell-seeded hydrogels into the base of the aortic cusp

3.5.1 Method of injection

First the isolated aortic root was mounted in an aortic root mount, as shown in Figure 30. Using the injection protocol previously established by a student in our lab, Allison Kennamer\(^49\), the aortic cusps were injected in the following manner. All materials were sterilized using either the autoclave (121°C) or ethylene oxide. Human adipose stem cells were passaged, as described in 3.3.1, and reconcentrated to the desired cell density of 3.2x10\(^6\) cells/mL. One mL of the cell dense hydrogel solution was pre-loaded into three separate syringes (one per cusp). Using a 27 GA x\(\frac{1}{4}\) inch needle connected to an air pump, sterile air was pumped into the base of the cusp at about 15-20 psi, which inflated the cusp. Using the same size needle, about 0.5 mL of the pre-loaded hydrogel solution was injected into the inflated aortic cusp. The same injection
method was repeated for all three cusps on the same mounted aortic root. The root was then transferred into the assembled valve bioreactor.
3.5.2 Bioreactor assembly and conditioning

The valve bioreactor was assembled based on the instruction of Dr. Lee Sierad, a former student in the BTRL. Once fully assembled, as shown in Figure 32:

Assembled bioreactor the bioreactor was filled to the fill line using 700-mL of culture media. A second round of antibiotic/antiomycotic was added to the media after 3 days of conditioning. The valves were pre-conditioned using a ramp up scheme as shown in. The valve was slowly subjected to higher pressures each day, reaching pulmonary pressures on day 3 and conditioned there for the next four days, for a total of one week (Figure 31).

<table>
<thead>
<tr>
<th>TIME (DAY)</th>
<th>PRESSURE (MMHG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/0</td>
</tr>
<tr>
<td>2</td>
<td>10/5</td>
</tr>
<tr>
<td>3</td>
<td>10/5</td>
</tr>
<tr>
<td>4</td>
<td>20/10</td>
</tr>
<tr>
<td>5</td>
<td>20/10</td>
</tr>
<tr>
<td>6</td>
<td>20/10</td>
</tr>
<tr>
<td>7</td>
<td>20/10</td>
</tr>
</tbody>
</table>

Figure 31: Bioreactor conditioning scheme.
Figure 32: Assembled bioreactor
3.5.3 Histological Analysis

The cusps were extracted and placed in 4% Paraformaldehyde overnight, and then processed using the Tissue Tek Processor. They were then embedded, sectioned, and stained for H&E and DAPI to determine if cells were viable in the bioreactor, as described in 3.1.3.1.
CHAPTER 4: RESULTS AND DISCUSSION
4.1 Study 1: Fabrication of hydrogel using decellularized aortic cusps

4.1.1 Decellularization Analysis

Figure 33 shows the histological tests to determine that there was proper decellularization of the aortic cusp. The H&E and DAPI stains both show that the nuclei of the cells have been removed fully through the decellularization process. The fresh aortic cusp H&E stain shows purple staining indicating the presence of cells in the ECM, while the decellularized stain does not show this. A DAPI stain, an immunofluorescent nuclear stain, shows the nuclei of cells as a bright blue fluorescence. Again, the fresh show that there is the presence of cells, while the decellularized cusp shows no presence of cells. Staining for Movat's pentachrome stains nuclei red, elastin dark purple, GAGs blue, and collagen yellow. It can be seen that there is a presence of GAGs in the fresh cusp, but there is no presence of GAGs in the decellularized cusp. The color of the gross, fresh cusp is shown to have a brownish tint, while the decellularized cusp is shown to be a ghost white color. The removal of DNA was examined using gel electrophoresis, as shown in Figure 34, and it is seen in lanes 2-5 that DNA is present in fresh aortic cusps, but is absent in lanes 7-11 corresponding to the decellularized aortic cusps. This absence indicates the proper removal of DNA and RNA during the decellularization process. Overall, the histology and gel electrophoresis indicates that the aortic cusps were completely and properly decellularized.
Comparison of fresh aortic cusp (top) and decellularized aortic cusp (bottom). H&E stains the extracellular matrix components pink and cell nuclei purple. DAPI (2\textsuperscript{nd} from left) a fluorescent stain that stains cell nuclei bright blue. Movat’s Pentachrome (3\textsuperscript{rd} from top) stains nuclei red, elastin dark purple or black, GAGs blue, and collagen yellow. Pictures of harvested aortic cusps (right).

**Figure 33: Decellularization analysis of porcine aortic cusps**

Verification of successful DNase/RNase via agarose gel electrophoresis. Lane 1 (left) holds 1kb ladder. Lanes 2-5 contain DNA extracted from fresh porcine aortic cusps. Lanes 7-11 contain DNA extracted from decellularized aortic cusps.

**Figure 34: Agarose gel electrophoresis of fresh and decellularized porcine aortic cusps**
4.1.2 Fabrication of decellularized porcine aortic cusp based hydrogel

The goal of this pilot study was to successfully isolate and decellularized aortic cusp tissue and then use the protocol described in 3.1.5 to solubilize the cusp and fabricate a hydrogel structure at physiological conditions. Figure 35 shows the successful fabrication of two different aortic cusp-derived hydrogels (ACG). The comparison between the two fabricated hydrogel reveal two basic, yet key properties of the hydrogel. Once cross-linked, this hydrogel is capable of maintaining the structure of container that the hydrogel was formed (or cross-linked) in. The hydrogel can be molded into both thick, 3-dimenional structures (A) with the potential for a 3-dimensional scaffold and molded to form a desired structure such as the shape of an aortic cusp (B). The hydrogel also shows the ability of the hydrogel to maintain its crosslinked structure once removed from physiological temperatures (37°C). This means that the gelation process is not spontaneously reversible at room temperature, once the initial bonding has formed.

Figure 35: Successful fabrication of ECM hydrogel
4.2 Study 2: Characterization of aortic cusp-derived hydrogel

4.2.1 Comparison of different concentrations of hydrogels

The goal of this study was to optimize the concentration of the aortic cusp-derived hydrogel through visualization of the reformed matrix, comparison of characterized properties, as well as using knowledge acquired from similar studies in literature. The first step in determining what the best concentration is of extracellular matrix in the hydrogel is to do histology on the structure that is produced via gelation (Figure 36). The H&E histology stains (36, top) reveal how the structures form after gelation, showing that as the concentration present in the cells decreases, the structure formed loses the distinct formation of the fibers. The highest concentration of extracellular matrix used was 6 mg of extracellular matrix per 1 ml of solution. This concentration showed distinct collagen fibers formed and noticeable pores formed within the matrix, which is important for cell-seeding in tissue engineered scaffolds. The lower concentrations (4.5 and 3 mg/ml) of extracellular matrix hydrogels do not show the same distinct fiber formation, nor have the same distinct pores. The lack of fiber formation in lower concentration leads to the conclusion that there would be limited interactions with the matrix, as there is not a distinct structure to adhere to. Their lack of distinct pores are most likely due to the lack of sufficient concentration of collagen to reform proper collagen fibers. Further analysis of the architecture using Masson’s Trichrome confirmed the formation of denser collagen fibers in the higher concentration hydrogel. Another interesting presence was discovered in the staining of the
hydrogels, the pink color that is present indicates muscle fibers are reforming in this matrix. This is interesting, and is probably the result of the isolation process of the porcine valves. There is one cusp in the porcine valve that contains a mix of muscle and collagen and elastin. What this shows is that the muscle fibers will also reform as a result of this gelation process.

The collagen content for all three hydrogel concentrations, as well as the fresh aortic cusp and decellularized aortic cusp was tested using a hydroxyproline assay, Figure 37. This test was run to see how much of the collagen was retained throughout the decellularization process, as well as the formulation of the extracellular matrix hydrogel. It was found that the native cusp has an average collagen content of 453 $\mu$g/mg and the decellularized cusp had an average of 445 $\mu$g/mg. These values were not found to be statistically significant, therefore the decellularization process does not degrade the collagen in any significant manner. The varying concentrations of aortic cusp hydrogel were also tested and found to have values as follows: 6 mg/ml has a concentration of 255 $\mu$g/mg, 4.5 mg/ml has a concentration of 317 $\mu$g/mg and 3 mg/ml has a concentration of 270 $\mu$g/mg. These were not found to be statistically significant across the three concentrations, but they were found to be statistically different from the fresh and decellularized cusp. This difference can be explained through the process of solubilization of the ECM. This process uses pepsin to break down and solubilize the tissue, which works by breaking down the triple helix structure of collagen and can then become soluble. Upon reformation, some of the collagen may not reform the triple helix structure,
resulting in the approximately 40% loss of total collagen content by weight. This also leads to the assumption that some other ECM components are preserved throughout the process of fabrication of the hydrogel, due to the dry tissue weight remaining is still around 700 μg/mg.

A summary of various other parameters of the hydrogels is shown in Figure 38, which summarizes the conditions at which these hydrogels are fabricated, the equilibrium water content percentage (%EWC), the swelling ratio, and the solubility percentage (%), as well as the collagen content. These parameters were all tested against PureCol®, a hydrogel made of pure collagen already on the market. The EWC of all the hydrogels was found to range from 97.5-99.1% indicating the high fluid content of these hydrogel scaffolds. The swelling ratio, which indicates the amount of fluid, relative to their own size, that a hydrogel can take in after the initial gelation process occurs. These values ranged from 15-20% with no significant difference across all samples, meaning that these hydrogels can take in an extra 15% of fluid while in the body. This parameter is important to take in to account, because the hydrogel should be swollen prior to injection to avoid uptake of undesirable fluids in the hydrogel upon injection into the body. The collagen content was also compared to PureCol®, which as its name suggests, is almost pure collagen. Advanced Biomatrix lists its collagen content as >99% collagen type I. While this leads to a reduction in variability in how the hydrogel performs, it means that there is no other ECM components present in the hydrogel for proper cell signaling and adhesion. From these studies, it was determined that a concentration
of 6 mg/ml for the hydrogels is best for cell encapsulation, due to its similarity in microstructure to that of the native ECM.

![Figure 36: Comparison of hydrogel concentrations using histology](image)

A comparison of different concentrations of tissue digest via H&E (top) and Masson's Trichrome (bottom). (left) A concentration of 6 mg of tissue/ml of solution. (center) Concentration of 4.5 mg of tissue per mL of solution. (Right) Concentration of 3 mg of tissue per mL of solution.

![Figure 37: Hydroxyproline assay for collagen content](image)

Collagen Content of Fresh Porcine Aortic Cusp, Decell Cusp, and various concentrations of the Aortic Cusp derived hydrogel (ACG). * denotes P<0.05, statistically significant when compared to the fresh aortic cusp.
<table>
<thead>
<tr>
<th>Sample</th>
<th>ACG (6mg/ml)</th>
<th>ACG (4.5mg/ml)</th>
<th>ACG (3mg/ml)</th>
<th>Pure Col</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelation pH</td>
<td>7.2-7.6</td>
<td>7.2-7.6</td>
<td>7.2-7.6</td>
<td>7.2-7.6</td>
</tr>
<tr>
<td>Gelation Temp(°C)</td>
<td>32</td>
<td>32</td>
<td>32</td>
<td>37</td>
</tr>
<tr>
<td>%EWC</td>
<td>97.5%±0.9</td>
<td>99.1%±0.2</td>
<td>97.9%±1.1</td>
<td>98.7%±0.3</td>
</tr>
<tr>
<td>Solubility (%)</td>
<td>68.3%±6.8</td>
<td>71.6%±21.0</td>
<td>77.4%±3.3</td>
<td>88.0%±14.5</td>
</tr>
<tr>
<td>Swelling Ratio (%)</td>
<td>15.3%±1.8</td>
<td>N/A</td>
<td>19.1%±3.2</td>
<td>10.8%±1.4</td>
</tr>
<tr>
<td>Dry Weight Collagen Content (μg/mg)</td>
<td>255±17.4*</td>
<td>316±70.1*</td>
<td>270±40.8*</td>
<td>933±48.3</td>
</tr>
</tbody>
</table>

*Figure 38: Summary of properties of different hydrogels. (* denotes P<0.05 compared PureCol®)*

**4.2.2 Analysis of porosity**

The porosity of a tissue engineered scaffold is a very important characteristic to account for in tissue regeneration. The porosity of this hydrogel was quantified using H&E sections and the ImageJ software (Figure 39), then compared to that of the decellularized aortic cusp and the PureCol hydrogel. The pore number, pore size, and pore area were all studied for each sample type and compared to for statistical significance. The aortic cusp derived hydrogel was found to have much larger pore size (47μm²) when compared to the decellularized cusp and the PureCol®. The pore number of the AC hydrogel was decreased over the same area. This increase in pore size seems to be inversely correlated to the pore number, which can be explained by the pore size increasing, leading to a decrease in the area available for other pores on the image. The pore size and pore number values were both statistically different from the PureCol® and decellularized cusp.
This larger pore size is conducive to the effectiveness scaffold for cell encapsulation and migration. Thus, the fabrication of this highly porous scaffold with cells already encapsulated in the hydrogel should help with cell migration in the scaffold, as compared with the decellularized cusp and PureCol®. This hydrogel also offers the advantage over a traditional tissue construct, as it is easy to encapsulate prior to reforming the ECM, thus a uniform distribution of cells should theoretically be attained during cell encapsulation.

Figure 39: Porosity analysis
4.2.3 Rheological studies for viscosity of hydrogel

Three rheological trials were run using different settings for the maximum RPM tested, plotting the shear stress vs shear rate and each fit with a power law model (Figure 40, A). The maximum viscosity recorded was found to be 76.80 mPa*s at a shear rate of 3.80 s\(^{-1}\) and the minimum viscosity recorded was 30.72 mPa*s at a shear rate of 57 s\(^{-1}\) (Figure 40, B). The decreasing viscosity of the fluid was plotted against the shear rate with a power law model trend line (Figure 40, C). These numbers were then compared to the literature value for PureCol\(^\circledast\), which was between 23 and 27 mPa*s.\(^50\)

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Shear Rate(s\(^{-1}\)) & Viscosity(mPa*s) \\
\hline
3.80 & 76.80 \\
3.80 & 76.80 \\
7.60 & 76.80 \\
11.40 & 59.73 \\
12.35 & 55.14 \\
15.20 & 57.60 \\
17.10 & 48.36 \\
19.00 & 51.20 \\
20.90 & 46.55 \\
25.45 & 41.29 \\
30.40 & 38.40 \\
38.00 & 37.12 \\
43.70 & 34.50 \\
57.00 & 30.72 \\
\hline
\end{tabular}
\end{table}

Rheological studies of the hydrogel precursor. (A) Power law model for viscosity of the hydrogel. (B) The instantaneous viscosities of the hydrogel, related to shear rate. (C) the plot of the viscosity (mPa*s) vs shear rate (1/s).

Figure 40: Rheological analysis of hydrogel
The rheological studies for viscosity of the aortic cusp derived hydrogel revealed a key property of the precursor. The precursor to the hydrogel does not behave like a Newtonian fluid, which exhibits a linear relation of shear stress (τ) to shear rate (ϒ), and thus a constant viscosity in different environmental conditions. Rather, the fluid precursor fit best to the non-Newtonian model known as the power law model. This particular model is indicative of a fluid that exhibits shear-thinning, or a pseudo-plastic behavior, which means that it has a decrease in the viscosity as it is subjected to larger shear stresses. This shear thinning property is characteristic of the Van der Waals and hydrogen bonds that are holding together the polymers of the hydrogel precursor solution, due to the breaking of those weak bonds under higher shear stresses. This characteristic is very important for injectable hydrogel, as this characteristic can determine the applications that the hydrogels are capable of. For a relevant comparison, this hydrogel was compared to PureCol®, a collagen solution already on the market with a viscosity of 23-27 mPa*s. The viscosity of the aortic-cusp derived hydrogel was found to be much higher at low shear stresses (76.8 mPa*s). The shear-thinning property of this hydrogel means that this material will not be well suited as an injectable hydrogel for drug delivery, due to its viscosity changing throughout the injection process. When the viscosity decreases, this subjects the drug that is imbibed to leaking out of the non-crosslinked solution prior to reaching its destination point. However, this shear thinning characteristic is what makes this hydrogel material very appealing for cell delivery. The viscosity coupled with the lubrication effect caused by
the stress shearing of the hydrogel of the solution should act to shield the encapsulated cells upon injection from the shear stresses subject to the hydrogel. This means that the hydrogel solution should be much better at shielding the encapsulated hADSCs from the shear stress when compared to PureCol®, theoretically resulting in a higher viability from injection. It should be noted that this test was performed at ambient room temperature and temperature has been shown to have an inverse effect on the viscosity of a polymer solution. Ambient room temperature (≈25°C) was used for two reasons: one, if this polymer solution is heated to >32°C then the solution will be subjected to spontaneous gelation, and two, the injection would most likely be taking place initially at room temperature, and therefore is a realistic representation of the application. However, this inverse temperature dependency of viscosity means that it may be possible to counteract this shear-thinning effect with cooling of the solution prior to injection.

4.3 Study 3: Encapsulation of hADSCs onto different hydrogel scaffolds

Human adipose-derived stem cells were encapsulated at a density of 400,000 cells/ml onto each hydrogel and placed inside a 24-well plate, as well as using the PureCol® hydrogel as a control and scaffolds with no cells encapsulated as the negative control. The tracking of this contraction of the hydrogels was qualitatively visualized over time (Figure 41, A). The AC hydrogel was found as the only hydrogel that exhibited a contraction over a 22 day study. This contraction was found to have a rate most closely resembling an exponential rate, as seen in Figure 41, B. The cell
viability of the scaffold was tested and found to be viable to cells, however it was revealed that there was not a high density of cells inside any of the 3-D constructs.

The contraction of the AC hydrogel with cells encapsulated into them prior to plating exhibited a contraction that is indicative if a material remodeling through cellular adhesion. This phenomena was not observed in the PureCol® hydrogel, with or without cells. This suggests one of two possibilities: that the PureCol®, is too dense for the cells to detach from the well-plate and therefore never contract. It also may suggest that there are biological cues present in the AC hydrogel that are signaling the cells to contract and remodel the hydrogel. This theory is furthered by the collagen content of the hydrogel only making up for about 30% of the dry weight, meaning there is probably elastin and other proteins retained from the aortic cusp. The low density of the cells inside the 3-D constructs, shown by a LIVE/DEAD assay, led to a further study with varying concentrations of cells, discussed in 4.4. This live/dead assay showed decent viability, but there were not enough cells present to draw a conclusion.
Figure 41: Contraction comparison of different hydrogels

Figure 42: Live/Dead of contractile study
4.4 Study 4: Hydrogel encapsulation of hADSCs in varying densities

4.4.1 Contraction of hydrogels with varying cell density

Based on the previous study, a contractile study of the AC-derived hydrogel was used to attempt to characterize the effect of cell density on the rate of the contraction (Figure 43, A). The rate of contraction was compared across all study groups over a course of 12 days (Figure 43, B), upon which the highest dense hydrogel was fully contracted and the study was stopped for viability testing. The rate of contraction for the group seeded with 3.2 million cells/mL was graphed and found to have a rate that seems to be trending towards an exponential rate (Figure 44, A), or otherwise has an initial surge of contraction then settling to a more linear rate of contraction (Figure 44, B). This initial surge can be explained by the detachment of the hydrogel from the well plate. This detachment process requires the cells to “pull” against the matrix much more to get the hydrogel to remodel, resulting to a higher rate initially once detachment occurs. This is further evidenced by the initial contraction in the lower cell density hydrogels seemingly not contracting until around day 4. This is probably how long it takes for the cells to accumulate enough force to break the adhesion to the well plate and begin remodeling the hydrogel.
(A) Days 1, 4, 7, 10 and 12 of contractile study. Succession of contraction of each hydrogel group (Groups A-D) in a 24-well plate. (B) Contraction comparison of cell-encapsulated hydrogels.

**Figure 43: Contraction comparison of cell-encapsulated hydrogels**

Analysis of the rate of contraction for cell-seeded hydrogel. The hydrogels seeded with $8 \times 10^5$ cells/well were found to behave with exponential growth initially (Left). After the initial contraction, a more linear contraction rate was observed.

**Figure 44: Contraction rate for cell-encapsulated hydrogels**
4.4.2 Live/Dead cytotoxicity assay

The cytotoxicity of each hydrogel was concentration was assessed using a live/dead assay (Figure 45). This assay showed there was an extremely high number of cells that were retained on the scaffold, relative to the number of dead cells. The viability was quantified using the live/dead images and ImageJ (NIH freeware) to analyze the cell count of each. The viability of the AC hydrogel was found to range between 91-95% at day 12 (Figure 46).

The live dead images in Figure 45 reveal the high viability of the hydrogel for cell encapsulation under static seeding conditions. The Live/Dead images also revealed an interesting tendency of the cells, which was to adhere to the edges of the scaffold and pull the scaffold inward. This suggests that the hADSCs encapsulated migrated to the edges of the scaffold and began the remodeling process, which resulted in a shrunken tissue like structure. The images in at 10x show a better view of this adherence, as the cells look as though they have stretched out along the edges of this valve to grab the collagen fibers. The quantified viability of 95% in the high-density seeded hydrogels, shows the extreme viability of this hydrogel, when compared to other similar scaffolds (Figure 46) shown to have success in hADSC encapsulation. This is probably do to the structural components more closely resembling the native ECM, thereby keeping the cell more likely to not initiate apoptosis. The higher density of cells also trended to a higher viability when compared to the low density of cells (95% vs 91% viability), which suggests that the
presence of other cells help viability and thereby suggests the initiation normal cellular processes indicative of proper cellular function.

### Figure 45: Live/Dead assay for cell encapsulation study

Live/Dead cytotoxicity assay. Group A (left) seeded at a seeding density of 3.2x10^6 cells/mL. Group B (2nd from the left) was seeded at a cell density of 1.6x10^6 cells/mL. Group C (2nd from the right) was seeded at a cell density of 200,000 cells/mL. Group D (right) had a cell density of zero and was considered the control for this experiment.

### Figure 46: Viability analysis and comparison of various hydrogels

Viability analysis of hydrogels. (Top) Live images. (Middle) Dead images. (Bottom) Comparison of different hydrogels' cell viability in static conditions. + denotes values found in literature. P < 0.05 denoted by * and #.
4.4.3 Biochemical analysis of cell-encapsulated hydrogels

The Glycosaminoglycan (GaGs) content was analyzed via a DMMB assay to determine if there was a restoration of GaGs in the cell-encapsulated hydrogels. It was found that there was a modest return of GaGs in the hydrogel, but it was not found to be statistically significant (Figure 47). Each group was also stained for α-smooth muscle actin (α-SMA), laminin, and vimentin using immunohistochemistry, all proteins found in the extracellular matrix important to the cell mobility and function (Figure 48). It should be noted that an alternative antigen retrieval process, due to the recommended one removing the hydrogel section from the slide, and it was very difficult to obtain a clear image as a result, and therefore are not reliable. IHC revealed the positive stain for Vimentin across all sample, a positive stain for α-SMA in Group B, and possibly group A. Laminin did not seem to be positive in any of the samples.

The presence of GaGs in cell-encapsulated hydrogels and not in decellularized cusps or non-encapsulated hydrogels, suggest that the cells are secreting GaGs while interacting with the scaffold. This is important to note, because the presence of GaGs is extremely integral in the tri-layered structure of the native aortic valve, and is important in the proper lubrication of the valve. As this study was a short term, two week study, these GaGs content would be expected to rise over time, as the cells continue to secrete the GaGs.

A positive stain for α-SMA is characteristic of activated VICs, and a positive stain for Vimentin is characteristic of the fibroblast phenotype. The positive stain
on the scaffold would indicate that the presence of cells on the hydrogel results in
the differentiation into activated VICs for α-SMA and into fibroblasts for vimentin.
The protein analysis of the hydrogels seeded with ADSCs shows that there was a
positive stain for α-SMA, which is indicative of activated VICs (Figure 49). The non-
descript protein commassiee stain shows more protein than just collagen type I is
present for cell-signaling. This is extremely important for the proper cell
differentiation signaling in stem cells.

Figure 47: GAGs analysis of tissue samples
Figure 48: Immunohistochemistry for Vimentin, Laminin, and α-Smooth Muscle Actin

Figure 49: Protein Analysis of aortic cusp derived hydrogel
4.4.4 **Electron microscopy for analysis of microstructure**

The morphology of the cell-encapsulated was investigated using electron microscopy to determine the cell interactions with the extracellular matrix. SEM was used to see the surface interactions of the cell with the collagen networks in hydrogel (Figure 50). Adhesion interaction of the cells to the matrix was visualized, with a specificity to the collagen, no cells were visualized binding to the elastin presence. Globular structures were visualized on the surface of the cells. Elastin was visualized inside the matrix morphology, further indicating the retention of extracellular matrix components. The collagen diameter was analyzed using ImageJ to confirm the reformation of the fibers is functional (Figure 51). The collagen diameter formation was found to be within a range of 64-73μm, indicative of a functional type I collagen fiber. Transmission electron microscopy was used to visualize inside the cellular membrane (Figure 52), showing the intact cellular components confirming the viability of these hydrogels.

The globular structures seen in Figure 50, (B and C) shows that the cell is secreting proteins, indicating the proper functionality of these cells. These globular structures may be the GaGs, which we found to be present in the DMMB assay, or they could be adhesion proteins aiding in their adhesion to the extracellular matrix. The functioning secretion of these proteins is evidence of an intact Golgi apparatus and other cellular components important in the creation and secretion of proteins. Further evidence of this is seen in Figure 52, with the presence of intact cellular
components. The functional collagen diameter reformation is extremely important to this scaffold (Figure 51), as this shows that the process of fabrication with the pepsin breakdown does not degrade the collagen irreversibly. This leads to the functional reformation of the collagen, and therefore a functional binding by the hADSCs.

Figure 50: Surface morphology of cell encapsulated hydrogel

SEM of hydrogel morphology. (A) Cells interaction with the collagen network at 5kX (B) Close up of surface of cell, revealing globular structures on the surface of the cell, 50kX (C) Cell interaction with collagen with more globular structures on surface, 10kX. (D) Visualization of possible elastin presence, 20kX.
Figure 51: Collagen fiber diameter analysis

Visual analysis of collagen fiber diameter using SEM (Left). (Right) Quantified collagen diameter was compared to collagen I fibers diameter from literature (*denotes literature value from Lodish, 2000)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average Diameter</th>
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<tr>
<td>Group A</td>
<td>63.55±8.76</td>
</tr>
<tr>
<td>Group B</td>
<td>65.27±6.33</td>
</tr>
<tr>
<td>Group C</td>
<td>71.27±4.56</td>
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<tr>
<td>Group D</td>
<td>73.09±6.85</td>
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<td>Collagen I Fibers*</td>
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Figure 52: Transmission electron microscopy

Transmission electron microscopy of cell-encapsulated hydrogel. An overview of the cell membrane can be seen at 15kX (Left). The contents of the cell can be seen at 30kX (Right).
4.5 Study 5: Injection of cell-seeded hydrogels into the base of the aortic cusp

The injection of the encapsulated hydrogel into the base of the cusp was investigated to determine if this scaffold would improve upon the viability that was previously investigated by Allison Kennamer\textsuperscript{49}, a former student in the BTRL. The initial visualization of the opening and closing of the valve in the bioreactor showed the valve to be functioning normally, meaning the injection method did not affect functionality of the valve. Unfortunately, there was contamination after day 5 of the study, when a leak occurred in the tubing. Figure 53 shows the H&E of the injected cusps, which shows there are no cells present at the end of day 7, DAPI confirms this.

This study shows how difficult it is to see cells effectively on the aortic cusp, as the cusp is subject to mechanical forces that wash the cells away. The hydrogel precursor was meant to protect the encapsulated cell, but the reverse seems to have been the case. The cells probably adhered to the hydrogel precursor, which did not bind to the spongiosa layer of the aortic cusp upon gelation and was washed away with the mechanical forces.
Figure 53: H&E and DAPI of injection study

H&E (Left) and DAPI (Right). H&E shows structural layers of injected cusp. DAPI shows nuclei stained bright blue.
CHAPTER 5: CONCLUSIONS
In cardiovascular tissue engineering, a more clinically relevant valve replacement needs to be achieved through the full recellularization of the heart valve scaffold. In the presented work, a new hydrogel material was developed, optimized, and characterized to reproducibly encapsulate of stem cells inside the scaffold. Through the analysis of the microstructure and biochemical properties of this hydrogel, it was found that this new hydrogel scaffold has a highly viable structure for the encapsulation of hADSCs. To support this conclusion, we compared the viability to hydrogels already on the market in similar conditions, and it was found to have a significantly higher viability in static conditions.
CHAPTER 6: RECOMMENDATIONS FOR FUTURE STUDIES
6.1 Determining diffusion of cells from hydrogel

Encapsulating cells inside the hydrogel allows the cells to bind to the scaffold and begin remodeling the hydrogel into a more tissue-like structure. Due to this fact, there is the concern that the cells are content with being contained inside the hydrogel and won't remodel the ECM of the surrounding damaged tissue. A simple rate of diffusion from hydrogel to a surrounding scaffold and viability test would be sufficient to determine the effectiveness of this material as a potential material for the delivery of cells to diseased tissue.

6.2 Viability of cells encapsulated in hydrogels: static vs. injection

It was found that this hydrogel is what is known as a shear-thinning material. Due to this property's presence, the cell-encapsulation capabilities of the hydrogel may be compromised, due to the viscosity being lessened in greater shear stress. The shear stress that is brought on by the injection through a needle may lead to the cells on the outer parts of the hydrogel precursor diffusing out much more readily than those hydrogels that are not subject to high shear stress prior to formation. Also, the cells encapsulated may not be as well protected after injection compared to prior to injection, resulting in a decrease in cell viability on the scaffold. The hydrogel should be tested using different gauge needles to determine the optimal needle size for retention of viability. The hydrogel should be compared to cells in cell culture media, as well as collagen gels already on the market. When using a material for cell delivery, the FDA requires at least 70% viability upon completion,
so it must be determined whether this hydrogel meets these requirements post-injection.

6.3 **Long term dynamic conditioning studies in the bioreactor**

The initial pilot bioreactor study was only performed for a short, one week period and only in one aortic root complex (3 cusps). Future studies should include the preconditioning of injected valves with this cell-encapsulated hydrogel to see if the cells differentiate into VICs under the mechanical cues of a bioreactor, while encapsulated in the hydrogel over the course of a month long study. This should be compared to the results of static controls, as well as to previous valve bioreactor injection studies without the presence of the hydrogel.

The effect on the mechanics of the valve should be tested. There is a possibility that the presence of a hydrogel could act as a reinforcement to the valve, allowing it to further withstand the stressful environment of the cardiovascular system. Using the Bose, or other means of fatigue testing, the valves mechanics should be compared to that of the native valve, as well as the decellularized valve without the hydrogel injection.

6.4 **Large animal studies to determine site-specific compatibility**

We have shown that this hydrogel is extremely viable for the encapsulation of cells. In order to determine if this hydrogel can translate into *in vivo* environment safely, the hydrogel should be tested in a large animal model, such as a sheep or pig, in order to see if the hydrogel would induce an immune response, as well as to test
to see if the stem cells would differentiate into the desired q-VIC phenotype while inside an animal model.
## APPENDIX

### Appendix A: Applications of hydrogels

<table>
<thead>
<tr>
<th>Application</th>
<th>Polymers</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound care</td>
<td>polyurethane, poly(ethylene glycol), poly(propylene glycol)</td>
<td>(Rozali &amp; Yoshida, 1999)</td>
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<tr>
<td></td>
<td>poly(vinylpyrrolidone), polyethylene glycol and agar</td>
<td>(Bennumar et al., 2006; Long &amp; Malissen, 2001; Rozali et al., 1995)</td>
</tr>
<tr>
<td>Nafion, methyl cellulose</td>
<td>(2006)</td>
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<tr>
<td></td>
<td>carboxymethyl cellulose, alginate, hyaluronic acid and other hydrocolloids</td>
<td>(Kim et al., 2005; Rozali et al., 1995; Rozali &amp; Yoshida, 1999; Walshe et al., 2005)</td>
</tr>
<tr>
<td>Drug delivery, pharmaceutical</td>
<td>poly(vinylpyrrolidone)</td>
<td>(Bennumar et al., 2006; Rozali et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>starch, poly(vinylpyrrolidone), poly(acrylic acid)</td>
<td>(Kumar et al., 2008; Spinelli et al., 2005)</td>
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<tr>
<td></td>
<td>carboxymethyl cellulose, hydromethyl methyl cellulose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>polyvinyl alcohol, acrylic acid, methacrylic acid</td>
<td>(Barbut et al., 2004; Porch &amp; Wittgen, 2013)</td>
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<tr>
<td></td>
<td>chitosan, oligo-glycosphosphate</td>
<td>(Zhou et al., 2008)</td>
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<td></td>
<td>κ-carrageenan, acrylic acid, 2-acrylamido-2-methacylamido-2-propenesulfonic acid</td>
<td>(Campos et al., 2006; Pourjavadi &amp; Zohuriaan-Malah, 2007)</td>
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<tr>
<td></td>
<td>acrylic acid, carboxymethyl cellulose</td>
<td>(El-Naggar et al., 2006; Sand et al., 2001)</td>
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<td>Dental Materials</td>
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<td>(Ab-Assali et al., 2009)</td>
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<tr>
<td>Tissue engineering, implants</td>
<td>poly(vinyl alcohol), poly(acrylic acid)</td>
<td>(Rozali et al., 1995)</td>
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<tr>
<td></td>
<td>hyaluronan</td>
<td>(Kim et al., 2005; Shin et al., 2004)</td>
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<td>collagen</td>
<td>(Grundy &amp; Moneghy, 2008)</td>
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<td>Injectable polymeric system</td>
<td>polyesters, polyphosphazenes, polyglycophosphate, chitosan</td>
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<tr>
<td></td>
<td>f-diargin peptide</td>
<td>(Yan et al., 2010)</td>
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</table>
REFERENCES


46. Instructions O. *BROOKFIELD DV3T Operating Instructions.* Vol 8139.


