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Neomycin-Enhanced Carbodiimide Cross-linking for Glycosaminoglycan Stability in Bioprosthetic Heart Valves

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NEOMYCIN-ENHANCED CARBODIIMIDE CROSS-LINKING FOR GLYCOSAMINOGLYCAN STABILITY IN BIOPROSTHETIC HEART VALVES

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Valvular heart diseases lead to over 290,000 heart valve replacements worldwide each year, and approximately half of these involve replacement with a bioprosthetic heart valve (BHV) [43]. BHVs exhibit excellent hemocompatibility, but suffer from inadequate long-term durability with most adult implanted valves failing within 12 to 15 years after implantation [62]. Although this may be adequate for some individuals, BHV implantation may be contraindicated in younger individuals to avoid reoperation. Even in elder recipients, valve dysfunction can still cause death or reoperation that could be avoided with increased BHV durability. Therefore, investigation into methods of increasing BHV durability can not only improve the quality of life for BHV recipients, but widen the accessible patient demographic as well.

All BHVs are currently treated with glutaraldehyde, which is used to stabilize collagen through crosslinking as well as reduce tissue immunogenicity. However, glutaraldehyde crosslinking potentiates BHV calcification and also causes undesirable alterations to tissue properties that are conducive to structural degeneration [5,78,143]. Considering that calcification and structural degradation are the foremost causes of BHV failure, there has been interest in alternative, non-glutaraldehyde fixation methods [5].

One phenomenon that can contribute to structural degeneration is the loss of glycosaminoglycans (GAGs) from cuspal tissue [15,19]. GAGs are large highly hydrophilic molecules that form a gel-like sheet within the center layer of valve cusps. This layer aids in stress absorption and reduction of shearing between the other tissue layers [18]. GAGs lack the amine functionalities to be crosslinked by glutaraldehyde and under current fixation methods are lost during preparation, in vivo implantation and
storage [18,19,75]. Carbodiimide crosslinking with EDC and NHS reacts with available carboxyl groups in addition to amine groups allowing for the crosslinking of both GAGs and collagen. These crosslinks may aid in GAG preservation and thereby contribute to improved mechanical function. However, it has been shown that crosslinking alone is not sufficient to fully preserve GAGs, as they are still prone to loss through enzymatic degradation [132].

Neomycin, a hyaluronidase inhibitor, has been shown previously to prevent the enzymatic degradation of GAGs [133]. Neomycin also contains amine functionalities that enable stable incorporation into carbodiimide-initiated crosslinks. This study investigates the effects of carbodiimide crosslinking in combination with neomycin on the stability of structural proteins, GAG preservation, calcification, and biomechanical properties.

The incorporation of neomycin into EDC, and NHS fixation (NEN) was found to improve GAG retention, fully preventing loss during storage, implantation, and direct enzymatic digestion. Additionally, the use of neomycin enhanced the elastin and collagen stability of EDC and NHS crosslinking alone (EDC). When compared to glutaraldehyde crosslinked tissues (GLUT), NEN treated tissues demonstrated comparable collagen and elastin stability but far superior GAG retention. EDC and NEN groups also displayed reduced stiffness and increased extensibility when compared to GLUT crosslinked tissues. These results suggest that NEN fixation and resulting GAG preservation may improve the mechanics of BHV tissues. In doing so, tissue stress and loads that occur during function may be reduced, preventing the onset of collagen damage and increasing the lifespan of BHVs.
DEDICATIONS

To all those who have helped me and encouraged me to work towards greatness

To all of my family and those who know they are just like family, their care and guidance has shaped me into the person I am today

To my parents for their unending support and for always believing in me

To my brother for whom I always strive to be the best role model

Finally, to my wife whose love, strength, and selflessness inspires me daily
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1. LITERATURE REVIEW

1.1. Anatomy of the Heart

The human heart is a physiological pump that ensures continuous transport of blood throughout the body for the entire lifetime of an individual. It contains four distinct chambers consisting of two ventricles and two atria, both left and right. The right chambers of the heart contribute to the body’s pulmonary circulation while the left side of the heart provides systemic circulation. Blood is driven through the body by pressure gradients that have varying intensities in different sections of the heart [1-3]. Due to the much greater resistance offered by systemic circulation, mean pressures are higher in the left side of the heart where average values are approximately 80 mm Hg during diastole and 120 mm Hg in systole upon entry into the aorta. Pressures in the right side are much milder amounting only to values of around 10 - 25 mm Hg in the pulmonary artery [1,2].

Blood transport in the heart begins at the right atrium. Deoxygenated blood is received from the inferior and superior vena cava before passing blood into the right ventricle for delivery to the lungs using the pulmonary arteries. Oxygenated blood from the lungs is then transported to the left atrium through the pulmonary veins and lastly into the left ventricle, which distributes blood to the rest of the body via the aorta. The heart itself is supplied with blood by the coronary arteries that originate in the left and right aortic sinus at the aortic root. Deoxygenated blood from the heart collects in the coronary sinus and empties into the right atrium to again to be cycled through the heart [1-3].

In order to ensure efficient and constant forward blood motion throughout the heart, there are four valves in place that function to prevent backflow. These valves are composed of connective tissue formed into “cusps” or “leaflets” that open and close to
regulate blood flow [1-3]. These valves are located at the entrance and exit of each ventricle and are each attached at their base to the cardiac skeleton. The cardiac skeleton separates the atria from the ventricles and although it is not a true skeleton, is composed of dense connective tissue and provides vital structural support to the heart. The fibrous rings that compose the cardiac skeleton serve as attachment points for the heart valves, muscular fibers, and major vessels in the heart. The attachment point for the heart valve cusps on the cardiac skeleton is known as the annulus, which is an important reference point when concerning native and artificial heart valve position [2].

Two of the four valves located in the heart are known as the atrioventricular (AV) valves, while the other two are identified as the semilunar valves [1-3]. Under normal circumstances, these valves function constantly throughout the approximately 3 billion cardiac cycles that occur in a single lifetime [4]. The AV valves separate each of the atria from their respective ventricles and prevent backflow from the ventricles during systole. The right AV valve is commonly known as the tricuspid valve, while the left is called the mitral valve [1-3]. These valves are anchored to the heart wall by thread-like structures called chordae tendineae. These collagen-based structures are attached to the heart wall at the papillary muscles, which contract during systole in order to pull the chordae tendineae taut and prevent over closure and inversion of the AV valves [1-2]. The closure of these valves is associated with the first heart sound, coined “lubb”, while the second heart sound results from closure of the two remaining heart valves after ventricular systole and is termed “dubb”. These other two valves, the semilunar valves, are named the pulmonary and aortic valves. They lead into the pulmonary arteries and aorta respectively and prevent retrograde flow into the ventricles [1-3].
1.1.1. Heart Valves

Heart valves are composed of cusps or leaflets that open and close to regulate blood flow. Each valve in the heart has three cusps that seal together during closure except for the mitral valve, which has only two. Although the basic function of heart valves is simple, the role they fill is quite demanding. Despite being in the extremely volatile environment of the bloodstream, each valve must exhibit nearly flawless function through an astounding number of cycles. The valves face constant blood flow, sharp pressure changes, and other environmental stresses that call for an extremely durable and specialized structure. Discussion in this section will primarily concern the aortic heart valve. It is the most commonly diseased and replaced heart valve, with many bioprosthetic heart valve replacements being constructed from xenogenic aortic valve...
tissue [5]. There are some morphological differences between the different heart valves of the body, yet their primary compositions are comparable. For our purposes, knowledge of the aortic heart valve will suffice.

The aortic valve is located at base of the aorta and marks the exit of blood from the heart into the body’s vasculature. The valve leaflets are an extension of the cardiac endocardium and fibrous skeleton. The fibrous ring on which they are positioned is known as the aortic annulus [5,6]. This is the main load bearing structure for the valve and serves as the transfer point of stresses to the aortic wall [5,11]. Adjacent to each cusp are round bulges in the aortic wall that are collectively called the aortic sinuses. These structures prevent valve leaflets from sticking to the aortic wall during opening, and also promote the formation of vortices in the blood flow that aid in valve closure [4,7]. Many structures contribute to overall aortic valve function, but the cusps will be the main topic since they are the primary structures of concern when discussing valve disease and replacement [5].

Aortic valve cusps are thin and translucent structures composed of connective tissue that is roughly semi-circular in shape. For the most part, they are avascular structures. They primarily support themselves by obtaining nutrients from the bloodstream via diffusion [5]. Although the cusps are microscopically complex structures, their surfaces contain very few noticeable macroscopic features. There exists a free edge that provides coaptation during closure with up to 40% of cusp surface area coming into contact with the other cusps [5]. The cusps are connected to the aortic annulus on the opposite side. This attachment is continuous until terminating at the free edge on both ends at the commissures. Besides these primary points of reference, one
side of the cusp is noticeably smoother than the other. Also, in the center of the cusp there is a thick nodular region, the *Nodule of Arantius*, where all three cusps meet in the center during closure [8]. Despite their bland external appearance, the internal composition of the aortic valve leaflet is much more interesting. It is a specialized blend of various structures including valvular interstitial cells (VICs), valvular endothelial cells (VECs), and large quantities of various extracellular matrix (ECM) components [5,6].

![Diagram of the aortic valve cusp and fibrous skeleton](image)

**Figure 2.** [A] Aortic valve cusp [4]. [B] Fibrous skeleton and valves of the heart [2].

1.1.1.1. Heart Valve Cell Types

1.1.1.1.1. Endothelial Cells

Endothelial cells (ECs) are a single layer of cells that are continuous throughout the entire cardiovascular system. This layer of cells lines the lumen of all blood and lymph vessels as well as other structures in the bloodstream filling many roles vital to normal cardiovascular function [1,2,7,8]. ECs provide the ideal smooth, non-adherent
surface that allows blood components to pass through the valve unimpeded and undamaged. Additionally, through the release of certain biomolecules, ECs also promote uninterrupted blood flow through the inhibition of thrombosis and maintenance of hemostasis. Other factors secreted by ECs, as well as certain EC cell surface markers, play essential roles in immune and inflammatory responses and contribute to management of local blood flow through vasomotion control. The endothelial cell layer also serves as a barrier between the lumen of the blood vessel and the exterior tissues. It not only provides a physical obstruction, preventing the exposure of internal valve components, but also controls the passage of nutrients, wastes, cells, and other materials by both diffusion and bulk transport into the underlying tissues from the bloodstream [1,8].

1.1.1.1.2. Valvular Interstitial Cells

The other main cell type existing in heart valves is the valvular interstitial cell (VIC). VICs have an elongated cell shape with narrow connecting projections that form a network throughout the valvular matrix [26]. These cells, also known as myofibroblasts, have the characteristics of both smooth muscle cells (SMCs) and fibroblasts and are thought to be primarily responsible for maintaining extracellular matrix homeostasis [6,26]. Similar to fibroblasts, VIC metabolic activities are highly geared towards producing, degrading, and reorganizing large amounts of matrix components [5,6]. However, their SMC-like properties enable them to display contractile capabilities which may aid in the maintenance of suitable internal stress levels [26]. The distinct contractile and metabolic characteristics of myofibroblasts allow them to
uniquely respond to various stimuli. The specialized nature of VICs enables them to respond to the constantly changing body environment and aid in the maintenance of the balance necessary for heart valve cusps to perform their intense physiological duties [6].

### 1.1.1.2. Extracellular Matrix

The majority of connective tissue volume is composed of extracellular matrix [1,7]. Heart valve cusps are no exception and are formed from a mixture of collagen, elastin and glycosaminoglycans [5,6]. Heart valve durability is strongly based upon the condition of the matrix and its ability to remodel efficiently [11]. Each matrix molecule has a specific structure and function within the valve, and when arranged accordingly, these molecules provide the structural support and flexibility heart valve cusps.

#### 1.1.1.2.1. Collagen

Collagen is the main structural molecule within heart valve cusp tissue and is also the most abundant protein. Collagen accounts for approximately 90% of heart valve cusp protein and about 55% of the young adult human cusp by dry weight [9,10]. There are multiple types of collagen present in cusp tissue, with the majority being type I collagen (~70%) and the remainder being mostly type III (~25%) [9]. Collagen organizes itself into larger fibrous formations, or aggregates, based upon a triple helix molecule subunit of three linear amino acid chains wound together. The resulting long fibrous formations enable collagen to exhibit very high tensile strength, but low resistance to torsional and flexural stress. When properly aligned, this organized structure enables collagen to provide most of the valve’s matrix strength and durability [13].
1.1.1.2.2. Elastin

Elastin is a branched and coiled protein with the ability to stretch when forces are applied [2,7,10]. If the forces are removed, elastin returns to its original state allowing it to impart flexibility onto the tissues it inhabits [10,13]. Elastin is rich in non-polar amino acids, making it insoluble and giving it a hydrophobic nature [12]. Unlike collagen, which can only withstand elongations of 1-2%, elastin can maintain strains of 150% without fracture. However, elastin may improve the elasticity of collagen through interfiber connections. These connections also allow collagen to return to its natural state when unloaded, giving aortic valve tissue great strength and extensibility. Elastin is the second most abundant protein in aortic valve cusps and it accounts for 13% of the dry weight in young adults [10].

1.1.1.2.3. Glycosaminoglycans

Glycosaminoglycans (GAGs) are long unbranching polysaccharides composed of repeating disaccharide units containing a hexosamine and a hexuronic acid, or hexose [16]. These molecules are found in many places throughout the body, including cartilage, tendons, vitreous humor, synovial fluid and aortic valve cusps. There are five main species of GAGs including hyaluronic acid (HA), chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate, and heparin/heparin sulfate (HS) [17]. Most GAGs are sulfated and can be covalently linked to core proteins in order to form proteoglycans (PGs) [18, 19]. However, non-sulfated HA does not covalently bind, but instead can act as a core connecting with proteoglycans, such as aggrecan, via link proteins resulting in large brush-like structures [18-23].
All GAG species have been found in human heart valves, but those primarily found are HA, CS, and DS, with significant amounts in PGs versican and decorin [18,19,26]. Approximately 60% of the GAGs found in cusp tissue are HA with the remainder being primarily CS (~30%) or constituents of the PG decorin (~10%) [24, 26]. GAGs are highly anionic due to elevated concentrations of uronic acid and sulfate groups making them very hydrophilic [24]. This allows them to absorb many times their weight in water and, while only representing 3.5% of cusp dry weight, this behavior allows GAGs to substantially affect valve properties, structure and biomechanics [25]. GAGs play many roles in aortic valve structure and function. This section includes only a brief overview of GAGs. For a more detailed discussion please refer to section 1.2.

**Figure 3.** [A] Large PG structure, aggrecan bound to an HA core. (adapted from [21]). [B] Disaccharide unit of the primary aortic valve GAGs (adapted from [17]).
1.1.1.3. Aortic Valve Layers

Heart valves exhibit three distinct layers that provide various structural and functional purposes that contribute to normal valve function. These three layers, the fibrosa, spongiosa, and ventricularis, differ both compositionally and morphologically from one another making each specialized in serving its particular purpose. The fibrosa is located on the superior aortic valve surface facing the aorta when the valve is closed. The ventricularis is located opposite of the fibrosa and faces the left ventricle while the intermediate spongiosa layer forms a barrier separating the other two layers [5,6,14,26].

![Diagram of aortic valve cusp section showing tri-layered structure including basic cell and molecular composition](image)

**Figure 4.** Depiction of aortic valve cusp section showing tri-layered structure including basic cell and molecular composition [46].

1.1.1.3.1. Fibrosa

The fibrosa faces the exit side of the aortic valve and is the primary load-bearing tissue layer [5,6,10,26]. This layer is continuous with the cardiac skeleton and like the fibrous ring is composed of high proportions of collagen [6]. The collagen is predominantly aligned parallel to the free edge giving it highly anisotropic properties
Consequently, the aortic valve cusps are extremely yielding in the radial direction, yet equally tough in the circumferential. The higher compliance in the radial direction allows valves to open with relative ease in response to blood flow while the strength imparted by the circumferentially aligned fibers makes the cusps stand firm when met by backflow. Additionally, the rigidity associated with the aligned fibers keeps the cusps taut, which helps promote coaptation and prevents regurgitation [5].

In order to provide support for the fibrosa’s high radial strains, corrugations are present to help mitigate the formation of high stresses [5]. The corrugations of the fibrosa facilitate large amounts of cusp expansion and contraction that occur during normal valve function [26]. Furthermore, microscopic collagen waves are also present, which makes the collagen fibers slightly more extensible and alleviate stresses in the circumferential direction [5,10]. Together, the features of the fibrosa make this layer the load-bearing workhorse of the aortic valve cusp.

Figure 5. [A] Radial cross-section of porcine aortic valve cusp showing tri-layered structure and fibrosa corrugation [5]. [B] Circumferential cross-section of aortic valve. View of microscopic collagen crimping [29].
1.1.1.3.2. **Ventricularis**

The ventricularis is the bottom layer of the aortic valve that faces the left ventricle when the valve is closed [5,10,11,27]. This layer is the thinnest layer and is composed primarily of dense collagen fibers, which are less orderly than those in the fibrosa [10]. The ventricularis is also smoother than the fibrosa and displays a significantly higher elastin content through a large proportion of radially aligned elastin fibers [5,11].

The abundance of elastin fibers is thought to support the maintenance of collagen fiber architecture as well as provide elastic recoil during valve function [10]. This recoil occurs because the ventricularis is naturally preloaded in tension making it contract when in the relaxed state [28]. This occurs at the beginning of systole and helps snap the valve open quickly. The preloaded state of the ventricularis also allows the valve to exhibit beneficial changes in cusp surface area during opening and closing, which helps maximize valve efficiency. Contraction during opening causes a reduction in cusp surface area, which minimizes interference with blood flow while elastic stretching during valve closure provides maximum coaptation [5,11].

**Figure 6.** Cross-section showing ventricularis and fibrosa changes from systole to diastole. (adapted from [4]).
1.1.1.3.3. Spongiosa

The spongiosa is the central layer of the aortic valve cusp that consists of loosely arranged collagen fibers and a plentiful quantity of glycosaminoglycan molecules and proteoglycans [5,11]. The high GAG and PG content of the spongiosa gives it an extremely high hydration capacity. So, despite its lack of structural strength, the spongiosa absorbs large amounts of water and takes on a gel-like structure with substantial thickness that demonstrates considerable contribution to overall valve function [5,18,29].

As mentioned above, the fibrosa and ventricularis undergo a large amount of expansion, contraction and bending during normal valve function. The differences in the deformations that occur between these two layers create large amounts of shear stress if uninhibited. The spongiosa acts as a flexible fluid barrier between the two main structural layers that minimizes shear stresses by lowering friction. The lubrication properties of the spongiosa allow for great mobility between the fibrosa and ventricularis. Alleviation of these high shear stresses that occur during flexure can prevent acute valve damage or even separation of the aortic valve layers [5,18,28,29].

The yielding nature of the spongiosa permits minimal interference with valve motion while still providing necessary thickness to the cusp. Its thickness ensures isolation of both the fibrosa and ventricularis and helps prevent tissue buckling [18,31]. Tissue buckling occurs during bending of the valve at sites of high compressive stress; however, GAGs and PGs express a viscoelastic response under compressive loading that results in the spongiosa’s high resistance to compressive force and capability of efficient shock absorption [5,18,19,30].
1.2. Glycosaminoglycans (GAGs) and Proteoglycans (PGs)

1.2.1. GAG and PG Structure

As mentioned previously in section 1.1.1.2.3, GAGs are long unbranching polysaccharides composed of repeating disaccharide units containing a hexosamine and a hexuronic acid or hexose [16]. These molecules are found in many places throughout the body including cartilage, tendons, vitreous humor, synovial fluid, and heart valve cusps. With the exception of hyaluronan, most GAGs are sulfated and can be covalently linked to core proteins in order to form PGs [18, 19]. The presence of these sulfate groups, as well as uronic acid residues, makes GAGs and their associated PGs highly anionic. This elevated concentration of charges results in the expression of extreme hydrophilicity [24].

There are approximately 30 different PG varieties that differ based upon the number, size, and type of the attached GAG chains as well as the structure of the protein core and location [17]. According to these characteristics PGs are grouped into five families. However, only two significantly associated with aortic valve cusps, hyalectins and small leucine rich proteoglycans (SLRPs) [17,19]. Hyalectins are large PGs capable of non-covalently associating with hyaluronan via link proteins and include aggrecan, versican, brevican, and neurocan [17,22]. Common SLRPs include decorin and biglycan which are small PGs consisting of one or two GAG chains attached to a protein core containing areas rich in leucine repeats [17,19].

Chondroitin sulfate (CS) and hyaluronan (HA) represent the large majority of GAGs present in aortic valve cusp tissue. Dermatan sulfate (DS) also has a significant presence in smaller amounts and, together with CS and HA, account for 90% of all cusp GAGs [19]. These GAGs are primarily found in the form of PGs as versican and decorin.
Versican, a large PG, is comprised of 12-15 CS chains that attach to their protein core using available serine residues [17]. It is mostly found in the spongiosa layer complexed with HA to form large brush-like structures. Decorin is the most frequently found small PG in aortic valve cusp tissue, although biglycan is also found in smaller amounts [19,131].

1.2.2. GAG and PG Function

The function of PGs is largely controlled by the characteristics of associated GAG side chains. The high concentration of anionic charges on large PG aggregates is a result of high CS expression and leads to the absorption and retention of large amounts of water [21]. Not only does this play a role in tissue hydration, but also nutrition and solute transport as well. These large, hydrated, brush-like structures take on a gel-like state that simulates a porous system limiting the movement of water as well as various solutes and nutrients [16]. The micro-flow of water within this networked gel structure, along with intra/intermolecular electrostatic repulsions, yields a contribution to the viscoelastic nature of cusp tissue [16,17,19,23,130]. These large PG structures have a large involvement in valve biomechanics by rendering occupied tissues resistant to compression, facilitating efficient load distribution, and enabling reversible deformation under mechanical load [16,17,19]. In fact, the osmotic properties of aggrecan have been established to provide greater than half of cartilage’s compressive modulus further demonstrating the biomechanical importance of these molecules [22]. Additionally, these structures can interact with collagen assemblies and have been stated to play a possible role in binding specific enzymes or other biosignaling molecules [16].
Small PGs are thought to be associated with the formation and organization of other ECM components [17]. Specifically, they interact with collagen through their core protein or side GAG chains and play a role in collagen fibrillogenesis [19]. SLRPs decorin and biglycan of aortic valve cusps utilize their leucine rich domains to bind to fibril-forming collagens. The full function of these is not known, but they are stated to modify the rate of fibril formation as well as play a role in end-stage production or termination [21]. Decorin and biglycan have also been suggested to aid in elastogenesis, which is supported by the fact that their highest expression is found in the ventricularis layer of cusp tissue [131].

1.3. **Native Heart Valve Disease**

Proper heart valve function is necessary to maintain normal blood flow, thereby ensuring the transport of vital nutrients throughout the body. Heart valve disease can cause the valves to become dysfunctional, therefore disrupting normal blood flow. This disruption can lead to many symptoms, including shortness of breath, fatigue, edema, heart palpitations, chest pain, and even death. A valve is termed dysfunctional when it cannot open properly (stenosis) or close fully (insufficient). Stenosis causes the valve to become a blockage that impedes normal blood flow exiting the heart, while a valve insufficiency is best described as a leaky valve that allows back flow or regurgitation during diastole. Stenosis and insufficiency are not mutually exclusive and can occur together, although, one usually predominates. These problems can stem from congenital or acquired sources, but regardless of the condition’s origin, diseased valves eventually contribute to an increased load on the heart, a common precursor to heart failure [32].
Diseased heart valves can often be diagnosed simply by listening to the heart. Disruptions in the regular flow produce irregularities in the normal sounds of the heart, which can be heard using a stethoscope. Diseases most commonly affect the aortic and mitral valves on the left side of the heart. Failure of the right side of the heart is usually a consequence of left-sided failure and is rarely an isolated incident. Valve disease, along with contributing factors such as heart tissue damage and hypertension, create an increased load on the heart forcing the heart to adapt to counteract. This adaptation involves hypertrophy of associated myocytes as well as disruption of the normal structure and composition of heart cells and extracellular matrix. If left untreated, continued presence of hypertrophy will commonly lead to heart failure [32].

**Figure 7.** Path to cardiac dysfunction [32].
1.3.1. Congenital Valve Defects

Congenital heart valve defects exist at birth and are not caused by environmental factors, such as diet, lifestyle, or aging. The most common congenital heart valve defects affecting the general population are bicuspid aortic valve and mitral valve prolapse (MVP) [11,32,33]. MVP is associated with various inherited disorders; however, the underlying disease mechanisms are not well understood and it may also be an acquired disease [11]. MVP affects up to 3% of the population and is usually asymptomatic leading to discovery during routine examinations [11,32]. Congenitally bicuspid aortic valves are similarly common, only affecting 0.5 to 2% of individuals [11,32,33]. In addition to defects that directly affect the valves, more systemic disorders can also cause valve dysfunction. These include, but are not limited to, autoimmune diseases and connective tissue disorders such as Marfan syndrome.

Mitral valve prolapse is described as a floppy mitral valve that overextends back into the left atrium during ventricular contraction. This disease is also called myxomatous degeneration of the mitral valve, due to the presence of a thickened spongiosa resulting from myxomatous material deposition [11,32]. This deposition causes the leaflets to become hooded, thick, and rubbery and is accompanied by a weakened fibrosa [32]. This disease occurs more often in young women and, though mostly asymptomatic, can cause valve insufficiency and lead to serious complications [11,32]. However, even asymptomatic mitral valve prolapse has recently been linked to serious harmful cardiovascular events including heart failure and death [11].

A bicuspid aortic valve is defined as an aortic valve that appears to only have two leaflets instead of the normal three. This abnormality can appear as one normal cusp and
a second cusp that is composed of two cusps fused together or, much less commonly, two symmetric cusps without signs of fusion. At early ages, this defect is also commonly asymptomatic. However, bicuspid aortic valve presence predisposes individuals to certain complications later in life, such as aortic root dilation, cusp prolapse, accelerated valve calcification, and increased susceptibility to infective endocarditis [32,33].

**Figure 8.** [A] Depiction of bicuspid aortic valve [33]. [B] Severe hooding and calcification in a patient with mitral valve prolapse [32].

### 1.3.2. Acquired Valve Diseases

Acquired diseases are those that are attributed to factors after birth, but can be affected by congenital disorders. Even though these diseases are acquired later in life, some congenital disorders can predispose individuals to certain acquired diseases or promote the progression of these diseases after they are contracted. These diseases may also be influenced by lifestyle choices, such as diet, activity level, drug use, stress, and surgical procedures. The primary acquired conditions that will be discussed in this section are rheumatic heart disease, infective endocarditis, and degenerative diseases.
Rheumatic heart disease (RHD) manifests as valvular defects due to an autoimmune response by the body. Development begins with the contraction of group A β-hemolytic streptococci bacteria and, although considered an acquired disease, genetic factors also play a role in its development [32,34,35]. Following infection, antibodies are produced by the body’s immune system to react with bacterial antigens. In susceptible individuals, these antibodies are also reactive with certain tissue proteins and can target the heart, joints, or central nervous system [32,35]. This is called rheumatic fever and clinical expression occurs 2-3 weeks after the end of the initial streptococcal infection [32]. Only 30-45% of rheumatic fever cases affect the tissues of the heart, while a much larger portion (75%) affects the tissues in the joints. This disease has been largely eliminated in industrialized nations. However, it still remains a large problem in developing countries and accounts for close to 250,000 deaths annually worldwide [35].

Infective endocarditis (IE) involves the growth of bacterial colonies on the heart valves and over 80% of cases are caused by particular strains of streptococci, staphylococci, or enterococci. These particular microorganisms have the necessary surface adhesins to overcome the hurdle of attachment to the heart valves and surrounding structures. IE can affect individuals with previously normal healthy valves, but valve damage promotes bacterial attachment at the site of injury [36]. Congenital valve defects, degenerative valve disease, rheumatic heart disease and mechanical wear all cause such valve damage. Intravenous drug users are also at a very high risk for IE, as well as people who recently had catheters or other surgical procedures and patients with prosthetic heart valves. The severity and speed of disease progression varies depending on virulence of the organism. Highly active organisms lead to rapid tissue destruction
causing obvious problems of valve insufficiency and heart failure [32,36,56]. More benign growths can lead to large vegetations that interfere with normal valve function causing leakage or stenosis [56]. In addition, large growths can form infected emboli that cause downstream destruction via local tissue necrosis and infection [32,36,56].

Degenerative valve diseases are those that occur passively with the wear and tear that accompanies age and most commonly include floppy damaged valves, aortic dilation, and valve calcification [37]. Individuals who experience congenital and acquired diseases that cause valve damage or increase the wear of valve tissues are more likely to develop these degenerative diseases and experience faster disease formation and progression. For example, individuals with bicuspid aortic valves develop aortic calcification up to two decades earlier than those with normal valves and are more prone to aortic dilation [11,32,37-39].

The calcification and thickening of the aortic valve is defined as aortic sclerosis and is present in over 25% of individuals older than 65 years of age [11,38,39]. Aortic sclerosis shares many developmental characteristics with atherosclerosis and is similarly influenced by hypercholesterolemia, hypertension, gender, diabetes, smoking, sedentary lifestyle and age. Aortic sclerosis can develop into calcific aortic stenosis, the most common cause of aortic stenosis [38-40]. Aortic stenosis is the most prevalent heart valve disease affecting 43% of patients with valvular heart disease [41].

Although valve calcification is still discussed as a degenerative disease, it has recently been discovered to also be an active process. This active process is due to macrophage activity and a shift in VIC phenotypes that causes them to express properties similar to osteoblasts, which are bone-producing cells [11,38,39,42]. Calcification of
valves is of particular interest in research due not only to its prevalence in native valves, but also because it is a primary mode of failure in prosthetic valves.

Together, each year, valvular heart diseases lead to the hospitalization of 93,000 Americans and are considered the primary cause of death for over 21,000 Americans. These diseases are also a major contributing factor in an additional 43,000 American deaths annually, not including instances of bacterial endocarditis or rheumatic fever. This problem becomes compounded in an aging population as the prevalence of valvular diseases is only 0.7% in individuals of ages 18 to 44 as compared to 13.3% in individuals 75 years or older [41]. Although valvular diseases are responsible for many deaths, numerous lives are saved by surgical intervention through heart valve replacement and repair. There are over 290,000 heart valve replacements performed every year worldwide with the demand increasing at a rate of 10 – 12% per year [43]. Current heart valve replacements are sufficient enough to prolong life in many cases, but they have their limitations and cannot yet be considered an equal replacement of the native valve.

1.4. **Artificial Heart Valves**

There are four primary types of prosthetic heart valves currently being researched that will be discussed in this section: mechanical valves, biological valves, polymeric valves and tissue engineered heart valves. Of these four types, mechanical valves and bioprosthetic valves are currently the only ones available for use and each of these has their various benefits and drawbacks. Since a perfect valve replacement has not yet been produced, valve type selection is a matter of balancing pros and cons. When evaluating the performance of an artificial heart valve it is important to keep in mind the criteria
necessary for an ideal valve substitute. The list is daunting, but a summary of these characteristics is displayed in Table 1, originally stated by Harken in 1962 [44].

Table 1. Desirable Characteristics of a Heart Valve Substitute [5].

<table>
<thead>
<tr>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonobstructive (Not stenotic in any way)</td>
</tr>
<tr>
<td>Quick and complete closure (No valve insufficiency or regurgitation)</td>
</tr>
<tr>
<td>Nonthrombogenic</td>
</tr>
<tr>
<td>Resistant to infection</td>
</tr>
<tr>
<td>Inert in the body (No inflammation or immune response)</td>
</tr>
<tr>
<td>Does not cause blood damage</td>
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<tr>
<td>Durable for the lifetime of the patient</td>
</tr>
<tr>
<td>Easily and permanently implanted</td>
</tr>
<tr>
<td>Tissue interface with the implant heals properly (No tissue overgrowth)</td>
</tr>
<tr>
<td>Not annoying to the patient (Noise free)</td>
</tr>
</tbody>
</table>

1.4.1. Polymeric Heart Valves

Polymers possess a wide range of properties that can vary based on molecular subunit types, subunit order, presence of cross-linking, molecular weight of macromolecules and the type processes used for manufacturing. We have the ability to control all of these things to a certain degree, allowing us to widely control the properties of polymeric materials and cater them to our specific needs. Many polymeric materials are well tolerated by the body and are commonly used in the medical industry for a variety of applications. In fact, polymeric materials are present in many cardiovascular devices such as catheters, vascular grafts, stents, blood oxygenators as well as current artificial heart valves in the sewing rings.

Polymeric heart valves are a promising topic of research with potential to produce valves that combine the best properties of current prosthesis types. The hemocompatibility of many polymeric valves is superior to mechanical heart valves and, likewise, there are materials available that are more durable than biological tissues [45]. However, developing a polymeric material with the proper combination of suitable
properties is a difficult task. Many polymers have been tested in order to find a suitable material for heart valve production, but polyurethanes have attracted particular interest due to their unique structure [45,46].

Polyurethanes have a two-phase structure of hard (crystalline) and soft (elastic) molecular subunits that gives them a favorable blend of compliance and strength [45,46]. This allows polyurethanes to closely match the stiffness of natural vessels, which is one basis of interest for their use in improved vascular grafts [47]. Polyurethanes also have excellent hemocompatibility, but studies using polyurethane in cardiovascular devices have suffered from durability problems with both in vivo and in vitro testing [45-47].

Figure 9. [A] Polyetheretherketone trileaflet valve. [B] Polycarbonate urethane trileaflet valve. [C] Polycarbonate urethane bileaflet valve [54].

Recent research of polyurethane materials has mainly focused on using different soft (elastic) segments to improve the long-term durability [45,46]. First generations attempted to use polyesters and polyethers; however, these materials degraded due to hydrolysis and oxidative damage. The use of polycarbonates solved some problems of these previous generations, but currently experience problems with valve calcification. More current polyurethane materials have shown promising results, but no polymeric materials are currently approved for prosthetic heart valve use [45,47].
1.4.2. Tissue Engineered Heart Valves

The basic premise behind this approach involves the use of a degradable three-dimensional scaffold upon which host cells are intended to grow. Cells may be intended to infiltrate \textit{in situ}, or tissue regrowth may occur partially or fully \textit{in vitro}. The host’s cells will eventually completely infiltrate the scaffold as it breaks down leaving behind only the living tissue. Ideally, this type of valve would be functionally identical to the healthy native valve resulting in the perfect valve replacement [5,6,11,26,46,54].

The material requirements for heart valve tissue engineering scaffolds are numerous and demanding. The scaffold itself must be a suitable valve replacement in its own right. It must meet all mechanical and biocompatibility requirements of current valve prostheses, with the exception of durability. This is to ensure that it can support
itself until cell infiltration is complete and the resulting tissue can function on its own. It must also support cell growth and degrade at a suitable rate to allow remodeling and regeneration [26,46,64]. Most importantly, at no point during the tissue regeneration process can mechanical failure occur, or death of the recipient is almost certain.

Materials from both natural and synthetic sources have been investigated as possible candidates for suitable tissue engineering scaffolds. The benefit of using natural materials is obvious since they offer a more familiar environment for cellular ingrowth [11,46]. Collagen, fibrin and small intestinal submucosal matrix along with decellularized xenogenic and allogenic valves are among some of the promising prospects, but so far none have emerged as a suitable material [46]. Human trials were attempted using tissue engineered decellularized porcine heart valves; though, these resulted in early failure [11,26,46,65].

This scaffold, Synegraft™, developed by CryoLife Inc. performed well in animal studies using the porcine model. Structurally whole valve cusps were found in valves explanted after 5 months. These valves showed significant host cell infiltration and lacked any signs of valve calcification [26]. Similar implants in the sheep model also demonstrated good results in three and six month explants [66]. However, implantations of four Synegraft™ valves into children were disastrous. Three of the children died at seven days, six weeks, and one year postoperatively while the last valve was explanted after only two days. The explanted valves showed signs of severe inflammatory response and experienced no cell repopulation. These devastating results call into question the reliability of results from current animal models and will have far reaching effects on future trials of any tissue engineered heart valves [65].
The use of synthetic materials is based around developing biodegradable polymer scaffolds. The ability to control many mechanical properties of these scaffolds as well as the degradation characteristics makes this approach particularly appealing. Polylactic acid (PLA), polyglactic acid (PGA), poly-4-hydroxybuturate (P4HB), polycaprolactone (PCL), polyurethanes (PUs), and their copolymers are some commonly investigated materials for use in synthetic scaffolds [26,54,64,67]. Hoerstrup et al. created scaffolds from PGA and P4HB that were seeded and implanted at the pulmonary position in the sheep model. These valves remained functional for 20 weeks and the typical tri-layered structure of the native valve was present in explanted valves. However, these valves also showed mild regurgitation and incomplete endothelial cell coverage [26,54,68].

The hope of tissue engineering research aims at creating the perfect heart valve replacement. Perfection of this technology could lead to the creation of a working living valve with lifelong durability that can remodel, repair, and grow with the patient. Many good results have made us optimistic; however, catastrophic results have also made it obvious that many obstacles still remain.

**1.4.3. Mechanical Heart Valves**

Mechanical heart valves (MHVs) are those made entirely from synthetic materials. However, for the purposes of this review, this excludes polymeric valves and tissue engineering scaffolds. All mechanical heart valves consist of three major parts: the occluder, valve housing, and sewing ring. The occluder is the only moving part of the valve that blocks and opens blood flow. The housing is the primary structure that maintains the integrity of the valve as well as provides vital guidance for the occluder.
The sewing ring is a flexible fabric, usually made from ePTFE or Dacron, and is used for attachment of the valve to the body. The main benefit of mechanical heart valves is their durability. Current valves have proven to be extremely long lasting and cases of structural failure are extremely rare [48]. However, the nature of MHV materials and geometries cause them to express lower hemocompatibility and patients that receive them must undergo strict lifelong anticoagulation therapy.

The level of required anticoagulation therapy is high and given the harshness of the required warfarin therapy, elderly patients, patients with bleeding disorders, or women who are pregnant or planning on conception should not be recommended for MHV replacement. This makes MHVs the replacement of choice for patients with a long life expectancy where anticoagulation therapy is not contraindicated [43,48-51]. However, even when MHVs are recommended, anticoagulant therapy is still undesirable and carries with it the risk of harmful side effects [49].

1.4.3.1. Background of Valve Types

The birth of mechanical heart valves occurred in 1952 with the implantation of a valve designed by Dr. Charles Hufnagel. This valve was not implanted at the site of the diseased aortic valve, but was instead inserted into the descending thoracic aorta [48,50,51]. The Hufnagel valve consisted of a silicone rubber coated nylon ball inside of a methacrylate chamber. This device was not a true valve replacement and was only designed to treat aortic valve insufficiency. The introduction of the Hufnagel valve was soon followed by the caged ball valve, which was the first device designed to actually replace the native valve [50].
Caged ball valves use a ball as the valve orifice occluder that is retained in a cage. After this evolution in design, there have been essentially no mechanical failures associated with these valves after over 250,000 implantations [48]. Problems associated with the caged ball valves include their bulkiness and risk of thrombus formation. These large valves are centrally occlusive to the bloodstream leading to high transvalvular pressure gradients and highly abnormal blood flow. Switching to the use of a disc occluder in caged disc valves proved to be less bulky; however, these also caused high central blockage of normal blood flow and experienced high complication rates, which sparked the development of tilting disc valves [50].

Tilting disc valves exhibit marked improvement over previous designs with respect to central flow and hemodynamics. These valves utilize protruding struts to tilt the disc during the open phase allowing better central flow [49,50]. The development of these valves coincided with the discovery of smooth pyrolytic carbon as an extremely tough, thromboresistant material, which quickly became the material of choice for the majority of all succeeding mechanical replacements [48,50,51].

The current gold standard of mechanical heart valve replacements is the bileaflet valve. When compared to other mechanical heart valves, bileaflet valves display a much flatter velocity profile and are heralded as the most hemodynamic valves available today. These valves utilize hinges built into the valve housing to guide the rotation of two half disc, semi-circle shaped occluders. This eliminates the need for protruding struts for occluder guidance making bileaflet valves the most compact valves offered. These valves have experienced enormous success and currently account for over 80% of implanted MHVs [43].
1.4.3.2. MHV Failure

1.4.3.2.1. Thrombus Related

Today’s mechanical heart valves are primarily composed of pyrolytic carbon, and although relatively inert in the body, is still a foreign material and can initiate blood coagulation through the intrinsic pathway [52]. This problem is compounded in areas where stagnant flow is prevalent or where recirculating flow gives time for coagulation to occur. Other flow abnormalities and high-risk areas for hemolysis and platelet activation further increase the risk of thrombus formation via the extrinsic pathway [43, 49, 52]. High-risk areas include regions where blood experiences high shear stress and places where blood damage occurs due to the closing of mechanical structures [43]. Once thrombus is formed it can attach and interfere with valve function or embolize and cause problems downstream. The overall incidence rate of these complications is 0.3% -1.3% and 0.6% - 2.3% per patient year, respectively [49].

1.4.3.2.2. Anticoagulant Related

Patients who receive an MHV replacement are required to undergo warfarin therapy to reduce the risk of thrombus related complications. The level of anticoagulation required by a patient is measured using INR, which stands for international normalized ratio. This is a ratio of the patient’s blood clotting time in reference to normal clotting time and must be monitored closely [53]. Since anticoagulant therapy increases the risk of bleeding complications, attempts are made to administer the lowest therapeutic amount. Still, anticoagulant related hemorrhages occur at a rate of approximately 1% per patient year [49].
1.4.3.2.3. Pannus Overgrowth

Failure from pannus overgrowth occurs when a hyperplastic tissue response causes abnormal growth across the sewing ring [54]. Chronic inflammation at the site of the implant causes macrophages to merge into multi-nucleated foreign body giant cells in an attempt to phagocytose the valve material [55]. This inflammatory response triggers the release of various growth factors and cytokines that prompt sustained tissue growth. The end result is tissue hyperplasia and a myointimal protrusion that causes narrowing of the lumen or prevention of normal valve function [54,55,89]. This occurs mainly on the ventricular side of aortic valves and is the primary cause of valve obstruction being much more common than thrombus related interference [54].

Figure 10. [A] Obstructive thrombosis in tilting disc valve. [B] Obstructive pannus overgrowth in bileaflet valve. [C] Strut fracture in Björk-Shirley tilting disc valve. [49]

1.4.3.2.4. Infective Endocarditis

Patients with prosthetic valve implants are predisposed to contraction of infective endocarditis, particular in the early post-operation period [36,56]. The incidence rate of infective endocarditis is similar in mechanical and bioprosthetic heart valves and is associated with a patient mortality rate of approximately 50%, even with surgical and medical intervention [32,56]. The disease itself is similar to that of the native valve and a
detailed discussion is provided in section 1.3.2. However, in mechanical valves organisms cannot attach to the surfaces of the valve structure itself, which cannot be destroyed. Attachment to the surrounding annulus and sewing ring is still possible, that can lead to detrimental tissue destruction and vegetation growth [56].

1.4.3.2.5. Patient Prosthesis Mismatch

Rahimtoola first described patient prosthesis mismatch (PPM) in 1978 and this phenomenon has been associated with increases in valve replacement mortality rates. PPM is used to describe circumstances where a patient has received a valve prosthesis that excessively impedes blood flow below the amount required by the patient [54,57]. High transvalvular pressure gradient is the primary consequence of PPM. This increases the workload of the heart and prevents regression of ventricular hypertrophy. These are, as mentioned before, common precursors to heart failure [32,57]. The indexed effective orifice area (EOAI), a common predictor of PPM, should ideally be no less than 0.85 to 0.90 cm²/m² in order to avoid PPM complications [43,57].

$$EOAI\left(\frac{cm^2}{m^2}\right) = \frac{Q_{rms}}{51.6\sqrt{\Delta p}}$$

BSA = Body Surface Area
$Q_{rms}$ = Root mean square systolic/diastolic flow rate
$\Delta p$ = Transvalvular pressure drop [43,57]

**Figure 11.** Equation for indexed effective orifice area.
1.4.3.2.6. Structural Failure

Structural failure in mechanical heart valves is extremely rare. Many models have experienced hundreds of thousands of implantations without experiencing a single instance of structural malfunction. However, certain models have been known to produce small, but significant, amounts of valves that succumb to some form of structural failure. Failure modes have included leaflet fracture, strut fracture, and leaflet escape [48]. Mechanical failures in MHVs are primarily attributed to three causes [54]:

- Elevated closing load that exceeds the valve material’s fatigue strength.
- Material fatigue
- Cavitation (Phenomenon that causes erosion and pitting via microbubbles [61].)

Experience from past models has lead to increased knowledge about these failure modes sparking necessary material and design improvements. For this reason, structural failure of MHVs has been largely abolished and continued efforts aim to eliminate it completely.

1.4.3.3. Current Developments

Current developments in MHVs aim to improve upon flaws in fluid mechanics by altering the structural designs. This will hopefully lead to increased hemocompatibility and, in turn, the amount of required anticoagulation therapy. The ATS Open Pivot® is a recent innovative bileaflet valve that claims to possess an improved hinge design. This valve has undergone trials with patients adhering to reduced anticoagulation (INR = 1.5 – 2.5) with good results [58]. Also, trileaflet valve designs are now emerging that promise better central flow, improved hemodynamics, lower impact stresses, lower amounts of regurgitation, and reduced risk of cavitation [59,60].
1.4.4. Biological Heart Valves

Biological heart valves are those that are fully or partially composed of biological tissues and include those derived from both human and xenogenic sources. Other than mechanical heart valves, bioprosthetic heart valves (BHV) are the only other replacement option currently available. These valves are hemodynamically superior to MHVs due to their anatomical and physiological similarities, and do not require anticoagulation therapy. Even so, the rate of thrombus related complications for BHV recipients is similar to that of MHV patients under full anticoagulant therapy [5]. However, biological valve replacements are far less durable with the majority of valves succumbing to structural dysfunction within 12-15 years [62].

1.4.4.1. Human Tissue Valves

Human tissue valve replacements consist of allografts (a.k.a. homografts) and autografts. Allografts are typically cryopreserved aortic valves that come from organ donors or cadavers [62,69]. The most common cryopreservation technique involves the use of 10% dimethyl sulfoxide (DMSO) as a cryoprotectant prior to storage in liquid nitrogen. However, it is debated whether the use of cryoprotectants improves cell viability or properly preserves ECM components [63]. Regardless, these valves experience extremely good hemodynamics, but suffer from problems with long-term durability, as with all biological valve replacements [62,69].

The most common form of autograft occurs when the patient’s diseased aortic valve is excised and their pulmonary valve is relocated to the aortic position in its place. This method is referred to as the Ross procedure and is normally used in children with
diseased aortic valves. This form of replacement gives the child a living aortic valve replacement of his or her own tissue with the ability to repair, remodel, and grow with the patient. However, a cryopreserved pulmonary valve allograft is still used in this case, which carries with it issues of long-term durability [62,69]. Regardless, this procedure may still be beneficial for children considering the drawbacks of pediatric BHV or MHV aortic valve replacements.

Both allograft and autograft procedures allow the patient to have freedom from anticoagulation therapy without significant risk of thrombus related events. However, they both also suffer from eventual structural degeneration and lack of long-term durability. These advantages and disadvantages are present for all biological valve replacements, though drawbacks of human tissue valves also include low availability [62,69]. Allografts and autografts may have their place in certain patients and procedures, but with the increasingly large volume of valve replacement procedures performed each year, this option cannot be viable as a solution for the general public.

Figure 12. Aortic Homograft after preparation [69].
1.4.4.2. Xenogenic Tissue Valves

Currently, there are two primary types of xenogenic tissue that are used in BHV fabrication. These include valves made from whole porcine aortic valves (PAVs) and those crafted from cuts of bovine pericardium (BPVs) \[5,6,71\]. Since xenogenic tissue is subject to severe immune rejection, the tissue antigenicity must be removed prior to implantation. Glutaraldehyde treatment sufficiently diminishes tissue antigenicity as well as crosslinks the tissue, which protects against proteolytic degradation \[5,71\]. Glutaraldehyde treatment of these valves is standard, but treatment methods and valve designs can vary greatly. There are many BHV models available today and, although widely studied, it is still largely unclear which valves yield the best clinical results.

1.4.4.2.1. Mounting Types

Valves of each tissue type can be mounted on a stent or manufactured without. BHV stents vary by model, but are made from metal or plastic covered in fabric and typically have three posts with a sewing ring around the base \[5,6,71,72\]. Rigid metal stents were initially used, however, tears at the commissures in proximity to the posts sparked a movement towards the use of flexible stents. These mostly polymeric stents still failed to the same tears, but now also suffered issues from stent creep and deformation. For the most part, this has now been eliminated due better material selection and technology, but the benefits of flexible stents are still debatable \[71\].

Stents are used to make valve handling easier, helping to maintain proper leaflet shape and alignment from the time of manufacture. This stability makes valve function more predictable allowing valves to function in the patient as they would *in vitro*. 

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Surgical implantation of stented valves is also much less technical, as these valves are relatively easily attached to the annulus using sutures [71]. Although the use of stents has many benefits, the resulting increased profile reduces valve EOA. This creates undesirable transvalvular pressure gradients and has sparked the use of stentless BHVs.

**Figure 13.** Various stented BHV valve designs. [A] Hancock II™ (PAV) [B] Carpentier-Edwards (PAV) [C] Perimount (BPV) [73].

The benefit of stentless BHVs lies simply in its ability to use a larger valve size than a stented valve for any given patient. This increases the EOA of the valve replacement, lowering transvalvular pressure gradients and better supporting ventricular mass regression [5,71]. In fact, a short-term follow-up demonstrated 30% lower ventricular mass index in patients implanted with stentless valves [74]. However, these valves lack the benefits of stented valves and are much more difficult to handle and implant. There is also evidence that these valves will be more prone to failure from aortic wall calcification and pannus overgrowth. This is because increased proximity and contact of the xenogenic wall tissue with the host may additionally increase immunological and inflammatory responses [5]. The clinical benefit of stentless valves is promising but uncertain and only the most highly skilled and experienced surgeons can perform implantations [71]. For these reasons, more follow-up studies are needed to justify the increased technical difficulties that accompany these valves.
1.4.4.2.2. Tissue Types

The use of porcine aortic valves to construct BHVs is possible due to the high amount of similarity between human and pig cardiac anatomy. In pigs, a muscle shelf supports the right coronary cusp and there is a lesser extent of mitral valve to aortic valve fibrous continuity, but otherwise, the aortic valves of humans and pigs are very comparable [70,72]. Valves made from porcine aortic valves (PAVs) use the aortic valve cusps and varying amounts of aortic wall. The primary structure of PAVs is based on the normal anatomy and remains mostly the same between models; however, there are still many variations in design as can be seen in the figure above (Figure 15: B and C).

Stented PAVs differ primarily in stent material and stent design. The fabric coverings and sewing rings are made from Dacron and PTFE with the occasional additions, such as silicone inserts. The stents themselves are made from various metals and polymers and most resemble the typical three-post design (Figure 14). However, some valves display vast differences in stent design, such as the BioImplant PAV valve that attempted a vast reduction in stent profile (Figure 16). Some valves also incorporate

Figure 14. Various stentless BHV valve designs. [A] Freedom Solo, Sorin Group (BPV) [B] Prima Plus, Edwards (PAV) [C] NR200, Shelhigh (PAV) [73].
metals into their sewing rings or stents to render them radioopaque [72]. Most stentless PAVs have very minimal additions to the aortic root or any portion of the porcine aortic valve, although some of these valves have minimal cloth coverings and diagrams that aid in trimming, fitting, and implantation [72,74]. Lower transvalvular pressure gradients, better LV mass reduction, and less structural tissue failure has been seen in stentless PAVs, but additional follow-up studies are still needed to validate these benefits [74].

As mentioned above, there is a muscle shelf supporting the right coronary cusp of the porcine aortic valve that is an extension of the ventricular septum [5,70]. This muscle tissue inhibits valve motion and is thought to be a promoting site for calcium deposition [5,71,72]. Therefore, in an effort to reduce these ill effects, some PAVs remove this valve cusp and replace it with an essentially congruent cusp from another valve. This type of PAV is referred to as a composite valve. Other variations of composite valves include tricomposite valves, which use three noncoronary cusps from separate valves. In this design, cusps are matched up for proper geometries to achieve optimal coaptation [5,71,72]. These valves have shown fine clinical performance, but more follow-up studies are needed to determine the long-term benefits of this approach [71].

Figure 15. Low-profile stent PAV, BioImplant [71].
Like the porcine aortic valve cusp, bovine pericardium is a specialized tissue with three distinct layers. The inner layer that faces the heart is a smooth layer protected by visceral mesothelium. The middle fibrosa layer contains collagen, elastic fibers, vasculature, lymphatics, and nerves that comprise the majority of bovine pericardial tissue thickness. Loosely arranged collagen and elastic fibers are abundant in the outer layer, which must be cut away from tissue that surrounds the heart. This trimming leaves the outer layer rough, especially when compared to the smooth visceral side. Pericardial BHVs are constructed so that this rough outer layer is positioned facing the inflow. This is to ensure that the rough pericardial surface is thoroughly washed, which helps to prevent thrombus related complications [5].

Sheets of bovine pericardium permit the production of virtually any needed shape allowing for enormous play in BPV design. Since they are not set in their primary structure, like PAVs, BPVs can make modifications as substantial as the number and shape of the cusps. A very popular stented BPV design is a folded cylindrical design (Figure 14: C) [71,72]. This design allows stented BPVs to be formed from multiple cuts of pericardium or molded from a single section (Figure 17) [6,71].

BPVs may have slightly better hemodynamics than PAVs due to the completely unobstructed flow offered by the cylindrical open position [71]. This may cause them to express slightly slower degradation giving them superior durability; however, BPVs often suffer from inferior biomechanics, making them prone to commissural tears and single leaflet prolapse [71-73]. Despite claims of valve superiority for each type, current overall rates of complication for both PAVs and BPVs are similar to that of MHVs, with a 50-60% rate of death or reoperation after 10 years [5].
1.4.4.3. Bioprosthetic Heart Valve Failure

Failure in BHVs occurs differently between types, but there are general events associated with contribution to valve failure that affects all varieties. These include non-calcific structural damage and deterioration, calcification, immunological and inflammatory responses, paravalvular leaks, pannus overgrowth, infective endocarditis, and thrombus related complications. Structural deterioration and calcification are the primary contributors to BHV valve dysfunction, with the majority of BHVs succumbing to some form of degenerative disease within 12-15 years [5,62].

Degenerative modes of BHV failure are highly age and time dependent with lack of long-term durability, presenting the biggest drawback for BHVs, especially in younger
patients. Short-term experience in adults has been excellent with less than 1% of implanted PAVs failing within five years. Failure rates increase to 20-30% at ten years and are capped at around 12-15 years, at which point 50% of implanted valves fail. These numbers are exacerbated in younger patients where nearly 100% of valves fail prior to 5 years in individuals under 35 years old. When also considering that ten-year failure rates fall to 10% in patients over 65, the age-dependence of BHV structural degeneration rates becomes obvious [5,62]. Failures from degenerative sources are most commonly expressed as valvular insufficiencies from cusp tears, affecting roughly 75% of PAVs. Purely stenotic valves, due to cusp damage or stiffening, occur much less often at a rate of approximately 10-15% [5].

Non-degenerative events also make contributions to valve failures and are important, but thrombus related complications, pannus overgrowth, and infective endocarditis are not discussed in this section. The rate of thrombus related failures in BHVs are similar to that of MHVs in patients under full anticoagulant therapy. This is an accepted risk, and when compared to other modes of BHV failure, this problem is minor. Infective endocarditis rates are also similar in both BHVs and MHVs at 1-6% and although this may still be cause for worry, thorough discussions of infective endocarditis are already provided in sections 1.3.2 and 1.4.3.2.4 [56]. Pannus overgrowth was also discussed earlier in section 1.4.3.2.3, but section 1.4.4.3.3 provides further discussion. All in all, the underlying cause for most modes of valve failure remains the loss of cell viability within BHVs. This disallows normal healing and repair patterns as well as removes the ability for the maintenance of cellular and extracellular matrix homeostasis; all of which are critical to preservation of normal valve function.
Structural Deterioration (Non-calcific)

Gradual structural deterioration of BHVs occurs primarily due to changes in natural valve biomechanics and loss of cell viability. These changes cause localized stresses in the tissue that can eventually lead to dysfunction in the form of tissue buckling or cusp tears [5]. This localized damage may progress slowly, but progresses unrestrained since BHVs lack the ability for repair. Although this form of cusp damage is sometimes difficult to discern from calcific effects, since they often occur together, non-calcific structural deterioration has been shown to play a major role in BHV failure, independent of calcification [81]. In fact, roughly 90% of PAVs fail from some form of structural damage with many showing little to no signs of calcification [76].

The initiation of tissue deterioration begins before implantation, during tissue harvesting and preparation where glutaraldehyde fixation is thought to play a large role. Glutaraldehyde causes loss of cell viability and, upon cell death, ECM degrading proteases may be released, which cause irreparable damage to the ECM ultrastructure. Degradation of collagen fibrils has been observed after fixation, as well as loss of collagen associated GAGs and PGs [75]. Increased levels of ECM degrading enzymes have also been observed in explanted valves, which further contribute to collagen damage and loss of GAGs [82,83]. Glutaraldehyde treatment does not fully prevent enzymatic activity and both early and chronic enzymatic presence could have devastating effects on ECM integrity [5]. Given the fact that loss of cell viability also eradicates the ability for ECM repair, current methods of tissue preparation seem inadequate to sufficiently protect the ECM in BHVs.
Changes in valve biomechanics also begin with glutaraldehyde fixation. Glutaraldehyde effectively removes tissue antigenicity and cross-links collagen to support preservation of ECM integrity. However, formation of collagen cross-links paired with lack of ECM remodeling capability results in a locked ECM architecture that is unlike its natural dynamic state [84,85]. This results in irregular cusp movement and increased cusp rigidity that cause increased stresses, principally at points of high tissue flexion [5,77]. In PAVs, this is compounded by the loss of GAGs from BHV cusps, which help cusp tissue cope with high compressive stresses. Glutaraldehyde treated BHVs have been shown to lose GAGs both in vitro and in vivo [19,30]. GAGs in the spongiosa also provide a shear stress dissipating buffer layer that accommodates motion between the ventricularis and fibrosa. Depleted spongiosa function has detrimental effects on BHV biomechanical function and further promotes increased tissue stresses and susceptibility to mechanical damage, such as delamination [5,15,18,31,77].

![Figure 17. Progressive delamination due to cyclic fatigue [77.]](image-url)
1.4.4.3.2. Calcification

Valve dysfunction due to calcification and related tissue damage is a prevalent mode of BHV failure, second only to non-calcific mechanical damage [54]. Calcification pathways are not all fully understood, though tissue damage, host responses, and implant characteristics are all known to contribute to the process. Calcification occurs in two phases: initiation/nucleation and propagation of calcific crystals. Initiation most commonly begins below the tissue surface and is referred to as intrinsic calcification [5,78]. Extrinsic calcification also occurs, which can lead to thromboembolic complications, but this is much more rare [78]. As calcific crystals propagate and coalesce, tissue microstructure begins to deteriorate and stiffness and/or cusp tears may occur [5,75,78]. Calcification contributes to structural failure over time and affects the long-term durability of BHVs. However, progression of calcification is greatly accelerated in younger patients and also contributes to the high short-term failure rate for this group [5,78]. Therefore, better understanding of how to prevent this mode of failure could greatly expand upon the useful applications of BHVs.

Formation of calcific nodes in BHVs is thought to be a result of loss of cell viability. Under normal circumstances, low intracellular calcium levels are maintained during standard cell metabolic processes. Upon cell death, this ability is lost allowing high concentrations of calcium to saturate remnant cell debris [5,75,78,79]. Mineralization begins when this influx of calcium reacts with sources of organic phosphorus that remain behind, including that from organelles, nucleic acid backbones, and cell membranes [78]. Red blood cells and inflammatory cells may also penetrate the tissue and accumulate, providing additional nucleation sites for crystal growth [5].
Cellular debris represents the most predominant nucleation site for BHV calcification. Extracellular matrix components are also subject to calcification, although modes are less understood [5]. Collagen fibers can serve as nucleation sites for calcification, however, only after crosslinking with glutaraldehyde [5,62,75,78,79]. Elastin calcification also occurs, but does not depend of the presence of crosslinking [5,75,78]. This may be a result of the loss of elastin-associated microfibrils, which shield elastin from calcification. These microfibrils are lost during tissue harvesting and preparation, possibly due to enzymatic hydrolysis by fibrillin degrading matrix metalloproteinases (MMPs). Conversely, glycosaminoglycans may inhibit calcification by chelating calcium to prevent nucleation or sterically through structural interferences with hydroxyapatite although this is controversial [75]. However, this has been supported by studies in the rat subdermal model where GAG loss was shown to promote calcification, whereas GAG fixation showed inhibition [86-88].

GAG retention may also reduce calcification through improvements in BHV biomechanics. Regions of increased mechanical stress are known to stimulate calcification, and these areas regularly harbor calcium deposits in BHVs. Maximum leaflet flexion occurs at the commissures and basal attachments, and both extrinsic and intrinsic calcification is prevalent at these sites of severe tissue deformation [5,62,78]. Loss of GAGs and glutaraldehyde treatment are thought to increase susceptibility to calcification; however, glutaraldehyde fixation is still a necessary standard for BHVs, and GAGs are not preserved through this process.
**Figure 18.** Flow of calcification as it progresses from nucleation to BHV failure [78]

**Figure 19.** [A] View of fibrosal side of porcine BHV showing calcification and tears after 8 years of implantation in the mitral position [62]. [B] Movat Pentachrome stained section of porcine BHV cusp showing calcification (9 years, aortic position) [62].
1.4.4.3.3. Inflammation and Immune Responses

The contribution of inflammatory and immune responses has been mostly ignored as a source of BHV failure. It has long been known that standard glutaraldehyde fixation using low concentrations does not eliminate antigenicity, but merely reduces it (approximately by 59%) [5,54,90,91]. Even though antibodies against tissue glycoproteins and inflammatory cells have been noted in failed valves, the link between these responses and BHV failure has been unclear [5,89]. These subjects have recently gained attention as being worth significant research, but the results of various studies have been conflicting. Still, enough evidence has been put forth to warrant further investigation into the role of inflammation and immune responses in BHV failure.

Remnant immunogenicity after BHV tissue fixation is a result of glutaraldehyde’s failure to alter membrane bound receptors or structural glycoproteins [54]. This insufficient removal of antigens is thought to result in an increased inflammatory response that contributes to decreased durability in BHVs. Almost monolayer coverage by inflammatory cells is found in most explanted BHVs, and it has been proposed that this is caused by proinflammatory characteristics of glutaraldehyde itself [89,92]. This has been refuted by many studies that demonstrate the use of increased glutaraldehyde concentrations to completely suppress immunogenicity will greatly mitigate recruitment of inflammatory cells [54,89]. Recruitment of phagocytes is proposed to occur via chemoattractants released through opsonization. This occurs through nonspecific mechanisms via the system of circulating complement plasma proteins as well as through more specific means that involve the humoral immune response and circulating antibodies against BHV tissue glycoproteins [54,55,89].
There is evidence that these phagocytes contribute to tissue damage leading to BHV failure. Studies have shown that intense amounts of phagocytes inhabit valves that have experienced failure from tearing [89,93,94]. Also, Dahm et al. demonstrated an increased percentage of patients showing BHV component specific antibodies in failed valves when compared to patients with functioning valves [95]. Direct observation of collagen phagocytosis by macrophages using transmission electron microscopy (TEM) was also found in 82% of failed BHVs [96]. To the same end, presence of digested collagen was discovered in the cytoplasmic vacuoles of invading macrophages during other studies [97].

Evidence has also suggested that inflammatory and immune responses play roles in other modes of BHV failure as well. The inflammatory potential of BHV tissue may add to the amplification of pannus growth [89]. This is supported by data showing a five times reduction in pannus length after six months of implantation in the sheep model with BHVs fixed using higher glutaraldehyde concentrations to suppress tissue antigenicity and associated inflammatory response [98]. Various groups have also demonstrated links to calcification. Vincentelli et al. showed a 35 times increase in calcification when comparing implanted xenogenic tissue to autologous tissue in the sheep model [99]. Another study provides further evidence of tissue antigenicity’s role in calcification by demonstrating that tissue from antigen knockout pigs expressed reduced calcification after incubation in human antibodies when compared to normal porcine tissue [100].
1.4.4.4. Current BHV Research

Since structural damage and calcification are the primary modes of BHV failure, most of the current subjects of BHV research concentrate on overcoming these obstacles. Main areas of research include: new tissue fixation methods, new BHV materials, and anti-calcification treatments. New fixation methods are currently being researched to supplement, alter, or even entirely replace glutaraldehyde fixation. This approach aims to improve the chemistry, micro-architecture, and biomechanics of the BHV ECM in order to achieve better long-term preservation. The search for new BHV materials is similar in that improved tissue structure and function is sought to achieve improved long-term durability. In fact, porcine vena cava is currently being researched by a member of our lab as a possible alternative to bovine pericardium.

Large investments of research have been placed on anti-calcification treatments due to the relative ease of integration when compared to completely changing material or fixation chemistries. Anti-calcification pretreatments primarily function by targeting the first phase of clinical calcification, the nucleation of calcific crystals. This can be done by using inhibitors to interfere with mineral development or through removal of sites conducive to crystal formation. Biphophonates, amino-oleic acid, and trivalent metal ions such as aluminum or iron chloride are used to prevent or inhibit calcium phosphate crystal formation; while ethanol, decellularization processes, and surfactants are applied to remove organic phosphates and other remnant cellular debris that is known to promote valve calcification [5,12,78,80].
Percutaneously implanted heart valve research represents a branch of improved BHV design. Instead of focusing on improving the durability of BHVs, the use of these valves merely enables us to better cope with current durability problems [54]. Insufficient durability of a BHVs leads to either failure of the valve or the need for reoperation and replacement of the diseased bioprosthesis. Current replacement surgeries are highly invasive and repeat operations are extremely undesirable. Application of catheter based BHV delivery could drastically reduce surgery related patient morbidity and mortality rates [102]. This would allow for easier replacement of diseased BHVs as well as enable a wider range of the population to be acceptable BHV candidates [101]. The process involves crimping a BHV down to deliverable size and expanding it at the desired site via a balloon expandable or self-expanding nitinol stent. Although simple in concept, all aspects of implantation, including valve compression, delivery, and valve function, present problems. However, new percutaneous valves are being developed and this tactic seems to represent the next generation of BHV design [54,101,102].

**Figure 20.** [A] Self-expanding nitinol pericardial BHV by CoreValve [101]. [B] Edwards Sapien® balloon expandable BHV [101]. [C] Depiction of catheter and transapical access in a BHV leaflet replacement procedure, ValveXchange® [54].
1.5. **Heart Valve Biomechanics**

Lack of durability is currently the primary drawback plaguing BHVs. Barring recipient death or malfunction from less prominent sources, failure due to calcification and/or structural deterioration is inevitable. Much of current BHV research has targeted reduction of calcification through alternative fixation methods and anti-calcification treatments. If anti-mineralization strategies progress to the point of 100% protection against calcification, stability and preservation of the collagenous skeleton would then become the limiting factor in BHV durability [122]. Already, 90% of current porcine aortic valve BHVs fail due to structural damage, many without signs of significant calcification [76,123]. As discussed previously, BHV tissues lack the ability to repair. This causes all mechanical damage to be cumulative and leaves prevention as the only solution [122]. This damage may also contribute to failure by facilitating proteolytic degradation, further suggesting that prevention of this damage may be critical to increasing the lifespan of BHVs [123]. The occurrence of this damage is highly dependent on cusp mechanics. Investigation into the biomechanics of both natural and fixed valves could provide not only insight into the mechanisms of structural degradation but also supply a knowledge base for the development of prevention strategies [124].

1.5.1. **Native Valve Biomechanics**

Normal aortic valve function is primarily passive, naturally opening and closing due to transvalvular pressure changes and direct interaction with the bloodstream. However, the term ‘passive’ seems terribly inadequate when describing the complex interactions occurring within the specialized tissue layers of aortic valve cusps during
normal function. Internally the tissue is very dynamic, constantly adapting to accommodate the changing mechanical loads [4]. There are three loading modes that accompany normal aortic valve function including tension, flexure, and shear. Tension occurs most prominently during valve closure when the valve cusps must expand in order to properly coapt. Shear forces are at their highest when the valve is open and blood is rushing parallel to the surface of the ventricularis at high velocities, while flexure occurs during the transitions [29].

Collagen, which is primarily found in the fibrosa, is the protein that most effectively deals with tension. Proper coaptation is passively facilitated through the characteristics and structure of collagen fibers in this layer. The circumferentially aligned collagen fibers provide resistance during full cusp expansion and help maintain proper shape. These taut fibers prevent sagging and prolapse while also aiding in stress transfer to the annulus [122]. The low resistance of collagen fibers to torsion and bending imparts flexibility in the radial direction, which aids in dealing with rapid radial strain changes. Macroscopic collagen corrugations as well as microscopic collagen crimp also lend additional extensibility further enabling cusp expansion and mitigating incurred stresses [4].

The natural curvature of aortic valve cusps is partly maintained by preload present in the ventricular and fibrosa. The fibrosa is naturally loaded in compression while the ventricularis is in tension [14,28]. The high elastin expression present in the ventricularis utilizes this to provide elastic recoil, which aids in proper opening. This action also minimizes contact with shear forces by reducing the available surface area during the open state [5,11]. Flexure occurring during the transition phases causes internal stresses

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that are attenuated by the spongiosa. Uneven deformations between the ventricularis and fibrosa cause high internal shear stresses to develop at the common boundary. The hydrated GAGs within the spongiosa provide lubrication that alleviates these stresses and promotes smoother bending. Areas of elevated bending that do manifest, harbor high amounts of compressive stress. When left unabated, tissue buckling can occur at these points of high stress causing tissue disruption that ultimately contributes to overall valve failure [126]. However, the hydrated state of GAGs also allows for absorption of compressive loads further providing protection against damage from flexure [15, 31, 125].

1.5.2. BHV Biomechanics

During glutaraldehyde fixation many alterations occur to aortic valve cusp tissue that may negatively affect tissue mechanics and, in turn, valve function and durability. The formation of crosslinks is essential to the preservation of the collagen structures. However, they also lock the microstructure into a static formation, which prevents the structural rearrangements that accompany normal function. The dynamic collagen rearrangements occurring during normal function, along with extension of crimp, are very responsive to transvalvular pressures. Rearrangement has been shown to lead to full collagen alignment at pressures as low as 4 mmHg, while collagen crimp is largely abolished by 20 mmHg [122]. Zero-pressure fixation is thought to minimize the consequences of a static ECM by preserving collagen crimp and stress free arrangement. While lower pressure fixation has been beneficial, it was demonstrated that the benefits of fixation below 4 mmHg is lost early under normal function. Substantial alignment of
collagen was shown to occur during the first 50 million cycles of *in vitro*, which also correlated with decreased tissue extensibility [123].

Increased cusp stiffness also occurs as a result of the crosslinked, static ECM and leads to increased compressive stresses during flexure. Areas of high flexural stresses have been demonstrated to generally coincide with increased calcification and have been stated by Broom to be the primary culprit of non-calcific tissue damage [124,127]. The penalty of these compressive stresses is compounded by the loss of GAGs, which diminishes resistance to tissue buckling [31]. Tissue buckling is further promoted by increased internal shear stresses resulting from GAG loss, which may also induce delamination of the tissue layers. The end result is the accumulation of damage to collagen fibers that eventually leads to BHV failure [15,31,75,77,125].

**1.5.3. Characterization of Biomechanics**

Basic characterization of the mechanics of BHV tissue begins with uniaxial tensile testing. When subjected to tensile loads, porcine aortic valve cusps display a non-linear stress strain curve demonstrating two clearly separate phases of mechanical response (Figure 22). The initial low-stiffness response is dominated by elastin contribution and presumably includes elastin stretching, collagen uncrimping, fiber rearrangement, and extension of corrugations. The secondary high-stiffness response is dominated by collagen’s input and the higher modulus in this phase is indicative of the properties of extended collagen in tension.
High-end (collagen) region:

Low-end (elastin) region:

**Figure 21.** Stress strain curve obtained from uniaxial tensile testing on fresh aortic valve cusps in the radial direction.

Simple tensile testing yields important information on the general extensibility, stiffness, and strength of the tissue. However, it is far from physiologic and cannot be a sole indicator of full-scale valve performance. Biaxial testing overcomes some of the downfalls of uniaxial testing and provides a more complete insight into planar tissue mechanics [28]. This testing procedure enables coupled analysis of the anisotropic behavior of the tissue, but again has its limitations. Flexural testing allows additional insight into tissue mechanics during bending behaviors, providing more information on the major deformation mode of aortic valve leaflets [128]. Studying the energy dissipation during loading and unloading, or hysteresis, may also be beneficial as it can shed light on the viscoelastic characteristics of the tissue [129]. This may be important in illustrating the significance of GAG loss and preservation in BHVs as hyaluronan and chondroitin sulfate have been demonstrated to affect the viscoelastic properties of the ECM and associated fluids [130]. Together these tools can provide us with a very comprehensive model for aortic valve cusp tissue mechanics.
1.6. **Aldehyde Fixation Methods**

1.6.1. **Formaldehyde Fixation**

Formaldehyde is a very commonly used biological tissue preservative for many lab practices. It is water soluble up to a saturation point of about 40% and aqueous solutions are commonly referred to as formalin. Prior to the induction of glutaraldehyde as the “gold standard” fixative for BHVs, formalin treatments were also considered as contenders. Like glutaraldehyde, formaldehyde crosslinks by reacting with the amine groups present on collagen molecules within BHV tissue. Tissues treated with formalin solutions in concentrations from 0.5% to 10% were found to possess the desired tissue stability for BHV construction. However, since formaldehyde is only monofunctional, the collagen crosslinks formed are unstable and can be reversed under dynamic conditions and during storage. This phenomenon lead to unsatisfactory long-term performance of formalin treated BHVs, heralding glutaraldehyde as a much superior BHV fixative. Still, the low concentrations of glutaraldehyde used for BHV fixation can be insufficient to completely sterilize the tissue, leaving formalin treatment to remain in use as a post-fixation step for glutaraldehyde fixed BHVs.

**Figure 22.** Simplified representation of collagen crosslinking by formaldehyde [103].
1.6.2. Glutaraldehyde Fixation

Glutaraldehyde is a water soluble, bifunctional aldehyde that is currently the industry standard fixative for bioprosthetic heart valve tissue. Glutaraldehyde renders BHV tissue resistant to autolytic degradation by the creation of Schiff-base crosslinks between primary amines present on lysine and hydroxylysine of collagen molecules [5,103]. Glutaraldehyde is also able to polymerize through aldol condensation, allowing the formation of crosslinks of variable length [103]. In addition, partial sterilization and immunogenicity masking is accomplished through glutaraldehyde treatment, which is necessary to prevent infection and immune rejection [5,6,54,71]. Various glutaraldehyde treatments have been used since the 1960s and continue to demonstrate their effectiveness, with the majority of adult implanted glutaraldehyde fixed BHVs currently lasting 12-15 years before failure [62,103]. However, glutaraldehyde fixation has been linked to various modes of BHV failure and this process has repeatedly been under fire as an area for improvement or replacement.

Glutaraldehyde is widely known to compromise the normal tissue mechanics of the aortic valve. However, various pressure fixation techniques have been developed to help reduce this phenomenon. First generation porcine BHVs were fixed under a backpressure of 80 mmHg, similar to natural diastole. This fixed the valve in the closed position, which yielded a visually pleasing, smooth cusp shape and proper coaptation. However, high-pressure fixed BHVs were soon found to have inferior biomechanics and durability due to excessively rigid cusps. The high-pressure fixation technique was found to extend macroscopic collagen corrugations as well as microscopic crimp, therefore locking collagen fibers into an elongated position and greatly reducing cusp flexibility.
Following generations have employed low-pressure and zero-pressure fixation techniques that possess improved tissue biomechanics and overall long-term durability [71].

Despite fixation process improvements, collagen microarchitecture still becomes locked into its initial fixed configuration, preventing the dynamic ECM restructuring that accompanies normal valve function. This results in stiffer, less compliant tissue that is more prone to tissue buckling and other localized mechanical stresses [5]. Tissue mechanics are further skewed by the loss of elastin and GAGs, other important cusp components that contribute to normal function. These molecules do not possess the amine functionalities necessary for crosslinking by glutaraldehyde and are lost during preparation, storage, and function. GAG loss stems primarily from the spongiosa layer, which normally functions to reduce internal shear stresses, absorb compressive stresses, and prevent tissue buckling [15,18,19,25,31,75]. The loss of these important functional molecules and the resulting changes in tissue mechanics may contribute to structural deterioration, the foremost cause of BHV failure.

Glutaraldehyde fixation has also been linked with calcification, the other major means of BHV failure, as was previously discussed in section 1.4.4.3.2. Although mechanical damage has a well-known synergy with calcification, glutaraldehyde fixation has also been directly linked to calcification through various other pathways [5,62,78]. Devitalization and other cell alterations result in the formation of cell debris, which function as nucleation sites for mineralization [5,6]. Glutaraldehyde is thought to stabilize these structures, although some studies using high concentrations of glutaraldehyde has shown shielding effects against calcification [78]. Residual unreacted glutaraldehyde has also been implicated in calcification and the use of amine
neutralization treatments have shown to nullify mineralization [6]. Many other anti-calcification strategies have been researched and were lightly discussed in section 1.4.4.4. However, a more in depth look at one approach, alternative fixation methods, is provided in section 1.8.

![Diagram of glutaraldehyde molecule and its polymeric form](image1)

**Figure 23.** [A] Free glutaraldehyde molecule and its polymeric form after aqueous uptake [B] Simplified example of crosslinking between two collagen molecules using glutaraldehyde (Both figures adapted from [103]).

1.7. **Alternative Fixation Methods**

The perceived contributions of glutaraldehyde treatment to BHV failure have lead to a search for suitable alternatives. Alterations to glutaraldehyde fixation methods as well as replacements have been pursued in recent years. Modifications have ranged from changes as simple as using higher concentrations to more involved strategies that reduce tissue reactivity, block residual aldehyde groups, or incorporate into the crosslinks [78,104]. Some non-glutaraldehyde tissue fixatives that have been investigated include: epoxy compounds, such as triglycidalamine [105]; carbodiimides [103,114-120]; acylazides [103]; poly-glycidyl ethers [106]; reuterin [107]; genipin [108]; glycerol [103]; sodium metaperiodate [88]; diisocyanates [103]; and dye-mediated photooxidation
Many of these fixatives have yielded promising results through preliminary testing, but there has been minimal conversion into clinical use.

The primary focus of many studies involving these new chemistries has been on the reduction of calcification, though there are additional benefits that may also accompany the nature of this strategy. Possible benefits include: potential enhancement to tissue stability, improved biomechanics, and reduction of fixative toxicity. However, the use of entirely new fixation chemistries also risks providing inadequate tissue durability as well as stimulating unknown host responses. BHVs crosslinked by dye-mediated photooxidation and carbodiimides have progressed to clinical trials; however, neither has yet further advanced to regular clinical use. Both of these particularly promising compounds do not become incorporated into the tissue, but merely catalyze the formation of covalent bonds within the tissue. This particular characteristic all but ensures reduced toxicity and early results have demonstrated reduced calcification in tissues crosslinked by each treatment [109-112,116-119]. The use of these new crosslinking methods, along with proper valve construction, may improve the average lifetime of BHVs representing the next evolution in their design.

1.7.1. Dye-mediated photooxidation

Fixation processes using dye-mediated photooxidation are fairly simple and include incubation in the photooxidative dye of interest, followed by exposure to specific wavelengths of light in an aerobic environment. However, the mechanisms behind this method of crosslink formation are not fully understood [111-112]. After light absorption by the dye, excitation initiates the crosslinking response that is theorized to include
reactions involving singlet oxygen and amino acids with the light-excited dye [112]. Methylene blue and methylene green are two dyes that have been investigated for use in BHV fixation [109-112]. Investigation of multiple dyes has also been augmented by exploration of differing wavelengths of light as well as varying lengths of exposure [112].

In addition to demonstrating resistance to enzymatic degradation and calcification, tissues crosslinked using dye-mediated photooxidation have also exhibited low immunogenicity, non-cytotoxicity and seemingly improved physical properties [109-112]. One particular fixation method, PhotoFix®, progressed to clinical trials but was cut short due to excessive regurgitation prior to two years. However, upon explantation, valves consistently exhibited signs of failure due to improper design while tissue performance seemed to be satisfactory [113]. Nonetheless, this has been a setback for the advancement of PhotoFix® and other alternative fixation chemistries; as the previously mentioned study suggests, current testing methods may be inadequate to properly predict clinical performance.

### 1.7.2. Carbodiimide Fixation

Water-soluble carbodiimides have also attracted a great deal of interest for use in alternative fixation methods. A popular pair of carbodiimide crosslinkers that have been investigated for use in BHV tissue fixation are 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). As mentioned previously, these reagents react to produce zero-length bonds within the tissue. This refers to the fact that neither EDC nor NHS remains in the bond after crosslink formation, but merely catalyzes the formation by utilizing available amino acid side groups [103,114-120].
Collagen crosslinking is initiated when EDC activates carboxyl groups on aspartic and glutamic acid, which then reacts with NHS to form a stable intermediate. NHS allows for increased crosslinking yield and facilitates reaction with nucleophiles, such as primary amines on lysine and hydroxylysine of collagen [114,115]. There is also evidence that these activated carboxyl groups can form ester crosslinks with hydroxyl groups, furthermore, in sufficient quantity to significantly affect tissue mechanics [114].

Tissue mechanics is a concern with carbodiimide crosslinked tissue as the zero-length bonds produced by crosslinks have been stated to yield undesirable collagen stiffness [116]. As such, some groups have investigated the incorporation of diamine spacer molecules into carbodiimide crosslinks that create “lengthened” bonds. These strategies aim to both enhance tissue stability and reduce stiffness, although, the majority of these alternative fixation studies have primarily focused on the reduction of calcification [116-119].

The amide bonds formed by EDC/NHS fixation are more stable than the Schiff-base bonds created by glutaraldehyde fixation, and it is theorized that carbodiimide crosslinking may offer superior collagen stability [117]. This theory is also suggested through a hypothesis submitted by Weadock et al., which proposes that improved stability is afforded due to the formation of a larger number of crosslinks using EDC/NHS treatment [115,121]. In addition to the collagen stability afforded by EDC/NHS fixation, GAGs can also be crosslinked through carbodiimide chemistry due to the high amount of available carboxyl groups situated on GAG molecules (Figure 3). The preservation of GAGs may further protect collagen sterically through the blockage of cleavage sites that are vulnerable to enzymatic attack [115]. GAGs may also improve
tissue stability through the improvement of tissue and valve biomechanics. Even slight improvements could delay the onset of fatigue damage and, as a result, slow structural degradation. Also, since EDC/NHS crosslinked tissues have exhibited resistance to calcification, carbodiimide crosslinking becomes an even more attractive alternative to glutaraldehyde fixation for improved BHV durability [116-119].

**Figure 24.** Representation of carbodiimide crosslinking in the presence of NHS [120].

### 1.8. GAG Loss in BHVs

Glutaraldehyde is currently the standard treatment used for the preservation of tissue structure. Although it is effective at collagen stabilization, it fails to preserve both GAGs and elastin because they lack the amine functionalities necessary to be crosslinked. The loss of GAGs has been demonstrated to occur during preparation, storage, cyclic fatigue, and implantation [15,18,19,30,75]. The effects of this GAG loss on the ultimate failure of BHVs is unclear, but has been theorized to contribute to both major modes of malfunction, including calcification and structural damage.

Versican and HA aggregates have been demonstrated as the primary contributors to overall GAG loss. This is most likely due to steric hindrance from side GAG chains preventing the crosslinking of protein cores within these aggregates. Also, these molecules are for the most part unbound, especially in the spongiosa, leading to loss by...
leaching. This is opposite smaller PGs, like decorin, which are better preserved. The protein cores of smaller PGs are less obstructed by GAG chains and are therefore more open to crosslinking [19]. However, given the function of these small PGs, it is unlikely that they would have any beneficial effect on the lifeless tissues of BHVs [17,19,21].

The role of GAG loss in structural damage is thought to be particularly significant and starts with detrimental changes to tissue biomechanics. This primarily concerns the loss of GAGs associated with the PG versican within the spongiosa. The loss of the primary component of this important tissue layer increases internal shear stresses and has been shown to cause increased flexural rigidity [18]. The increased flexural stiffness also brings about increased compressive stresses during flexure, and GAG depleted tissues have been shown to be more prone to tissue buckling [31]. Versican expression has been demonstrated to be highest in the spongiosa, and losses of versican and HA aggregates have been shown to be the primary contributors to overall GAG loss [19,131]. This further supports the fact that versican aggregate loss from the spongiosa is the primary contributor to the deterioration of valve biomechanics.

The loss of GAGs may also contribute to calcification, although indirectly, as areas of high flexural stresses have also been demonstrated to generally coincide with increased calcification [124,127]. More direct relationships are also possible as versican is associated closely with elastin networks and versican expression has been verified to be second highest in the ventricularis [19,131]. Unlike collagen, purified elastin has been shown to calcify heavily, regardless of crosslinking; while versican binding, under normal conditions, has been implicated as an inhibitor to elastin calcification [19]. GAGs
may also prevent hydroxyapatite nucleation through the chelating of calcium, which inhibits reaction with extracellular phosphate [75].

1.9. **GAG Preservation Strategies**

As mentioned in the previous section, it has been demonstrated that GAGs are lost from BHV tissues during preparation, fatigue, implantation, and storage [15,18,19,30,75]. GAGs are thought to be vital to the proper biomechanical function, and discussion of their structure and function has been laid out in sections 1.1.1.2.3, 1.1.1.3.3, 1.2, 1.5, and 1.8. They reside primarily within the central spongiosa layer of cusp tissue and their loss has been attributed to increases in tissue buckling depth as well as flexural rigidity [18,31]. These changes may cause additional undue stresses on BHV tissue, which damage collagen fibers and ultimately lead to BHV failure.

Our lab has attempted to improve GAG preservation using various crosslinking strategies. Sodium metaperiodate and carbodiimide crosslinking using EDC/NHS have exhibited improved GAG stabilization when coupled with glutaraldehyde fixation [30,31,88,132,133]. Sodium periodate has been evaluated without the addition of glutaraldehyde, but was found to provide insufficient collagen stability [88]. Regardless, it has been shown that none of these fixation chemistries were adequate to fully preserve GAGs when subject to enzymatic degradation [30,88,132,133]. Therefore, it was hypothesized that a GAG degrading enzyme inhibitor needs to be employed in order to provide full protection against GAG loss.

Neomycin trisulfate, a hyaluronidase inhibitor, has an ideal structure for this function and possesses multiple amine functionalities that allow it to be incorporated into
BHV tissue by using numerous crosslinking strategies. Neomycin prevents the enzymatic digestion of GAGs both by the blocking of active cleavage sites as well as through binding and interacting with hyaluronidase. The presence of hydrophilic groups in combination with lipophilic residues facilitates this binding and allows neomycin to sterically interfere with the active site of hyaluronidase, subsequently causing a conformational change that renders the enzyme ineffective [31,133].

Neomycin has been demonstrated to supplement GAG protection in cusp tissue when subjected to both cyclic mechanical fatigue and storage. This was demonstrated in tissues crosslinked with both EDC/NHS and glutaraldehyde as well as in tissue fixed using glutaraldehyde alone [30,UD*]. Reduced tissue buckling was found in these tissues, providing further evidence of the importance of GAG preservation [31]. In addition to improved GAG stability, collagen and elastin were also further stabilized by neomycin through unidentified mechanisms [30,UD]. Altogether, the incorporation of neomycin into BHV tissues helps prevent GAG loss, which may contribute to better overall ECM stability. This may increase BHV durability by delaying the mechanisms that lead to structural damage and ultimately BHV failure. (*UD=unpublished data)

**Figure 25.** Structure of neomycin trisulfate (www.sigmaaldrich.com).
2. RESEARCH RATIONALE

2.1. Overview

Lack of long-term durability remains the primary concern for BHVs with the majority failing within 12-15 years after implantation into adults. Although this may be adequate for many patients, BHV implantation can be contraindicated in younger individuals in order to avoid reoperation. Even in elder recipients, valve dysfunction can still cause death or reoperation that could be avoided with increased BHV durability. Therefore, investigation into methods for increased BHV durability is warranted in order to both widen the patient demographic as well as improve the quality of life for BHV recipients.

Improving BHV durability requires prevention of the two foremost causes of failure, calcification and non-calcific structural degradation. Glutaraldehyde crosslinking has been implicated as a contributor to both of these phenomena and has long been a target of improvement or replacement. Investigation into alternative fixation methods has yielded many options, many of which have demonstrated increased resistance to calcification. However, even with a fixation method that offers 100% reduction of mineralization, structural damage from mechanical sources would still lead to failure. This requires the development of fixation strategies that not only reduce calcification, but also resist the accumulation of structural damage.

The accumulation of non-calcific damage is highly dependent on cusp mechanics, and even minor biomechanical alterations can have profound implications on the fatigue life of BHVs. This stems from the lack of tissue repair mechanisms that leads to a cumulative effect for all incurred mechanical damage. Important biomechanical
molecules, GAGs, are not crosslinked by glutaraldehyde fixation and are consequently lost during implantation, storage, and cyclic mechanical fatigue. The increased flexural rigidity and tissue buckling that results from GAG loss may have direct repercussions on collagen integrity, which leads to cusp tears that cause valve insufficiency and failure.

Carbodiimide crosslinking using EDC and NHS is a particularly promising alternative fixation treatment. This method of crosslinking is capable of reacting with carboxyl groups as well as amines, enabling the crosslinking of both GAGs and collagen. This difference in crosslinking chemistry also causes changes to the ECM microarchitecture that may affect mechanical properties. As a result, improvements to tissue mechanics may occur through increased GAG preservation along with differing crosslink architecture. Additionally, tissues crosslinked using EDC and NHS have demonstrated improved resistance to calcification using *in vivo* models.

The crosslinking of GAGs alone may not be sufficient to preserve them, since they are still be prone to loss through enzymatic degradation. The use of neomycin, a hyaluronidase inhibitor, additionally protects GAGs by preventing enzymatic cleavage. Integration of neomycin into tissues is facilitated by the presence of multiple amine groups that permit integration into carbodiimide-initiated crosslinks.

We propose that the addition of neomycin into EDC/NHS fixation will demonstrate superior GAG stability while also exhibiting reduced calcification when compared to glutaraldehyde crosslinked tissue. Cusp tissue will be treated as such and assessed for general tissue stability as well as for the ability to resist GAG loss from all sources. Basic mechanical testing will also follow in order to obtain initial characterization on the effects of this crosslinking method on tissue mechanics.
2.2. Specific Research Aims

2.2.1. Aim I: What is the optimal concentration of neomycin needed to improve GAG preservation during *in vitro* degradation and storage?

**Hypothesis:** GAGs may be stabilized through EDC crosslinking due to bonding of available carboxyl groups to free amine groups on collagen. The presence of bound neomycin may further stabilize GAGs through the prevention of enzymatic degradation.

**Experimental Plan:** Porcine aortic valve cusps will be treated using various concentrations of neomycin and then subjected to enzymatic degradation. Tissues crosslinked using the optimal treatment will be compared to EDC treated tissue with an inert amine functional molecule added in order to verify the specific contribution of neomycin to GAG preservation. GAG stability will be measured after enzymatic degradation and storage. Tissue GAG content will be quantified using hexosamine and DMMB assays, in addition to being visually verified via histology.

2.2.2. Aim II: Does neomycin further enhance EDC crosslinking of collagen and elastin in porcine aortic valve cusp tissue?

**Hypothesis:** The available amine functionalities on neomycin may enable interaction with EDC/NHS during crosslinking. The incorporation of neomycin into crosslinks may affect the stability of collagen and elastin.

**Experimental Plan:** NEN treated porcine aortic valve cusps will undergo collagenase and elastase treatment. Resistance to enzymatic degradation will be used as an indicator of
elastin and collagen stability. Differential scanning calorimetry will also be used to detect any changes in the collagen denaturation temperature of the tissue. Data will be compared to that of a GLUT control as an indicator for baseline performance.

2.2.3. **Aim III: Does neomycin used with EDC crosslinking demonstrate improved in vivo performance?**

**Hypothesis:** The physical crosslinking along with neomycin protection in the NEN treatment may prevent the loss of GAGs due to in vivo degradation. Calcification may also be reduced for the NEN group due to the lack of glutaraldehyde crosslinks.

**Experimental Plan:** Rat subdermal implantation will be used to assess the performance of various crosslinking strategies. Hexosamine assay will be used to quantify the remaining GAGs after implantation, while atomic absorption spectroscopy will be used to quantify mineralization. Results will be also verified visually via histology.

2.2.4. **Aim IV: Does neomycin used with EDC crosslinking affect the stiffness and extensibility of porcine aortic valve cusp tissue?**

**Hypothesis:** The unique crosslinks formed within the NEN treated groups may cause the cusp tissue to exhibit different levels of stiffness and extensibility when compared to fresh tissue and GLUT fixed tissue.

**Experimental Plan:** Uniaxial tensile testing will be used to analyze stress-strain relationships for all groups in both the radial and circumferential directions.
3. MATERIALS AND METHODS

3.1. Materials

Ammonium acetate, calcium chloride, chondroitinase ABC from Proteus vulgaris, collagenase type VII from Clostridium histolyticum, 1,9–dimethylmethylene blue (DMMB), (D+) glucosamine HCl, hyaluronidase type IV-S from bovine testes, chondroitin sulfate C sodium salt, and neomycin trisulfate hydrate were all purchased from Sigma-Aldrich Corp (St. Louis, MO). Acetyl acetone, TRIS buffer, glycine, hydrochloric acid, sodium azide, p-dimethylaminobenzaldehyde, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchase from Fisher Scientific (Fair Lawn, NJ). Sodium Chloride, sodium carbonate, and 1,6-hexane diamine was purchased from Acros Organics (Morris Plains, NJ). Elastase from porcine pancreas (135 U/mg) was purchased from Elastin Products Company (Owensville, MO). EM Grade Glutaraldehyde- 8% wt. in H2O was purchased from Polysciences Inc. (Warrington, PA). 2-(N-morpholino) ethanesulfonic acid (MES) and Ultra II ultra pure hydrochloric acid was purchased from J.T Baker (Phillipsburg, NJ). n-hydroxysulfosuccinimide (NHS) was obtained from Pierce Biotech (Rockford, IL). Lanthanum(III) oxide was purchased from Alfa Aesar (Ward Hill, MA). 10% buffered formalin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and isopropyl alcohol were purchased from VWR International (West Chester, PA). Sodium Phosphate (dibasic) was purchased from EMD Chemicals (Gibbstown, NJ). Porcine aortic valves from Snow Creek Meat Processing (Seneca, SC).
3.2. Methods

3.2.1. Porcine Aortic Valve Acquisition

Fresh porcine aortic valves were harvested on site at a local slaughterhouse, Snow Creek Meat Processing (Seneca, SC). Valves were excised immediately upon animal death and were removed by cutting just below the aortic cusps and just above the aortic sinuses. The valves were transported on ice back to the laboratory where the valves were rinsed thoroughly using physiological saline. The valve leaflets were then separated and excess muscle tissue, aortic wall, and fat was removed leaving only the leaflet tissue and approximately 0.5 cm to 1 cm of aortic wall connected beyond leaflet to wall interface. After rinsing and trimming, all tissue was subject to immediate fixation.

3.2.2. Tissue Fixation Methods

For all fixation steps, 33 mL of fluid was used per valve cusp. Fixation processes were carried out at room temperature within 4 hours of harvesting and were as follows:

\textbf{CONTROL GROUPS:}

\textbf{GLUT:} Tissue was placed in 0.6\% glutaraldehyde in 50 mM HEPES buffered saline (pH 7.4) for 24 hours. Solution was then replaced with 0.2\% glutaraldehyde in 50 mM HEPES buffered saline (pH 7.4) for another 6 days.

\textbf{EDC:} Tissue was placed in 30 mM \textit{1-ethyl-3-(3-dimethylaminopropyl) carbodiimide} (EDC) and 6 mM \textit{N-hydroxysuccinimide} (NHS) in 50 mM MES buffered saline (pH 5.5) for 24 hours. Valves were then placed in 10\% formalin (pH 7.4) for another 6 days.
EXPERIMENTAL GROUPS:

NEN: Tissue was placed in 0.5 mM neomycin trisulfate in 50 mM MES buffered saline (pH 7.4) under constant orbital shaking for 1 hour. After incubation in neomycin, fixation proceeded identical to the EDC fixation method.

NOTE:

- NEN refers to valves that underwent fixation as above. If a concentration is specified, this refers to a change in neomycin trisulfate concentration only. If left unspecified, 0.5 mM neomycin trisulfate concentration should be assumed.

EXPERIMENT SPECIFIC CONTROL GROUPS:

HEX: Tissue was placed in a 1.5 mM solution of 1,6-hexanediamine in 50 mM MES buffered saline (pH 5.5) under constant orbital shaking for 1 hour. Fixation then proceeded identical to the EDC fixation method. ***HEX was used to provide a control for neomycin’s amine functionalities and their contribution to ECM preservation.***

NEX: Tissue was placed in 0.5 mM neomycin trisulfate in 50 mM MES buffered saline (pH 7.4) under constant orbital shaking for 1 hour. After incubation in neomycin, tissue was placed in 30 mM (EDC) and 6 mM (NHS) in 50 mM MES buffered saline (pH 5.5) for 24 hours. Tissue was then stored in a solution of 80% 50 mM HEPES buffered saline (pH 7.4) and 20% isopropyl alcohol. ***NEX was used to provide a control for formaldehyde and its contribution to collagen stability.***
NOTES:

- Valves not used immediately were stored in their final fixation solution until needed.
- After fixation, immediately prior to all experiments, aortic wall tissue was separated from leaflet tissue and discarded.

3.2.3. Enzymatic Degradation of GAGs

GAG degrading enzyme solution (GAGase): 5 U/mL hyaluronidase and 0.1 U/mL chondroitinase in 100 mM ammonium acetate buffer (AAB, pH 7.0). Leaflets were thoroughly washed in 100 mM AAB for 3 changes of 5 minutes each. Leaflets were then approximately cut into two equal halves and briefly blotted dry. Half of each cusp was put into 1.2 mL of GAGase, while the other half was put into 1.2 mL of AAB as a control. Both groups were incubated under high agitation at 37°C for 24 hours. After incubation, leaflets were removed from solution and thoroughly rinsed in distilled water. All solutions were saved for both DMMB and hexosamine analysis, while the tissue was blotted dry, frozen, and saved for hexosamine analysis (Sections 3.2.4 and 3.2.5). (n=6)

3.2.4. GAG Quantification: Hexosamine Assay

Tissues were lyophilized, weighed, and then acid hydrolyzed in 2 mL of 6 M HCl for 20 hours at 95°C. Resulting solutions were then dried under nitrogen gas and re-suspended in 2 mL of 1 M NaCl. These were reacted with 2 mL of 3% acetylacetone in 1.25 M sodium carbonate solution for 1 hour at 95°C and let cool to room temperature. The following were then added to each sample followed by vortex mixing: 4 mL of 100% ethanol and 2 mL of Ehrlich’s reagent (.18 M p-diethyl-aminobenzaldehyde, 50%
ethanol in 3.0 N HCl). Samples were allowed to sit at room temperature for 45 minutes to allow sufficient reaction time. The resulting color product indicative of hexosamine quantities was read for absorbance at 540nm. A set of D (+) glucosamine solutions (1-200µg) was used to produce a standard curve, and all values were normalized to their respective dry tissue weights. It was previously determined that resulting hexosamine values include ~90µg /10mg of non-GAG related hexosamines. Thus, this assay was performed as it has been previously, and this value was subtracted from all data [18,30,31,131,132]. Spectrophotometry was performed using the µquant spectrophotometer (BIO-TEK Instruments, Winooski, VT).

In addition to tissues, hexosamine was also performed on the resulting lysate solutions obtained during the enzymatic degradation of GAGs, per section 3.2.3. Methods were identical with only one change; the lyophilized solution was acid hydrolyzed using only 1 mL of 2 M HCl. (n=6)

### 3.2.5. GAG Quantification: DMMB Assay

DMMB assay was performed, as it has been previously, in order to quantify sulfated GAGs leached from tissue into the corresponding enzyme or buffer solution as outlined in section 3.2.3. The following were pipetted into the individual wells of a 96 well plate: 20µL of each sample, 30 µL of PBE buffer (100 mM Na₂HPO₄), and 200 µL of DMMB reagent (40mM NaCl, 40mM glycine, 46 µM DMMB, pH 3.0). Chondroitin sulfate solutions (0 - 1.25µg) were used to produce a standard curve. The resulting purple-like color change was indicative of sulfated GAG presence and was read for absorbance at 525 nm. This is done immediately to avoid degradation of the unstable
DMMB dye-GAG complex [30,31,133,134]. Spectrophotometry was performed with the µquant spectrophotometer (BIO-TEK Instruments, Winooski, VT). (n=6)

3.2.6. Collagen and Elastin Stability

Collagen and elastin stability is assessed using resistance of leaflet tissue to enzymatic degradation. Leaflets were cut in half, rinsed in distilled water, blotted dry, and then frozen at -80°C. Samples were then lyophilized for 24 hours and weighed prior to treatment with either porcine pancreatic elastase or Type VII collagenase, as described previously. Six half leaflets were placed in either 1.2 mL of 5.0 U/ml elastase (100 mM Tris buffer, 1 mM CaCl$_2$, .02% NaN$_3$) or 1.2 mL of 75 U/ml collagenase (50 mM CaCl$_2$, .02% NaN$_3$, pH 8.0). Elastase samples were incubated for 24 hours while collagenase samples were subject to 48 hours. Both groups were carried out at 37°C under constant shaking at 650 RPM. Samples were then removed from solution, rinsed, blotted dry, and frozen at -80°C. They were then lyophilized and the final dry weight was taken. This weight along with initial dry weight was used to determine the percent weight loss. (n=6)

3.2.7. Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was used to determine the collagen denaturation temperatures ($T_d$), previously described as the temperature at the endothermic peak [135]. Small (3 – 10 mg) samples were cut from generally the same region of different cusps, immediately adjacent to the nodule of arantius. These samples were carefully cut to size to enable them to lie flat within the DSC pans. Samples were then weighed and briefly blotted dry before encasing them within hermetically sealed
DSC pans. In the interest of conserving time, a pilot experiment was run with each group to determine an approximate value for $T_d$. In this pilot run, samples were heated from 25°C to 105°C at 2.5°C/min. All subsequent runs were heated at 10°C/min. until a point 20°C before the pilot run $T_d$, at which point a one-minute isothermal was held before proceeding to 105°C at 2.5°C/min. The collagen denaturation temperature that was recorded was taken from the most extreme value within the endothermic peak. All samples were run on DSC 2910 (TA Instruments, New Castle DE). (n=4)

### 3.2.8. Neomycin Optimization for NEN treatment

NEN fixation was performed using various concentrations of neomycin trisulfate from 0.05 mM to 2 mM. EDC and GLUT groups were used as controls. All tissues were then subjected to GAG-degrading enzymes, as outlined in section 3.2.3. GAG stability was assessed by hexosamine assay and DMMB analysis (Sections 3.2.4 and 3.2.5). (n=6)

### 3.2.9. Histological Assessment

Radial cusp cross-sections were taken from the cusp center and stored in 10% buffered formalin (pH 7.4) until use. Samples were then embedded in paraffin wax and sectioned for light microscopy analysis. For GAG analysis, alcian blue staining with a nuclear fast red counterstain was used, where blue staining is indicative of GAG presence. For calcium analysis, Dahl’s alizarin red staining was used with a light green counterstain, where red staining is indicative of calcification. Digital photographs were taken with a Zeiss Axioskop 2 Plus (Carl Zeiss MicroImaging, Inc., Thornwood, NY) in conjunction with SPOT Advanced software.
3.2.10.  **Uniaxial Tensile Testing**

For uniaxial tensile testing, the MTS Synergie 100 (MTS Systems, Eden Prairie, MN) was used with a 10 N load cell along with Testworks 4 software. Sections were cut from whole leaflets, approximately 4 mm wide using a rectangular tissue stamp. Sections were taken from the center of the cusps in both the radial and circumferential directions and were only cut immediately before they were to be run. The samples were then placed within custom grips that were lined with fine sandpaper to protect the tissue from tearing. The tissue was then preloaded to a small tension (0.01 N - radial, 0.1 N – circumferential) and the tissue’s dimensions were taken. The tissue was then stretched at a constant rate of 12.5 mm/min and the resulting stress-strain curve was used for analysis. (n=6)

3.2.11.  **Rat Subdermal Implantation**

All tissues were rinsed thoroughly through three changes of sterile saline for 30 minutes each and remained in sterile saline until implantation. Male juvenile Sprague-Dawley rats (35-40g, Harlan Laboratories, Indianapolis, IN) were anesthetized by inhalation of 3% isoflurane gas. Two incisions were made on the dorsal side of each rat, one on each side of the sagittal plane, and the skin was carefully lifted up to form a pocket. Cusps were blotted dry and positioned in the pockets to lie as flat as possible. Incisions were closed using surgical staples and tissues were retrieved at three weeks. Half of each cusp was used for hexosamine analysis (section 3.2.4) and the other half was analyzed for mineral content (section 3.2.12). Small middle sections were taken for histology and immediately immersed in 10% buffered formalin. All other samples were immediately put on dry ice and frozen at -80°C as soon as possible. (n=10)
3.2.12. Calcium and Phosphorus Analysis

**Sample Preparation:** First, samples were lyophilized for 24 hours and then weighed. Tissues were then acid hydrolyzed in 2 mL of 6N Ultrex II HCl for 20 hours at 95°C. Samples were then dried under nitrogen and resuspended in 1 mL of 0.01 N Ultrex II HCl. Only small amounts of the sample are used for both calcium and phosphorus assays. Dilution is used to bring samples near the range of the standard curve for each analysis.

**Calcium and Phosphorus Analysis:** A small amount of each sample was used to make a 100x dilution in distilled water and Clemson University faculty performed calcium and phosphorus analysis at the Clemson University Agricultural Service Laboratory. Analysis was performed using the Spectro Arcos ICP Spectrometer (SPECTRO Analytical Instruments, Kleve, Germany). Dilution ratios were used to determine the total mineral content of the sample and values were normalized to the dry tissue weight.

3.2.13. Storage Studies

The GAG content of leaflets was analyzed using hexosamine assay (section 3.2.4) after two, four, and six months of storage at room temperature. All valves were stored in the respective final solutions that were used in their fixation process. Short-term storage effects on resistance to enzymatic digestion of GAGs were also tested. (n=6)
3.2.14 Statistical Analysis

All results are expressed as the mean ± standard error of the mean (SEM). All statistical analysis was performed using single factor analysis of variance (ANOVA). Differences between the means were determined using least-significant difference with a significance level of $\alpha = 0.05$. 
4. RESULTS

4.1. Neomycin Optimization for NEN Treatment

To determine the most effective concentration of neomycin to use with the NEN treatment, porcine aortic valve leaflets were crosslinked by NEN using various concentrations of neomycin ranging from 0.05 mM to 2 mM. These tissues were then assessed for resistance to GAG degrading enzymes and compared to performance by EDC and GLUT groups. Hexosamine and DMMB analysis were performed after enzyme digestion was completed. It is important to note that the hexosamine assay measures non-sulfated and sulfated GAGs, while the DMMB assay is only capable of quantifying sulfated GAGs.

Hexosamines remaining in the tissue were measured and all concentrations of neomycin above 0.5 mM in the NEN groups retained high amounts of GAGs within the tissues (Figure 4.1). Low neomycin concentration NEN groups (0.05 mM – 0.25 mM) exhibited significantly lower GAG levels for both buffer and enzyme treatments with no significant difference between the two. GLUT offered the least resistance to GAG loss with only 79.48 ± 17.09 µg GAGs / 10 mg dry tissue remaining after enzymatic digestion compared with 141.54 ± 11.14 µg GAGs / 10 mg dry tissue remaining after storage in buffer. All GAG digested NEN groups with higher than a 0.5 mM neomycin concentration yielded significantly higher tissue GAG contents than both GLUT and EDC (p<0.03). EDC offered some protection against enzymatic attack preserving an average of 40.68 ± 30.82 µg GAGs / 10 mg dry tissue more than GLUT (p=0.03). The 0.5 mM neomycin was the optimal concentration to fully preserve GAGs, exhibiting an average of 104.4 ± 30.41 µg more GAGs / 10 mg dry tissue than GLUT (p=0.001).
Figure 4.1: Quantification of GAGs remaining in the tissue after GAG digestion by hexosamine assay. “Red star” indicates significant difference between digested and undigested tissue (p<0.05, n=6 per group).

Hexosamine assay was also performed on the solutions that were used to digest the tissues (Figure 4.2), and general trends appeared to be the same. No significant differences were detected in the GAG levels of the buffer solutions between any groups. The 0.5 mM NEN treatment again seemed to be optimal, losing negligible amounts of GAGs to enzymatic attack. Differences from both 1 mM and 2 mM NEN groups were not significant. Significant differences in the amount of GAGs lost between EDC and low concentration NEN groups (0.05 – 0.25 mM) were not found. This suggests that such low neomycin concentrations add little GAG protective effect. The EDC group
seemed to offer some resistance to enzymatic degradation, losing only $10.75 \pm 4.52 \mu g$ GAGs/10 mg dry tissue. On the other hand, GLUT offered the least protection, losing $69.95 \pm 4.17 \mu g$ GAGs/10 mg dry tissue.

**Figure 4.2:** Quantification of GAGs lost into solution during GAG digestion by hexosamine assay. “Red star” indicates significant difference between digested and undigested tissue ($p<0.05$, $n=6$ per group).

DMMB assay was used to further confirm results and was performed on the solutions. With the exception of the GLUT and 0.5 mM NEN groups, all other tissues subjected to GAG degrading enzymes lost significantly more sulfated GAGs into solution than those stored in buffer solution. This suggests protection against enzymatic degradation by the 0.5 mM NEN group, considering the small amounts of GAGs lost.
However, lack of protection is suggested by the GLUT group as the average GAG losses were significantly higher than 0.5 mM NEN, with 25.3 ± 6.55 µg/10 mg dry tissue (p=0.0015) more sulfated GAGs lost to buffer and 29.27 ± 5.94 µg/10 mg dry tissue (p<0.001) more lost to enzymatic digestion. In agreement with hexosamine assay results, no significant difference was found between GAGs lost for the low neomycin concentration group and the EDC group; suggesting again, that such low neomycin concentrations little GAG protective effect. Once more, the 0.5 mM NEN treatment was determined to be the most effective at preventing sulfated GAG loss, losing negligible amounts on both accounts. The 0.5 mM group also preserved 7.52 ± 4.86 µg/10 mg (p=0.051) more sulfated GAGs than the 2 mM NEN, group when subjected to GAGase.

Figure 4.3: Quantification of sulfated GAGs lost into solution during GAG digestion by DMMB assay. “Red star” indicates significant difference between digested and undigested tissue (p<0.05, n=6 per group).
The combined results from hexosamine and DMMB assays led to the choice of 0.5 mM neomycin concentration to represent the NEN group for all other experimentation. The choice of a 0.5 mM neomycin concentration for NEN was further verified by histological evaluation. Intense blue staining, indicative of GAGs, was seen for both GAG-digested and undigested valves in the 0.5 mM NEN group (Figure 4.4). EDC again seemed to offer some resistance to GAG digestion as lighter blue staining was seen, while GLUT offered the least resistance and demonstrated almost an entire absence of blue staining. Alcian blue staining agrees with the values of Figure 4.3, except for the progressive decreases in blue staining that are present in the undigested tissue pictures. This is possibly due to excessive time in storage prior to embedding and sectioning, and may be indicative of resistance to GAG loss in storage.

4.2. **1,6-Hexane Diamine as a Control for Neomycin**

Substances with multiple amine functionalities have been commonly used by certain groups to enhance EDC/NHS crosslinking. These molecules perform this function by acting as bridge molecules during crosslink formation that lengthen carbodiimide-initiated zero-length bonds [116-119]. Neomycin is incorporated to carbodiimide crosslinks in the same manner and may similarly result in enhanced EDC/NHS crosslinking. To prove our hypothesis that neomycin, when bound to tissue, inhibits enzymatic activity and does not just improves EDC crosslinking, we used 1,6 hexane diamine as a control. 1,6-hexane diamine is not an inhibitor of GAGase enzymes, but provides similar amine functionalities to improve EDC crosslinking.

1,6-hexane diamine has been previously used as an EDC/NHS crosslink coupler and is incorporated into HEX fixation [117]. Since neomycin has three times the amine groups per molecule, a triple (1.5 mM) concentration of 1,6-hexane diamine was used to provide control for amine functionalities. HEX and NEN were both subjected to enzymatic degradation and GAG retention and losses were analyzed using hexosamine assay, DMMB assay and histology.

NEN was shown to overwhelmingly prevent GAG loss better than HEX. There was no significant GAG loss observed for the digested NEN group, however the HEX group showed a significant amount of GAG loss. The HEX group showed only 75.3 ± 6.02 µg GAGs / 10 mg dry tissue in the GAG digested group compared to 117.44 ± 11.14 µg GAGs / 10 mg dry tissue remaining in the buffer stored group (p=0.01). The NEN group had significantly higher GAG amounts than HEX, with 148.46 ± 8.86 µg GAGs / 10 mg dry tissue remaining after GAGase digestion (p<0.001).
Figure 4.5: GAGs lost into solution after enzymatic digestion per section 3.2.3. [A] Sulfated GAGs lost for HEX and NEN as measured by DMMB assay. [B] GAGs lost by HEX and NEN as measured by hexosamine assay. “Red star” indicates significant difference between digested and undigested tissue (p<0.05, n=6 per group).

Figure 4.6: GAGs remaining in NEN and HEX tissue after digestion per section 3.2.3. Measured using hexosamine assay. “Red star” indicates significant difference between digested and undigested tissue (p<0.05, n=6 per group).
DMMB assay demonstrated that NEN lost only $2.09 \pm 0.94 \mu g$ GAGs / 10 mg dry tissue to enzymatic degradation accounting for a loss of only $1.47\% \pm 0.66\%$, while HEX lost $27.25 \pm 6.16 \mu g$ GAGs / 10 mg dry tissue or a loss of $23.2\% \pm 5.25\%$. These losses between NEN and HEX were significantly different ($p=0.01$). This is supported by almost identical results from hexosamine assay when performed on the solutions. However, results for the NEN group exhibited one discrepancy where significantly more GAGs to enzymatic digestion when compared to buffer, $8.07 \pm 1.39 \mu g$ GAGs / 10 mg dry tissue compared to $1.78 \pm 0.63 \mu g$ GAGs / 10 mg dry tissue ($p=0.02$).

**Figure 4.7:** Histological sections of porcine aortic valve cusps after storage in buffer and GAGase (Alcian blue staining, blue indicates the presence of GAGs). [A] NEN – Buffer stored [B] NEN – GAG digested [C] HEX – Buffer stored [D] HEX – GAG Digested
DMMB demonstrated that both fixation methods lost negligible amounts into buffer solution. Hexosamine assay performed on the buffer solutions showed higher amounts of GAG loss, $1.78 \pm 0.63 \, \mu g \, GAGs / 10 \, mg \, dry \, tissue$ lost by the NEN group and $2.92 \pm 0.47 \, \mu g \, GAGs / 10 \, mg \, dry \, tissue$ lost by the HEX group which were not significantly different ($p=0.18$). As expected, these losses are extremely small and pale in comparison to those lost by enzymatic degradation. Results obtained from all three of the performed assays agree and alcian blue staining, as seen in Figure 4.7, visually supports these trends.

### 4.3. GAG Loss During Storage

The effects of valve tissue storage on GAG loss were tested. Prior to storage all groups demonstrated similar levels of GAGs within the tissue with no significant differences detected. After two months of storage, only GLUT lost a significant amount of GAGs ($p<0.001$). GLUT valves lost an average of $53.59 \pm 19.52 \, \mu g \, GAGs / 10 \, mg \, dry \, tissue$ in the first two months of storage. After four months of storage there was little change for any groups when compared to data from the two-month storage time point, with no significant differences detected. After 6 months of storage both GLUT and EDC received sharp drops in average GAG levels. From 4 to 6 months storage, EDC lost an astounding $108.06 \pm 27.19 \, \mu g \, GAGs / 10 \, mg \, dry \, tissue$ ($p=0.005$) while GLUT lost $80.23 \pm 9.82 \, \mu g \, GAGs / 10 \, mg \, dry \, tissue$ ($p<0.001$).

Overall, NEN experienced no significant loss after 6 months of storage when compared to the initial time point, suggesting full protection against GAG loss due to storage. After 6 months of storage, EDC lost $118.34 \pm 33.3 \, \mu g \, GAGs / 10 \, mg \, dry \, tissue$
overall, which is equivalent to $76 \pm 21.4\%$ loss of all tissue GAGs. GLUT experienced similar catastrophic GAG loss after 6 months of storage, although the leaching of GAGs in the GLUT group started as soon as 2 months. When compared to the initial time point, GLUT lost $125.77 \pm 22.28$ µg GAGs / 10 mg dry tissue or $88.9 \pm 15.7\%$ of all tissue GAGs. This same loss was seen visually using alcian blue staining where the GLUT group displayed almost a complete lack of blue stain. Matching hexosamine assay results, EDC showed light blue staining suggesting partial GAG preservation and the NEN group demonstrated superior preservation of GAGs after 6 months of storage.

**Figure 4.8:** GAGs remaining in NEN, EDC, and GLUT tissue after 0, 2, 4, and 6 months of storage as measured using hexosamine assay. “Red star” indicates significant difference between the two groups (p<0.05, n=6 per group).
The effect of short-term storage on resistance to enzymatic degradation of GAGs was also tested. Enzymatic digestion was performed on tissue after two months of storage. The NEN group demonstrated high resistance to GAG degradation losing only negligible GAG amounts into solution (Figure 4.10) and retaining high amounts in the tissue (Figure 4.11). The EDC group offered some protection against enzymatic attack, losing only $5.33 \pm 1.65 \mu\text{g GAGs} / 10 \text{ mg dry tissue}$. The GLUT group offered the least resistance to GAG loss from both treatments, losing $8.87 \pm 0.93 \mu\text{g GAGs} / 10 \text{ mg dry tissue}$ into buffer solution and significantly more, $29.75 \pm 4.04 \mu\text{g GAGs} / 10 \text{ mg dry tissue}$ to enzymatic attack ($p<0.001$). GLUT exhibited the least amount of GAGs remaining in the tissue, much less than NEN, with $42.87 \mu\text{g GAGs} / 10 \text{ mg dry tissue}$ less in the buffer group and $68.19 \pm 15.54 \mu\text{g GAGs} / 10 \text{ mg dry tissue}$ less when subjected to GAGase.

![Figure 4.9: Histological sections of porcine aortic valve cusps after 6 months of storage](image)

(A) NEN – 6 months of storage  
(B) EDC – 6 months of storage  
(C) GLUT – 6 months of storage
Figure 4.10: Quantification of sulfated GAGs lost into solution during GAG digestion by DMMB assay after two months of tissue storage. “Red star” indicates significant difference between digested and undigested tissue (p<0.05, n=6 per group).

Figure 4.11: Measure of GAGs remaining in the tissue after GAG digestion by hexosamine assay after two months of storage. “Red star” indicates significant difference between digested and undigested tissue (p<0.05, n=6 per group).
4.4. **Resistance to Collagenase**

Resistance to collagenase digestion is used as a common measure of collagen stability within BHV tissues. Tissues were subjected to collagenase digestion as per section 3.2.6 and % weight loss after treatment was used to assess performance (Figure 4.12). The HEX group was used as a control for similar reasons that were explained in section 4.2. HEX was used to determine if the amine groups or bridging effects were responsible for enhanced crosslinking instead of functions specific to neomycin. There was no significant difference in percent weight lost between GLUT, HEX and NEN groups which lost 16.86 ± 0.94%, 17.16 ± 1.66% and 17.9 ± 1.82% weight after treatment, respectively. This suggests similar collagen stability between these 3 groups, but all of these groups exhibited significantly lower weight loss than the EDC group (p<0.013). The NEN group lost 5 ± 2.74 % less weight than the EDC group (p=0.001). Comparatively, the HEX group also lost 5.75 ± 2.58% less weight than the EDC group (p=0.013). This suggests that neomycin enhances collagen stability in EDC/NHS crosslinked tissue. However, considering that the HEX group also experienced similar improvements, this suggests that effects of amine bridging enhance the collagen stability of EDC/NHS crosslinked tissue instead of functions specific to neomycin.

4.5. **Resistance to Elastase**

Resistance to elastase was used as a measure of elastin stability as per section 3.2.6 and % weight loss was used to assess performance (Figure 4.13). The HEX group was used as a control for the same reasons described in sections 4.2 and 4.4 above. The NEN and GLUT groups had statistically insignificant differences in percent weight loss
at 26.69 ± 0.68% and 27.36 ± 0.62%, respectively. The NEN group exhibited 5.72 ± 1.78 less percent weight loss than EDC (p=0.002), suggesting that neomycin improves the elastin stability of EDC/NHS crosslinked tissue. Also, given that NEN demonstrated 3.81 ± 1.83 less percent weight loss than HEX, this suggests that the improved stability is due to functions specific to neomycin and not due to effects from amine coupling.

**Figure 4.12:** Results of collagenase digestion per section 3.2.6 expressed as % weight loss. “Red Star” indicates significant difference from EDC (p<0.05, n=6 per group).

**Figure 4.13:** Results of elastase digestion per section 3.2.6 expressed as % weight loss. “Red Star” indicates significant difference from EDC, “Green Star” indicates significant differences from HEX (p<0.05, n=6 per group).
4.6. **Differential Scanning Calorimetry (DSC)**

The collagen denaturation temperature ($T_d$) for various tissue groups was assessed as discussed in section 3.2.7. All groups exhibited a sufficiently high degree of crosslinking as the $T_d$ for EDC and NEN were significantly higher than that of GLUT ($p<0.001$). The $T_d$ of GLUT was only measured to be $86.59 \pm 0.56 ^\circ C$, while EDC and NEN were measured as $92.83 \pm 0.36 ^\circ C$ and $93.6 \pm 0.38 ^\circ C$, respectively (Figure 4.14). The insignificant difference between the measured $T_d$’s for the EDC and NEN groups suggests that neomycin has no affect on the degree of crosslinking for EDC/NHS fixed cusp tissue. Examples of DSC curves that were obtained are displayed in Figure 4.15, the endothermic peaks are highlighted and the $T_d$ was taken at the point of minimum value within this peak. Visual overview of these DSC curves, demonstrates the similarities in collagen thermal stability between EDC and NEN groups.

![Figure 4.14: Collagen denaturation temperatures as measured by differential scanning calorimetry (DSC). “Red Star” signifies significant difference when compared to GLUT. ($p<0.05$, n=4 per group).](image)
Figure 4.15: Overlay of example DSC curves for each tissue group. Data was obtained per section 3.2.7. Endothermic peaks where $T_d$ was recorded are within the red circle.

4.7. NEX as a Control for Formaldehyde Crosslinking

Formaldehyde is a well-known tissue fixative that is used widely throughout laboratory practices. As discussed in section 1.6.1, formaldehyde is capable of forming crosslinks with collagen molecules using available amine functionalities. These crosslinks may affect resistance to collagenase treatment as well as collagen thermal stability. In order to determine these effects, the NEX group (section 3.2.2) was used as a control for both collagenase and DSC experiments (sections 3.2.6 and 3.2.7).
Results after collagenase treatment showed no significant difference in the percentage of weight loss amongst any groups (Figure 4.16). Weight loss by the NEX group was $13.02 \pm 1\%$ while the NEN group lost $14.01 \pm 1.76\%$ weight after collagenase. This suggests that formaldehyde crosslinking had no affect on the enzymatic stability of collagen. On the other hand, formaldehyde was found to have an effect on the degree of crosslinking within the tissue.

This effect was characterized by measuring the average collagen denaturation temperature using DSC (Figure 4.17). The NEX group was found to have a much lower $T_d$ than the NEN group ($p<0.001$), measured at $87.225 \pm 0.45°C$. This was $6.38 \pm 0.82 °C$ lower than the measured $T_d$ for the NEN group at $93.6 \pm 0.38 °C$ and suggests that formaldehyde greatly increased the degree of crosslinking within cusp tissue. However, the $T_d$ of NEN was not found to be significantly different from GLUT suggesting that the NEN treatment without formalin (NEX) may provide a sufficient degree of crosslinking for use in BHVs. A visual overview of this can also be seen through the similarities between the DSC curves of GLUT and NEX displayed in Figure 4.15.

![Figure 4.16](image_url): Results of collagenase digestion per section 3.2.6 expressed as % weight loss. No significant differences were detected ($p<0.05$, $n=6$ per group).
“Red Star” signifies significant difference from NEN (p<0.05, n=4 per group).

4.8. Rat Subdermal Study: GAG Preservation and Calcification

Rat subdermal implantation was performed to study calcification and in vivo GAG stability. The amount of GAGs remaining in the tissues after explant was measured by hexosamine assay and verified visually using histology. The NEN group exhibited the highest GAG levels remaining within the tissue at $152.74 \pm 16.42 \mu g$ GAGs / 10 mg dry tissue. This is similar to levels measured from in vitro studies after storage in buffer, suggesting near full protection against GAG loss from in vivo degradation. This was significantly higher than levels for both the EDC and GLUT groups, which measured $112.57 \pm 8.95 \mu g$ GAGs / 10 mg dry tissue and $97.64 \pm 6.50 \mu g$ GAGs / 10 mg dry tissue, respectively. There was no significant difference detected between GAG amounts in the EDC and GLUT tissues (p=0.20). The NEN group preserved $40.17 \pm 25.37 \mu g$ GAGs / 10 mg dry tissue more than the EDC group, which was found to be significant (p=0.045). This suggests that the addition of neomycin into EDC/NHS crosslinked tissue provides additional protection against GAG degradation from in vivo sources.
Figure 4.18: Measure of GAGs remaining in the tissue after rat subdermal implantation by hexosamine assay. “Red star” indicates significant difference from GLUT. “Green Star” signifies significant differences from EDC. (p<0.05, n=10 per group).

Figure 4.19: Histological sections of porcine aortic valve cusps after rat subdermal implantation (Alcian blue staining, blue indicates the presence of GAGs). [A] GLUT [B] EDC [C] NEN.

Visual review of histology sections largely corresponds with hexosamine assay results, except that EDC is suggested to offer slight improvements on GAG retention. Again, this may be due to brief storage in formalin prior to embedding and sectioning. However, it is still very clear that NEN tissues retained a superior amount of GAGs as indicated by intense blue staining, while GLUT exhibits very light blue staining.
Rat subdermal implantation was also used to study the effects of the various fixation methods on calcification. Calcium and phosphorus levels were measured after tissues were explanted, as calcium and phosphate are key players in BHV mineralization [5,6,78,79,147]. No significant differences were detected between any groups when analyzed for calcium content. Calcium levels for the EDC and NEN groups were found to be similar at 63.96 ± 6.12 µg/mg dry tissue and 64.16 ± 11.95 µg/mg dry tissue, respectively. These values were both lower than the GLUT group at 83.60 ± 6.12 µg/mg dry tissue, but not significantly so, with p=0.115 when compared to EDC and p=0.199 when compared to NEN (Figure 4.20).

Measured phosphorus content in the cusps demonstrated an identical trend. There were no significant differences observed between any of the groups although the EDC and NEN groups appeared to be slightly lower than GLUT (Figure 4.20). GLUT phosphorus levels were measured as 41.2 ± 2.61 µg/mg dry tissue, while EDC and NEN groups seemed to be lower on average (p=0.06 and p=0.14, respectively). EDC phosphorus levels were measured as 29.9 ± 4.52 µg/mg dry tissue and NEN was found to be 30.4 ± 5.89 µg/mg dry tissue.

The molar ratios of calcium to phosphorus were also calculated to further characterize the tissue mineralization. The Ca:P molar ratios were similar for all groups at 1.57, 1.65, and 1.63 for GLUT, EDC, and NEN groups, respectively. This suggests similar progression of calcification for all groups and is indicative of hydroxyapatite formation, which has a Ca:P molar ratio of 1.67. Despite the appearance of lower mineralization in EDC and NEN, no differences found were significant. This suggests that NEN treatment did not inhibit BHV cusp mineralization.
Figure 4.20: Calcium and phosphorus content of cusp tissue after rat subdermal implantation as measured using atomic absorption spectroscopy (section 3.2.12). No significant differences were detected for any groups. (p<0.05, n=10 per group).

Histology was used to visually analyze the presence of calcification using Dahl’s alizarin red stain (Figure 4.21). Observed tissue sections exhibited varying amounts of calcification, as indicated by the presence of red staining. There appeared to be no noticeable difference in the location of calcified areas, and the morphology of calcified regions for all groups appeared to be very comparable to one another. This is expected as the modes of mineralization where suggested to be the same, with hydroxyapatite formation indicated for all groups. Visual analysis of all tissue section supports the hypothesis that mineralization for all groups progressed similarly.
Figure 4.21: Histological sections of porcine aortic valve cusps after rat subdermal implantation (Dahl’s alizarin red staining, red indicates the presence of calcification).


4.9. **Characterization of Tissue Mechanics: Uniaxial Tensile Testing**

4.9.1. **Stiffness (Modulus of Elasticity)**

Modulus of elasticity (E) was used as a measure for tissue stiffness. Uniaxial tensile testing was performed on all groups and the slope of the resulting stress-strain curve was utilized to assess tissue stiffness. NEN was compared to GLUT and untreated tissue (FRESH) for baseline controls. NEN was also compared to EDC to determine the effects of neomycin and, likewise, was evaluated against NEX to ascertain the effects of formaldehyde crosslinks. Stiffness was tested in both the radial and circumferential directions, and both the low-end and high-end stiffness responses were recorded (Figure 22 and Figure 23).
The stiffness of GLUT tissue was significantly higher than FRESH on all accounts, as expected (p≤0.025). Also, stiffness in the circumferential direction was found to be much higher than in the radial direction for every treatment group. The stiffness of NEN tissue was found to be statistically higher than FRESH on all accounts (p<0.001), except for the high-end response of the radial direction, which appeared to be a very close approximation to the E of FRESH tissue (p=0.505). Stiffness for GLUT was found to be significantly higher than NEN for the high-end response in both the radial and circumferential direction (p≤0.026). High-response E values for the NEN group were 49.59 ± 11.93% lower than GLUT in the circumferential direction and 30.91 ± 9.53% lower in the radial direction. On the other hand, for the low-end response, no significant difference was detected between NEN and GLUT groups for either direction.

Neomycin was only found to have a significant effect on stiffness for the low-end response in the circumferential direction. In this one case, E for EDC was measured to be 32.88 ± 11.58% lower than NEN (p=0.044). This is at odds with results for all other responses, where a trend seemed to form in which NEN stiffness appeared to be slightly lower than EDC, although not significantly so. Formaldehyde only seemed to play a role in stiffness for the low-end response, as no significant differences were found between NEN and NEX for the high-end E values. Low-end response E values for NEX were found to be significantly lower than NEN for both directions (p≤0.048). E for NEX was measured to be 56.07 ± 10.91% lower in the circumferential direction and 28.28 ± 10.23% lower in the radial direction. Overall, GLUT tissues appeared to be the stiffest while FRESH tissues were, by far, the least stiff. Stiffness for NEN tissue seemed on the middle ground, lower than or equal to GLUT and higher than or equal to FRESH.
Figure 4.22: Modulus of Elasticity for porcine aortic valve cusps for the circumferential direction.  

[A] High-end response.  [B] Low-end response.  “Red Star” indicates significant difference from GLUT.  “Green Star” indicated significant difference from FRESH (p<0.05, n=6 per group).
Figure 4.23: Modulus of Elasticity for porcine aortic valve cusps for the radial direction.

[A] High-end response.  [B] Low-end response.  “Red Star” indicates significant difference from GLUT.  “Green Star” indicated significant difference from FRESH (p<0.05, n=6 per group).
4.9.2. Extensibility

For this study, extensibility is defined as the amount of strain the tissue allows before transition to the collagen dominated high-end stiffness response. Uniaxial tensile testing was performed on all groups, and the results from the stress-strain curve were extrapolated to find extensibility. The slope line used to determine the high-end modulus of elasticity was extended down to the x-axis, and the strain at this point was recorded as the transition point, or extensibility (Figure 4.24).

Extensibility in the radial direction was much higher, overall, than in the circumferential direction (Figure 4.25). This is expected as the tissue in the aortic valve cusp has adapted to be much more yielding in the radial direction to aid in proper function. Extensibility of the tissue seemed to decrease after GLUT fixation, with average GLUT extensibility values measuring $12.08 \pm 10.4\%$ less than FRESH in the radial direction and $16.31 \pm 3.17\%$ less in the circumferential direction. However, only the difference in the circumferential direction was found to be significant ($p<0.001$).

None of the EDC/NHS crosslinked groups were found to have any statistically significant change in circumferential extensibility when compared to FRESH tissue ($p>0.12$). All EDC/NHS crosslinked groups experienced increases in extensibility in the radial direction, although EDC was not found to be statistically different from FRESH ($p=0.083$). Extensibility for NEN was measured to be $27.62 \pm 13.98\%$ higher than FRESH ($p=0.020$) in the radial direction and $39.7 \pm 7.36\%$ higher than GLUT ($p<0.001$). Formaldehyde crosslinking appears to play no role in these measured extensibilities, as no statistically significant differences in extensibility were found in either direction between NEX and NEN.
Figure 4.24: Stress-strain curve for GLUT tissue in the radial direction. The point at which the thin red line crosses the x-axis was recorded as the strain for extensibility.

Figure 4.25: Extensibility for various groups in both the radial and circumferential direction. “Red Star” indicates significant difference from GLUT. “Green Star” indicates significant difference from FRESH. (p<0.05, n=6 per group)
4.9.3. Stress-Strain Relationship Observations

During stiffness and extensibility analysis, a unique attribute of certain stress-strain curves was noticed that distinguishes the different groups from one another. As mentioned previously in section 1.5.3, the stress-strain relationship for heart valve cusp tissue is highly non-linear and clearly separated into two regions. This was also verified firsthand during uniaxial tensile testing on FRESH and GLUT tissue, where both groups were found to express this relationship exactly (Figure 26A). However, all other groups were found to demonstrate an additional third region (Figure 26B and 26C).

The third region appeared at the transition between the low-end response and the high end-response, but only in the radial direction. Instead of the normal bend during transition that is seen in GLUT and FRESH stress-strain curves, a clear “hump” is seen in the stress-strain curve for all EDC/NHS crosslinked groups in the radial direction. This hump is noticeable in the EDC group, but is much more pronounced in the NEN and NEX groups. This suggests that not only does EDC/NHS crosslinking play a role in this unique response, but neomycin does as well.

Figure 4.26: Comparing a normal two-region stress-strain curve two those with a third-region (This region is circled in red). [A] FRESH [B] EDC [C] NEN.
5. DISCUSSION

Calcification and non-calcific tissue damage are the two primary causes of structural deterioration that lead to clinical BHV failure [5,76]. Collagen, elastin, and remnant cell debris within BHV tissue are all recognized nucleation sites for calcification [5,12]. Elastin calcification is thought to occur regardless of chemical modification [12]; however, there is evidence that mineralization at other sites is potentiated by glutaraldehyde treatment [137,138]. Calcification is also exacerbated at sites of increased mechanical stress, which highlights another major contributor to BHV failure, mechanically induced tissue damage [124,127].

Non-calcific structural damage has been shown to occur independently of calcification and roughly 90% of porcine aortic valve BHVs fail from structural damage while showing little to no signs of calcification [76,81]. Degeneration in cyclic fatigue testing suggests that mechanical stresses are a major source of non-calcific tissue damage [124]. This may be due in part to glutaraldehyde fixation, which increases stiffness, reduces extensibility, and fails to preserve GAGs within cusp tissue.

GAG loss has been connected to both tissue buckling and delamination and has been documented in failed BHVs [19,31,77]. Even minor deviations from normal biomechanics or tissue stress levels can lead to premature BHV dysfunction since damage progresses unrestrained without the ability for BHV tissues to repair. This is substantiated by cases of BHV failures that occurred as a result of only minor design changes [139]. Therefore, the loss of GAGs in BHVs may indirectly lead to the accumulation of collagen damage over time, ultimately contributing to BHV failure.
5.1. **GAGs in BHVs**

GAGs in BHV cusp tissue are primarily associated with the PGs versican and decorin [19,131]. The functions of decorin are not fully understood, but have been proposed to include roles in collagen fibril formation and organization as well as roles in elastogenesis [140,141]. These functions cater to viable tissues, and the loss of these GAGs may not significantly impact BHVs durability. Versican aids in tissue hydration, helps control internal shear stress levels, absorb compressive loads, and promotes smoother bending which helps prevent tissue buckling [31,140]. Versican also contains elastin binding regions that may deter elastin calcification. This further establishes the case that GAGs associated with versican play a much larger role in BHV durability [19].

5.2. **GAG Loss and Consequences**

GAGs associated with decorin are more likely to be preserved by glutaraldehyde due to a relatively unblocked protein core that is available for crosslinking. On the other hand, GAGs related to versican are thought to remain unbound since the numerous attached GAG chains hinder reaction by protein core with crosslinkers. Fluorophore-assisted carbohydrate electrophoresis performed on clinical BHVs that failed due to structural deterioration helped confirm these hypothesis [19]. Highly reduced levels of chondroitin-6-sulfate were expressed, a GAG normally associated with versican [19]. Conversely, a high proportion of remaining GAGs were found to be chondroitin-4-sulfate, which is commonly associated with decorin [19]. This is consistent with the theory that GAG loss facilitates BHV failure, since versican loss would be much more detrimental than decorin loss.
Versican expression is highest in the spongiosa, and GAG loss associated with versican leads to thinning of this tissue layer, a phenomenon observed in failed, explanted BHVs [19,131]. The spongiosa is responsible for providing lubrication that reduces shear stresses formed between the fibrosa and ventricularis that could otherwise lead to delamination. Cyclic flexural fatigue demonstrated that delamination of the tissue layers was the primary cause of BHV failure beginning at 10 million cycles [77]. Taken together with another study that demonstrated that the majority of GAGs are loss in glutaraldehyde fixed tissues by 10 million cycles, this advocates the role of GAGs in preventing failure from delamination [15]. The onset of GAG loss at 10 million cycles is also prior to collagen structural damage, which makes GAG loss a possible contributor to structural deterioration [15]. GAG depleted tissues have also demonstrated greater tissue buckling depth as well as increased flexural rigidity, both undesirable traits [18,31]. This further demonstrates the importance of GAGs in BHV tissue, and again implicates versican as a major source of GAG loss.

GAGs are not only lost during implantation and fatigue, but are also lost during preparation and storage [31,75]. Loss can occur from the liberation of unbound GAGs, but is also driven by enzymatic degradation since glutaraldehyde does not fully inactivate GAG-degrading enzymes [75,83,142]. In addition to biomechanical repercussions, GAG loss has also been suggested to impact BHV durability in other ways. The bulky nature of versican has been stated to offer protection from proteolytic degradation by steric interference [136], however this has been disputed [18]. Moreover, GAG loss was shown to stimulate calcification in the rat subdermal model [86], and conversely GAG binding was demonstrated to hinder mineralization [88,133], but this also faces debate [18].
5.3. **GAG Preservation**

Various fixation methods have been used to chemically stabilize GAGs to improve preservation during storage, fatigue, and implantation. The uses of EDC/NHS and sodium metaperiodate have both demonstrated improved GAG stabilization when compared to glutaraldehyde treated tissues [88,132]. However, they were also established to be only partially effective at protecting GAGs from enzymatic degradation. Considering the fact that GAG degrading enzymes infiltrate BHV tissues after implantation [25], additional protection against enzymatic degradation is needed.

Neomycin was employed by our lab using various fixation strategies and offered improved GAG stability in tissues that were subjected to enzymatic degradation [133,UD*]. The NEG group (Neomycin + EDC/NHS + Glutaraldehyde) demonstrated superior GAG preservation but excessive crosslinking sacrificed the compliancy of cusp tissue, which was demonstrated by biaxial and uniaxial analyses [30,133,UD]. The NG group (Neomycin + Glutaraldehyde) demonstrated better compliancy along with improved GAG preservation, but still lost GAGs during storage as lack of covalent GAG crosslinking led to loss by leaching. [UD]. NG and NEG were both also subject to calcification in the rat subdermal model, presumably, due to the use of glutaraldehyde [133,UD]. We proposed the use of formaldehyde instead of glutaraldehyde, would yield tissues with better mechanical properties since formaldehyde storage has previously been demonstrated to better maintain tissue compliance [143]. Additionally, formaldehyde performs many of the beneficial functions of glutaraldehyde, but may produce tissues with reduced tendency to calcify. Therefore, we proposed that the use of EDC/NHS
fixation in combination with neomycin and formaldehyde would provide the desired stability to ECM components without sacrificing tissue compliance.

5.4. **Resistance to Enzymatic GAG Digestion**

Past fixation strategies, NEG and NG, have utilized a 1 mM concentration of neomycin to effectively shield GAGs from *in vitro* enzymatic degradation [133,UD]. The optimal neomycin concentration for the NEN treatment was found to be 0.5 mM, which is in close agreement with unpublished data for both NEG and NG fixation methods. The NEN group demonstrated complete resistance to GAGase digestion noting no difference in the amount of GAGs remaining in cusp tissue after GAGase treatment. GAG preservation was confirmed by analyzing the GAG content of the solutions, as well as through histology. These results are similar to those previously found using NEG fixation suggesting that the use glutaraldehyde is unnecessary to provide neomycin and GAG stability when used with EDC and NHS [133].

EDC/NHS crosslinking, without the addition of neomycin, offered some protection against *in vitro* enzymatic degradation, although not as complete as NEN fixation. Carboxyl groups on the hexuronic acid repeats of GAGs have been shown to be the initiating site for enzymatic attack by GAGases, and are the same groups used by EDC and NHS for crosslink formation in NEN treated tissues [144]. Blockage of these sites was shown to prevent enzymatic hydrolysis of β-(1,4) glycosidic linkages, which are present on both hyaluronan and chondroitin sulfate (Figure 3B) [144]. According to enzyme supplier’s website (www.sigmaaldrich.com), both the hyaluronidase and chondroitinase used for these studies are capable of initiating cleavage at β-(1,4)
glycosidic linkages. Therefore, steric interferences caused by these crosslinks are likely the cause of EDC treated tissue’s resistance against GAGase degradation. However, protection against GAG loss is only partial due to incomplete blocking of the activation site along with insufficient crosslinking to safeguard all associated carboxyl groups.

All neomycin containing groups, NEN, NEG, and NG, have demonstrated protection against both chondroitinase and hyaluronidase [133,UD]. This is probably due to the fact that both enzymes exhibit similar cleavage mechanisms. According to the enzyme supplier’s website, as stated above, both enzymes are capable of hydrolyzing β-(1,4) glycosidic linkages on hyaluronan and chondroitin sulfate (Figure 3B). Protection against both hyaluronidase and chondroitinase is critical for the full preservation of versican aggregates in the spongiosa since they contain large amounts of both CS and HA [17,19,131]. These aggregates have been established as a significant source of GAG loss [19] and are likely the most important GAG structures when concerning protection against mechanical fatigue damage in BHVs [18,30,31].

Protection against the enzymatic degradation of GAGs that is offered by neomycin carried over into an in vivo model with the NEN treated tissues demonstrating full GAG preservation after rat subdermal implantation for 3 weeks. There appeared to be no significant amount of GAGs lost during 3 weeks of implantation when compared to baseline GAG content for undigested tissues. This mirrors results previously obtained for the NEG group, which demonstrated similar GAG preservation after 3 weeks of rat subdermal implantation [133]. Again, this suggests that the use of glutaraldehyde treatment is unnecessary to provide neomycin and GAG stability for tissues crosslinked using EDC and NHS.
5.5. **Controls for Neomycin**

The inhibitory effect of neomycin on GAG degrading enzymes has previously been demonstrated by both NEG and NG fixation methods [30,UD]. In order to verify that neomycin was responsible for inhibiting GAGase activity, the GAG preserving characteristics of 2-deoxystreptamine dihydrobromide (DOS), a molecule with comparable structure to neomycin, were evaluated. Tissues treated with DOS along with EDC, NHS, and glutaraldehyde failed to demonstrate matching enzymatic protection against GAG loss to that of the NEG group, and exhibited significant amounts of GAG loss after GAGase treatment [30].

For this study, a triple concentration of 1,6-hexane diamine was used as a control for neomycin instead of an equal concentration of DOS. Previously, 1-6-hexane diamine has been used to enhance EDC/NHS crosslinking [117], and a triple concentration was used in order to provide a control for the number of provided amine functionalities (HEX fixation). HEX treated tissues failed to provide protection against enzyme-initiated GAG loss equal to that of NEN, losing a significant amount of GAGs after GAGase treatment. This further demonstrates the GAG preserving properties of neomycin, and rules out the contribution of amine functionalities to the protection of BHV tissues against enzymatic GAG degradation.

5.6. **GAG Preservation During Storage**

BHVs may be stored for up to three years from the moment of sterilization, in a clinical setting [145]. During storage for a period up to 5 years, progressive GAG loss has been shown to occur in glutaraldehyde treated BHV tissues. The addition of
neomycin to glutaraldehyde was shown to slow the rate of GAG loss during storage, as demonstrated by NG fixation for storage up to 400 days [UD]. This demonstrates the importance of protection from enzyme-initiated GAG degradation during storage, considering that enzymatic activity is not abolished by glutaraldehyde fixation [75,83,142]. However, GAG loss was only slowed by neomycin, not prevented, and GAGs were still lost due to leaching. The additional use of EDC/NHS crosslinking further preserved GAGs, and NEG treated tissues demonstrated negligible GAG losses after 1 year of storage [30].

NEN treated tissues demonstrated similar characteristics by showing complete GAG preservation throughout storage periods spanning 2, 4, and 6 months. Resistance to direct GAGase digestion for the NEN group was also retained after 2 months of storage, suggesting that the protective properties of neomycin do not degrade during short-term storage. The effects of further storage on the decay of neomycin’s functions were not tested for the NEN group, but a previous study has demonstrated that it still provides total protection from direct GAGase digestion after 1 year of storage [30].

5.7. **Collagen and Elastin Stability**

Collagen is the most prominent protein in BHV tissue and collagen fibers are recognized as the most important structures regarding the overall integrity of BHVs. The most common cause of collagen fiber disruption is non-calcific damage, which can be mechanically induced or initiated by enzymatic activity [76,81,83]. Collagen crosslinking due to glutaraldehyde fixation helps protect against autolytic degradation [5,6]. This may occur during storage and implantation, as active proteolytic enzymes
have been noted in BHV tissues after glutaraldehyde fixation [83], and have also been found to inhabit clinically explanted BHV tissues [146].

Resistance to collagenase digestion has previously been used as a measure of collagen stability [30] and was also used to assess the viability of NEN treated tissues. Tissues crosslinked using the NEN treatment were found to demonstrate similar collagen stability to those crosslinked using glutaraldehyde. Neomycin improved upon the collagen stability of EDC/NHS crosslinked tissue, with the NEN group showing less weight loss than the EDC group. However, this is due to the addition of amine functionalities into the tissue and not a function specific to neomycin since the use of 1,6-hexane diamine yielded similar improvement. Two hypotheses are offered as the reason for the enhanced collagen stability offered by neomycin and 1,6-hexane diamine addition:

- The bridging effect offered by polyamine molecules yields enhanced collagen crosslinking via the ability to form different length crosslinks instead of only the zero-length bonds formed by EDC and NHS.

- The addition of more amines into the tissue allows additional crosslinks to be formed, which leads to higher stability. More carboxyl groups are readily available on collagen than amine groups [115], leaving the number of amine groups on collagen as the limiting factor in the number of possible crosslinks.

Previous use of 1,6-hexane diamine along with suberic acid, EDC, and NHS has also been demonstrated to provide tissues with similar collagenase resistance to that afforded by glutaraldehyde [117]. However, this treatment was not equally compared to EDC/NHS fixation alone and therefore, enhanced EDC/NHS crosslinking by 1,6-hexane diamine could not be confirmed.
Since formaldehyde reacts with free amine groups on collagen molecules, the effect of formaldehyde crosslinking on collagen stability was also evaluated. Despite the monofunctional nature of formaldehyde, formaldehyde crosslinks actually provide greater resistance to collagenase than the bifunctional glutaraldehyde at a concentration of 1% [148]. This is most likely because of deeper penetration into the layers of collagen structure allowed by formaldehyde’s small size [148]. The NEX group, identical to the NEN group with the omission of formaldehyde, was found to have similar collagen stability to NEN treated tissues. This suggests that formaldehyde does not affect the collagenase resistance of tissues crosslinked using EDC, NHS, and neomycin. Formaldehyde crosslinks may have no effect because the majority of collagen amines have already reacted with EDC and NHS during NEN fixation. The deeper level crosslinks that are afforded by formaldehyde may also be inaccessible to collagenase and fail to contribute to enzymatic resistance.

Assessing the loss of elastin is also important as it may augment the onset of collagen damage indirectly through changes to BHV tissue mechanics. The stability of elastin was also measured using resistance to elastase digestion, as done previously [30]. Like with collagen stability, similar elastin stability by both NEN and GLUT groups was observed and the elastin stability of EDC tissue was improved by the addition of neomycin. However, in this case, the action appears to be exclusive to neomycin, as the HEX group did not experience the same effect. The mechanism behind neomycin’s protection against elastase digestion is unknown, but was also previously demonstrated in NEG and NG crosslinked tissues [30,UD].
5.8. Collagen Denaturation Temperature

The collagen denaturation temperature ($T_d$) of BHV tissues is an indirect measurement of the degree of collagen crosslinking. The $T_d$ for all groups was measured using differential scanning calorimetry, which was first described in 1995 [135]. The measured $T_d$ for GLUT was found to be around 86 °C, which is very similar to previously reported values [135]. The measured $T_d$ for NEN and EDC were statistically higher than GLUT, but not statistically different from one another. This suggests that neomycin has little effect on the thermal stability of collagen structures.

The large increase in $T_d$ for EDC and NEN tissues was found to be a result of the contribution from formaldehyde crosslinking. The small size of the formaldehyde molecule allows deep penetration into collagen structures initiating crosslinks all the way down at the pentafibril level [148]. The increased amount of crosslinking allowed by formaldehyde causes tissues to display a higher $T_d$. This is consistent with previous studies that demonstrated a higher $T_d$ for formaldehyde crosslinked tissue than glutaraldehyde crosslinked tissue [148].

The effect of formaldehyde crosslinking was confirmed as the NEX group was measured to have a much lower $T_d$ than the NEN group. The measured $T_d$ of NEX tissue was consistent with previously measured values for EDC/NHS crosslinked tissue [135]; further supporting the fact that neomycin does not affect thermal stability. The $T_d$ for the NEX group was not statistically different from that of GLUT tissue, suggesting that EDC/NHS crosslinking with neomycin provides sufficient crosslinking without the use of formaldehyde.
5.9. Calcification

Glutaraldehyde crosslinking has previously been implicated to render BHV tissues more conducive to calcification [5,62,78], and alternative crosslinking strategies, using EDC and NHS, have demonstrated improved resistance to calcification in the rat subdermal model [116,117,119]. Despite this fact, the EDC and NEN tissue groups did not display significantly reduced calcification when compared to the GLUT group. This may be due to the use of formaldehyde, which is part of the same family of chemicals as glutaraldehyde and reacts with amine groups in an identical manner. These crosslinks may aggravate the calcification of BHV tissues in a similar manner to glutaraldehyde contributing to the calcification of EDC and NEN tissues. There was also no observed difference between NEN and EDC tissue groups suggesting that neomycin does not affect the calcification of cusp tissue. This is consistent with previous data, which demonstrated that neomycin does not affect the calcification of EDC/NHS crosslinked tissue when used with glutaraldehyde [133].

The calcium to phosphorus molar ratios observed for all tissue groups were comparable to those found in explanted BHV tissue, suggesting similar progression of mineralization in both models [147]. The observed Ca:P molar ratios for all groups were also close approximations of the molar ratio for hydroxyapatite, which is a known player in BHV calcification [78,79,147]. Similar progression of calcification was also viewed using histology, which noted no obvious differences in the morphology of calcified regions. The observed ratio values were slightly lower than the known ratio for hydroxyapatite and may indicate increased proportions of dicalcium phosphate dihydrate.
and octacalcium phosphate. These are precursors to hydroxyapatite that have been documented in calcified BHV tissues [147].

It has previously been hypothesized that GAGs may, in some way, function to inhibit BHV calcification and various studies have provided evidence to support this claim [75, 86-88]. It has previously been demonstrated that the removal of GAGs leads to heightened levels of calcification [86]. Likewise, various methods of covalent GAG binding have reduced calcification [87, 88]. NEN tissues were found to fully preserve GAGs during rat subdermal implantation; however, no reduction in calcification was observed. This again may be due to the role of formaldehyde crosslinks in EDC and NEN tissue calcification. These crosslinks may exacerbate tissue calcification to such a degree that any inhibitory effects offered by GAG preservation are negated.

5.10. Tissue Biomechanics

Tissue stiffness and extensibility were analyzed using uniaxial tensile testing since they may have direct implications on stress levels and the amount of shock loading experienced by BHV cusp tissue. Stiffness increase in cusp tissue is undesirable and has been correlated with increased mechanical damage and lower BHV lifespan. The initial generation of BHVs used high-pressure fixation to achieve proper coaptation and aesthetic appeal. These valves had excessively stiff cusps and suffered from far inferior durability when compared to later models [71]. Dermal collagen stiffness has been found to rise with age and has also been proposed to affect heart valve cusp tissue [13, 148]. This may further provide a correlation between stiffness and failure as incidences of native heart increase valve disease increase with age [5].
Glutaraldehyde treatment is known to cause an undesirable stiffness increase and GLUT tissues were found to display a significantly higher stiffness than FRESH on all accounts [152]. NEN tissue was significantly less stiff than GLUT for both directions in the high-end response region. The high-end response in the radial direction was a close approximation of natural stiffness, which is promising since cusp tissue experiences the greatest amount of deformation in the radial direction. On the other hand, cusp tissue experiences the highest amount of tension in the circumferential direction so a reduction of high-end stiffness in this direction may be equally beneficial. NEN tissues did not experience the same excessive stiffness increase that was observed in NEG tissues [UD]. This is most likely due to storage in formaldehyde, which was previously shown to better preserve tissue compliance than glutaraldehyde [143].

Decreasing extensibility may also be related to failure and, like increased stiffness, has also been found to occur with age in native valves [13,149]. Decreased extensibility is also a result of elastin damage, which has been hypothesized to be a possible contributor to BHV failure [150]. Finally, decreased extensibility has also been documented in failed BHV explants [19,151]. When compared to FRESH tissue, GLUT tissues demonstrated a significant decrease in extensibility in the circumferential direction as observed previously [152]. All groups that utilized EDC/NHS crosslinking performed better by demonstrating significantly higher extensibility than GLUT in both directions and higher than FRESH in the radial direction. Also, the circumferential extensibility for all EDC/NHS crosslinked groups was measured to be no different than FRESH. This is consistent with previous data that also demonstrated EDC/NHS crosslinked tissues were more extensible than untreated tissues [115].
The differences in the mechanical properties of EDC/NHS crosslinked tissue from GLUT and FRESH tissues may be explained by a single phenomenon. Bond formation during carbodiimide crosslinking may cause the tissue to shrink offering an explanation for increased extensibility as well as reduced high-end stiffness. Tissue shrinkage after fixation would cause the initial length measurement used in strain calculations to be smaller. Therefore, if two samples of similar size, one sample that underwent shrinkage and a one that did not, were measured for extensibility at a common final length, the resulting strain and extensibility would be larger for the tissue that underwent shrinkage. This may also provide an explanation of the reduction in high-end stiffness since tissue shrinkage may also cause collagen molecules to crimp or fold. When tension reaches the high-end response, collagen will not be fully unfolded resulting in a lower stiffness as collagen straightens and becomes more taut. Altered stiffness response may also simply be due to differences in ECM microarchitecture, caused by differing crosslinking chemistries.

The third response region that was demonstrated on the stress-strain curve may be due to the formation of collagen-GAG crosslinks. Evidence for these crosslinks was given by GAG preservation data, and would be a cause for a unique strain response under tension. Since GAGs have little resistance to tensile force, this would only be viewed under low tissue loads. This would account for the appearance of this unique response in the lower stiffnesses of the radial direction.

Since tissue dimensions were not studied before and after fixation, talk of tissue shrinkage is all conjecture. The effects of observed extensibility and stiffness changes on BHV tissue durability are also not known, and cyclic fatigue or full-scale implantation
models would be needed to study this. The effect of strain rate was also not investigated and stress-strain responses were only analyzed at an arbitrary strain rate for relative ease. It has previously been demonstrated, however, that the use of differing strain rates up to physiological values has no affect on the stress-strain behavior of valve leaflet tissue during biaxial tensile testing [129]. The effect of the third-response region is unknown, and further study on all mechanical responses from biaxial mechanical testing or flexural testing may be warranted. Lastly, the effects of GAG preservation by the NEN group on tissue biomechanics have also not been elucidated and deserve further investigation.
6. CONCLUSIONS

The current average lifespan of implanted BHVs is inadequate in many cases, causing contraindication of implantation, undesirable reoperation and even catastrophic failure. Increasing BHV durability will not only improve the quality of life for BHV recipients, but also open the door for more patients to become suitable candidates for BHV replacement. In order to accomplish this, the foremost causes of BHV failure need to be addressed. It has been determined that structural dysfunction due to the accumulation of collagen damage is the primary cause of BHV failure, with calcification being a common co-contributor. Current standard fixation methods, using glutaraldehyde, may facilitate these modes of failure. Therefore, development of an alternative fixation method may avoid the drawbacks of glutaraldehyde treatment, yielding tissues with better properties that will increase the lifespan of BHVs.

Glycosaminoglycans are large, hydrated molecules that are present within the specialized tissue layers of bioprosthetic heart valve tissue. The functions of these molecules may be vital to preservation of proper tissue biomechanics and, consequently, their loss may facilitate premature BHV failure. GLUT tissues were demonstrated to experience GAG loss when subjected to enzymatic degradation, rat subdermal implantation, and storage. EDC crosslinking enables the crosslinking of GAGs and reduced losses due to enzymatic degradation and storage. However, protection against GAG loss was incomplete and further protection against enzymatic degradation was required. The use of neomycin in NEN treated tissues drastically improved resistance to GAG loss, showing full preservation during enzymatic degradation, rat subdermal implantation, and six months of storage.
GAG preservation may be important, but preservation of collagen integrity is the ultimate goal. Neomycin was also demonstrated to improve both the collagen and elastin stability of carbodiimide crosslinked tissue. The NEN group displayed superior collagen and elastin stability compared to EDC and was also a comparable match for GLUT. The degree of crosslinking for NEN tissues was also found to be sufficient. The calcification potential of NEN tissues was not determined to be significantly different from GLUT tissues, and progression of tissue mineralization appeared to be identical for both groups. This is thought to be due to the similar crosslinking mechanisms of formaldehyde and glutaraldehyde.

On the other hand, analysis of biomechanics demonstrated clear differentiation in responses between NEN and GLUT groups. GLUT tissues experienced an increase in stiffness during fixation and were the stiffest tissues on average. A reduction in extensibility was also observed in the GLUT group. NEN tissues were measured to be less stiff than GLUT on some accounts and were never found to be statistically stiffer. NEN tissues also demonstrated vastly improved extensibility over GLUT tissues and were even statistically higher than FRESH tissue in the radial direction.

We have demonstrated, that NEN tissues offer superior GAG preservation while still providing the necessary stability for structural ECM components. NEN fixation was not successful at reducing the calcification potential of cusp tissue, but yielded tissues improved compliance and extensibility. NEN fixation, and its associated GAG preservation, may improve valve biomechanics and slow the onset of collagen damage. As a result, the rate of structural degradation may be reduced resulting in improved durability for BHV tissues.
7. FUTURE RECOMMENDATIONS

GAGs were demonstrated to be preserved using NEN fixation, but the effects of their preservation were not investigated. Analysis of valve biomechanics with and without selective GAG removal could help reveal this. Any effects cause by longer storage periods should also be studied as valves may be stored clinically for up to three years. EDC and NHS utilize the carboxyl groups on GAGs to form the crosslinks that aid in preservation. However, these same groups are necessary to retain the hydrophilic properties of GAGs. Effects on tissue hydration should be studied along with any associated changes this may cause to tissue biomechanics.

Since formaldehyde is suspected in the calcification experienced by the NEN group, storage in an alternative reagent should be investigated. However, if formalin storage is still determined to be favored, the use of anti-calcification treatments could also be studied. Specific attention could also be paid to the areas in which calcification occur. As GAGs have been associated with possible prevention of calcification, specific tissue layers may be more resistant to calcification than others. Other segments of BHV tissue, such as the aortic wall, should also be tested for calcification potential.

Further exploration into the mechanisms for the reduced stiffness and increased extensibility is needed. Tissue dimensions should be measured before and after fixation to determine the legitimacy of the tissue shrinkage hypothesis. Further research into the effects of NEN fixation should also be performed including flexural testing, biaxial tensile testing, and cyclic mechanical fatigue testing. Changes to valve biomechanics that may occur during storage or fatigue should also be assessed. Lastly, these enhanced biomechanical studies should also include studies into the effects of GAG preservation.
8. REFERENCES


3. Cabin HS. The heart and how it works. The Heart and Circulation: Chapter 1, 3-9.


