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Characterisation of Bovine Pericardium and Alternative Treatments for its Application as a Biomaterial

A thesis submitted to the School of Chemistry
College of Science
National University of Ireland

In fulfilment of the requirements for the
Degree of Doctor of Philosophy

by

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November 2019

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**AFM:** Atomic Force Microscopy;

**ANOVA:** Analysis of Variance;

**AS:** Aortic Stenosis;

**ATR:** Attenuated Total Reflectance

**BHV:** Bioprosthetic Heart Valve;

**BP:** Bovine Pericardium;

**BSE:** Bovine Spongiform Encephalopathy;

**Cp:** Heat Capacity;

**CSA:** Cross Sectional Area;

**Decell:** Decellularised;

**DMEM:** Dulbecco's Modified Eagles Medium;

**DMSO:** Dimethyl sulfoxide;

**DSC:** Differential Scanning Calorimetry;

**DTGS:** Deuterated Triglycine Sulfate;

**ECM:** Extracellular Matrix;
**E-modulus:** Modulus of Elasticity;

**F:** Force;

**FBS:** Fetal Bovine Serum;

**FDA:** Food and Drug Administration;

**FTIR:** Fourier Transform Infra-Red Spectroscopy

**GAGs:** Glycosaminoglycans;

**Genp:** Genipin;

**Glut:** Glutaraldehyde;

**H&E:** Hematoxylin and Eosin;

**HMDS:** Hexamethyldisilazane;

**hMSC:** Human Mesenchymal Stem Cells;

**ISO:** International Standards Organisation;

**LLDPE:** linear low density polyethylene;

**MCT:** Mercury-Cadmium-Telluride;

**MTDSC:** Modulated Temperature Differential Scanning Calorimetry;

**MS-5:** Murine Bone Marrow Stromal Cells;
**PBS:** Phosphate Buffered Solution;

**PFA:** Paraformaldehyde;

**PTFE:** Polytetrafluoroethylene;

**QiMTDSC:** Quasi-Isothermal Modulated Temperature Differential Scanning Calorimetry;

**SDS:** Sodium Dodecyl Sulfate;

**SEM:** Scanning Electron Microscopy;

**TAVR/TAVI:** Transcatheter Aortic Valve Replacement/Implantation;

**T_d:** Denaturation Temperature;

**TEM:** Transmission Electron Microscopy;

**T_g:** Glass transition Temperature;

**T_m:** Melting Temperature;

**T_s:** Shrinkage Temperature;

**UTS:** Ultimate Tensile Strength;

**vCJD:** Variant Creutzfeldt-Jakob disease;

**VEC:** Valvular endothelial cells;

**VIC:** Valvular interstitial cells;
Declaration

I hereby certify that the thesis has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a degree or qualification.

Karl Joyce
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Abstract

Bovine pericardium is an extensively used biomaterial utilised in a wide range of biomedical devices such as bioprosthetic heart valves. The characterisation techniques employed for the analysis of bovine pericardium and similar natural soft tissue biomaterials, vary across both information sought, preparation of sample and the method itself. Natural biomaterials differ from traditional synthetic types in that additional steps are required during the production process to make them suitable for their application in vivo. These steps can include chemical fixation using glutaraldehyde to preserve stability, strength and prevent against enzymatic degradation. This thesis assessed the biomechanical, physical, chemical and cytotoxic methods available for the analysis of bovine pericardium.

Biomechanical assessment of bovine pericardium is varied across the literature in both techniques and methods used. The thesis investigated uniaxial testing by focusing on two standard test parameters of strain rate and preconditioning number of cycles, to elucidate recommendations for the standardisation of a uniaxial method, while also measuring not so common parameters of low modulus and hysteresis. The results demonstrated that an extension rate of 10 mm/min and 5 preconditioning load-unload cycles as a reference point for the standardisation of a uniaxial testing method. Imaging analysis of the collagen structure post mechanical testing using scanning electron microscopy, displayed the crimped pattern present after the removal of the stress. This provided a quantitative assessment of bovine pericardium post mechanical testing, that can be applied to further the understanding of the behaviour of the tissue under stress.

Glutaraldehyde is an extensively used sterilant and fixative for the crosslinking of natural soft tissue biomaterials like bovine pericardium. There is significant debate around the reaction mechanism of this crosslinker with natural
biomaterials. This section explored the reaction mechanism using a derivative calorimetry technique, quasi-isothermal modulated differential scanning calorimetry, most commonly used for the analysis of polymorphic transformations in pharmaceuticals, to measure the rate of crosslinking between glutaraldehyde and bovine pericardium. The analysis showed that crosslinking reaction was completed after approximately 10 min and provided further evidence of the changing monomeric chemistry of glutaraldehyde. Additional characterisation of crosslinked bovine pericardium using Ninhydrin assay, proved to be a fast and convenient method to qualitatively demonstrate the crosslinked status of the tissue. Also, the structural assessment of the tissue using electron microscopy methods of scanning and transmission, and atomic force microscopy provided further insight into the directionality of collagen, ultrastructure analysis of cellular components and quantitative measurements of the D-banding pattern of collagen fibrils.

The final phase of the thesis compared glutaraldehyde fixation with that of alternative treatments using genipin and decellularisation. A concentration of 3 mM of Genipin was recommended due to its increase in the thermal denaturation temperature (T_d), producing mechanical properties similar to those of glutaraldehyde. The decellularisation protocol using sodium dodecyl sulfate produced a favourable cytotoxic evaluation compared to glutaraldehyde fixed bovine pericardium. While decellularisation used in combination with glutaraldehyde or genipin improved both its mechanical and cytotoxic properties. Together this data demonstrated that Genipin and the decellularisation protocol are viable alternatives for the treatment of bovine pericardium.
For, Grace and Liam
Chapter 1: Introduction

1.1 Biomaterials

The general classifications of biomaterials have moved from the traditional metal, ceramic and polymer to keywords like scaffolds and tissue engineering where a single class does not reflect the complexity of highly structured tissues and demonstrates how focus has evolved from technologically processed biomaterials towards biologically inspired design [1]. Natural biomaterials and their uses date as far back to ancient Egypt with animal sinew used for sutures, to the use of a biodegradable suture called “catgut” in 1885 by Joseph Lister and William Macewen [2] developed from ovine intestine. Synthetic materials were used through the ages in the forms of iron dental implants in 200AD to Adolf Fick’s research into glass contact lens in 1860[3] A key change point came at the end of WWII when materials developed for the military were available for general use [1] such as Ripley’s development of the intraocular lens from the poly(methyl methacrylate) fragments of fighter planes dome coverings[3]. From the 1960’s and the development in the fields of chemistry and engineering, synthetic materials were seen as the biomaterial of choice[3]. However with the advancement in molecular biology, led through the pivotal Watson and Crick 1953 paper on the structure of DNA[4] and the increasing ability to understand the organization, architecture and workings of the extracellular matrix (ECM) of natural tissues, interest in them as biomaterials has increased. The field of toxicology also progressed with leachable’s from synthetic polymers causing concern and where natural materials do not generally have this issue[3, 5]. Biomaterials derived from natural sources have found applications and have developed from the 60’s and 70’s in varied areas as bioprosthetic heart valves,[6-8] wound repair [9] and vascular grafts [10, 11]. Naturally derived biomaterial tissues have increased biocompatibility and a greater degree of tissue remodeling than a synthetic polymer type[12]. Biomaterial scaffolds derived from natural sources consist of an ECM that is composed of the structural polymeric proteins collagen and elastin and
glycosaminoglycan’s (GAGs) [9]. Table 1-1 displays various sources of natural biomaterials, their applications, components and organization. Synthetic polymeric materials have the advantage of known and repeatable mechanical and thermal properties through closely monitored manufacturing methods. The sourcing of natural biomaterials can be limited for certain types of tissue such as bovine or porcine where guidelines state that they should be free of contaminants like transmissible spongiform encephalopathy (TSE) agents and so are restricted to countries such as Australia and New Zealand [13]. Also the availability of allografts and autologous sources are limited. Naturally sourced biomaterial after harvesting must undergo a series of treatments whether to sterilize, alter mechanical properties or to remove the immunological response of the body to the material. Cardiovascular applications such as prosthetic heart valves and vascular grafts are serviced by both biomaterial types – prosthetic heart valves are broken into 2 categories of either mechanical[14, 15] (Dacron® valve on a metallic frame –caged ball type using silicone ball or a tilting disc consisting of a carbon disc with an expanded polytetrafluoroethylene (ePTFE) covering) or xenogeneic natural tissue of porcine heart valve or bovine pericardium (BP) [16, 17].

Bovine pericardia are used extensively in bioprosthetic devices from heart valves to soft tissue repair and abdominal wall defect repair. The variety of naturally derived materials is outlined in Table 1-1 and shows how tissue from different sources and different functions, whether dermal, pericardial or submucosal in nature can be applied to the same application such as bioprosthetic heart valve or abdominal wall repair [18]. Patches derived from BP provide several advantages over synthetic prosthetic patches like PTFE and polyester (Dacron®) by allowing functional host tissue integration, greater biocompatibility, reduction of infection rates and also no requirement for lifelong anti-coagulation treatment that is associated with synthetic prosthesis [10, 19]. Also in the area of vascular grafts synthetic materials such as PTFE or Dacron also are not suitable for smaller diameter vessels, less than 6mm, as they can prove to be more susceptible to thrombogenicity and so natural materials
are the preferred choice.[10] The choice of natural materials can vary for this type of vascular graft where typically considered, autologous sources such as the likes of a saphenous vein could be applied to repair a small diameter arterial bypass or in the case of a coronary artery an arterial graft such as radial artery and internal thoracic artery could be used [20, 21]. The structure of the natural materials may be the key factor in why they a desirable choice of biomaterial. Collagen and elastin of the ECM among other components are the chief structural framework for natural materials which allows for the material to function biologically on both the molecular level and macroscopic level, [5] whereas synthetic materials are only capable of performing on macroscopic level. It is due to this biofunctionality that these are the still go-to biomaterial despite the limitations with naturally derived materials mentioned previously.

Bulk and surface properties are critical in determining the application of any biomaterial. Surface properties are important for implant blood contacting materials, with the macromolecular microstructure important in determining the interaction between the functional groups of the biomaterials surface and the surrounding medium [22]. The bulk properties determine the physical properties such as the mechanical strength, density and elasticity. Benefits of BP over mechanical and synthetic heart valves can be identified through both surface and bulk properties.

1.1.1 Surface Properties
Surface topography is known to influence biological processes such as protein adsorption and conformation, cell behaviour, blood-contacting properties and bacterial adhesion [23]. Other surface factors like energy, hydrophobic, hydrophilic and chemical composition are also important considerations. Blood compatibility is governed through its interaction of its negatively charged platelets and the surface of the biomaterial [24].

Mechanical valves have evolved from the 1950’s Dr. Hufnagel aortic ball valve of methacrylate to the Pyrolytic Carbon introduced in the 1970’s; it consists of
covalently bonded atom layers stacked in a disorderly fashion, that create wrinkles and distortions [25]. An example of this type effect is in the area of bioprosthetic heart valve replacement. While this material is an improvement on previous models, the requirement for anticoagulation treatment remains. Even with the superior profile of new generation mechanical bileaflets to previous generations of prostheses, localized regions of turbulent flow can still develop and lead to stasis and thrombus formation [26]. Mechanical valves are associated with the risk of thromboemboli and thrombotic occlusion due to non-physiologic surfaces and flow abnormalities caused by obstructions on the surface [27].

The bioprosthetic heart valve does not require the post-operative immune suppressant medication, as pericardium tissue valves maintain a low rate of thromboembolism without anticoagulation [27][28]. The central pattern of flow of the pericardium valve is similar to that of the natural heart valve and the cusps typically more thromboresistant than synthetic materials [29]. Figures suggest that thromboembolism of the tissue valve at approximately the same rate as a fully anticoagulated mechanical valve [30]. The degree of risk and cause of thromboembolism has been credited to the interaction of Virchow’s triad of factors such as the trans-prosthetic-induced endothelial dysfunction, prosthetic material electrical surface charge properties, prosthetic material-induced platelet aggregation and alteration of the thrombotic/fibrinolytic milieu [26, 31, 32]. Elimination of post-operative anticoagulation medication for bovine pericardium valves is also due to the surface charge properties of the collagen dominant material. The zeta-potential for a glutaraldehyde treated pericardium tissue has been quoted as -4.9 ± 0.6mV while for a synthetic polymeric material like PTFE is -20.12mV which is a significantly higher than the value for the natural heart valve of -1.9 ± 0.5mV [33, 34].
1.1.2 Bulk Properties

While the surface properties affect the biomaterial-host interaction the physical properties and some of the key selection criteria are dependent on the bulk properties. Physical properties of biomaterials depend on the type and strength of the interatomic bonds. This is evident when synthetic biomaterials are applied to replace elements of the cardiovascular system such as aortic valves. The aortic valve structure of three leaflets and sinuses that operate in an environment of variable pressures and experience three main physical modes during the opening and closing of the valve, tension, shear and flexure [35, 36]. The constant shutting and opening movement of the valve is difficult for man-made materials to replicate in vivo as the structure is far removed from the native valve’s arrangement.

Native leaflet’s, tri-layer architecture of predominantly collagen type I fibres in the main load bearing layer of the fibrosa, and the opposing ventricularis layer that consists of collagen and radially aligned elastin, responsible for the leaflets ability to close under the backpressure of the outflowing blood [37-39]. The spongiosa layer, sandwiched between the fibrosa and ventricularis layers, contain proteoglycans that act as a buffer between the outer two layers providing lubrication as they shear and deform relative to each other during leaflet bending and pressurization [40, 41]. Bovine pericardium is constructed of the same collagen, elastin and GAG structural foundations of the native valve. The hierarchical architecture of the collagen type I fibres replicate the biomechanics of the native valve, which the mechanical valve cannot.
Table 1-1: Selection of naturally-derived biomaterials, their key-components and structural organisation

<table>
<thead>
<tr>
<th>Origin of Material</th>
<th>Application</th>
<th>Key Components</th>
<th>Organization</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td><strong>Porcine Sources</strong></td>
<td></td>
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<tr>
<td>Porcine Aortic Valve</td>
<td>Artificial Heart Valve</td>
<td>Collagen Type I and III, Proteoglycans</td>
<td>Layered structure of fibrosa, spongiosa and ventricularis. Thickness: 0.36-0.86mm</td>
<td>[29, 42, 43]</td>
</tr>
<tr>
<td>Porcine small intestinal submucosa</td>
<td>Abdominal wall repair devices, Skin repair/wound dressing, Vascular prostheses, Ligament repair</td>
<td>Collagen I, III, and V, Elastin and GAGs</td>
<td>Single layer of naturally cross-linked collagen based ECM, Thickness: 0.1-1.0mm</td>
<td>[44-48]</td>
</tr>
<tr>
<td>Porcine Urinary Bladder mucosa</td>
<td>Abdominal wall repair, Bladder augmentation</td>
<td>Collagen I, III and IV and GAGs</td>
<td>Layered structure with surface, intermediate and basal cell layers, Thickness: 1.4mm</td>
<td>[49-52]</td>
</tr>
<tr>
<td>Porcine Dermal Graft</td>
<td>Abdominal wall repair, Skin repair/wound dressing, Rotator Cuff Repair</td>
<td>Collagen and Elastin</td>
<td>Crosshatch pattern of woven collagen fibres</td>
<td>[48, 53]</td>
</tr>
<tr>
<td>Porcine Bladder</td>
<td>Urethral Repair, Wound repair</td>
<td>Collagen I, II, III, IV, V and VII, GAGs</td>
<td>Multi-layered with both longitudinal and circumferential directed collagen fibres, Thickness: 4-5mm</td>
<td>[49, 54-56]</td>
</tr>
<tr>
<td><strong>Bovine Sources</strong></td>
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</tr>
<tr>
<td>Bovine Pericardium</td>
<td>Artificial Heart Valve, Patch Angioplasty, Abdominal Wall repair</td>
<td>Collagen Type I, Elastin, GAGs</td>
<td>3 layers of wavy collagen bundles and elastin. Thickness: 0.2-1.2mm</td>
<td>[48, 57, 58]</td>
</tr>
<tr>
<td>Bovine Ureter</td>
<td>Vascular Prostheses</td>
<td>Collagen I</td>
<td>Fibrous and muscular components arranged in a loose helical structure.</td>
<td>[59-61]</td>
</tr>
<tr>
<td>Bovine Fetal Skin</td>
<td>Wound repair</td>
<td>Collagen Type I, III and XIV</td>
<td>Layered structure, Thickness: 1mm</td>
<td>[48, 62-64]</td>
</tr>
<tr>
<td><strong>Allografts</strong></td>
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<td></td>
</tr>
<tr>
<td>Dermal Allografts</td>
<td>Breast Reconstruction, Ligament Repair, Skin Repair/wound dressing</td>
<td>Collagen III, IV, VII and Elastin</td>
<td>Collagen bundles in a loose meshwork Thickness: 0.4 – 4.0mm</td>
<td>[48, 65, 66]</td>
</tr>
</tbody>
</table>


1.2 Heart and Heart Valves

The heart is a delivery system that distributes the body with oxygen enriched blood and provides blood to organs to remove waste products from the body such as carbon dioxide and other metabolic waste. Its location is in the middle of the thoracic region. The structure consists of four chambers, the left and right ventricle and atrium. The septum divides the heart in half with the left side pumping oxygenated blood from the lungs to the body while the right side receives deoxygenated blood pumping it to the lungs. The circulation of the oxygenated blood around the body is composed of the systematic circulation that controls the oxygenated blood pumped through arteries and veins to the organs and limbs while the pulmonary circulation transports the deoxygenated blood to the lungs.

There are four valves of the heart, the aortic, pulmonary (semilunar valves) and the tricuspid and mitral valves (atrioventricular). The mitral valve and tricuspid valve lie between the atria (upper heart chambers) and the ventricles (lower heart chambers), Fig. 1-1.

Figure 1-1: Cross section of the heart displaying the pulmonary, aortic, tricuspid and mitral valves.
Each heart valve has different structures and features in relation to their position and function within the heart i.e. valves structure can vary due to pressure gradients and direction of flow. The aortic and pulmonary valves (semilunar valves), separate the ventricles from the great arteries while the mitral and tricuspid (atrioventricular valves) separate the atria from the ventricles [38]. The valves provide unidirectional flow to and from each of the chambers [67]. Significant differences between the two valve types include the attachment of collagenous cords, chordae tendineae, to the atrioventricular valves to prevent them collapsing back into the atria during closure. The semilunar valves opening and closure is dependent on the pressure difference across the valve. Structurally the semilunar valves and the tricuspid valve contain three cusps while the mitral valve contains two cusps [68].

The valve of interest for this research is the aortic valve, with increasing developments around bioprosthetic heart valves and the treatment of related diseases.

1.3 Aortic Valve
The aortic valve consists of 3 cusps the left coronary, right coronary and non-coronary cusps, named according to their relationship to the coronary arteries [69, 70]. It is part of the aortic root that also contains a crown shaped annulus, the 3 sinuses of Valsalva and the aortic valve leaflets [67]. The aortic valve and pulmonic valve lie between the ventricles and the major blood vessels leaving the heart. The aortic valve undergoes approximately 30-40 million cycles per year which averages approximately 3 billion cycles in a single lifetime [71]. The volume of blood passing through can range between 1 and 20 l of blood per minute depending on rest, exercise or other physiological or pathological conditions [67]. The structure of the aortic valve has developed so that it is robust to function under these conditions.
1.3.1 *Structure*

The aortic valve comprises of three cusps each of which have a three-layered structure of thickness ranging from 300-700 µm. These cusps of the aortic valve are approximately less than 1 mm in thickness with the base and tip the thickest sections. They are thin enough to receive nourishment through hemodynamic convection and diffusion [38, 72]. The three layers consist of a spongiosa central layer is sandwiched between an endothelial cell covered fibrosa layer located on the aorta side of the valve and an endothelial covered ventricularis found on the ventricular outflow side **Fig. 1-2**.

*Figure 1-2:* Aortic valve cusp structure and composition. The cusp has a thickness of approximately 300 – 700 µm.
The fibrosa layer contains mainly Type I and Type III collagen fibres that are circumferentially orientated, densely packed and arranged parallel to the cuspal free edge [29]. The ventricularis, consists of largely collagen that are organised with less directionality than in the fibrosa when fully relaxed and also filamentous elastic fibres aligned radially [29, 72]. The spongiosa is composed of proteoglycans and collagen fibres. This spongiosa layer that acts as a shock absorber between the outer ventricularis and fibrosa layers through systole and diastole [29]. This central layer is also found in porcine valves that contain proteoglycan deposits that help reduce friction with the outer layers [73] but is absent from pericardium. The unique characteristics and organisation of collagen and elastin contribute to the surface and bulk properties of the tissue. The fibrosa collagen delivers tensile stiffness while the elastic fibres of the ventricularis allow for the extension and recoil movement of the valve. The whole leaflet structure is efficient for the large pressures when the valve is closed and reduced pressure gradients when the valve is opening during systole [38, 67, 74, 75]. It has been suggested that elastin and collagen fibres, Fig. 1-3, are mechanically coupled together in a honeycomb or sponge-like that maintains the collagen fibre orientation and geometry during release of external forces [67]. Between the extracellular components of the collagen, elastin and the proteoglycans, interstitial cells exist.

![Figure 1-3: Structure of collagen and elastin during systole and diastole. The fibrosa undergoes a corrugation effect during systole with elastin fibres extending. As collagen is fully stretched it takes over the main load bearing of the cusp from elastin during closure [76]](image-url)
1.3.2 Cellular components

Although the valvular extracellular matrix behaves like a dense planar connective tissue of the musculoskeletal system both structurally and mechanically, due to the blood contacting environment it is coated with an endothelial cell monolayer [37]. The communication between the endothelial and the interstitial cells is necessary for the ECM homeostasis. The main cells for the aortic valve leaflets are endothelial and interstitial cells [29, 67, 76]. The valvular endothelial cells (VEC) are located on the fibrosal and ventricular surfaces of the leaflets and their function is to provide a non-thrombogenic blood contact surface and also communicate to and regulate the activity of the interstitial cells [77, 78]. VEC are different endothelial phenotype to vascular endothelial cells (EC) [79] and also behave different to shear stress by aligning perpendicular to the direction of flow compared to the parallel arrangement of EC [80]. The valvular interstitial cells (VIC) are found in the spongiosa layer and are responsible for the valve structure through remodelling and organising of matrix proteins [76, 78, 81]. The VICs consists of different cell phenotypes such as smooth muscle cells, fibroblasts and myofibroblasts [67, 76]. Research has shown that VICs have a higher generation of proteins and glycosaminoglycans than other cell types and suggests that they increase the durability of the valve through the repair of mechanically induced damage [76, 82]. Their function is dependent on the requirements of the valve at various stages of development as the interstitial cells are phenotypically plastic and can be dormant in a healthy valve and developing during wound healing or remodelling. VIC induced remodelling and synthesis has been shown to increase in accordance with the scale of the mechanical forces and the resultant local signalling. This was reported by Ku et al. [83] who showed that collagen synthesis by VIC was related to the length of time and extent of strain with an increase in [3H]-proline incorporation into stretched valve cells at 10%, 14% and 20% stretch. The communication of the VEC and VIC enable the valve tissue to adapt to any change in conditions and maintain a stable environment as demonstrated by endothelial cells increasing protein synthesis of co-cultures after the addition of steady shear stress [79]. The valve opens in systole and
closes during diastole, blood flows approximately at a peak acceleration of 1.35 ± 0.35 ms⁻¹. Mechanical effects on the valve have an impact on both a tissue and cellular level with the long-term growth/durability determined by the mechanical interaction with the biomechanics of the valve [37].

1.3.3 Mechanics

Stresses and strains within the aortic valve vary depending on the phase of the cardiac cycle [84] Fig. 1-4. During diastole, stresses on the order of 500 kPa and 50 kPa on systole have been approximated on the leaflets assuming that deformation on the valve from blood pressure is a combination of axial stretch and bending [85]. Also areas of the attachment of the valve to the aortic wall, have reported stresses in the diastole from 76 – 95 kPa in the circumferential direction and from 37-44 kPa in the radial direction [36]. The fibrosa took the higher diastolic load while the ventricular surface took the systolic load.

**Figure 1-4**: Schematic of biomechanical forces during systole (A) and diastole (B) and the resulting impact on the VICs and VECs [85].
Other studies have estimated the physiological stresses on the leaflets during diastole and systole to be in the region of 200-400 kPa [76, 86, 87]. Leaflet strain in vivo have been calculated approximately 0.1 in the circumferential direction and 0.4 in the radial direction [88]. The biomechanics of the leaflets show that they extend compliantly before reaching a critical strain where they then become rigid. The leaflet extensibility is greater in the radial direction (perpendicular to the free leaflet edge) with a Lagrangian strain of $\varepsilon_R$ 0.6-0.8 compared to circumferentially $\varepsilon_C$ 0.2-0.3 [76]. This transition from high compliance at low strains of systole and increase in rigidity at higher strains due to diastole allows the leaflets of the valve to perform. Ultimate tensile strength (UTS) loads of aortic valve leaflets range from 2.0 - 4.0MPa which is approximately 10 times greater than the stresses that occur in vivo [89]. Fibre rearrangements during closure allow crimps and corrugations expand in the radial direction and permit an increase in dimension with minimal stress [29]. Elastin operates in the ventricularis by expanding when the cusps stretch to enlarge the coaptation area and recoil to make the leaflet smaller in the open phase [29, 41, 76].

1.3.4 Aortic Valve Disease

Over 27 million of American adults have been diagnosed with heart disease [90], with global valve replacements at approximately 290,000 per year and predicted to triple by 2050 [91]. The aortic valve is the most commonly diseased [38, 92] and transplanted valve [93, 94]. Aortic stenosis (AS) is the predominant valvular heart disease in developed countries, with onset of severe symptoms producing a mortality rate of 25% per year [95]. Elderly patients, experience aortic stenosis at a rate of 2% of the population over 65 years with 4% of patients over 85 years experiencing the condition [96, 97]. Younger patients experience aortic valve issues through congenital defects, infections such as rheumatic fever or problems with bicuspid valve’s [93, 98, 99]. The effect on the heart valve is the incomplete opening or closure of the valve, producing an impediment in blood flow, Fig. 1-5 [100].
Regurgitation is the resulting effect of incomplete closure and occurs when tissue is damaged from conditions such as endocarditis [101] and causes backflow of blood from the aorta to ventricle. Other defects happen when the tissue is damaged or torn due to calcification and plague formation leading to stenosis or failure for the valve to completely open. Calcific disease of the aortic valve, now the primary cause of aortic stenosis in developing countries and is a common reason for the stenosis of the valve leading to its need for eventual replacement [102-104]. A general indicator is the development of calcific minerals such as calcium phosphates in the fibrosa region of the aortic valve [105]. Aortic valve sclerosis is a primary process starting deep within the tissue before leading to stenosis and the reduction in functionality of the valve [106]. Rajamannan et al. observed that valvular aortic stenosis is due to bone formation in the valve as a result of an osteoblast-like phenotype [107]. Lipids too are involved in the calcification process with oxidized low-density lipoprotein (LDL) evident in calcified valves [106]. Also patients with aortic stenosis have indicated higher concentration levels of cholesterol than a control group [108, 109]. The calcification cascade for a natural valve is illustrated in Fig. 1-6 [110].

Figure 1-5: Normal and damaged heart valve in open and closed positions. Note the limited movement of the heavily stenosis valve and the gap in its closed position.
Heart issues can lead to symptoms including shortage of breath, lack of energy and requires medical intervention for successful treatment. The available options for the treatment of AS range from full open-chest surgical procedures to the selection of the minimally invasive replacement devices known as transcatheter aortic valve replacement (TAVR) using bioprosthetic heart valves [104, 111-114]. These can be broken down into three main categories, cryopreserved homograft heart valves, mechanical valves and bioprosthetic

Figure 1-6: Aortic valve calcification mechanism. Modified from Merryman et al. [110]
heart valves [115]. Homograft pulmonary valve replacement through the Ross procedure, where the damaged aortic valve is replaced with the patient’s own pulmonary valve, offer advantages for younger patients [116]. However, limited availability and challenging surgical procedure restrict their use [117].

1.4 Prosthetic heart valves
Prosthetic heart valves consist of two main categories, the mechanical and bioprosthetic heart valves. A selection of both mechanical and bioprosthetic heart valves are listed in Table 1-2. Mechanical valves whereas, have the benefit of a structurally stable lifespan and are used frequently in younger patients however they are thrombogenic, can be more audible than those of bioprosthetic heart valves and require anticoagulation medication [104]. While mechanical valve replacement was the benchmark treatment bioprosthetic valves have seen an increase in their use rise to over 45% of 300,000 valve replacements globally per year compared to 55% for mechanical valves [27, 118]. The main reasons for the increase in usage of bioprosthetic heart valves will be explored further.

Research in other synthetic polymeric materials like silicones, polyurethanes and flouroethylene’s have proceeded for over 50 years without significant clinical success [117, 118]. TAVR bioprosthetic heart valves consist of biological xenografts of porcine heart valve, porcine or bovine pericardium, sewn onto a rigid or semi-rigid mechanically expanded frame such as nitinol, and delivered via a catheter [29, 104]. They have increased in use over the past 15 years to 200,000 procedures across 65 countries [104] and predictions of heart valve replacements to triple by 2050 [119] indicate even further increased demand. The use of bovine pericardium as a bioprosthetic heart valve material, despite a number of limitations, has earned widespread suitability [120, 121].
1.4.1 Mechanical heart valves

Mechanical valves have been around for almost 70 years, with Charles Hufnagel implanting a ball-and-cage designed valve into the descending aorta [15]. Developments in both the areas of surgery and biomaterials have seen innovations in the designs of mechanical valves progress from the ball-and-cage to the Bjork-Shiley tilting disc model to a bileaflet design [114, 122]. Ball and cage components evolved from stainless steel to titanium cages with a silicone ball [123]. With increasing knowledge of flow dynamics, the design of the mechanical valve changed to the tilting disc model in the 1970’s. Mechanical valves are typically constructed of a metal alloy or pyrolytic carbon material [123]. This valve type is durable and robust, but the key limitation is its effect on the flow dynamics that can lead to systemic thromboemboli and thrombotic occlusion [124, 125]. The lifelong anti-coagulation medication also presents possibilities of haemorrhaging complications [29].

1.4.2 Bioprosthetic heart valves

Bioprosthetic valves evolved from a stainless steel frame to the superelastic shape memory nitinol stent, that provides ease of catheter delivery, to investigational stentless devices [126]. Unlike the mechanical valves there is no requirement for anti-coagulation treatment due to similar blood flow to that of the native valve and the less thrombogenic tissue material compared to the biomaterials used in mechanical valves [29]. However limitations present themselves in the form of decline in integrity of the tissue through either calcific [93, 127] and non-calcific damage [128-130].

Bioprosthetic heart valves have seen a steady increase in their selection as treatment of diseased aortic valves since their introduction in 2002 [131]. The benefits over mechanical valves include minimally invasive procedure and no requirement for lifelong anticoagulation medication. The procedure involves delivery of the valve via the femoral or subclavian artery resulting in a quicker recovery than other valve replacements.
1.4.3 Next generation

The limitations of both mechanical and bioprosthetic have seen extensive research on the next generation of valve that can somehow provide the best qualities of each but also increase the lifespan and age profile of patients. The main types of synthetic type materials include polyurethanes [132-134], silicones [135], fluoroethylenes [135, 136] and composites like polycarbonate ureas and silsequioxane [137]. Polymeric leaflets in theory would provide the lifelong stability avoiding calcification and mechanical damage issues [117]. However, even with the significant development in hybridising polymer chemistry with valve design and all potential benefits like reduced cost, reduction in delivery size and recently hemodynamically [138] they are still not considered a viable option. Whereas, BP for all its disadvantages, is still the main material for use in bioprosthetic heart valves.
<table>
<thead>
<tr>
<th>Category</th>
<th>Device</th>
<th>Details</th>
<th>Ref</th>
<th>Valve Image</th>
</tr>
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<tr>
<td>Allografts</td>
<td>Cryoperserved pulmonary and aortic valves from human donors.</td>
<td>[139, 140]</td>
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<tr>
<td>Autograft</td>
<td>Ross Procedure Pulmonary autograft</td>
<td>[141]</td>
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<td></td>
<td>Starr Edwards Ball in cage type, with Silicone rubber</td>
<td>[114]</td>
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<td></td>
<td>Magovern-Cromie Caged ball valve</td>
<td>[142]</td>
<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>Mechanical Valves</td>
<td>Bjork-Shiley Monoleaflet valve (tilting disc)</td>
<td>[143, 144]</td>
<td><img src="image3.png" alt="Image" /></td>
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<td></td>
<td>Medtronic-Hall Monoleaflet valve (tilting disc)</td>
<td>[145, 146]</td>
<td><img src="image4.png" alt="Image" /></td>
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<tr>
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<td>Bileaflet Valve</td>
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<td>Porcine Valve</td>
<td>152</td>
<td><img src="image" alt="Hancock II™ Porcine Valve" /></td>
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<td></td>
<td>Epic™ (St. Jude Medical)</td>
<td>Porcine Valve</td>
<td>153</td>
<td><img src="image" alt="Epic™ Porcine Valve" /></td>
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<tr>
<td>Category</td>
<td>Device</td>
<td>Details</td>
<td>Ref</td>
<td>Valve Image</td>
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<tr>
<td>Bioprosthetic Heart Valves</td>
<td>Lotus™ (Boston Scientific)</td>
<td>Bovine Pericardium</td>
<td>[154, 155]</td>
<td><img src="image" alt="Lotus Valve Image" /></td>
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<tr>
<td></td>
<td>Portico™ (St. Jude Medical)</td>
<td>Bovine Pericardium</td>
<td>[156]</td>
<td><img src="image" alt="Portico Valve Image" /></td>
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<tr>
<td></td>
<td>Sapien™ (Edwards Lifesciences)</td>
<td>Bovine Pericardium</td>
<td>[157, 158]</td>
<td><img src="image" alt="Sapien Valve Image" /></td>
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<td>Engager™ (Medtronic)</td>
<td>Bovine Pericardium</td>
<td>[159, 160]</td>
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<td></td>
<td>Symetis Acurate Neo™ (Boston Scientific)</td>
<td>Porcine Valve</td>
<td>[161]</td>
<td><img src="image" alt="Symetis Valve Image" /></td>
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</table>
1.5 Bovine pericardium structure

BP is the outer sack membrane that surrounds a cow’s heart. Its main purpose is to protect the heart by acting as a shock absorber and providing lubrication to minimise friction that may arise during its movement. The heart's location is in the thoracic cavity, between the lungs. It is cone shaped with a broad top end where the blood arteries and major vessels allow for the exit and entrance of blood into the heart. The heart narrows towards the bottom where the apex is closest to the sternum. It consists of two layers, a deep layer visceral serous pericardium or also known as the epicardium layer that is closely adherent to the heart [162]. The outer layer is called the parietal layer and acts to prevent distension of the heart due to excessive stretching of the heart muscle fibres [163]. In between these 2 layers there is a small cavity, the pericardial cavity that contains a fluid that acts as a lubricant to reduce friction on the heart during movements. The bovine parietal pericardium has three layers (1) the serosa which is a thin layer of mesothelial cells (2) the fibrosa containing multidirectional orientated wavy bundles of collagen and elastic fibres and (3) the epipericardial connective tissue layer which is partly continuous with the pericardiosternal ligaments [58, 164, 165].

Its extensive application as a biomaterial is multifactorial, including availability, structure and resulting biomechanics, and proven historical uses for applications like vascular patches [10], vaginal [166] and abdominal wall repair [167]. It has also found use in the manufacturing of bioprostheses for over 30 years [168]. Its thickness is varied depending on the location of measurement and can range from 0.3 – 1.5 mm [169]. Bovine pericardium is a non-linear, anisotropic, multilaminate composite pliable material [170], consisting of a collagen, elastin and GAGs extracellular matrix. The collagen fibres and there respective bundles are orientated in a weave structure where they can criss-cross one another and align in different directions [165].
1.5.1 Collagen

Collagen and collagen based natural materials are widely used for biomedical applications. Collagen combines strength and flexibility due to covalently bonded chains and cross-links that give added strength like a vulcanized rubber. It provides tension support for soft tissues and provides a platform for the mineralization of hard tissues that undergo compression such as bone. There are at least 28 different forms that perform various functions from mechanical structural support to initiation of the blood coagulation cascade and can be located in areas like tendons and pericardium[171, 172]. The family of collagen types are genetically distinct molecules that have a unique triple-helix configuration of 3 polypeptide subunits known as α-chains[173]. Molecular forms and organization of these alpha chains determines the function of each type of collagen [174]. The fibrillar type I collagen found in natural tissues such as bovine pericardium and vascular grafts, are the quintessential collagen as it provides a structural role, contains the right-handed triple helix without imperfections that allow for the arrangement of its molecules into a fibrillary organization[175]. The structure of type 1 collagen, Fig. 1-7, displays a hierarchical structure that forms a banded fibril structure.
Figure 1-7: Hierarchical structure of collagen type I with banding structure evident in SEM image of a sample of bovine pericardium.
Collagen fibres have a quaternary structure with the final structure of a collagen fibril containing a characteristic banding pattern called the D spacing where a staggered arrangement of tropocollagen molecules are arranged together to form the pattern [176]. This “stagger” is a combination of the strongest polar and hydrophobic interactions through moving neighbouring molecules by 67 nm and the gap between the ends of consecutive molecules forming this periodic pattern evident through electron microscopy and x-ray diffraction [177]. The gap between adjacent molecules can be calculated to 35 nm and the overlap to about 32 nm. This molecule is formed through the right-handed twist formation of 3 α chains that wrap around one another. The chain consists of two heterotrimer chains α1(I) and one α2(I) [174]. The helix-forming (Gly-X-Y), where X and Y are predominantly proline and hydroxyproline respectively, repeat in triple helical domains of a physiochemical stable molecule with dimensions of 300nm in length and 1.5nm in diameter corresponding to approximately 1000 amino acids. Proline, hydroxyproline and glycine as every third residue provide stability to the triple helix with the hydroxyproline responsible for the intramolecular bonding [172, 174, 178, 179]. Hydrogen bonding of the glycine NH- group of one chain to the carbonyl C=O of a neighbouring chain hold the helix together. A closely packed structure is possible due to the glycine residues located in the centre of the triple helix allowing the bulkier side chains of the other amino acids position themselves on the outside of the helix [172]. The graded structure order of collagen, primarily collagen fibres Type I, which are typically found in skin, tendon and bone and type III found in blood vessels are in the order of n=4 with the next level depending on its final biological application. The scale of the organization starts at the nanometre level for the single polypeptide chain and continues at this level to form the triple helical collagen macromolecule attached with a single hydrogen bond per triplet [180]. From here it moves to the fibril staggered arrangement of the fibrils of the micrometre scale and then to the fibre structure in the millimetre scale, as shown in Fig. 1-7. Fibril formation is produced by the covalent intermolecular cross-linking between the lysine and hydroxlysine residues at the carboxyl-terminus (C-terminus) of a molecule and its adjacent
molecule’s amino-terminus (N-terminus), this intermolecular bonding produces the fibril [181]. The head-to-tail organisation of the amino acids is what provides the fibrils with its strength. In tendon and ligaments the organisation can be quite one-dimensional such as for tendons where the collagen fibres can run almost the length of the tendon. However, for structures such as skin, blood vessels, internal mucosa and pericardium the fibres are organised into a more complex 2 and 3-dimensional structures. Collagen type IV and type VII while both displaying similar hierarchical structure of type I, they have particular additions to the structure that allow them perform their biological functions. Type IV collagen has the ability to create sheets and form interlaced networks such as the basement membrane of the kidneys that perform crucial molecular filtration functions [182]. Regions of the triple helical conformation are interrupted with large non-helical domains as well as with the short non-helical peptide interruption [183].

Collagen type VII is what is known as an anchoring filament that links the epithelial basement membrane to the underlying fibrillary tissue [176]. The type VII subunit polypeptide chain of α1(VII) is a complex modular protein consisting of a central 1530-amino acid triple helical domain [184]. The repeating Gly-X-Y motif is interrupted by 19 imperfections due to insertions or deletions of amino acids in the sequence [185].

The importance of the triple helix structure for the properties of collagen is evident with the effect of denaturation of collagen into gelatin. Gelatin[186-188] is obtained from collagen through the unfolding of the triple helix molecules into random coils via an acidic or basic hydrolysis process of denaturation [189]. Gels of gelatin are formed through the cooling of the gelatin passed its gelling transition temperature and where the kinetics of the cooling and the thermal history determine the structure of the gel. Renaturation of the gelatin gel relates to the formation of the triple helix after being unfolded,[190] and can take a number of forms depending on the cooling rate and the concentrations. Tightly packed structures are formed with low concentrations
combined with slow cooling rates while for high concentration and fast cooling rate intermolecular clusters form producing a disordered gel structure. The formation of the triple helix is the determining factor for the properties of the gel [191]. Although gelatin has similar chemical composition of collagen its mechanical properties are not comparable with lower strength and stiffness than collagen’s 120 MPa and 1.2 GPa tensile strength and stiffness respectively[188, 192-194]. Comparison of collagen and gelatin scaffolds showed that the fibrous structure of collagen provided collagen its strength and stiffness and through the denaturation process, gelatin loses this fibrous structure resulting in a lower strength and stiffness compared to collagen scaffold. However, the absence of stiff fibres provides improved compressive resistance properties than collagen [193].

1.5.2 Elastin
The elastic extracellular protein elastin found in blood vessels, skin, lung and ligaments [195-198] is a stable and insoluble protein that forms a three-dimensional network of fibrous monomers[199]. Where collagen provides the tensile strength, elastin maintains the resilience of the tissue [198, 200]. The structure of elastin is not as clear as that of collagen with some suggesting an amorphous structure and others a level of order [198]. Elastin fibres and as an extracellular matrix component in derived natural materials has uses from tissue engineering [195, 200] to autografts, allografts and xenografts [10]. The elastogenesis process is complex and multifaceted with the precursor to elastin the tropoelastin molecule, 72 kDa, synthesized, forming filaments through coacervation [201, 202] before aligning and covalently crosslinking causing the elastin network to grow [198, 203, 204]. The cross-linking and molecular architecture is responsible for the elasticity and stability of elastin providing excellent properties for biomaterials such as dermal grafts and arterial prosthesis [205-208]. These properties present in elastic fibres and laminae are responsible for the elasticity and resilience of tissues [209]. It is this elasticity property along with biological and biochemical aspects that make elastin a widely used biomaterial [195]. The biomechanics of elastin are similar to those of a rubber
with a low young’s modulus, high extensibility and a sharp rise of the stress-strain curve at high elongations [210, 211]. The modulus of elastin is in the region of 0.3-1.5MPa [212, 213] compared to a synthetic polymer’s used for similar applications such as vascular grafts poly(ethylene terephthalate) (Dacron®)[10] and polyurethane have Young’s modulus values of 2.5GPa [214] and 4.7-7.4MPa depending on moisture content [215] respectively. Even though these synthetic vascular graft biomaterials are used for larger diameter sizes >6mm, natural materials like autologous vein and arterial grafts [10, 20] are the preferred choice for smaller sizes as they reduce the threat of thrombogenicity.

1.5.3 Role of water

The interaction of water with the structures of these natural polymers is central to their properties and behaviour. Water plays a critical role in maintaining the conformation of collagen molecules and mechanical properties of collagen fibrils [216]. The presence of water in the molecular structure determines properties relating to tensile strength and thermal stability of collagen and its absence affects this structure leading to an increase in the rigidity of the molecule [217]. Also water is heavily involved in the process of collagen self-assembly [218] and affects the stability of collagen in a complex way[219]. Gelatin’s transition from gel-to-sol (liquid) is affected by rising temperature with the degree of hydration increasing and the number of hydration water molecules decreasing with the increase in concentration of amino acid composition [220]. Water in proteins can be generally be classified into bulk, bound or hydration. Bulk water is free to move and assists in protein diffusion, bound water has multiple contacts that provide stabilization for the protein and hydration is in direct contact with the protein and keeps it in solution [221]. Hydration water in elastin directly affects the elasticity of the biopolymer through the formation of hydrogen bonds with the main chain atoms which provides elastin its dynamic properties in both the extended and relaxed states [222]. The water in elastin through interaction with the polar amino acids forms internally bonded structures of clathrate cages [223] that force the protein chains
apart and produce an open helical structure with the clathrates through the centre [224]. These structures of water allow sections of the elastin to rotate freely to produce an elastic response and the changes in the backbone yield an entropic elastomeric force that is responsible for the elasticity mechanics of elastin [225].

1.5.4 Glycosaminoglycans
The other key component of bovine pericardium that is assembled in the ECM along with collagen and elastin, are glycosaminoglycans GAGs. They are long unbranched polysaccharides that form when attached to a protein core of proteoglycans [226]. Polysaccharides contain approximately 10 monosaccharides, which are a simple sugar (like sucrose or fructose) that do not hydrolyse to give another sugar. The role of proteoglycans involves the regulation of collagen [227] and water, with the later cause for the large concentration within the highly hydrated spongiosa of the aortic valve [226]. Proteoglycans and GAGs typically make up approximately 1% of the bovine pericardial ECM [58, 228]. Biomechanical effects have been debated with reports in the reduction in shear forces within the aortic valve [29, 229] and the role in viscoelasticity and hysteresis behaviour of BP [230, 231].

1.5.5 Mechanics
The mechanical properties of BP are generated through its complex structure of an amorphous matrix surrounding the network of unidirectional collagen and elastin fibres. From an ultrastructure level groups of the collagen fibres can be identified to be in a vertical z direction where others are in a horizontal or planar x-y direction. However, from a microstructure perspective a more ordered orientation appears in that there is a definite difference in the orientation of the fibres with respect to the apex-base of the pericardium and circumferentially around the pericardium [232-234]. This orientation difference, and also the thickness differences across the tissue has a significant impact on the structural and mechanical properties of the pericardium [235, 236].
Uniaxially tensile tested collagen-based biomaterial like BP produce a characteristic J-shaped graph Fig. 1-8.

The applied force produces a non-linear stress-stiffening graph due to the uncrimping of the collagen fibres. The curve consists of a toe region, due to the uncrimping of the collagen fibres. This then transitions into a heel region of the curve, which represents the end of the uncrimping of the fibres and the aligning of the molecules that now have the ability to slide past one another [199]. Stress in the linear section of the curve is high and is difficult to fracture the collagen fibres under normal physiological conditions [212, 237]. The complete stress-strain process represents the rearrangement and movement of the collagen molecules without the breaking of bonds. This ability of the crimped fibres to reorganize, straighten out and then return to their original wavy structure upon
release of a tension is a characteristic biomechanical feature of BP, but if tension exceeds the straightening of the waviness then fibre damage can occur [199]. The tensile strength of elastin is lower than that of collagen but it has an order of magnitude greater elongation. Elastin which is considered the most “linearly” elastic biosolid material known is found in arteries, skin and connective tissues in combination with collagen. The ligamentum nuchae such as that present in the necks of horses and is composed of mainly elastin and a small degree of collagen [238] is an example of its elasticity and elastic-energy storage capabilities. Elastin’s capability of molecular rearrangement after the application of a force provides the mechanical properties of low stiffness, high extensibility and high resilience [192]. The durability of elastin is evident in that it is not synthesized after development and is present throughout a lifetime. Table 1-3 displays several mechanical properties of elastin and collagen through various hierarchical stages. The decrease of E-modulus from the collagen molecule to the final functioning heart valve displays a reduction by three orders of magnitude.

<table>
<thead>
<tr>
<th>Biomaterial</th>
<th>E (MPa)</th>
<th>UTS (MPa)</th>
<th>εf</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen Molecule</td>
<td>4800±2000</td>
<td>-</td>
<td>-</td>
<td>[239]</td>
</tr>
<tr>
<td>Collagen Microfibril (Dehydrated)</td>
<td>3260</td>
<td>-</td>
<td>-</td>
<td>[232]</td>
</tr>
<tr>
<td>Collagen Microfibril (Hydrated)</td>
<td>600±200</td>
<td>-</td>
<td>-</td>
<td>[239]</td>
</tr>
<tr>
<td>Collagen (along fibres)</td>
<td>1000</td>
<td>50-100</td>
<td>0.09</td>
<td>[238]</td>
</tr>
<tr>
<td>Collagen (tendon)</td>
<td>1200</td>
<td>120</td>
<td>0.13</td>
<td>[192]</td>
</tr>
<tr>
<td>Native Heart Valve (Circumferentially)</td>
<td>15±6.4</td>
<td>2.6±1.2</td>
<td>0.22±0.11</td>
<td>[240]</td>
</tr>
<tr>
<td>Native Heart Valve (Radially)</td>
<td>2±1.5</td>
<td>0.42±0.24</td>
<td>0.30±0.14</td>
<td>[240]</td>
</tr>
<tr>
<td>Elastin (bovine ligament)</td>
<td>1.1</td>
<td>2</td>
<td>1.5</td>
<td>[211]</td>
</tr>
<tr>
<td>Elastin</td>
<td>0.3-1.5</td>
<td>0.65</td>
<td>&gt;2</td>
<td>[5, 212, 241, 242]</td>
</tr>
</tbody>
</table>
These changes in properties are also illustrated in the Ashby plot in Fig. 1-9. The hierarchical structure (molecule to leaflet), organization (from tendon to valve leaflet) and environment (hydration level) all influence the mechanical properties of the natural biomaterial.

![Figure 1-9: Ashby plot of Young’s Modulus versus density for elastin and various structures of collagen.](image)

The mechanical stress that the bovine pericardium can withstand is ultimately down to the fibre architecture and its orientation with the collagen fibres integral to the mechanical properties of the tissue.

The collagen fibre and its waviness can be represented by an idealised curve Fig. 1-10 [199]. Where $2\ l_0$ is the wavelength of the fibre and angle $\Theta_0$. 

32
This idealised representation of a collagen fibre and the ratio between the amplitude and wavelength can be used to calculate the maximum strain that the collagen fibres can undergo without damage. As collagen fibres uncrimp and stretch when a tensile force is applied they can return to their original shape when the load is removed. However, if the fibre is stretched beyond the straightening of the waviness it can break, Eq. 1 [199].

\[ \epsilon = \frac{\pi}{4} \left[ \frac{3}{2} (1 + 2r) - (2r)^{\frac{3}{2}} \right] - 1 \quad \text{Equation 1} \]

With \(2r\) related to the ratio of the amplitude and crimp period \((b/a)\). Using Liao et al. [232] synchroton small angle X-ray scattering (SAXS) measurements for the crimp period and amplitude of approximately 30 \(\mu\)m and 15 \(\mu\)m respectively for bovine pericardium. The nominal strain \(\epsilon=0.22\) can be estimated and above this strain the molecules will break.
1.6 Bovine pericardium sourcing and treatment

Sourcing a natural biomaterial like BP, depends on several factors like the country of origin, species and age all play a part in the selection process [48]. Country of origin of the material is of significance for soft tissue biomaterials like bovine sources where guidance is provided by the various regulatory bodies such as the Food and Drug Administration (FDA) for medical products with components derived from bovine sources and where the country of origin poses a risk of prions such as bovine spongiform encephalopathy (BSE) [243]. Age of the animal is an important factor with neo-natal (4-7 days old) has been shown to have higher E-modulus and UTS than adult pericardium (18 - 24 months old) due to the alignment and organization of its collagen [236, 244].

The goal for the processing of natural materials is to produce a material that is biocompatible for its chosen purpose, is immunogenic, durable and retains or has its mechanical properties altered so that it is functionally fit for purpose. The common methodology to the processing of the natural biomaterial can be seen in Fig. 1-11, where the tissue is sourced then destructed down before final processing to the finished biomaterial. The process is different from a synthetic biomaterial processing approach where the material, like a polymer, is developed from the bottom up through various chemical and polymeric reactions, that form the building blocks for the material and then ultimately leading to the extrusion or moulding of the final biomaterial. Natural material follows a top to bottom approach where the material is stripped of excess material surplus to requirements such as fat and appendages and cleaned to produce the base material. This base material then depending on its application is built up through crosslinking, decellularisation and/or cell implantation. While the raw extracellular matrix necessary for a successful biomedical application is present the processing involves critical steps that will ultimately determine the success of the material. Guidelines are provided from standard authorities like the International Standard Organization (ISO) on the utilizing of materials of an animal origin for the manufacture of medical devices including the collection, handling, storage and transport of animals and tissues [245]. The
elimination of any possible immunogenic response of the biomaterial is normally carried out through the removal of all cellular and nuclear remnants via decellularization [246, 247].

1.6.1 Decellularisation

Decellularisation can be completed by physical, chemical or enzymatic methods with an aim of removing cellular material and also reducing any possible

Figure 1-11: Flow of typical processing steps for a natural biomaterial like bovine pericardium, with dashed arrows optional steps that can be added depending on application
negative effects on the composition, biological activity and mechanical integrity of the ECM [248]. Physical methods include temperature [249] and pressure [250] changes that interrupt and burst the cell membrane respectively. The enzymatic approach with the likes of nuclease, Trypsin and chelating agents such as EDTA are frequently used in a number of different combinations [56, 251-253] with varying degrees of success. Chemical methods of decellularisation include non-ionic detergents like Triton X-100 [254-256] and the ionic detergent sodium dodecyl sulfate (SDS) [257, 258]. All these methods are generally not used individually but rather in combinations where initially ionic solutions or physical methods proceed an enzymatic agent to separate any cellular component from the ECM is then followed by a detergent process to solubilize cellular components and to finish the decellularisation procedure a final washing cycle to remove all residual chemicals from the tissue [162, 248].

Crosslinking can occur after, in place of or in combination with the decellularisation process.

1.6.2 Crosslinking

The ECM material is crosslinked to increase strength but is mainly carried out to prevent degradation [45, 259, 260] where it has been reported of a non-crosslinked naturally occurring ECM degrading by as much as 60% after 4 weeks with complete degradation occurring by 3 months [261, 262]. Crosslinking methods come in many forms with glutaraldehyde (Glut), despite its cytotoxic and calcification disadvantages, [263] is still the predominant “gold-standard” [33] where it’s stabilizing properties, cost and availability [260, 264] make it the go-to choice for many commercially available natural biomaterials such as Mosaic®, Freestyle® (Medtronic Inc.), PrimaTM (Edwards Lifesciences) which are Glut crosslinked porcine heart valves and Perimount® (Edwards Lifesciences LLC) a bovine pericardium glutaraldehyde crosslinked heart valve [246, 265]. Other crosslinkers both at research and commercial stages include carbodiimides [266] found in the CuffPatchTM (Organogenesis Inc.) porcine SIS, [267] genipin a natural plant extract [268-270] and epoxy compounds [271].
1.6.3 Sterilisation
Sterilisation of bovine pericardium is of critical importance for its use as a biomaterial. The main infectious agents that have to be monitored and minimized, if not eliminated include, prions which are a known agent causing BSE in cows and variant Creutzfeldt-Jakob disease (vCJD) in humans, along with viruses, bacteria and parasites. A fundamental step in sterilisation of the tissue is in the selection of its country of origin where there is a negligible risk of BSE and good animal health management systems in place[245]. Once the tissue is harvested the sterilisation process continues with storage and transportation of the tissue in a timely manner critical in reducing the bioburden. Final medical devices can undergo a variety of sterilisation techniques like ionizing radiation (X-ray, ultraviolet, γ-radiation and E-beam) to chemical sterilants (ethylene oxide, hydrogen peroxide and peracetic acid) [272-274]. These techniques are not suitable for pericardium as they can damage the physiological and biomechanical properties of the ECM [275, 276]. The most common treatment for the sterilisation of BP tissue is through the use of Glut, where along with its crosslinking benefits it can significantly reduce its bioburden [162, 277].
1.7 Glutaraldehyde

Glutaraldehyde (Glut) was introduced in the early 1900’s as a general lab reagent [278] but it was not until the 1960’s and the industrial scale production [279] that interest in its capabilities grew. During the 50 – 60’s its benefits over formaldehyde as a sterilant/disinfectant was evident [280, 281]. The ability of Glut to crosslink and fix proteins was utilised for electron microscopy sample preparation where superior protein fixation than the heavy metal Osmium Tetroxide was suggested [282]. Further applications followed from tanning leather and wool [283], embalming [284] to biomedical applications as wound repair [9] and vascular grafts [10, 11]. More recently it has found applications in bio-catalyst design [285] and protein crystal fixation [286].

Research conducted in late 60’s eluded to the crosslinking ability of Glut with collagenous networks [287]. Since the introduction of Glut preservation of xenografts in 1969 by Carpentier et al. [288], the reagent has been used successfully in the preparation of bioprosthetic heart valves (BHV) [260]. The technique of fixing and storing the prostheses in buffered Glut solution has been around since 1975 when the “Stabilized Glutaraldehyde Process” was introduced and is still performed today [289, 290]. Processing of biological tissues with Glut is required to produce a material that is resistant to enzymatic degradation, [260, 291] reduce antigenicity [268] while retaining mechanical properties so that it is functionally fit for purpose [162]. It is the main choice for fixation of BHV and other collagenous tissues, often considered the “gold standard”[162]. However, limitations include loss of interstitial cell viability [29], calcification and non-calcific structural failure leading to a short 15-20 year life span in adults, and even less again in children and young adults [128, 292].
1.7.1 Chemistry

Glut is a 5-carbon di-aldehyde with a linear structure that is water and alcohol soluble. The ability for it to polymerise freely in solution is well documented [293, 294]. The polymerisation makes it difficult to find it in a pure monomer form [295, 296] with various forms of Glut existing in a neutral buffered solution Fig.1-12. These include a free aldehyde (I), a hemihydrate (II), a dihydrate (III), cyclic hemiacetal cis and trans isomers (IV) and polymers of Glut (V) [296]. This multicomponent behaviour of commercial Glut is beneficial rather than a hindrance and may be a reason for its success as a crosslinking fixative with a number of forms present in the solution[293].

![Chemical structures of Glut](image)

**Figure 1-12**: Equilibrium structures of Glut in aqueous solution [289].
The properties are solution dependent with the key attributes of temperature and especially pH critical to Glut's various structures, Fig. 1-13. Farris et al. [297] proposed that acidic pH conditions of 4.5 produce covalent bonds between the aldehyde of Glut and the hydroxyl groups of hydroxyproline and hydroxylysine to produce hemiacetals.

It would be acceptable to think that the understanding of a crosslinking reagent’s base structure and behaviour in various solutions would be well-known. However, Glut and its forms found in solution have been investigated for over 50 years indicating a highly investigated, researched reagent with a debatable history. The variation of Glut in solution leads to further debate over its possible crosslinking mechanisms.

Figure 1-13: Forms of Glut in aqueous solution and polymerising outcomes with increase in pH [286].
Reaction with the primary amine groups in proteins leads to inter- and intra-molecular crosslinking via covalent bonding Fig 1-14 [260]. Numerous crosslinking mechanisms have been reported between Glut and collagen based materials. [293, 296, 298] The formation of Schiff bases is the initial mechanism occurring between the ε-amino group of a lysine or hydroxylsine [285, 297], with all the various forms of Glut present in solution available for reaction with lysine residues [293]. Mechanism of crosslinking post this Schiff base formation is contentious, with the stability of the base key to the debate. Stable Schiff bases from Glut polymers through aldol condensation [299], to intermediate Schiff bases forming additional crosslinks [300, 301] have been reported. The unstable nature under acidic conditions of Schiff bases is known with suggestions that they undergo further reactions during the crosslinking process, Okuda et al. [302] suggested that the Schiff base is the central intermediate where reactions occur before a crosslink is formed. This was also proven by Daminik et al. [301] by monitoring the primary amine groups present with respect to the duration of crosslinking. It was found that only after the hydrolysable Schiff bases were stable that crosslinking of the collagen could occur. The overall effect of the crosslinking is to provide a structure that is biocompatible, non-thrombogenic, prevent against structural deterioration while also maintain the mechanical properties like that of a native aortic valve and its hemodynamic properties.

Figure 1-14: Crosslinking mechanism between Glut and collagen
1.7.2 Glutaraldehyde limitations

The lifespan of bioprosthetic heart valves are one of the significant disadvantages of bioprosthetic heart valves and can be attributed to failure modes such as mechanical and calcification due to the treatment of the tissue with glutaraldehyde [93, 128, 292].

A key factor is Gluts cytotoxicity [296], leading to a loss of interstitial cell viability [29] with the bioprosthetic heart valve tissue not viable for new cell growth to allow for the co-adaptation of the valve to the in vivo environment.

Calcification of the bioprosthetic heart valve is one of the most common failure modes [303]. Some of the individual or combination of factors at play include, the highly capable immune system of younger patients [128], mechanical stresses [93] and cellular damage [93, 110]. The role of Glut and its impact on the calcification of tissue heart valves is well documented [29, 93, 260, 263, 304]. The most common location for such calcific deposits is on the commissural and basal areas of the cusp [128]. The mechanism involves calcium containing extracellular fluid and membrane associated phosphorous combine to start the calcification of the cells. Calcium ions are unable to be extruded from cells that have become non-viable due to the treatment of glutaraldehyde. Along with the high concentration of phosphorous within the cell membranes the calcium and phosphorous combine to form nucleators that can grow and coalesce to form mineralised nodules that stiffen and weaken the valve leading to failure [93]. This calcification effect is one of the main reasons for the limitation of the valve’s lifespan and the lack of suitability for younger patients. Either through genetics or hemodynamic calcific damage is expediated [76]. The estimated failure of a bioprosthetic aortic heart valve in a 21-40 year old patient after 15 years is 70% while for a patient >70 years this is reduced to 5% [305]. Calcification effects of the valve can involve stenosis of the valve which leads to cuspal stiffening and or tearing of the valve which results in regurgitation [93]. Natural calcification process is like that of the process occurring on the valves [128].
Another limitation of Glut fixation is that the tissue is locked in a static geometry. It is not capable of adapting to the in vivo environment. This means that any damage or impact on the valve is permanent whereas a natural valve has the capability to change and grow new cells in a response to the stress effects of its environment [306, 307]. The Glut does not allow for such a cellular interaction to occur as it is toxic to its surrounding environment [260]. The limitations of Glut have not stopped it remaining the go-to choice fixative for bioprosthetic heart valves. However, this has not stopped other alternative crosslinkers to be evaluated, with a goal to identify a fixative that can match Gluts biomechanical effects, maintain the structural integrity of the valve while also reducing the toxicity to allow cellular interactions to occur.

1.7.3 Alternative crosslinkers
Fixation of biological tissues with alternatives has been investigated by many groups [33, 308]. Some of the most common methods include, epoxy compounds [309, 310], carbodiimides [311, 312], hexamethylene-diisocynate [313], acyl azide [314] and transglutaminase [315] either solely or in combinations. A crosslinking agent that has found applications with a range of biomaterials, from chitosan to collagen and gelatin is Genipin (Genp).
1.8 Genipin

Genipin (Genp) is obtained from geniposide found in the Gardenia jasminoides Ellis fruits and is traditionally used in Chinese medicine as antiphlogistic’s and cholagogue’s and as a food dye [316-318]. Genp has found crosslinking applications in cellular materials and biomaterials such as collagen [319-321], chitosan [322, 323] and gelatin [324] also research in wound dressing [325], drug delivery [326, 327] and as a bioadhesive [328].

1.8.1 Genipin Chemistry

Genp has a molecular formula of C11H14O5 and a molecular weight of 226.23 g/mol and contains a dihydropyran ring [329], Fig.1-15.

![Genipin structure](image_url)
It is a crystalline white solid that is soluble in organic solvents such as 100% Ethanol, DMSO and dimethyl formamide. It turns a characteristic blue colour on reaction with amino acids in the presence of oxygen [330]. The reaction of genipin with primary amines of lysine, hydroxylysine or arginine residues of biological materials produces covalent crosslinks that involves two sites of the Genp molecule, the ester group and the opening of the dihydropyran ring [291, 331, 332]. Both intermolecular and intramolecular crosslinks are formed in the collagen fibres, Fig. 1-16.

Figure 1-16: Crosslinking mechanism between Genipin and collagen [326].

Genp fixed tissue has shown superior stability during storage compared to Glut fixed tissue [333] and increases elongation and endothelial coverage than formaldehyde [334]. Sung et al. also demonstrated greater tensile strength and toughness compared to glutaraldehyde [335]. The crosslinking of genipin had been reported to be 5000 – 10,000 times less cytotoxic than Glut [333] while still having comparable mechanical results.
1.9 Characterisation of bovine pericardium

The characterization of a natural biomaterial like BP is different to that of a synthetic biomaterial. Sourced materials from a biological origin put the architectural and organizational structure as the foundation from which their suitability is assessed. Microscopy techniques are prevalent and come in a number of different forms from analysis of ECM organization and cellular presence such as histology and staining [45, 56, 336-338] to electron microscopy. Both transmission (TEM) and scanning electron microscopy (SEM) [276, 339, 340] are fundamental aspects of the characterization of natural derived materials. Polarized, fluorescence and confocal microscopy have been applied to understand the organization of the collagen and elastin components of soft tissues [341]. Other techniques for the immunological analysis are also of primary importance. Surface parameters are a priority for both natural and synthetic polymers but differ in that natural materials are focused more on the cellular viability [276, 342] and diffusivity of oxygen [43, 343] while synthetic polymers focus on surface energy [344] which include the hydrophilic and hydrophobic properties [345] and surface chemistry [346, 347]. The topography [348, 349] analysis of both natural and synthetic biomaterials share some common techniques like SEM and AFM [206, 350, 351].

1.9.1 Biomechanics

Mechanics of native bovine pericardium has been studied both pre- and post-treatment with various crosslinkers such as Glutaraldehyde. The principle methods of testing for tensile mechanical properties have involved both biaxial and uniaxial tensile testing. The biaxial testing approach has several more parameters involved which provide a closer physiological result but with this comes added complexity [352]. Uniaxial testing is more common as it produces reliable reference values without the requirement of controlling a few testing variables. A typical stress-strain curve for a native valve leaflet or indeed a bovine pericardium tissue sample shows a non-linear curve. The tensile stress (σ) is defined as the force (F) over the cross-sectional area (A);
\[ \sigma = \frac{F}{A} \quad \text{Equation. 2} \]

While the strain \( \varepsilon \) is;

\[ \varepsilon = \frac{\Delta L}{L_0} \quad \text{Equation. 3} \]

Where the \( \Delta L \) is the change in length and \( L_0 \) is the original length. The ratio of this stress versus strain is the E-modulus.

\[ E = \frac{\sigma}{\varepsilon} \quad \text{Equation. 4} \]

Some of the key measurement values taken from uniaxial testing consist of the high modulus, ultimate tensile strength, and strain at failure [353, 354]. A uniaxial test consists of a sample placed between a static clamp and another clamp that moves in a vertical direction pulling on the sample with a constant force. The clamping and the size of the sample is important according to Saint-Venants principle where gripping imposes local stresses at the grip-sample interface and the influence of these local stresses becomes negligible at some distance away from the grip this is the principle behind the use of long slender samples for uniaxial testing [355]. The anisotropic properties using the uniaxial technique where the difference in the E-modulus of a tissue sample orientated circumferentially or axially of the pericardium can be clearly identified.

Biaxial testing generally involves a square shaped tissue sample clamped on each of its four sides and typically a uniform force applied on each of the sides. The test involves the characterisation of the material in two perpendicular directions that provides multi-directional data to be generated that can be used to quantify and model the mechanical properties of a biological tissue. In the centre of the tissue sample small markers such as particles of sand are arranged in a square formation with a marker in the centre. This is the central target region [356] and should be small and located away from the edges to avoid any tethering effects. This central target region with its markers is used for the
generalized large deformation biaxial strain fields through the video tracking of the markers. The sample can be gripped in a number of ways such as clamping or suturing and the choice of applied sample boundary conditions should be carefully addressed prior to mechanical testing [355]. Tissue samples are generally analysed while completely immersed in phosphate buffered normal saline (pH 7.4) at room or body (37 °C) temperature.

1.9.2 Physical and chemical
Characterization of a synthetic biomaterials surface properties involve the investigation of surface free energy [357, 358], contact angle [359], chemical structure and topography. Synthetic biomaterials are suitable for the technique due to their solid, stable and homogeneous surface however challenges arise for the evaluation of the surface energy for a natural material like BP. While the contact angle approach for the measurement of collagen and elastin based materials has been researched [206], it is not ideal. Most biological sourced biomaterials natural environment is that of an aqueous media and combined with the complexity of their biological molecules [4] and their heterogeneous surface they are not ideally suited for these surface techniques.

Surface topography can determine the functional introduction of cellular activity. With this interaction the importance of a surface’s properties and the benefit of their characterization led to the increasing use and development of surface analysis techniques. How a biomaterial “transduces” its structure at the surface interface and influences the host response is necessary for the success of the implant material.

Spectroscopy methods such as Attenuated Total Reflectance-Fourier Transform Spectroscopy (ATR-FTIR) is a common method for the analysis of the surface chemical structures of both synthetic and natural biomaterials [4, 340, 360-363]. The surface characteristics of biomaterials of natural origin are also analysed from a topographical and structural interest. SEM is an example that is commonly used for the imaging of a biomaterials surface rather than its
chemical analysis. SEM does have the capability for chemical analysis through Energy Dispersive X-Ray Analysis (EDX) however the penetration depth of the signal is significantly higher than the surface depth [361]. Another surface technique used for the structural characterization of soft biological biomaterials is that of Atomic Force Microscopy (AFM) in which topographical and mechanical information can be produced down to the nanometer level, however chemical characterization is limited [206, 364, 365].

1.9.3 Thermal characteristics

After the sample is sourced and treated, indicators of the degree of crosslinking are significant. The goal for taking a natural material and treating it in any number of ways is to not only make it robust for its application but also create a reproducible biomaterial with a reduced sample to sample variation in properties. The measurement of the degree of crosslinking can take a number of forms but typically are a combination of techniques to determine that the tissue is completely crosslinked and is of an adequate standard for use.

A typical method to confirm that the tissue is fully crosslinked is the measurement of its shrinkage temperature or denaturation temperature (T_d) using a water or saline bath of increasing temperature of approximately 1 °C, with the sample held in a load cell of a tensometer [366] [367]. Another method of assessing the crosslinking efficacy and an alternative to the shrinkage temperature method, to measure the T_d is that of Differential Scanning Calorimetry (DSC) [368]. This is a common technique that allows the quick assessment of the degree of crosslinking based on an increase of the endothermic peak by approximately 20 °C from the untreated state to the fixed state [301]. Denaturation of tropocollagen occurs as the tissue is heated beyond 60 °C and the helical structure unfolds to produce random chains of gelatin [354, 369]. The DSC technique is typically used for the analysis of a polymers thermal profile, providing information on first and second order transitions of polymeric chains like glass transitions (T_g), melting temperatures (T_m) and crystallisation. Further development of the technique in 1993 by Gill et al. established a
variation on DSC with the introduction of modulated temperature DSC (MTDSC) [370]. The development allowed MTDSC to superimpose a sinusoidal modulation over the DSC's standard linear rate [371]. The equation that describes the components of both DSC and mDSC is shown in Eq. 5

\[
\frac{dH}{dt} = C_p \frac{dT}{dt} + f(T, t) \quad \text{Equation. 5}
\]

The total heat flow \(dh/dt\) as measured by conventional DSC, is equal to the heat capacity \(C_p\) (J/C) and its heating rate \(dT/dt\) (C/min) and the kinetic component of the heat flow that is a function of \(T\), temperature and \(t\), time.

The overlay of the modulating temperature and the linear rate lets the total heat flow signal be deconvoluted into its reversing element of the specific heat from the \(C_p\) and heating rate and its non-reversing element due to the kinetic component of temperature and time [372]. Each of these heat flow signals provide information on different thermal events, Table 1-4.

This method produces an increase in both resolution and sensitivity, through the separation of overlapping signals from the baseline [373]. For example, hard to

<table>
<thead>
<tr>
<th>Reversing Heat Flow</th>
<th>Non-Reversing Heat Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melting (some)</td>
<td>Enthalpic Relaxation</td>
</tr>
<tr>
<td>Glass Transition (Tg)</td>
<td>Melting (some)</td>
</tr>
<tr>
<td>Heat Capacity</td>
<td>Evaporation</td>
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<tr>
<td></td>
<td>Crystallisation</td>
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<td></td>
<td>Denaturation</td>
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<td>Decomposition</td>
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Table 1-4: Reversing and Non-Reversing heat flow signals and the thermal events detected
detect Tg, in polymers can be separated to clearly identify other features such as enthalpic recovery beside the Tg that would typically give the inaccurate impression of a melting peak.

The MTDSC model adds the additional parameters of the amplitude of the temperature modulation $A(T)$, angular frequency $\omega$ and phase shift $\theta$, to those of temperature $T$, time $t$, initial temperature $T_0$ and underlying heating rate $\beta$, to produce Eq. 6.

$$T = T_0 + \beta t + A_T \sin(\omega t - \theta) \quad \text{Equation. 6}$$

Further evolution of MTDSC has seen the development of quasi-isothermal modulated temperature differential scanning calorimetry (QiMTDSC). Like MTDSC a modulated temperature profile is applied but across a constant underlying temperature or by collecting a series of quasi-isothermal data points by increasing the underlying temperature incrementally [371]. This has found applications in pharmaceutical industry for the analysis of polymorphic transformations through the changes in heat capacity (Cp)[374, 375]. The removal of a heating rate creates an increase in the sensitivity to the reverse Cp (Rev Cp) and the removal of melting effects due to the large number of modulations [376].

The measurement of heat capacity in situ is of great benefit in the analysis of the molecular mobility within the crosslinking or curing reactions of a sample [377]. An example of this would be the curing of an epoxy, as it cures, the heat capacity will decrease because the crosslinking of molecules reduces their mobility [378]. This reduction in mobility is similar to that of the crosslinking reaction between biological tissues like BP and Glut, where there is an increase in stiffness and strength of the tissue due to the bonding between the amino acids.
1.10 Project Hypotheses

The characterisation of natural biomaterials like BP can be broken down into three general groups. Biomechanical characterisation focuses on the direct properties of the biomaterials performance and include, modulus, ultimate tensile strength and strain at failure. While there are only a few key measurements to deem the tissue acceptable, the range in both techniques and parameters across both industry and research result in ambiguity when comparing these performance properties.

The physical characterisation including chemical analysis demonstrate the processing of the tissue has been successful and indicates that the biomaterial will perform as expected. Indicators for crosslinking success include the determination of the $T_d$ through DSC providing a direct measurement of the degree of fixation of the BP. A modified version of this technique, QiMTDSC, can be applied to assess the interaction between the Glut and the BP and provide a better understanding of the crosslinking mechanism. Also assessing the amino acid content using a quick and versatile technique would be of significant benefit.

The third characterisation area is that of a biological assessment of the biomaterial, including the comparison of the standard Glut fixation protocol against alternatives of Genp and decellularisation. While numerous crosslinkers have been researched, Glut is still the go-to fixative for soft tissue biomaterials. However, Genp has shown promise as a viable alternative, warranting further investigation. The reduced cytotoxicity of the material in both its native and treated states is required to determine if the tissue is suitable for in vivo application and that treatments have not caused any undesired effects. Following on from this, recommendations can be made suggesting the suitability of the tissue as a scaffold candidate for the application of a cell sheet.

The goal of this project was to recommend experimental parameters for uniaxial tensile testing of BP, gain further understanding of the crosslinking reaction
between Glut and BP using a novel calorimetry technique and also compare alternative treatments with the standard Glut method across biomechanics, $T_d$ and cytotoxicity to determine their viability as possible replacements. The approach was to assess the BP biomaterial in the three characterisation areas as follows;

1.10.1 Part I – Chapter 3: Biomechanical Assessment

**Aim:** Assessment of the uniaxial experimental parameters utilised for the mechanical testing of bovine pericardium.

**Hypothesis:** A recommendation of mechanical test parameters using uniaxial tensile testing can be suggested, to provide a more accurate basis for the comparison of mechanical data using this technique.

**Objectives:**
- Suggest a recommendation for the mechanical testing of BP using the uniaxial tensile testing focusing on the number of preconditioning cycles and extension rate.
- Visually evaluate the effects of uniaxial tensile testing on the collagen fibre structure.

1.10.2 Part II – Chapter 4: Physical and Chemical Characterisation

**Aim:** Investigate the use of a novel calorimetry technique to assess the crosslinking reaction between Glut and BP, trial an assay for the quick identification of a fixed tissue and assess the microstructure of collagen using several microscopy techniques.

**Hypothesis:** Measuring the reverse heat capacity of the crosslinking reaction between Glut and BP can provide an insight into the rate of reaction.
Objectives:

• Measure the effect of reaction time and volume of Glut between Glut and BP on the $T_d$.

• Assess the use of a Ninhydrin assay for the quick determination of a fixed BP tissue.

• Analyse the microstructure and architecture of BP and collagen using different microscopy techniques.

1.10.3 Part III – Chapter 5: Characterisation and cytotoxicity evaluation of decellularised bovine pericardium and treatments with a natural crosslinker–Genipin

Aim: Assess the biomechanics and cytotoxicity of Decell and Genp treated BP and combinations of, in comparison with Glut crosslinked BP.

Hypothesis: The Genipin fixed tissue will be less toxic than the Glut tissue and be a viable option for further studies into the attachment of cell sheets.

Objectives:

• Compare the biomechanics, degree of crosslinking using $T_d$ and structures using histology analysis of Genp and Decell protocols against the Glut fixed BP.

• Compare the cytotoxicity of all fixed tissue types using both alamarBlue® and Live/Dead assay utilising the different techniques of elusion and contact methods.
1.11 References


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Chapter 2: Materials and Methods

This materials and methods chapters provides background information on the techniques used in this research. Further details such as specific parameters will be included in the corresponding individual specific chapters to follow.

2.1 Bovine pericardium fixation

2.1.1 Bovine pericardium

BP was prepared and utilised for testing using two different sources and methods.

Method 1: A local vendor (Bradys butchers, Athenry, Galway, Ireland) provided the BP. The pericardium was harvested upon slaughter of the animal, using an incision to keep the pericardium intact and stored in an icy PBS solution (10 x diluted 1:2 water). The BP was then transported to the CÚRAM culture labs where it was then processed using the below protocol;

1. Tissue rinsed in PBS solution
2. Excess fat removed from pericardium
4. Tissue is continually washed in PBS during processing and for a final step it is washed three times in fresh PBS for 5 mins each.
5. Tissue is either used immediately or then stored in fresh PBS at 4°C for a maximum of 4 days before fixation.
Method 2: The tissue was sourced and processed from a proprietary vendor in Australia/New Zealand, following the below steps Fig. 2-1.

Required to ensure that the animal is disease free and is fit for human consumption

- Animals age confirmed using dentition (< 24 mths).
- Tissue from animal must be traceable back to the farm

Pericardium removed and inspected

Pericardium dimensions are approximately 10 cm x 12 cm with a thickness range of 0.25 mm - 0.5 mm

Excess fat removed from tissue

Solution consisting of disodium ethylene diamine tetra acetate dihydrate (EDTA) and PBS. Stored in solution for <96 h and then fixed or stored at -80 °C

Tissue rinsed, stored in solution and insulated shipping container

Figure 2-1: BP flowchart from abattoir to final state for fixation or storage
2.1.2 Glutaraldehyde
Glut was prepared from 25% electron microscopy grade solution (Merck, Darmstadt, Germany). Solutions of varying concentrations ranging from 0.1%, 0.6%, 1% and 6% were prepared using 0.1M PBS (Sigma Aldrich).

2.1.3 Genipin
A Genipin (> 98% Sigma Aldrich) stock solution was prepared using 0.1 ml of 100% Ethanol (Sigma Aldrich) placed into the 25 mg vial of Genp. This solution was then further diluted using PBS, to create a range of concentrations of solutions 0.1 mM, 0.3 mM and 0.6 mM and 1.0 mM.

2.2 Physical and Chemical Characterisation
2.2.1 Uniaxial testing
A uniaxial tester measures the tensile behaviour of a material by applying a force. The sample is clamped between two clamps and is then extended using a load cell of known mass, until a pre-defined point or failure. The rate of extension depends on the sample and the measurements of interest and can range from 0.1 to 500 mm/min. The values are presented in a force v extension format and can be converted into stress v strain values by taking into account the cross-sectional area of the sample and the change in length of the sample respectively. Stress \( \sigma \) is calculated by dividing the force by the cross-sectional area (width x thickness) of the sample Eq.2-1, and the strain \( \varepsilon \) from the change in length \( \Delta L \) of the sample at the end of the experiment divided by the original length \( L_0 \), Eq.2-2.

\[
\sigma = \frac{\text{Force}}{\text{Cross Sectional Area}} \\
\varepsilon = \frac{\Delta L}{L_0}
\]

\( \text{Equation: 2-1} \)
\( \text{Equation: 2-2} \)
The equipment used here was a Zwick Z005 tester (ZwickRoell, Germany) with a 100 N load cell. Samples were placed between two pneumatic pressure clamps with roughened grips at 3 Bar.

2.2.2 Atomic Force Microscopy (AFM)

AFM is a scanning probe microscopy technique which is used for surface and morphological analysis [1]. The key components of the instrument include a tip and cantilever that are typically constructed of silicon (Si) or a doped version such as silicon nitride (Si$_3$N$_4$). The cantilever either triangular or rectangular shape can come in various lengths (100 – 200 µm), width (10 – 40 µm), thickness (0.5 – 4.5 µm) dimensions. This cantilever behaves like a spring where a laser pointing at the back of it responds to any deflections of the tip and cantilever and is directed to a photodetector. Topographical images are produced as the tip scans the surface in the x–y plane direction and moves up and down in the z plane caused by the tip and sample – force interactions. The interaction of the tip with the sample is dependent on the distance to the surface resulting in either repulsive or attractive forces. These tip interactions create three possible mode types of analysis. Contact mode when the tip is in “soft physical contact” where the electron clouds of the tip and surface essentially overlap [2] and is typically used for hard, flat samples at high resolution and can produce atomic resolution images. Non-contact mode can be used for soft specimens including liquids, where the tip sample separation is approximately 1 nm. The tip operates in the attractive region of the force-distance curve through mainly Van der Waals and electromagnet static forces. The third mode is tapping mode and allows for high resolution images even for a soft material. Forces acting between the tip and sample will result in, high cantilever amplitudes, when the tip is not in contact with the sample, or low amplitudes when the tip is in contact or “taps” the surface of the sample for a short period of time. The force distance curve is represented in Fig. 2-2 and displays how each of the modes fit into respective attraction or repulsive regions. Three forms of data are generated, height data on the z-axis produce topographical map of the samples surface. A phase image is produced from the varying dampening
effect of the sample surface on the tip, while an amplitude map shows the surface features and is plotted from the change in amplitude of the cantilever.

AFM images were produced using a Veeco Dimension 3100 AFM, with a Pointprobe® silicon probe, detector side coated in aluminium (Nanoworld Group, Switzerland) and a cantilever length of 125 µm and force constant of 42 N/m. Samples were located and tested in tapping mode with a amplitude of 2.0 V, a scan rate of 0.5 Hz for 512 x 512 points in a single scan. Image sizes ranged from 10 µm x 10 µm to 500 nm x 500 nm. Images and phase data were processed using WSxM software (Nanotec Electronica, Spain) [3].
2.2.3 Fourier Transform Infra-Red Spectroscopy (FTIR)

This technique provides a spectral fingerprint of a sample. The mechanism involves an Infra-Red (IR) beam that can pass through a sample resulting in molecular vibrations, which are then processed into a spectrum. Depending on the samples molecular composition the bonds will stretch, wag or bend with each response corresponding to a specific location and intensity on the spectrum.

This research used a Bruker Vertex 70 with Hyperion 3000 microscope. Samples were analysed using a micro-ATR accessory with a liquid nitrogen cooled mercury-cadmium-telluride (MCT) detector. It consisted of a diamond crystal with testing performed using a scan number of 64 with a resolution of 4 cm⁻¹.

2.2.4 Differential Scanning Calorimetry (DSC)

DSC was used to determine if the tissue is crosslinked based on the Td, where a value of approximately 80 °C would confirm a fully crosslinked sample. The Td for all samples were obtained using a DSC (Model Q1000, TA Instruments, Delaware, USA). Circular samples of 3 mm diameter were cut using a biopsy punch, patted dry and hermetically sealed into aluminium pans. The test method followed a heating rate of 5 °C/min as suggested by Loke et al. [4] in a temperature range of 30 °C to 110 °C. An empty pan was used as the reference. Heating was carried out in a nitrogen atmosphere of 50 ml/min. The Td can be calculated from the extrapolated onset or the peak temperature of the endothermic phenomena, here the peak temperature was used to determine the Td [4, 5]. Calibration of the instrument was carried out using an Indium standard (Melting point, (Tm) 156.6 °C).
2.2.5 *Quasi-Isothermal Modulated Temperature Differential Scanning Calorimetry (QiMTDSC)*

While conventional DSC is used to evaluate the $T_d$, QiMTDSC was utilised to measure the relationship between the change in $C_p$ and the resulting $T_d$. Two methods were followed, Fig. 2-3. The difference between the two methods is based on volume of solution used (BP in 10 µl Glut) and the time the BP is in contact with the Glut solution (BP placed in 10 µl of solution for 30 s). After each QiMTDSC test the sample’s $T_d$ was assessed using a conventional DSC test method following a heating rate of 3 °C/min in a temperature range of 30 °C to 100 °C. The lower heating rate was chosen as to allow for greater resolution for any changes that may be occurring at the lower isothermal times.

The QiMTDSC and post $T_d$ protocols are listed in Table 2-1. Both method 1 and method 2 followed the same post $T_d$ protocol. The isothermal time ranged from 10 min to 720 min depending on each test.

![Figure 2-3: Method 1 and 2 for the BP and Glut sample preparation for QiMTDSC analysis](image-url)
A control sample of an epoxy resin (Loctite M-31Cl, Henkel Technologies) consisted of a Part A and B components of epoxy and hardener respectively. A 2:1 mix was tested over a range of isothermal temperatures consisting of 25 °C, 50 °C, 100 °C and 200 °C for a period of 720 min. All samples were run under a modulation of +/- 1 °C every 100 s. The thermograms of the Cp (mJ/ °C) as a function of temperature were used to measure the total change in Cp after 30 min to the end of the time period. Curing was confirmed using DSC at 10 °C/min from 30 °C to 100 °C of the sample post QiMTDSC method with a glass transition (Tg) of >70 °C indicative of a fully cured epoxy.

Table 2-1: QiMTDSC and post-test, T_d protocols

<table>
<thead>
<tr>
<th>QiMTDSC Protocol</th>
<th>T_d Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Equilibrate at 25.00°C</td>
<td>1. Equilibrate at 30.00°C</td>
</tr>
<tr>
<td>2. Modulate +/- 1.00°C every 100 s</td>
<td>2. Data storage: On</td>
</tr>
<tr>
<td>3. Isothermal for 5.00 min</td>
<td>3. Ramp 3.00 °C/min to 100.00°C</td>
</tr>
<tr>
<td>5. Isothermal for 240.00 min</td>
<td>5. End of method</td>
</tr>
<tr>
<td>6. Data storage: Off</td>
<td></td>
</tr>
<tr>
<td>7. End of method</td>
<td></td>
</tr>
</tbody>
</table>
2.2.6 Ninhydrin assay

The free amino content of the crosslinked BP was qualitatively assessed using Ninhydrin assay. The assay (2, 2-dihydroxyindane-1, 3-dione) is a chemical that reacts with α-amino acids to produce a distinctive purple colour, Ruhemans purple, Fig 2.4. The colour intensity is proportional to the amino acid concentration within the sample. A non-crosslinked BP will have high concentration of free amino acids and should produce a deep blue colour. Conversely, after crosslinking with Glut the BP should have few amino acids remaining and therefore should have no colour change.

![Ninhydrin assay reaction](image)

**Figure 2-4:** Ninhydrin assay reaction with amino groups and the resulting Ninhydrin amino acid complex which produces the purple colour.

The protocol for the qualitative assessment of the BP is listed below;

**Ninhydrin assay qualitative protocol**

1. 3 mm samples of BP were cut with a biopsy punch.
2. 10 samples each were placed into three vials of 5 ml PBS, 0.6% Glut and 2.5% Glut.
3. Samples were left at room temperature for 2 d.
4. A 2.5% (w/v) Ninhydrin assay was prepared by adding 2.5 g to 100% ethanol.
5. 3 x BP samples from each vial were placed onto separate glass slides.
6. 15 µl of the 2.5% ninhydrin solution was added to each BP sample.
7. The slides were then placed into an oven at 80 °C for 5 mins.
8. Images taken pre and post oven treatment.
2.3 Imaging Techniques

2.3.1 Scanning Electron Microscopy (SEM)

Scanning electron microscopy uses a high energy electron beam (5 – 100 keV) to interact with a sample resulting in the emission of different signals like, secondary and backscattered electrons to produce an image. Benefits over optical imaging include high magnification (> 500,000 x) along with high resolution and a superior depth of focus. However, an intensive sample preparation is required for biological samples to ensure that a true image of the sample can be achieved and that moisture from the sample does not interfere with the instrument itself. The SEM used was a Hitachi S-4700 Scanning Electron Microscope (Hitachi, Japan), samples were analysed in high vacuum mode, at an accelerating voltage of 15 kV and using the SE2 detector. The protocol for the preparation of BP samples for SEM is as follows;

1. Samples were cut into 3 to 4 mm diameter discs or square sections depending on the experiment.
2. Samples were initially fixated in SEM grade Glut at a concentration of 2.5% (Merck, Darmstadt, Germany), for 2 h at room temperature.
3. Samples were then rinsed in 0.1 M PBS for 5 min, twice.
4. After the final rinse the samples were then dehydrated using ethanol in a gradient series.
   - Replace PBS buffer with 50% ethanol for 5 min at 4 °C.
   - Replace 50% ethanol with 75% ethanol for 5 min at 4 °C.
   - Replace 75% ethanol with 80% ethanol for 5 min at 4 °C.
   - Replace 80% ethanol with 90% ethanol for 5 min at 4 °C.
   - Replace 90% ethanol with 100% ethanol for 5 min at 4 °C.
5. The 100% ethanol was then removed and replaced with another 100% ethanol for a final 5 min.
6. After this the 100% ethanol was removed and the samples were added to hexamethyldisilazane (HMDS) for 30 mins.
7. Samples were then removed and allowed to air dry overnight.
8. Samples were then placed onto double-sided adhesive carbon tab sheets and
gold sputtered using an Emitech™ K550X gold sputter coater, after which
the samples were analysed using SEM.

2.3.2 Transmission Electron Microscopy (TEM)
Similar to the SEM this technique uses an electron beam to generate an image,
however instead of interacting with the surface the beam passes through a thin
section of the sample. Also, for biological samples an extensive protocol is
followed to prepare the samples for imaging, Table 2-2. TEM was used to
assess and compare the ultrastructure of the native and the Glut crosslinked BP.
Table 2-2: Each stage of sample preparation for TEM imaging of BP samples.

**Primary Fixation**
- Native BP and Glut crosslinked BP were cut into 10 x 10 mm sized sections.
- The samples were fixed with 2% Glut + 2% paraformaldehyde (PFA) (Sigma Aldrich) in 0.1M sodium cacodylate/HCl (Sigma Aldrich) buffer pH7.2, for 60 min at room temperature.
- Samples were then rinsed twice with cacodylate buffer, for 5 min per rinse.

**Secondary Fixation**
- Samples are then fixed in 1% osmium tetroxide (Agar Scientific) in 0.1M sodium cacodylate/HCl buffer pH 7.2 for 60 min.
- The osmium solution was extracted using a Pasteur pipette and placed into 2% ascorbic acid solution to neutralise the osmium solution toxicity.

**Dehydration**
- Samples were then dehydrated in a series of graded ethanol solutions at room temperature.
  - 50% ethanol for 15 min
  - 70% ethanol for 15 min
  - 90% ethanol for 15 min
  - 95% ethanol for 15 min
  - 100% ethanol for 15 min
- The 100% ethanol solution is then replaced with 100% propylene oxide for 20 mins, and repeated.

**Embedding**
- Samples were initially placed into a 50:50 mix of low viscosity embedding resin (R1078A, Agar Scientific) and polypropylene oxide and left on a rotator overnight.
- The 50:50 mix was then replaced with a 75:25 mix and placed onto a rotator for 6 h.
- The 75:25 mix was then replaced with a pure 100% resin and placed on a rotator overnight.
- The samples were then transferred to silicon mould cavities and placed in an oven at 65 °C for 48 h to allow the resin to polymerise.

**Sectioning and Imaging**
- “Scouting sections” of samples were taken by removing samples from the moulds and slicing thin sections, 1 µm thickness, staining with 1% Toluidine Blue, and viewing under a light microscope to ascertain structure and components.
- Regions of interest were selected and then slices of ultrathin sections 80 – 100 nm thickness were taken using an ultramicrotome (Leica Reichert Jung Ultracut) and placed onto a 3 mm copper grids.
- The grids are stained for 30 min in 1.5% aqueous uranyl acetate for 10 min in lead citrate using an automated contrasting apparatus (Leica EMAC 20).
- Sections were air dried and then viewed in a TEM (Hitachi 7000 TEM)
2.3.3 Fluorescent microscopy

Fluorescent microscopy in combination with Live/Dead assay, was used to analyse the cytotoxic effect of Glut and Genp crosslinked BP on cells. The microscope stimulates a fluorescent source in the sample which produces a weak signal that is then detected [6]. Samples were analysed with an Olympus IX81 fluorescence microscope (Olympus) with a DP72 CCD camera (Olympus) attachment that was linked to CellSens Dimension software (Olympus).

2.3.4 Histological Analysis

The BP samples were treated with both Hematoxylin and Eosin (H&E) (Sigma Aldrich) and Masson Trichrome (Sigma Aldrich) staining, to assess the overall structure of the tissue and specifically the collagen fibres, respectively. The protocol for histological sample preparation is detailed in Table 2-3.
Table 2-3: Embedding, staining and imaging protocol for histological analysis of BP

**Embedding**
- BP samples are fixed in 10% formalin for 24 h.
- Then embedded in paraffin using an automated processor, Leica ASP300 (Leica Biosystems, UK).
- Resultant sections were then sectioned using a Leica RM 2235, Rotary Microtome (Leica Biosystems, UK) to a thickness of 5 µm.

**Deparaffinisation**
- Samples dried, deparaffinised and rehydrated by the following steps;
  - Xylene with agitation – 2 min x 2 times
  - 100% ethanol with agitation – 2 min
  - 95% ethanol with agitation – 2 min
  - 70% ethanol with agitation – 2 min
  - Distilled H₂O with agitation – 2 min x 2 times

**Staining and Mounting**
- Samples were stained using both H&E and Masson’s Trichrome following manufacturers instructions
- Samples were then dehydrated through a graded series of ethanol solutions;
  - 70% ethanol – 2 min
  - Replaced by 80% ethanol – 2 min
  - Replaced by 90% ethanol – 2 min
  - Replaced by 100% ethanol – 2 min
  - Replaced by Xylene – 5 min
- Samples then mounted using DPX Mountant (Merck, Germany)

**Imaging**
- Samples imaged using an Olympus BX51 with DP70 Colour Camera (Olympus, Japan).
- Images taken at a magnification of 40 x with scale bar added to images using ImageJ (NIH, Bethesda, MD, USA)

2.3.5 Phase contrast microscopy
Microscopic analysis of all cell types grown on the control culture dishes were captured using phase contrast microscopy. The microscope used was an Olympus IX81 inverted microscope (Olympus, Japan).
2.4 Cell Culture

2.4.1 Freezing and thawing of cells

The two cell types used were human mesenchymal stem cells (hMSCs) and murine bone marrow stromal cell line (MS-5) cells. They were received in a cryo-preserved state, from storage in liquid nitrogen, and so below details the protocol for the thawing of cells;

1. The vials of cells were removed from cryo-preservation by placing in a heated water bath of 37 °C.
2. They were then transferred to a T75 tissue culture flask.
3. 10 ml of media pre-heated to 37 °C was added immediately dropwise, and the flask was placed into an incubator at 37 °C, 5 % CO₂ for 24 h.
4. After 24 h the media was replaced with fresh media to remove any toxic effect of the freezing media dimethylsulfoxide (DMSO).

2.4.2 Storing and cryopreservation of cells

Cryopreservation is a commonly used method for long-term storage of cells. The below protocol outlines the process of preserving the cells for future use;

1. Cell suspension is harvested by trypsinization and then spun down to a pellet by centrifugation.
2. The pellet was re-suspended in a freezing medium containing 10% DMSO in 90% fetal bovine serum (FBS).
3. This cell suspension was then aliquoted into sterile cryogenic vials and placed in a -80 °C freezer overnight.
4. The cells were then transferred to liquid nitrogen for long-term storage.
2.4.3 Materials

The cell types MS-5 and hMSC were both received from CÚRAM. The culture medium was prepared by supplementing DMEM (Lonza, Switzerland) with 10% FBS (Sigma-Aldrich) and 1% penicillin-streptomycin antibiotic (Sigma-Aldrich). Cells were cultured in T75 plastic flasks (Starstedt) and solutions exchanged using disposable serological pipettes of 5, 10, and 25 ml volume. To remove cells 0.5% Trypsin (Sigma- Aldrich) was used. Cells were subcultured in an incubator at 37 °C of a 95% air and 5% CO2 atmosphere.

2.4.4 Sub-culturing of cells

The protocol for sub-culturing and counting of cells is detailed in Table 2-4.
### Table 2-4: Sub-culturing protocol.

| Assessment of confluency | Cells were assessed daily to determine confluency, using an Olympus IX81 microscope.  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells of approximately 80% confluency required sub-culturing, every 2-3 days.</td>
</tr>
<tr>
<td>Sub-culturing</td>
<td>Cells were subcultured using asceptic technique by aspirating media from the T75 flask and then rinsed gently in approximately 2 ml of pre-warmed, (37 °C) HBSS, three times.</td>
</tr>
<tr>
<td></td>
<td>1 - 2 ml of Trypsin is then added, ensuring to spread over the surface of the flask, and then stored in the incubator for 5 min.</td>
</tr>
<tr>
<td></td>
<td>Upon removal the flask is then tapped gently before 2 – 4 ml of pre-warmed (37 °C) culture media is added to neutralise the Trypsin.</td>
</tr>
<tr>
<td></td>
<td>The suspension was added to a 15 ml tube and centrifuged at 1500 rpm for 5 min.</td>
</tr>
<tr>
<td></td>
<td>This pellet was then reseeded into a T75 flask with 10 ml of culture media</td>
</tr>
<tr>
<td>Counting of cells</td>
<td>10 µl of Trypan Blue (Sigma-Aldrich) was used to stain the cells.</td>
</tr>
<tr>
<td></td>
<td>A hemocytometer was used to determine the number of cells present.</td>
</tr>
</tbody>
</table>
2.5 Viability Assessment

2.5.1 *alamarBlue®* assay

For the metabolic assessment of the cells a Resazurin assay, *alamarBlue®* (Thermo Fisher Scientific, USA) was used. *alamarBlue®* is an oxidised, blue coloured, non-fluorescent assay that upon contact with oxygen in viable cells is reduced to a pink fluorescent form *Fig. 2-5*. The change in colour and fluorescence can be measured either via colourimetry or fluorimetry, which can then be used to determine the metabolic activity of cells or the cytotoxicity of a material. The benefits of the assay over the similar 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay is that *alamarBlue®* is nontoxic and cells can be washed free of the assay and reused for further studies [7].

![Figure 2-5: Resazurin in alamarBlue® reacts with oxygen in live cells reducing to a highly fluorescent pink, resorufin.](image)

The assay and cytotoxicity experiments were performed as per protocol in *Table 2-5*. 

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A 10% alamarBlue® assay solution was prepared according to manufacturer’s protocol, by adding it to HBSS.

To avoid any contact with light, the solution was prepared in a darkened laminar fumehood and the tube containing the solution was covered in tin foil.

All cytotoxicity tests were performed using 96 well plates in a spectrometer (Perkin Elmer, Victor³ V™, Boston, MA) using fluorescence mode at wavelengths of 530 nm and 590 nm for excitation and emission respectively.

The assay was applied using both the contact and elusion method.

### Contact Method

- Both cell types of MS-5 and hMSCs were used for the contact method for two timepoints of 48 h and 7 d.
- 3 mm BP samples of various treatment groups were sterilised using 1 ml of 70% ethanol and placed under UV light for 40 min. Respective groups assigned 3 wells each in a 12 well plate.
- Ethanol solution replaced with approximately 1 ml of media and incubated for 24 h at 37 °C at 5% CO₂.
- This media was then replaced with 1 ml of fresh media and 100 µl of cell solution, cell number of approximately 10 x 10⁴ cells.
- The 12 well plate then incubated at 37 °C at 5% CO₂ for the respective timepoints, with 600 ul of fresh media added to the 7 d timepoint.
- After both the 48 h and 7 d timepoints the media from each cell was removed, washed with HBSS to remove any presence of media, and then 300 µl of 10% alamarBlue® was added to each well.
- The 12 well plate was then covered in tin foil and incubated at 37 °C at 5% CO₂ for 1 h.
- After incubation 100 µl of solutions from each well were removed in triplicate and added to a 96 well plate and fluorescence then measured.
- Controls consisted of a positive control of cells in DMEM with the negative based on cells in DMSO.

### Elution Method

- Media incubated for 24 h at 37 °C at 5% CO₂, with various treated BP samples was extracted and added to both cell types at concentrations of 100%, 50% and 25% for 48 h at 37 °C at 5% CO₂. Each concentration prepared by adding the 100% DMEM at the respective volumes.
- After incubation the media was removed, cells washed with HBSS, to remove any traces of media. A 300 µl of 10% alamarBlue® assay added to each well, with the plate then covered in tin foil and incubated for 1 h.
- A 96 well plate was then prepared by extracting 100 ul in triplicate of each solution to each well and fluorescence then measured.
- Controls consisted of a positive control of cells in DMEM with the negative based on cells in DMSO.

---

**Table 2-5:** Protocols for alamarBlue® for both contact and elution methods
2.5.2 *Live/Dead assay*

The cell viability assessment after contact with the various treated groups of BP was performed using Live/Dead assay (Invitrogen, US). The assay consists of calcein-AM and ethidium homodimer-1 that stain cells green and red respectively. Calcein-AM can permeate through viable cells and emit a green fluorescence while the ethidium homodimer-1 is able to penetrate through damaged cell membranes and interact with the DNA to emit a red fluorescence.

The assay was prepared according to the manufacturer's protocol where a solution of 1 µM calcein-AM and 2 µM ethidium homodimer-1 in HBSS was mixed thoroughly. Samples were imaged after contact of 48 h and 7 d and using the elusion method with concentrations of 100%, 50% and 25%. The elution method samples were also analysed using a spectrometer (Varisokan Flash, Thermo Scientific, Finland) under fluorescence, with live cells detected at an excitation and emission wavelength of 485 nm and 530 nm respectively and dead cells at excitation and emission wavelengths of 530 nm and 645 nm respectively. The Live/Dead solution was added to the cells, protecting it from the light and incubated for 20 min at 37 °C at 5% CO2. Samples were prepared in triplicate with five images taken for each sample using an Olympus IX81 fluorescence microscope (Olympus Corporation) with a DP72 CCD camera (Olympus Corporation). Images were analysed using ImageJ software [8].

2.5.3 *Trypan Blue*

Trypan Blue dye is routinely used during the subculturing of cells to assess the viability of the cell line. The dye is negatively charged and cannot penetrate intact membranes of healthy cells and thus stains only dead cells. Using a hemocytometer and microscope for the counting of cells, where non-viable cells will be stained blue and viable cells not stained. The cell suspension was diluted 1:5 with 0.4% Trypan Blue solution, with 10 µl then added to the hemocytometer. Cells were counted in the five big squares of the hemocytometer on the right hand and bottom boundaries. The number of cells
was per ml was determined using **Eq. 2-3** while the total number of cells in the total solution was calculated from **Eq. 2-4**.

<table>
<thead>
<tr>
<th>Number of cells counted</th>
<th>Number of squares</th>
<th>Dilution of solution</th>
<th>Dilution of trypsin solution</th>
<th>$\times 10^4$</th>
</tr>
</thead>
</table>

**Equation: 2-3**

\[
\frac{\text{Number of cells counted}}{\text{Number of squares}} \times \text{Dilution of solution} \times \text{Dilution of trypsin solution} \times 10^4
\]

\[
\text{Total number of cells/ml} \times \text{Total volume of solution} \quad \text{Equation: 2-4}
\]

### 2.6 Statistical analysis

Statistical analysis was performed using MINITAB® 17.1.0 (Minitab Inc., US). Numerical data is presented as mean ± standard deviation (SD) unless otherwise stated as ± standard error of the mean (SEM). Comparison of each sample group was completed using one-way analysis of variance (ANOVA) with a Tukey post-hoc test. Statistical significance was set at $p < 0.05$. 
2.7 References


Chapter 3: Biomechanical Assessment of Fixed Bovine Pericardium

3.1 Introduction

Soft tissue biomaterials such as BP, porcine pericardium and porcine aortic valves are utilised in bioprosthetic heart valve manufacturing. Number of heart valves replaced worldwide each year have been reported as over 250,000 with 45% of these bioprosthetic heart valves [1] and projection of heart valve replacement to triple by 2050 [2]. Their structural components of collagen, elastin and glycosaminoglycans (GAGs) provide the basis for the mechanical properties that prove suitable for bioprosthetic heart valves [3]. The collagen and elastin are the main structural components that respond to the various stresses of a heart valve. The native heart valve beats approximately 100,000 times a day, during which the valve undergoes different mechanical stresses including shear, flexural and tensile [4]. The biomechanical characterisation of the tissue for the valve is wide ranging across both the research literature and from an industrial standpoint. Industrial standard involves the minimum of 200,000,000 beats (5 years accelerated) in a simulated model to deem the valve acceptable ISO 5840/2005 [5] or the shear stress analysis of textile designed prosthetic valves [6]. Mechanical values for in vivo operation of a healthy heart have been reported as 8.3% - 14.5% and 0.71 – 1.22 MPa for bending stress and strain respectively [7]. However, the most common techniques used in the literature include uniaxial [8-11], and biaxial [12-15] testing. Also, from an industrial perspective the standards around cardiovascular implants have been purposely not specified to allow for development and innovation [16]. The mechanical specifications reported for the raw materials of tissue engineering applications, include more characteristic properties of polymer materials such as elastic modulus and ultimate tensile strength (UTS) [17]. Inconsistencies arise when the standard parameters such as UTS, strain at failure and modulus values are quoted yet variations in method or technique mean that they cannot be fairly compared across the literature, Table 3-1. Taking a uniaxial tested BP
sample modulus of 10 MPa reported by Hulsmann et al. [8], while for a biaxial test 250 ± 100 MPa was a reported value from Langdon et al [18]. Not only can the methods vary but also the conditions of that test. Taking one of the most accessible techniques, uniaxial testing, for mechanical assessment of heart valves [19] extension rates can vary from 2 mm/min to 25 mm/min [20, 21]. The UTS for BP uniaxially tested have been reported at 17.7 ± 3.0 MPa and 7.7 ± 4.3 for 2 mm/min [20] and 10 mm/min [22] rates respectively. Other differences such as the terminology for directionality of the tissue, in the above example Adraidano specified the directions of the samples taken as 90° (y-axis) 45° and 0° (x-axis) while Sung et al., described the samples as horizontal and vertical. There are others too that include axial [11], root-to-apex [23], longitudinal [24]. Preconditioning of the tissue where the soft tissue undergoes several loading and unloading cycles to allow collagen fibres realign in the direction of the applied force also has no standard protocol. While the typical result is that the first load unload cycle is greater than the subsequent cycles, where the hysteresis is the lost mechanical energy per unit volume and can be represented as a percentage Eq. 3-1.

\[
\% h = \frac{\text{loading area} - \text{unloading area}}{\text{loading area}} \times 100 \quad \text{Equation: 3-1}
\]
<table>
<thead>
<tr>
<th>Technique</th>
<th>Protocol</th>
<th>Key Parameters</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uniaxial and</td>
<td>Load until failure</td>
<td>Stress at Fracture and Hysteresis</td>
<td>[379]</td>
</tr>
<tr>
<td>Biaxial</td>
<td>Stepwise at mean loads of 2, 3, 4, 5, 6, 8 and 10kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uniaxial and</td>
<td>Cyclic loading – 30Hz max strain of 16%</td>
<td>Extensibility – peak stretch ratios</td>
<td>[380]</td>
</tr>
<tr>
<td>Biaxial</td>
<td>Low cycles – 30x10⁶ and High-65x10⁶ cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biaxially tested after cycling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uniaxial</td>
<td>Loading and Unloading for 10 cycles</td>
<td>Energy loss – difference between stress strain areas of upward and downward</td>
<td>[381]</td>
</tr>
<tr>
<td>Hardness</td>
<td>250N load cell, 4cm test zone, 50mm/min speed,</td>
<td>parts for each cycle and each sample.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ISO 6383-2:2005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uniaxial</td>
<td>Tensile test 20N load cell</td>
<td>Ultimate tensile stress, maximum strain at break and modulus of elasticity of</td>
<td>[240]</td>
</tr>
<tr>
<td></td>
<td>Strain rate of initial sample length per minute (l₀/min)</td>
<td>the linear part of the curve.</td>
<td></td>
</tr>
<tr>
<td>Uniaxial</td>
<td>Tensile tester – pre-loaded to 0.02N before acquiring data</td>
<td>Force extension curves converted to stress and strain.</td>
<td>[382]</td>
</tr>
<tr>
<td></td>
<td>Extended to failure (first significant decrease in load) at rate of</td>
<td>Elastic slope (initial low stress slope), collagen slope (high stress),</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mm/min. Sample size of 5x10mm</td>
<td>transition stress, transition strain, ultimate tensile strength, Strain at</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>failure</td>
<td></td>
</tr>
<tr>
<td>Technique</td>
<td>Protocol</td>
<td>Key Parameters</td>
<td>Ref</td>
</tr>
<tr>
<td>-----------</td>
<td>----------</td>
<td>----------------</td>
<td>-----</td>
</tr>
<tr>
<td>Uniaxial</td>
<td>Tensile tester (Instron 4411) with chamber of PBS at 37°C. Sample size of gauge length 20 mm and 5 mm width. Samples cut in root-to-apex (0°) and circumferential (90°). Stress relaxation – tissue stretched to stress of 100 kPa at a crosshead rate of 50 mm/min. Crosshead stopped, and load recorded over 100s. After this relaxation tissue unloaded and then restretched to failure at rate of 1.8 mm/min. Before and after stress relaxation tests a “few” load cycles between 0 and 0.1N applied until a reproducible response with a negligible hysteresis loop.</td>
<td>Stress relaxation data – ratio of stress at time t to the initial stress was plotted vs time on a logarithmic scale. Stress-strain data – tissue elastic modulus at linear stiff region (high stress), Stress at break and strain at break determined as sudden drop in load where first tissue layer failed, tangent modulus determined as the first derivative of the stress-strain curve for the initial region (0 and 0.6 MPa).</td>
<td>[255]</td>
</tr>
<tr>
<td>Uniaxial</td>
<td>Tensile tester in PBS 37C. Strain rate 0.1 mm/s.</td>
<td>Strain at fracture, UTS, modulus, stress relaxation and hysteresis.</td>
<td>[383]</td>
</tr>
<tr>
<td>Uniaxial</td>
<td>Uniaxial testing with a digital force gauge. Loading speed 100 mm/min.</td>
<td>Tensile strength and strain percentage.</td>
<td>[308]</td>
</tr>
<tr>
<td>Constitutive Modelling</td>
<td>Cycling loading effects on lifetime of crosslinked BP.</td>
<td>Long term cyclic loading.</td>
<td>[384]</td>
</tr>
</tbody>
</table>
The number of these cycles vary from three [9], ten [12, 13, 24, 33] or when a stable response is evident [22]. The characteristic J-shaped curve of a collagen dominant soft tissue like BP, Fig. 3-1, allows for the analysis of other parameters not typically assessed such as the low modulus and the hysteresis of the preconditioning cycles. The curve consists of four phases, applied force produces a non-linear stress-stiffening graph due to the uncrimping of the collagen fibers known as the “toe-region”. The heel region of the curve represents the end of the uncrimping of the fibers and the aligning of the molecules that now have the ability to slide past one another [34]. Stress in the linear section of the curve is high and is difficult to fracture the collagen fibers under normal physiological conditions [22, 28, 35-37]. The complete stress-strain process represents the rearrangement and movement of the collagen molecules without the breaking of bonds. Elastin’s function within the native heart valve is to support collagen when the external forces are removed returning the valve to its original configuration [38].

Most modulus values are taken from the linear section of the graph and will provide information on the stiffness of the tissue, and while values can vary from 10 MPa [8] to 77 ± 23 MPa [28] they are typically in the region beyond which the peak stresses of 1.0 MPa associated with a heart valve in vivo [39]. Therefore, assessment of the low modulus would provide more applicable data regarding the mechanics of the tissue in vivo.

Extension rate effects on polymers during uniaxial testing is that at slower rates the polymer chains can extend gradually favouring the viscous properties with elastic properties preferred at higher rates. Another factor in selecting an extension rate is the length of time, while a slow rate of 2 mm/min may be ideal,
Figure 3-1: Idealised stress-strain curve for a collagen based biological tissue such as bovine pericardium. Key parameters of interest include the low modulus in the elastic “toe” phase, where the initial linear slope at low stress is related to the uncrimping of the collagen fibres and the elongation of elastin. The high modulus of the linear elastic region linked to the collagen fibres extension. The high modulus will be of greater value than the low modulus, due to this engagement of the collagen fibres. Both the ultimate tensile strength (UTS) and strain at failure at the rupturing of the sample. The inset displays an example of five preconditioning cycles, where the percentage hysteresis is calculated from the area between the load and unloading of an initial force.
As number of samples increase the practicality would require a faster rate of 20 mm/min.

Assessing the extension rate impact on the mechanical properties of a soft biological tissue, BP, this chapter will compare three extension rates of 5, 10 and 20 mm/min, while in tandem measuring the effect of three different preconditioning cycles, one, two and five. The standard parameters of UTS, modulus and strain at failure along with the lesser measured mechanical values for low modulus and hysteresis. Another feature of the report will be to provide quantitative analysis of the mechanically tested samples post testing using SEM and an imaging software, where the orientation and crimping structure of the collagen fibers will be assessed.

The effect of preconditioning cycles and the extension rates on the uniaxial testing of BP will be investigated. The BP will be treated with the industrial standard fixative and sterilant, Glut that maintains the structural integrity of the pericardium and provides resistance to enzymatic degradation [40, 41]. Phosphate buffer solution (PBS) treated BP will act as the control. The standardizing of parameters for the testing and analysis of soft biological tissues such as BP are relevant from both a research and an industrial perspective for the design and production of medical devices like bioprosthetic heart valves. It would provide a more consistent platform for the comparison and quality assessment of mechanical properties across soft biological tissue biomaterials. The quantitative analysis of the samples post testing will further the knowledge on the behaviour of these tissues under mechanical loads.
3.2 Materials and Methods

3.2.1 Tissue fixation

Bovine pericardium (BP) tissue harvested from an animal less than 24 months of age, was acquired from a proprietary vendor defatted and stored in EDTA/PBS solution at 2 – 8 °C. Samples were washed prior to use twice with saline. Sections of BP were placed in 0.6% Glut prepared from an electron microscopy grade solution of 25% GLUT (Merck, Darmstadt, Germany) in 0.1 M phosphate buffered solution (PBS) (Sigma Aldrich)[25, 42] for 3 d at room temperature. The same method was applied to the PBS group but were stored in a solution of 0.1 M PBS.

3.2.2 Uniaxial testing

Tissue samples of 5 mm x 50 mm were cut using a scalpel in axial and circumferential (Circum) directions, Fig. 3-2, and were fixed in 0.6% GLUT or PBS as detailed earlier in tissue preparation. Thickness measurements were taken on three locations across each sample using a digimatic indicator (Mituyou Absolute ID-S, Toronto, ON, Canada). The average thickness value was used for the calculation of the cross-sectional area for each sample. Uniaxial testing was performed on a Zwick Roell tensile tester using a 100N load cell. Each sample (n=5) was held in position with an air pressurized roughened surface clamp, gripped at 3 Bar with a gauge length of 15mm. Samples were tested under a range of pre-conditioning cycles, 1, 2 and 5 in a loading range of 0 N to 1 N. Extension rates of 5, 10 and 20 mm/min were utilized with each sample was extended to failure after the last preconditioning cycle. Failure was set at 80% drop in the peak load. The force extension curves produced were converted into stress strain curves using the cross-sectional area of each sample. The parameters calculated include, 1) the low modulus of the sample taken as the slope of last preconditioning cycle, 2) the high modulus as characterized by the slope of the linear portion of the stress/strain curve 3) the ultimate tensile stress (UTS) calculated as the peak stress of the curve before failure, 4) the percentage strain of failure is the strain at the point of UTS and 5) the hysteresis of the first and 6) final preconditioning cycle.
3.2.3 SEM imaging and analysis

Post uniaxial testing samples were dried in increasing concentrations of ethanol from 50% to 100% where they were then treated with Hexamethyldisilazane (HMDS) for 30 mins and then allowed to air dry. Samples were then gold sputtered. SEM (Hitachi, S-4700) imaging was carried out at accelerating voltages of between 5 kV to 10 kV.

Analysis of the SEM images was carried out using ImageJ software (NIH, Bestheda, Maryland, USA)[43]. Measurements of the collagen fibre bundles included the straight-line distance between three crimps of a collagen bundle (Lo), the actual length of this bundle (Lf), the thickness (t) averaged across three areas of the fibre bundle and the angle of the crimp (θ), as shown in a representative image Fig. 3-3. These measurements were used to calculate two parameters, waviness, and the orientation angle of the fibres [44]. The waviness or straightness parameter (Ps) is calculated from the actual distance and the straight-line distance of three crimps on a collagen bundle, Eq. 3-2. The

Figure 3-2: Schematic of pericardial sac and the orientation of samples taken for uniaxial testing.
straighter the fibre bundle the closer to a value of 1 while a wavier sample would measure 0.

\[ P_3 = \frac{L_0}{L_f} \]  

Equation: 3-2

The global angle (\( \Theta \)) is measured in respect to the axial (0°) and circumferential (90°) direction of the sample.

Figure 3-3: Representative SEM image with the key measurements analysed using ImageJ. The measurements include the crimped fibre length (\( L_f \)) calculated along the collagen fiber including three peaks of the fiber and the straight fiber length (\( L_0 \)) between the first and third peaks of the collagen fiber. The other parameters is the angle (\( \Theta \)) based on the orientation of the sample.
3.2.4 Differential Scanning Calorimetry
The $T_d$, provides a gauge to the degree of crosslinking within a fixed tissue. The $T_d$ for both Glut and PBS samples (n=3) were obtained using a differential scanning calorimeter (DSC) (Model Q1000, TA Instruments, Delaware, US). Circular samples of 3 mm diameter were cut using a biopsy punch, patted dry and hermetically sealed into aluminium pans. The test method followed a heating rate of 5 °C/min as suggested by Loke et al. [45] in a temperature range of 30 °C to 110 °C. An empty pan was used as the reference. Heating was carried out in a nitrogen atmosphere of 50 ml/min. The $T_d$ can be calculated from the extrapolated onset or the peak temperature of the endothermic phenomena, here the peak temperature was used to determine the $T_d$ [28, 45, 46]. Calibration of the instrument was carried out using an Indium standard (Melting point, Tm 156.6 °C).

3.2.5 Statistical Analysis
Measurements across all groups were averaged and presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to compare the differences across all samples. A p-value of less than 0.05 was deemed statistically significant. Statistical analysis was carried out using Minitab (Minitab 17) statistical software.
3.3 Results

3.3.1 Denaturation temperature and thickness

The denaturation temperature as analysed with DSC, showed that a non-treated sample displayed a temperature of 64.5 °C ± 1.2 °C while the PBS and GLUT samples had significantly higher values of 75.2 °C ± 2.9 °C and 84.7 °C ± 0.2 °C respectively. The Glut prepared samples were also statistically higher than those of the PBS treated group, Fig 3-4.

![Figure 3-4: DSC denaturation temperature (Td) for non-treated, samples stored in PBS for 2 h and Glut fixed BP sample. The Glut treated sample displayed a Td significantly higher than both the PBS treated and non-treated BP (p < 0.05)](image-url)
Pericardium thickness measurements after treatment in PBS and Glut resulted in statistically significant higher values of $0.62 \pm 0.11 \text{ mm}$ and $0.55 \pm 0.10 \text{ mm}$ respectively, compared to the native tissue $0.44 \pm 0.08 \text{ mm}$. The PBS had a statistically greater thickness than the Glut BP, Fig 3-5.

**Figure 3-5:** Thickness measurements of fresh tissue pre-PBS, post-PBS and Glut fixed tissue. Statistically significant increase in thickness after both PBS and Glut treatment. Also, PBS statistically higher than the Glut fixed samples. ($p < 0.05$).
3.3.2 Mechanical results

The UTS samples displayed a statistical difference for the 5 mm/min extension rate at 5 cycles for the higher PBS BP compared to the Glut BP with the trend of higher PBS values evident across both directions Fig. 3-6 (A) and (B). Also, in the circumferential direction there is significant differences across the extension rates at 5 cycles with 5 mm/min displaying higher values than those

![Upsilon Tensile Strength (UTS) Graphs](image)

**Figure 3-6:** Mean and SD of the UTS in the axial (A) and circumferential (B) directions for both Glut and non-fixed PBS samples. The axial direction values for UTS (A) showed a statistically higher value for the PBS BP compared to the Glut BP. Circumferential direction, (B) PBS 5 mm/min at 5 cycles has statistically higher values than 10 and 20 mm/min 5 cycle PBS samples. (*) indicates a statistical difference (p < 0.05).
of both 10 and 20 mm/min. Fig. 3-7 (A) and (B) display Glut BP with higher values than PBS samples for strain at failure, statistically greater at the higher strain rates and cycling numbers. The Glut BP had higher values for 5 cycles at 10 mm/min than that of its respective settings of 20 mm/min and 5 mm/min in the axial direction.

Figure 3-7: The strain at failure values were statistically higher for the Glut against PBS samples over all strain rates at 1 cycle in both the axial (A) and circumferential (B) direction. There were also statistical differences over the 10 mm/min and 20 mm/min at 5 cycles in the axial direction and in the circumferential direction at 1 cycle. (*) indicates a statistical difference (p < 0.05).
Low modulus, Fig. 3-8 (A) and (B) for the Glut BP did not display a significant difference across extension rates or pre-conditioning cycling number. The PBS BP did however display significantly greater low modulus values than the Glut BP across all settings. Also, PBS BP had a statistically lower value in 20 mm/min 2 cycles compared to the 5 cycles at 20 mm/min, and 2 cycles at 5 mm/min in the axial direction. In the circumferential direction PBS BP at 5 cycles 5 mm/min was statistically greater than that of the same cycle number at 10 mm/min.

Figure 3-8: Mean and SD of the low modulus for Glut and PBS in the (A), (B) axial and circumferential directions respectively, showed higher values for the PBS across all extension rates and cycle numbers compared to the Glut samples.
Comparing the Glut and PBS samples, the PBS BP samples displayed slightly higher values than the Glut BP samples with statistically different values evident across the 5 mm/min extension rate for 2 and 5 cycles, the 10 and 20 mm/min extension rates at the 1 cycle for the high modulus in the axial direction Fig. 3-9 (A). The circumferential direction, Fig. 3-9 (B) displayed the same significantly higher differences at 5 mm/min 2 and 5 cycles along with the 10 mm/min 5 cycle between the PBS and GLUT samples. There were significantly

Figure 3-9: The high modulus (A) and (B) for both Glut and PBS samples in the axial and circumferential directions also displayed significantly higher values for the PBS samples to Glut for all extension rates and cycle numbers. (*) indicates a statistical difference (p < 0.05).
higher values across the extension rates for PBS with 5 mm/min 5 cycle group showing higher values than those of both the 10 and 20 mm/min groups.

Combining the results of each cycle number for each extension rate displayed statistical differences across the directionality of the BP but not within each direction group, **Fig 3.10** (A) – (D). The axial direction showed statistically higher values for the UTS and High modulus parameters for each extension rate. While the axial direction showed higher values for the percentage strain at failure, there was no statistical difference and both directions displayed similar results at the low modulus.
Figure 3-10: Mean and SD for the (A) UTS, (B) high modulus, (C) percentage strain at failure and (D) low modulus for Glut BP at each extension rate after combining all cycle numbers. The UTS and high modulus Glut samples in the axial direction displayed significantly greater values than the circumferential direction across all extension rates. The low modulus and strain at failure did not show any statistically significant differences in axial or circumferential directions. (*) indicates a statistical difference (p < 0.05).
The hysteresis values across all the extension rates for both the Glut and PBS samples in the axial directions are displayed in Fig. 3-11 (A) – (C). The increase in extension rate shows an increase in variation between the Glut and PBS samples with values overlaying at 5 mm/min compared to the statistical significant differences at 20 mm/min. The trend across all rates and sample types is that the hysteresis for the first cycle is significantly greater than that of the 2nd cycle were the values begin to stabilise.
Figure 3-11: Hysteresis graphs comparing Glut and PBS samples of the axial direction for all three extension rates (A) 5 mm/min, (B) 10 mm/min and (C) 20 mm/min. There was significant difference across the 2 – 5 cycle numbers for the 10 and 20 mm/min extension rates, while there was also a reduction in variation for the Glut samples across these higher rates.
The treatment of the BP with Glut and the standardisation of the tissue is evident in Fig. 3-12, where a representative selection of the final pre-conditioning cycle display reduced variation compared to the PBS treated samples.

![Figure 3-12: Representative hysteresis curves for the 5th load-unload cycle for the Glut BP and PBS samples across the range of extension rates. Larger variation evident in the PBS samples compared to the Glut BP samples.](image-url)
When the data of all the extension rates for the Glut BP are combined, Fig. 3-13, for each cycle number the 1st cycle is over two times that of the 2nd cycle, 44.63% compared to 19.31% respectively for the axial direction. The circumferential direction displays similar results of to 44.46% and 19.63% for the 1st versus 2nd cycle respectively. The 2nd cycle of both the axial and circumferential directions shows a statistical difference with the 5th cycle of each direction.

**Figure 3-13:** Hysteresis for Glut BP in both the axial and circumferential directions across each of the cycle numbers with the results for each extension rate combined. Statistical significance between the first cycle and the remaining cycles for both directions, while also a statistical difference between the 2nd cycles and the 5th cycle also for each direction. (*) indicates a statistical difference (p < 0.05).
3.3.3 Image analysis and results

Key values from the SEM images are displayed in Table 3-2, with the representative images for each of the parameters and circumferential and axial directions displayed in Fig. 3-14 and Fig. 3-15 respectively. The straightness parameter of Ps consisted of a scale of 0 – 1 representing a highly crimped and straight collagen fibre respectively. The Ps values ranged from a low of $0.62 \pm 0.05$ for 5 mm/min 5 cycles in the circumferential direction compared to the highest value of 0.89 at 10 mm/min for 2 cycles. The axial direction samples values did not fluctuate as much with a lowest value of $0.69 \pm 0.01$ at 10 mm/min 5 cycles to a maximum of $0.82 \pm 0.1$ on average for 10 mm/min 2 Cycles and 20 mm/min 5 cycles. With a statistically significant difference at 10 mm/min 5 cycles when compared to 1 and 2 cycles at the same extension rate and the 5 cycles of the other extension rates. The circumferential fibre had on average lower values than the axial direction at rates and cycle numbers of 5 mm/min 5 cycles and 20 mm/min 5 cycles.

The orientation angles of the fibres showed variation in both directions. The axial displayed fibres at a low of $22.54^\circ \pm 0.82^\circ$ and a high of $148.82^\circ \pm 15.32^\circ$ for 5 mm/min 2 cycle and 20 mm/min 2 cycle respectively. Circumferential fibres ranged from a low of $34.46^\circ$ to a high of $135.24^\circ \pm 23.96^\circ$. 
### Table 3-2: SEM images quantification of a collagen bundle waviness, size and orientation

<table>
<thead>
<tr>
<th>Extension Rate Number of Cycles</th>
<th>5mm/min</th>
<th>10mm/min</th>
<th>20mm/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>θ Axial (±SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>86.07 ±</td>
<td>22.54 ±</td>
<td>106.20 ±</td>
</tr>
<tr>
<td></td>
<td>4.66</td>
<td>0.81</td>
<td>22.91</td>
</tr>
<tr>
<td>Circumferential (±SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.03 ±</td>
<td>118.18 ±</td>
<td>133.28 ±</td>
</tr>
<tr>
<td></td>
<td>20.56</td>
<td>81.91</td>
<td>7.65</td>
</tr>
<tr>
<td>P3 Axial (±SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75 ±  0.05</td>
<td>0.80 ± 0.02</td>
<td>0.76 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
<td>n=1</td>
<td>0.01</td>
</tr>
<tr>
<td>Circumferential (±SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.78 ±  0.11</td>
<td>0.68 ± 0.17</td>
<td>0.62 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>n=1</td>
<td>±n=1</td>
</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>
Figure 3-14: Representative SEM images of circumferential direction samples with selection of fibres highlighted in yellow. Scale bar 50 µm.
Figure 3-15: Representative SEM images of axial direction samples with selection of fibres highlighted in yellow. Scale bar 50 µm.
3.4 Discussion

The mechanical properties of Glut fixed BP and how the effect extension rate and preconditioning cycles had on mechanical parameters were investigated. The mechanical parameters measured included the standard properties UTS, modulus and percentage strain measured. The effect of extension rate and preconditioning cycle did not have a significant effect on UTS and the high modulus results, Fig. 3-6 (A) (B) and Fig. 3-9 (A) (B) respectively for the Glut fixed group. However, this was not the case with the PBS group, where statistically higher values of modulus in the axial direction for the 10 and 20 mm/min rates at 1 preconditioning cycle, and higher values at 5 mm/min for the 2 and 5 cycles were evident. The circumferential direction at 5mm /min also displayed significant differences in the 5 mm/min group at 5 cycles compared to the 10 and 20 mm/min rates. Fixation with Glut did not significantly alter the UTS of the tissue compared to the PBS group except for the axial and circumferential direction at 5 mm/min 5 cycles and across both 10 and 20 mm/min respectively. This too was reported by Hulsmann et al, [8] who observed a reduction in the high modulus between fixed and native BP tissue, also evident in this study. The author suggested that the fixation without any loading reduces the elasticity of the tissue [8]. Another reason for the reduction in modulus is that native non-fixed tissue is thinner than Glut BP and therefore the cross-sectional area is smaller yielding higher values after calculating [22]. This however is not the case here where the PBS samples tissue were also statistically higher than those of the Glut BP.

Low modulus values for PBS tissue were significantly higher than the Glut tissue across all extension rates and cycle numbers for both directions, Fig. 3-8 (A) (B). As elastin is the primary load bearer during this toe elastic region due to the uncrimping of the collagen fibers [12], the Glut appears to have a significant effect on the elasticity of the elastin. The biomechanics of elastin are like those of a rubber with a low E-modulus, high extensibility and a sharp rise in stress-strain curve at high elongations [47]. The extension rate and cycle number did not have a significant difference across all the low modulus samples.
The effect of crosslinking was also evident with the percentage strain at failure, where across all the extension rates the strain at failure was greater than the PBS samples in both directions. The cycle number also reported higher values with only 5 cycles at 20 mm/min displaying lower values, but this was not significant. The increase in extension after crosslinking is similar to what occurs in the vulcanisation of a rubber but depending on the crosslinking density there can also be a significant increase in the stiffness and UTS of the rubber. Crosslinking of collagen fibres with Glut, involves the formation of both inter- and intra-molecular crosslinks that form a network creating a stable tissue with an increase in extensibility [22]. This also is evident with the PBS samples where the H2O bound within the tissue stabilizes the collagen and elastin [48] which accounts for the increase in the Td temperature and the comparable UTS and high modulus values but is limited in its extension affect. It is suggested that the elastic limit is a much more reliable mechanical reference for bioprosthetic heart valve tissue, than the UTS as the valve should never approach this value [49].

The anisotropic effect was present on the UTS and high modulus with the axial direction displaying statistically higher values than circumferentially. Reported retention or removal of the anisotropic effect varies with Paez et al., suggesting that the tissue directionality differs for both strength and stiffness [49] with others suggesting that the anisotropy is removed [22].

The directionality effect Fig. 3-10 (A)-(D) across all the extension rates after the combination of all cycles values showed that axial direction is statistically higher for the UTS and high modulus by two-fold and between 10-20% respectively. This is typically the scenario in the literature where the axial direction has reported higher values [22, 28] contrary to the anisotropic effects of Glut fixation where the circumferential direction is stiffer [39, 49]. There was no statistical difference in the hysteresis, low modulus or strain at failure between both directions in this study. There was no statistical difference across the extension rates.
Analysis of the hysteresis curves showed a wider variation for the PBS samples compared to the Glut BP, Fig. 3-12 in the 5th load-unload cycle. The effect of extension rate is evident at that higher rates of 10 and 20 mm/min, where there are statistically different after the 1st and 2nd cycle when compared to the PBS samples, with the Glut samples displaying lower hysteresis of around 10%. The 5 mm/min rate did not show a significant difference across each cycle. The Glut samples also displayed less variation per each cycle compared to the much wider spread of the PBS samples, this is most evident at the 20 mm/min rate, Fig. 3-11 (A)-(C). Comparison of hysteresis cycle number for each direction regardless of extension rate, Fig. 3-13. showed that the directionality of the tissue did not influence the hysteresis and that both directions had no significant difference after the 2nd cycle. It was also noted by Daar et al, that hysteresis values tended to stabilise after approximately 5 cycles [30]. From this examination the 1st and 2nd cycles are critical in the conditioning of the tissue. Also, the Glut samples did not have significant differences across the extension rates as too was noted by Trowbridge et al. [50] who reported that hysteresis was extension rate independent.

Analysis of the samples post mechanical testing assessed the crimping structure of the collagen fibres and their respective orientation to the axial and circumferential direction. The crimping structure of the fibres ranged from 0.62 to a highest of 0.89 for the circumferential direction at 10 mm/min. This shows that the fibres are not fully extended after the tensile test but still retain elements of their crimp structure. The extension rate variation did not have a significant effect which was due to the viscous properties of the tissue that allow it to return to a cramped structure while retaining elements of the applied stress exertions after the stress has been removed [49]. Therefore while the samples were treated for SEM analysis in a timely manner, they had the opportunity to recoil on removal of the extension load.

Pericardium is known as an anisotropic material, the analysis of the fiber orientation here confirms this with variation across both directions. Given that
the analysis is at a microlevel and looking at individual fibre bundles, the variation is evident and a possible cause for the variation in both here and across the literature. Uniaxial testing exert an extension in one direction so if the tissue in the axial direction is misaligned then inconsistencies will occur.

Across the literature the characteristic mechanical parameters of UTS, high modulus and strain at failure are assessed. However, the range of methods and in turn the reported results are varied [51]. Here the extension rate of 10 mm/min produced similar UTS, modulus and strain values compared to other studies [22, 28] and would be recommended for the application as a standardised extension rate. The preconditioning number of cycles showed that after the first two there is a plateau effect suggesting that 5 cycles would be an adequate number for the preconditioning of BP tissue prior to the uniaxial test.
3.5 Conclusion

Comparing the mechanical properties of Glut BP through a range of parameters provided insight into the importance of the number of preconditioning cycles and a suggestion for a standardised extension rate. The effect of the number of preconditioning cycles on the hysteresis of Glut BP was shown with a significant drop from the first cycle to the 2nd cycle, with a plateau in hysteresis values after the 3rd cycle, indicating that 5 load-unload cycles is a required standard for the preconditioning of BP. The directionality of the fibres was not a factor in hysteresis with no significant difference between the Glut BP axial and circum samples, while a significant difference is evident between the Glut and PBS samples at the higher extension rates of 10 and 20 mm/min. The effect of extension rate showed minor variations but no statistically significant differences across the mechanical properties of BP. However, the 10 mm/min rate produced values close to those in the literature at the same rate and would satisfy the time requirements for large scale sampling and is recommended as a standardised rate. Orientation effects and crimping of the collagen fibres as assessed by SEM post mechanical testing provides quantitative data on the orientation of the fibres and its correlation to the mechanical properties of uniaxial tested BP. The similar crimped pattern across all extension rates provide evidence of the viscous behaviour of the BP and how the structure remains upon removal of the stress. This quantitative method could be employed exclusively or used to complement other techniques such as small angle light scattering or second-harmonic imaging microscopy for the assessment of BP.
3.6 References


Chapter 4: Physical and Chemical Characterisation of Bovine Pericardium

4.1 Introduction

Characterisation of natural biomaterials can be broken into the areas of surface chemistry, assessment of treatment effects, structural and topographical properties. Techniques traditionally used for synthetic materials can be applied directly to natural materials whereas others require extensive sample preparation. Natural biomaterials like BP are heterogeneous, multi-layered and anisotropic [1]. They are vulnerable to degradation when removed from their natural environment, which limits the use of certain methods of surface analysis where testing conditions call for high temperatures, contacting probes and electron beams. Spectroscopy is an area where some methods are better suited to the analysis of BP. FTIR is better suited for the general molecular structure analysis of a biological sample as it requires little sample preparation compared to the more surface sensitive techniques typically used for surface chemical structures of synthetic biomaterials of X-Ray photoelectron spectroscopy (XPS) and Time-of-Flight Secondary Ion Mass Spectroscopy (TOF-SIMS) [2-4]. Post treatment characterisation of crosslinked BP are varied from shrinkage temperature [5] to calorimetric free amine assay assessment [6, 7]. While these techniques can inform on the effectiveness of the crosslinking reaction between Glut and BP, they do not provide a direct measurement of the rate of reaction. DSC is a technique that is associated with the analysis of polymers [8], nitinol [9] and pharmaceuticals [10]. With the increasing scale of bioprosthetic valves on the market the requirement for an efficient and reliable test technique for the determination of a fully crosslinked tissue sees it increasingly used for $T_d$ measurement in place of the traditional shrinkage method [5]. A variation of the technique, QiMTDSC, can be utilised to measure the behaviour of a material or reaction with another substance isothermally over a period and determine transitions that would normally not be measurable [11]. The crosslinking protocol between Glut and BP is similar in areas relating to concentration, where
0.6% is standard for most biomaterial applications, providing the required balance between desired mechanical, collagen stabilising and sterilant properties [12] and temperature, typically between 4 °C to room temperature. However, the time for the process is varying from hours to days [13-15]. This reaction time is investigated here using QiMTDSC. First step was to determine its feasibility as a method to assess the rate of reaction between Glut and BP. Taking the model of an epoxies reaction between its epoxy and hardener components, the crosslinking interaction between Glut and BP was measured, to see how long the fixation process takes for the BP to be deemed fully crosslinked.

Measuring the free amine content of a Glut treated tissue, is another method to determine if a sample is adequately crosslinked or not. The number of free amines is inversely proportional to the degree of crosslinking, and a number of different assays are available to perform this assessment, though can be time consuming and involve lyophilisation and spectroscopy [7, 16]. Ninhydrin assay also measures the free amine groups and has found applications from the fields of forensics [17, 18] to measuring the level of Glut crosslinking [19]. This assay will be applied in this study in a quicker more qualitative method and will be based on the visual assessment of BP.

The surface and microstructure of BP in bioprosthetic heart valves are important considerations that can minimise the chance of thrombosis, with the rough side, free of excess fat and appendages facing the inflow of blood [20]. SEM analysis can provide detailed images of both fibrous and serous surfaces of the valve and examine the effect of processing on the tissue surface. The ultrastructure of the tissue can provide information on the orientation of the collagen fibrils, distribution of elastin complexes and the integrity of its cellular components. This was completed through TEM where samples of BP were compared pre- and post-fixation. Another surface technique used for the structural characterisation of soft biological biomaterials is that of AFM in which the
topographical and quantitative measurements can be made at the nanometre level [21-23].

4.2 Materials and Methods

4.2.1 Tissue preparation

Bovine pericardium (BP) tissue of an age less than 24 months was acquired from a proprietary vendor defatted and stored in EDTA/PBS solution at 2 – 8 °C. Samples were washed prior to use twice with saline. Samples of BP were cut using a 3 mm diameter biopsy punch and consumed in the protocols detailed in the methods. A fixation solution of 0.6% Glut prepared from an electron microscopy grade solution of 25% Glut (Merck, Darmstadt, Germany) in 0.1 M phosphate buffered solution (PBS) (Sigma Aldrich) was used. For the QiMTDSC a Loctite M-31Cl epoxy (Henkl Technologies) was used where a 2:1 mix of Part A epoxy and Part B hardener was mixed and tested at different temperatures over a 12 h period.

4.2.2 QiMTDSC

The BP sample was cut into 3 mm diameter sections using a biopsy punch and followed one of two sample preparation methods for QiMTDSC as detailed in Chp_2, Fig. 2-3. Method 1 applied a 10 µl volume of 0.6% Glut to the 3 mm BP sample in an aluminium pan and was then hermetically sealed. Method 2 involved immersing the 3 mm BP sample in 10 µl of 0.6% Glut for 30 s. The sample was patted dry and hermetically sealed in an aluminium pan. All QiMTDSC and conventional DSC testing were performed using a DSC, (Model Q2000, TA Instruments, Delaware, USA). Both method 1 and 2 followed the same protocol of equilibrating the sample at 25 °C and holding isothermally for a selection of isothermal periods from 10 min, 120 min, 240 min, 360 min and 720 min. The Loctite M-31Cl epoxy resin samples were also tested over a range of isothermal temperatures consisting of 25 °C, 50 °C, 100 °C and 200 °C for a period of 720 min. All samples were run under a modulation of +/- 1 °C every 100 s. The thermograms of the heat capacity (mJ/ °C) as a function of temperature were used to measure the total change in heat capacity after 30 min
to the end of the time periods. Directly after each QiMTDSC test the samples were verified using a conventional DSC to determine the $T_d$, the degree of crosslinking within the fixed tissue. The test method followed a heating rate of 3 °C/min as suggested by Loke et al.[5] in a temperature range of 30 °C to 100 °C. An empty aluminium pan was used as the reference. Heating was carried out in a nitrogen atmosphere of 50 ml/min. The $T_d$ can be calculated from the extrapolated onset or the peak temperature of the endothermic phenomena, here the peak temperature was used to determine the $T_d$ [5, 24, 25]. Calibration of the instrument was carried out using an Indium standard (Tm 156.6 °C).

4.2.3 Ninhydrin assay
Sections of BP, 3 mm diameter, were placed into vials of PBS, 0.6% and 2.5% Glut solutions, n=3 for each vial, at room temperature for 2 d. A 2.5% (w/v) Ninhydrin assay was prepared by adding 2.5 g to 100% ethanol. The three samples from each vial were placed onto a separate glass slides, where a 15 µl of the 2.5% Ninhydrin assay solution was added to each sample. The slides were then placed into an oven at 80 °C for 5 mins. Images were taken pre and post oven treatment.

4.2.4 Fourier Transform Infra-Red Spectroscopy (FTIR)
Spectroscopy was performed using a Vertex 70 with Hyperion 3000 microscope (Bruker, Germany) using a micro-ATR accessory. Both ATR’s consisted of a diamond crystal with samples tested at 64 scans with a resolution of 4 cm⁻¹. Crosslinked samples were washed with PBS post crosslinking to remove any residual Glut components.
4.2.5 Scanning Electron Microscopy (SEM)
BP samples were prepared following a dehydration protocol of increasing concentrations of ethanol from 50% to 100% before a final 30 min treatment in HMDS and allowed to air dry. Samples were gold sputtered and imaged using a SEM Hitachi, S-4700 (Hitachi, Japan) at an accelerating voltage of between 5 to 15 kV.

4.2.6 Transmission Electron Microscopy (TEM)
Native and Glut fixed BP ultrastructure’s were compared using TEM. Samples were fixed in 2% Glut solution in 0.1 M cacodylate buffer at room temperature for 60 min. Secondary fixation was carried out using a 1% osmium tetroxide in 0.1 M cacodylate buffer for 60 min at room temperature. Samples were then dehydrated in a series of increasing ethanol solutions from 50% - 100%, before been embedded in low viscosity resin (R1078A, Agar Scientific). After finding regions of interest using “scouting sections” ultrathin sections of 80 – 100 nm thickness were cut using an ultra-microtome (Leica Reichert Jung Ultracut). Samples were stained in 1.5% aqueous uranyl acetate and lead citrate (Leica EMAC 20) and then analysed using a TEM (Hitachi 7000 TEM, Japan).

4.2.7 Atomic Force Microscopy (AFM)
Samples were placed onto a glass slide and imaged using a Veeco Dimension 3100 AFM (Digital Instruments, Santa Barbara, CA, US). A silicon Pointprobe® with a cantilever length of 125 µm and force constant of 42 N/m was used in tapping mode with an amplitude of 2.0 V and a scan rate of 0.5 Hz for 512 x 512 points in a single scan. Image sizes ranged from 10 µm x 10 µm to 500 nm x 500 nm. Height and phase image data were processed using WSxM software (Nanotec Electronica, Spain).
4.3 Results and Discussion

4.3.1 QiMTDSC

Demonstration of the QiMDSC technique and the proof of concept for its application to the analysis of a crosslinking reaction is illustrated by the thermogram of the epoxy across various isothermal curing temperatures, Fig. 4-1. The extrapolated onset of the Rev Cp curve was used as an indicator for the start of the epoxy hardener crosslinking reaction. The epoxy samples held isothermally at 25 °C and 50 °C displayed a drop in the Rev Cp after 227.85 min and 96.81 min respectively. While the higher temperature curve of 100 °C produced a drop at 15.84 min. The drop could not be calculated at 200 °C as the baseline appeared flat almost immediately. The total drop in the Rev Cp baseline was also measured between 10 min and 720 min. There was a difference evident across all the cure rates with both the 25 °C and 50 °C cured samples displaying a similar drop of 3.511 and 3.312 mJ/ °C respectively. However, these were considerably greater than the values measured at the higher isothermal temperatures with 0.4329 and 0.0676 mJ/ °C measured for 100 °C and 200 °C respectively. This would indicate that at the lower curing temperatures there was a greater degree of molecular mobility during curing, resulting in a longer curing time.
The measurement of the Tg of each epoxy post curing are shown in Fig. 4-2. The lowest curing temperature of 25 °C displayed a Tg value of 37.15 °C, while also displaying an exothermic peak at 83.73 °C suggesting some unreacted components present within the epoxy. The epoxy cured at 50 °C showed an increase in the Tg to 57.66 °C. However, this temperature is significantly lower than those for the higher cure temperatures of 100 °C and 200 °C with both displaying temperatures above 74 °C. Both these temperatures are above the reported value of the typical physical properties of the cured epoxy with a Tg of 70 °C.

**Figure 4-1:** Comparison of curing behaviour at different isothermal temperatures for an epoxy adhesive. The Rev Cp was taken as the drop in the baseline from 10 min to 720 min. Also extrapolated onset was used to determine the drop in the baseline as an indicator of crosslinking reaction occurring between epoxy and hardener.
Analysing Method 1 protocol over 10 min, 360 min and 720 min for BP and 0.6% Glut reaction there is a continuous drop in the baseline, Fig. 4-3. Applying the same principle from the epoxy analysis, this would indicate that the reaction is continuing between the BP and Glut and the sample is not fully crosslinked. However, on testing the samples once the QiMTDSC method is complete using conventional DSC and analysing the $T_d$, it is evident that all samples are fully crosslinked even after 10 min, Fig. 4-4.

**Figure 4-2:** Measurement of $T_g$ for each epoxy sample after their respective isothermal curing temperature

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156
Figure 4-3: QiMTDSC thermograms after 10 min, 360 min and 720 min for BP and 0.6% Glut following method 1.

Figure 4-4: Measurement of $T_d$ post QiMTDSC method that suggests BP and Glut reaction produced a fully crosslinked material, even after 10 min.
This suggests that while the Glut reaction with the amide bonds is swift and occurs before 10 min, there is still a continuing reaction taking place. This quick reaction was also reported by Damink et al. who reported that free amine groups in collagen of dermal sheep react quickly with Glut to form Schiff bases [5]. When looking at the control samples of Glut only, the continuous drop in the baseline is present Fig. 4-5.

![Image](image.png)

**Figure 4-5:** Overlay of two runs of 10 ul 0.6% Glut only using QiMTDSC for 720 min. Both samples display good reproducibility with similar Rev Cp decreasing value of over 2 mJ/°C.

This suggests that the Glut polymerisation is a continuous process over time. When the BP was dipped into 0.6% Glut the similar drop in the Rev Cp was again present, however the T_d analysis post-test showed a gradually increasing T_d of value with increase in time stabilising at 720 min Fig. 4-6.
Using the technique to assess the individual components of BP, Glut and PBS showed that there is a continuation in the decrease of the Rev Cp, **Fig 4-7**. This would indicate that there are continuing reactions occurring with the Glut itself, pointing to its reputation of continuous monomer transformations in aqueous solutions [4, 18]. While both BP + Glut and BP + PBS show that reactions with the proteins continues with no specific endpoint. The change in the Rev Cp for the BP + PBS also points to the reaction mechanism between the Hydrogen bonds and the $T_d$ increases significantly from its native temperature. This continuing change in Rev Cp is direct evidence of molecular mobility within the samples and suggests that the reaction between BP + Glut is continuous.

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**Figure 4-6**: $T_d$ measurements of BP + Glut post QI/MTDSC using method 2. Displays an increase of over 10 °C from a native untreated BP sample to BP + Glut tested after 720 min. (n=3) for all but 720 min which due to time constraints (n=1).
A component of the technique is the generation of Lissajous plots that provide information on the effect of the modulation on the sample. The modulated heat flow is plotted against the modulated temperature and ideally with no phase shift the plot would be a straight line, but due to the effects of heat dissipation and phase lag the plot is an ellipse shape [26], where the slope represents the heat capacity and the width provides information on the heat dissipation. The curves in Fig. 4-8 and Fig. 4-9 display the Lissajous plots for BP + Glut following method 1 for 10 min - 720 min and the epoxy samples respectively. The curves of the BP + Glut reaction display equilibration after approximately 40 min. This measure of reaction equilibration is evident with the epoxy where at lower temperatures of 25 °C and 50 °C, it displays a broad curve while at higher temperatures the curves are overlaid after 5-10 min at 100 °C and immediately at 200 °C.

Figure 4-7: Overlay of individual Glut component, BP+Glut and BP+PBS. The BP and Glut drop in reverse Cp is double than that of Glut only and multiple times greater than the BP+PBS interaction. All display a drop in the Rev Cp indicating reactions ongoing.
Figure 4-8: Lissajous curves (A) – (C) for 10 min, 360 min and 720 min for BP in 10 µl of 0.6% Glut following method 1, respectively. The curve undergoes equilibration after approximately 40 min where the ellipses for each modulation start to overlay.
Figure 4-9: Lissajous curves for the epoxy resin heated at 25 °C (A), 50 °C (B), 100 °C (C) and 200 °C (D). The modulations for the lower temperatures of 25 °C and 50 °C are varied and do not overlay until towards the end of the experiment. While the higher temperatures of 100 °C and 200 °C, display ellipses that overlay after an initial period and immediately respectively.
4.3.2 Ninhydrin assay

The number of free amines in a crosslinked sample were measured using a qualitative method with Ninhydrin assay. Samples of Glut crosslinked BP and PBS treated BP after an oven treatment with Ninhydrin assay are displayed in Fig. 4-10. The PBS sample turned the characteristic Rheumans purple colour while both the 0.6% and 2.5% Glut samples turned a darker shade due to the heating and drying effect of the oven. The assay was a quick and convenient method to gauge the free amine content of the BP that in turn determines if the tissue is crosslinked.

![Fig. 4-10: Samples of 3 mm of BP in PBS, 0.6% Glut and 2.5% Glut for 2 d analysed using Ninhydrin assay. Images (A) and (B) are pre- and post-oven at 80 °C for 5 min respectively. Images (C) and (D) display the pre- and post-oven treatment of samples, respectively, that have not been treated with Ninhydrin assay.](image)
4.3.3 FTIR

Analysis of the BP pre and post reaction with Glut was performed using micro-ATR FTIR. Average spectra of non-fixed BP and fixed BP are displayed in Figure 4-11.

(A)

(B)

(C)

Figure 4-11: FTIR average spectra (n=4) overlay of non-fixed BP, non-fixed BP washed, Glut fixed BP and Glut fixed BP washed in the 4000 – 600 cm⁻¹ region (A) 4000 – 2500 cm⁻¹ (B) and 2000 – 600 cm⁻¹ region (C), with major bands are assigned in (B) and (C). Amide bands in blue shaded regions with the phospholipid peak in the non-fixed BP identified with the dashed line. Helical ratio identified between Amide III and red shaded region.
The spectra display the characteristic five Amide bands associated with collagen-based materials. The wavenumbers of the key peaks for all spectra are listed in Table 4-1. Amide A band at 3300 cm$^{-1}$ from amide (N-H) bonds and the Amide B bands at 2900 cm$^{-1}$ due to the asymmetric stretching of CH2 bonds [27, 28]. Other amide bands identified are amide I, found at 1630 cm$^{-1}$ and is assigned to the carbonyl group (C=O) while amide II detected at 1550 cm$^{-1}$ is due to N-H bending. The amide III present at 1241 cm$^{-1}$ is due to the stretching of C-H. The relationship between the amide III and the peak at 1453 cm$^{-1}$ provides information on the helical structure found in collagen, with a ratio of 1 indicative of the triple helix assembly [29-31]. Peaks identified in the non-fixed sample at 2900 – 2850 cm$^{-1}$ and 1741 cm$^{-1}$ are assigned to the CH$_2$ stretching and esters respectively of phospholipids of the pericardium.
<table>
<thead>
<tr>
<th>Band Type</th>
<th>Non-fixed BP_Frozen (cm⁻¹)</th>
<th>Non-fixed BP_Washed (cm⁻¹)</th>
<th>Fixed BP_Non-washed (cm⁻¹)</th>
<th>Fixed BP_Washed (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amide A</td>
<td>3299</td>
<td>3288</td>
<td>3288</td>
<td>3285</td>
</tr>
<tr>
<td>Amide B</td>
<td>2918</td>
<td>2959</td>
<td>2961</td>
<td>2923</td>
</tr>
<tr>
<td></td>
<td>2850</td>
<td>2879</td>
<td>2879</td>
<td>2851</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>1741</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amide I</td>
<td>1644</td>
<td>1633</td>
<td>1632</td>
<td>1629</td>
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<tr>
<td>Amide II</td>
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<td>1549</td>
<td>1550</td>
<td>1549</td>
</tr>
<tr>
<td></td>
<td>1463</td>
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</tr>
<tr>
<td>Amide III</td>
<td>1242</td>
<td>1241</td>
<td>1241</td>
<td>1241</td>
</tr>
</tbody>
</table>

**Helical Ratio**

1.18

1173
4.3.4 SEM

The microstructure of the BP was assessed using SEM. Both the fibrous, rough and serous smooth side were imaged. A sample received directly from the abattoir and followed Method 1, went through a stripping and cleaning process of the main appendages on the surface. The surface displays random loose fibres spread across the surface of the collagen wavy structure Fig. 4-12.

![Image of fibrous side of BP sample after main appendages removed. Inset scale bar 200 µm and main pic scale bar 20 µm.]

**Figure 4-12:** Fibrous side of BP sample after main appendages removed. Inset scale bar 200 µm and main pic scale bar 20 µm.
A fibrous sample received from the vendor, following Method 2, displays a surface that is more uniform with very few appendages evident on the surface and the collagen wavy structure clearly visible Fig. 4-13.

Figure 4-13: Fibrous surface of BP received from the vendor. The collagen wavy structure is visible with loose fibers removed from the surface. Inset image scale bar 200 µm and main image scale bar 20 µm.

The fibrous side of the BP is placed on the inflow side of a bioprosthetic heart valve so that the surface will be continually washed and minimise thrombosis [32].
The serous side of an abattoir sample is imaged in Fig. 4-14. Here the surface is smoother in appearance with all appendages removed. This side of native BP is in contact with the heart and is required to be frictionless on its surface to accommodate the interaction during beating [33]. It is worth noting that as this side of the BP is naturally smooth, there is no requirement for mechanical processing of the excess fat appendages that is required with the fibrous side.

Figure 4-14: Serous side of BP with no evidence of appendages or loose fibers on the surface. Inset and main pic scale bar 200 µm and 20 µm respectively.
The cross section reveals the anisotropic and laminate structure of the tissue. Fibers are seen in linear direction across the tissue and other cross sections of collagen bundles in perpendicular orientation to these, **Fig. 4-15**.

**Figure 4-15**: Cross section of BP with main region displaying the laminate structure of the tissue while the zoomed image shows the multi-directionality of the collagen fibers. Inset and main pic scale bars 200 µm and 100 µm respectively, highlighted region scale bar 20 µm.
Further inspection of the tissue under higher magnification reveals the banding structure of the individual collagen fibers Fig. 4-16. Also, the crimping structure of the fibers and their organisation into bundles are evident.

Figure 4-16: Main picture displays a bundle of fibres from the inset image that also shows the wavy structure of the tissue. The highlighted region focuses on the individual collagen fibers and their banding structure. Inset and main pic scale bars are 20 µm and 5 µm respectively. The highlighted region scale bar is 1 µm.
Sections of non-fixed and Glut fixed BP were imaged using TEM, to analyse the tissues ultrastructure. The non-fixed tissue displayed intact cells, and the matrix of collagen and elastin, Fig. 4-17. The variation in directionality of the collagen was clear to see with bundles in a linear pattern and other fibrils cross sectioned indicating a respective perpendicular orientation. The cellular components of the tissue appear intact, fully surrounded by collagen fibrils probably fibroblasts as these synthesise collagen Type I [34]. The collagen banding structure is evident in the linear directed fibrils. The presence of elastin complexes are evident as large dark spots interspersed among the collagen bundles.

The Glut fixed images showed similar structural components to those of the non-fixed tissue. The varied directionality of collagen was also evident with the elastin present throughout the tissue. The structure also displayed the presence of fibroblast cell’s, however there was a void evident between it and the collagen fibers, suggesting damage to the cellular structure. This would point at the crosslinking treatment with Glut as the cause for this effect. Both tissue types have been prepared using the same protocol which involve the use of Glut at a higher concentration of 2.5%. However, Glut used as a microscopy fixative is used to preserve the samples structure without altering the size or shape of its cellular components [35]. As a microscopy fixative treatment, it is used in tandem with Osmium Tetroxide so that the cell will be fixed or “frozen” in its natural state without any morphological changes. The collagen fibrils, while orientated in different directions they are situated beside each other and arranged in groups that would suggest inter-fibrillar bonding. Evidence of this is evident in the cross section of the fibrils where fibre like structures cross between each fibril, Fig. 4-18 (D).
Figure 4-17: TEM images of non-fixed BP tissue. Images display an overview of the ultrastructure (A), displaying the directionality of the collagen fibres, linear (rectangle) and cross section (circle) (B), elastin complex (thick arrow) embedded throughout collagen and presence of fibroblast cell (C), cellular components including nucleus (N) within the tissue (D), fibroblast surrounded by collagen fibers in various directions (E) and collagen banding structure (F). Scale bar: A, B, C and D, 2 µm; E and F, 500 nm.
Figure 4-18: Glut crosslinked BP TEM images of the collagen directionality of the tissue linear (rectangle) and cross section (circle)(A), fibroblast cell with void between the collagen fibres (thin arrow) and elastin complex (thick arrow) (B), collagen banding (C) and elastin complex (D). Scale bar: A, 10 µm; B, 2 µm; C and D, 500 nm.
4.3.6 AFM

The analysis of the non-fixed BP tissue on the fibrous surface was performed using AFM, with the deflection images displayed in Fig. 4-19. The collagen fibrils display a high degree of criss-crossing and directionality on the larger scan areas of 10 x 10 µm and 3 x 3 µm. On the lower scan size of 1 x 1 µm the fibrils appear tightly packed and run parallel to one another. The banding structure of various collagen fibrils show alignment that suggests a form of interfibrillar coupling [36]. Measurements of the D-banding period were taken across various sections of a BP sample, using profiles taken across the height.

**Figure 4-19:** Deflection images of a section of BP displaying the tightly packed collagen fibres and their range in directionality.
images, Fig. 4-20. The size of the repeating pattern ranged from 65 – 69 nm, which is a similar range as seen in other bovine pericardium samples [37]. The profile of the respective lines taken from the images show the periodicity of the collagen structure and how the height difference changes across the groove depth of each fibril, from 2 – 14 nm. Also, there are changes in the peak height for some fibrils that can be possibly attributed to the sloping of the fibrils in located in different sections.
Figure 4-20: Height images with the respective profiles and their average (n=3) measurements taken from profiles of the collagen fibrils banding structure, from various sections of the BP sample.
4.4 Conclusions

The novel technique of QiMTDSC proved a feasible option to analyse the reaction between Glut and BP. Proof of concept using the crosslinking reaction of the epoxy resin, where it showed that the reaction kinetics over time could be measured using the QiMTDSC and the resultant thermal properties measured immediately post-test. Applying the technique to a biological application of Glut and BP interaction, demonstrated that the crosslinking process is continually ongoing. Measurement of the $T_d$ directly after the experiment confirmed that the sample was fully crosslinked. It was shown that the BP and Glut is crosslinked after approximately 10 min, with the volume of solution important. Also, further evidence of the widely documented changing monomeric chemistry of Glut was provided. QiMTDSC has been utilised in the pharmaceutical and food industry, and the research here-in demonstrates its benefits when applied to soft tissue biomaterials and their fixation rate. Determination of the crosslinking state of a BP tissue using Ninhydrin assay, was shown to be a simple technique for the efficient objective visualisation of a tissue and whether it is crosslinked or not. Spectroscopy analysis of the tissue using micro-ATR provides the opportunity to pin-point a region of interest on the tissue and determine its molecular fingerprint. Microscopy of BP using the varied methods of SEM, TEM and AFM, displayed advantages and disadvantages for each method, depending on the information required. For an assessment of the microstructure its surface orientation SEM is an excellent technique. It can provide detailed images of the collagen banding structure and the organisation of the fibrils. The sample preparation involves several key steps regarding the fixation, dehydration and conductive surface coating. However, while the method is time consuming it is not as highly involved as that for TEM, where sample preparation for TEM is on the scale of days compared to hours for SEM. The benefits though are striking where the complete ultrastructure of the tissue can be viewed. Not only was the anisotropic directionality of the collagen fibres evident and the D-banding pattern, but also elastin complexes and cellular components were easily identified. Through TEM, the impact of Glut crosslinking treatment on the cellular structures of BP was identified. The
topography of BP was assessed using AFM. This technique provided, through little sample preparation, qualitative information in the form of high-resolution images that showed the overlapping and variation in directionality of the collagen fibrils and quantitative data on the dimensions of D-bands across an area of the tissue. The combination of these techniques provides an overall assessment of the structural and fixation state of the BP tissue.
4.5 References

11. Qi, S. and D.Q. Craig, *The development of modulated, quasi-isothermal and ultraslow thermal methods as a means of characterizing the α to γ


Chapter 5: Characterisation and Cytotoxicity Assessment of Decellularised Bovine Pericardium and Treatments with a Natural Crosslinker-Genipin

5.1 Introduction
The success of Glut has been well documented with it considered to be the “gold-standard” treatment for the crosslinking of collagenous based biomaterials like porcine and bovine pericardium [1, 2]. Despite its success it has its limitations with its toxicity [3] and has been suggested to contribute to the calcification of collagen-based biomaterials [4]. Alternative treatments have led to the investigation of epoxies [5, 6] and carbodiimides [7] but still Glut is the fixative of choice. Other treatment options that have been tested include decellularisation processes [8] and the natural occurring crosslinker Genipin [9, 10]. The decellularisation of biological materials to produce scaffolds is a common practice where the extracellular matrix (ECM) ultrastructure is preserved with all cellular material removed [11, 12]. Resulting in the possible reduction of the tissues cytotoxic properties. Genipin is a fixative that has seen success with applications in the area of chitosan [13], bovine serum albumin [14], gelatin [15, 16] and collagen [17]. It is a naturally occurring molecule found in Gardenia jasminoides fruit extract [18] and has been reported to have a cytotoxicity level less than 5000-10,000 times to that of Glut [19]. Both treatments warrant further investigation, with the interaction of decellularised BP in combination with the crosslinking treatments of Glut or Genp in comparison to the standard Glut to determine if they can challenge as a possible alternative. Herein, native BP (BP_Non-Treat) was compared to Glut treated (BP_Glut), Genp (BP_Genp), decellularised (BP_Decell) and combinations of decellularised BP with Glut and Genp (BP_Decell_Glut and BP_Decell_Genp). The BP_Genp was first compared to BP_Glut using DSC to assess the denaturation temperature ($T_d$) and the appropriate concentration of Genp for further analysis. The biomechanics of all groups were compared using uniaxial testing. Structural comparison of BP_Glut and BP_Genp were compared using
histological analysis to assess the integrity of the collagen architecture before and after mechanical testing. Also, the cytotoxicity of all samples were compared with respect to their metabolic activity using alamarBlue® before further additional toxicity testing with Live/Dead assay was carried out on the BP_Glut and BP_Decell groups.
5.2 Materials and Methods

5.2.1 Bovine pericardium

BP tissue was acquired from a proprietary vendor, defatted and stored in EDTA/PBS solution at 2 – 8 °C. Samples were washed prior to use twice with saline. Samples were treated with either Glut or Genp. While other samples were decellularised and then treated with Glut or Genp.

5.2.2 Glutaraldehyde fixation

Sections of BP were cut accordingly to their respective test and placed in various concentrations of Glut, ranging from 0.1%, 0.6%, 1% and 6%. The Glut was prepared from an electron microscopy grade solution of 25% Glut (Merck, Darmstadt, Germany) in 0.1 M phosphate buffered solution (PBS) (Sigma Aldrich) for 3 days at room temperature.

5.2.3 Genipin fixation

Solutions of Genp (>98% Sigma Aldrich) were prepared into a range of concentrations of 0.1 mM, 0.3 mM, 0.6 mM, 3.0 mM and 10.0 mM. A stock solution of Genp was first prepared using 0.1 ml of 100% Ethanol (Sigma Aldrich) in a 25 mg vial of Genp. The solution was then diluted down using PBS (Sigma Aldrich) to the respective concentrations.

5.2.4 Decellularisation

The decellularization of the BP followed a previous method developed by Oswal et al.[20] with some modifications. The sample was washed in PBS (Sigma Aldrich) supplemented with 1% penicillin-streptomycin antibiotics and left in sodium dodecyl sulfate (SDS, 0.1% w/v) for 24 h, both in the presence of protease inhibitors. The tissue was finally treated with a nuclease solution (RNase/DNase) and then washed in sterile PBS supplemented with 1% penicillin-streptomycin antibiotics for 24 h with agitation. The decellularized BP was then treated with 0.6% Glut and 0.3 mM of Genp.
5.2.5 Denaturation temperature
The $T_d$ for both Glut and Genp samples (n=3) were obtained using DSC (Model Q1000, TA Instruments, Delaware, USA). Samples of 3 mm diameter were cut using a biopsy punch, patted dry and hermetically sealed into aluminium pans. The test method followed a heating rate of 5 °C/min as recommended by Loke et al. [21] in a temperature range of 30 °C to 110 °C. An empty pan was used as the reference. Heating was carried out in a nitrogen atmosphere of 50 ml/min. The peak temperature of the endothermic phenomena was used to determine the $T_d$ [20-22] and the enthalpy, taken as the area of the endothermic peak, was also measured. Calibration of the instrument was carried out using an Indium standard (Tm 156.6 °C).

5.2.6 Uniaxial testing
Rectangular tissue samples of approximately 5 mm x 50 mm were cut using a scalpel in the axial and circumferential directions. The thickness value used for the cross sectional area was an average value taken from three locations across each sample using a digimatic indicator (Mituyou Absolute ID-S, Toronto, ON, Canada). Testing was performed on a Zwick Roell tensile tester with a 100 N load cell. Each sample (n=5) was held in place with an air pressurised roughened surface clamp, gripped at 3 Bar. Each sample was pre-conditioned through cyclic loading at a rate of 20 mm/min in a loading range of 0N to 1N. After preconditioning, the sample was extended at a rate of 20 mm/min until failure. Failure was set at 80% drop in the peak load. The force extension curves produced were converted into stress strain curves using the cross-sectional area of each sample. Four parameters were calculated, 1) the E-modulus, taken as the slope of the linear portion of the graph, 2) the ultimate tensile stress (UTS) calculated as the peak stress of the curve before failure, 3) the percentage strain of failure is the strain at the point of UTS and 4) the percentage strain at 1MPa.
5.2.7 Histology
BP samples were embedded in paraffin (Leica ASP300) and sectioned using a microtome (Leica RM2235). Samples were stained with H&E (Sigma Aldrich, St. Louis, USA) and Masson’s Trichrome (Sigma Aldrich) imaged using a Leica DM500 microscope at 40 x with LAS Core software, following the protocol in section 1.3.4. Sections of BP both before and after mechanically testing were stained across regions labelled top, middle and bottom of the sample, with n=4 for each region.

5.2.8 alamarBlue® assay
The metabolic activity of cells was assessed using an alamarBlue® assay according to the manufacturer’s instructions. Two different cell types were used, human mesenchymal stem cells (hMSC) and murine bone marrow stromal cell line (MS-5). The fluorescence was measured using a Thermo Scientific Varioscan FlashMultimode (Thermo Fisher Scientific Inc., USA) plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The assay was applied via the elution and contact methods detailed in section 1.5.1. To summarise the elution method was performed after cells were incubated for 48 h while in the presence of tissue extracts of 100% extract, 50% extract and 25% extract. Eluates were prepared using DMEM to dilute the 100% extraction to the respective concentration. The contact method involved seeding the cells on top of tissue samples and incubating for 48 h and 7 d. Treated sample groups were compared against the BP Non-Treat sample group with cells in DMEM and DMSO acted as positive and negative controls respectively.

5.2.9 Live/Dead assay
Viability of cells were measured using a Live/Dead cell viability assay. Titration was performed for both Calcein-AM and ethidium homodimer-1 to define optimal dye concentration according to the manufacture protocol. A solution of 1 μM Calcein-AM and 2 μM ethidium homodimer-1 in HBSS was mixed thoroughly and then added to the cells for 20 min at 37 °C. Samples were assessed using the contact and elution methods. The fluorescence of live and
dead cells were measured at an excitation wavelength of 530 nm and emission wavelength of 645 nm respectively using a Thermo Scientific Varioscan Flash Multimode plate reader (Thermo Fisher Scientific Inc., USA). The fluorescent staining of the cells were observed using an Olympus IX81 fluorescence microscope (Olympus) and images captured using a DP72 CCD camera (Olympus) linked to CellSens Dimension software (Olympus). Images (n=4) of each sample group were assessed for the quantity of live and dead cells, which appear as green and red respectively and were manually counted using the multiphoton feature on ImageJ [23].

5.2.10 Statistical analysis
Statistical analysis was performed using MINITAB® 17.1.0 (Minitab Inc., US). Numerical data is presented as mean ± standard deviation unless otherwise stated. Comparison of each sample group was completed using one-way analysis of variance (ANOVA) with a Tukey post-hoc test. Statistical significance was set at p < 0.05.
5.3 Results and Discussion

5.3.1 BP treatment

A profile of Genp was first analysed to determine the optimal concentration for comparison with Glut’s typical 0.6%. Concentration of Genp in the literature varies from 10 mM [13] to 1 mM [24]. This research looked initially at concentrations of 0.1 mM, 0.3 mM, 0.6 mM, 3 mM and 10 mM. The reaction with a 3 mm section of BP after 3 d at room temperature display a striking colour change as the concentration of the Genp increase’s Fig. 5-1. The change in colour can be attributed to the reaction between the Genp and the primary amines in the presence of oxygen. Only a slight colour change was evident at approximately 0.3 mM and the characteristic Rheuhmann’s purple not apparent until 3 mM. This suggests that the Genp solution is concentration dependent and at low values cannot react with all amine groups in the BP sample. It is not until 3 mM that the primary amines react to produce the full colour change, with this effect plateauing to 10 mM. Similar evidence of this colour change was evident in Hwang 2011 et al. that showed comparable profiles between 1 mM and 10 mM Genp on collagen hydrogels tested using one-photon fluorescence [24]. The lower 1 mM colour concentration compared to 3 mM identified here is possibly due to the thicker, darker and higher collagen content than the semi-translucent collagen hydrogels used for the fluorescence analysis.
Glut treated BP was performed over a range of concentrations of 0.06%, 0.1%, 0.6%, 1.0% and 6.0%. Visually the change in tissue colour was not as dramatic as that for the Genp with the tissue turning a dark brown shade but the profile was not as linear as that for the Genp Fig. 5-2. The control samples of fresh BP and BP in PBS, Control 1 and 2 respectively, show the effect of the PBS has on the tissue with an increase in thickness and white colour present.

Figure 5-1: BP samples treated with Genp of various concentrations. The colour change can be seen to increase in darkness until 3 mM and 10 mM display the characteristic Rheuhmann’s purple.
Figure 5-2: BP samples treated in increasing concentrations of Glut. Control samples of BP and BP in PBS are also included. Right side of images display the 1 mm increments on a ruler.
5.3.2 Denaturation temperature \((T_d)\)

The representative DSC thermograms show the increase in peak endotherm across the concentrations of Glut, Fig. 5-3. The non-treated BP displays a \(T_d\) of 64 °C, similar to reported values in the literature [25]. The increase in temperature to 70 °C for the PBS treated sample suggest that water influences the collagen fibrils with some form of crosslinking taking place. Literature suggests that the peptide backbone bind on average C=O groups of Glycine (Gly) to one water, while the OH of hydroxyproline (Hyp) can bond to two water molecules, bridging either two carbonyl groups or one carbonyl with a hydroxyl group of Hyp [26]. The Glut treated BP samples produced a further increase in \(T_d\) to 80 – 86 °C. The lowest concentration of Glut increased the \(T_d\) to 86.03 °C ± 0.16 °C, while the highest concentration of 6.0%, reported a significantly less value of 80.35 °C ± 0.16 °C. Further analysis of \(T_d\) values along with the enthalpy for all samples (n=3) are displayed in Fig. 5-4.

**Figure 5-3:** Representative thermograms for control and Glut groups with increasing concentration. All fixed samples display similar \(T_d\) values taken from the endotherm peak value.
Figure S-4: Peak $T_d$ values (A) and enthalpy values (B) for non-treated BP, PBS and Glut treated BP (B). Both sets of values display a significant difference after Glut treatment, regardless of concentration. (*) indicates a statistical difference ($p < 0.05$).
Genipin treatment of BP produced a more gradual increase in the $T_d$ relative to the concentration used, Fig. 5-5. The lower concentrations of 0.1, 0.3 and 0.6 mM displayed values of $< 75 ^\circ \text{C}$ signifying values comparable to PBS treated BP. However, there is a significant increase at the higher concentration of 3 mM to $78.82 \pm 1.69 ^\circ \text{C}$. The multiple increase to the 10 mM concentration yielded only a slightly higher value of $80.04 \pm 0.29 ^\circ \text{C}$ with no significant difference between the lower value for 3 mM. The value from 3 mM is consistent to values reported by Hsing-Wen Sung et al. of $76.6 \pm 0.9 ^\circ \text{C}$ and $80.6 \pm 1.1 ^\circ \text{C}$ [9, 27].

Figure 5-5: Graph of $T_d$ as a function of concentration of Genipin. Low concentration groups with a slow rise in temperature, followed by the sharp increase for 3.0 mM. There was no significant difference between 3.0 mM and 10.0 mM $T_d$. Points signify mean and bars standard deviation. $(p<0.05)$. 

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Comparison between BP_Genp and BP_Glut showed that there was a significant difference between both the T_d and enthalpy values, Fig. 5-6. The T_d for Genp 3.0 mM was measured as 78.82 ± 1.69 °C compared to 85.01 °C ± 0.7 °C for Glut. While the enthalpy of Glut fixed BP was almost double at 12.33 ± 3.22 J/g to 6.893 ± 1.28 J/g reported for Genp 3.0 mM.

Genp 3 mM treated BP sample showed that the T_d increased significantly when compared to the BP_Non-Treat and PBS BP with measurements of 65.46 °C ± 1.15 °C and 70.43 °C ± 0.10 °C respectively. The increase in T_d was not reciprocated in the enthalpy values with no significant difference evident

Figure 5-6: Comparison of T_d (A) and enthalpy (B) for Genp 3 mM, Glut 0.6% and control samples of non-treated and PBS treated BP. Significant difference across all samples with respect to T_d while enthalpy only the BP_0.6% Glut sample displayed a significantly higher value than those of the other samples, including the Genp 3 mM. (*) indicates a statistical difference (p < 0.05).
between BP_Genp (6.893 ± 3.22 J/g), BP_Non-Treat (5.63 ± 2.28 J/g) and PBS treated BP (5.487 ± 0.419 J/g). The increase in the T_d would indicate that the collagen fibrils have been crosslinked and the BP is stabilised. The reaction mechanism for Genp and biological tissues is not fully understood with Fujikawa et al.[28] suggesting that a nitrogen-iridoid undergoes dehydration to form an aromatic monomer.

Another method involves the polymerisation of Genp molecules prior to amino acid crosslinking through the introduction of an oligomeric-Genp spacer [29-31]. The fixation mechanism within the collagen fibres in BP may be through a cyclic structure of intra- and intermolecular compared to a network crosslinking structure for Glut fixation [27]. The formation of intramolecular bonds within the tropocollagen molecule with Genp give increased stability against denaturation however the network formation of Glut is evident with the increase in the BP’s enthalpy measurements.

This assessment demonstrates that there was not a significant increase in T_d with the 10 mM concentration, and along with the high cost of Genp, the 3 mM was chosen as the optimal concentration to pursue the comparison of mechanical properties with Glut.
5.3.3 *Uniaxial testing*

Samples of BP_Glut, BP_Genp, BP_Decell and decellularised samples also treated with these crosslinkers, BP_Decell_Glut and BP_Decell_Genp were investigated for their mechanical properties. Traditional parameters of UTS, E-modulus, percentage strain at 1 MPa and strain at failure were all compared for each sample type. The UTS, Fig. 5-7, of the samples showed that the BP_Non-Treat in axial direction (10.75 ±2.7 MPa) was significantly higher than all samples except for the BP_Decell (7.89 ± 1.52 MPa) and BP_Glut (10.21 ± 1.23 MPa) axial orientated samples.

The BP_Glut had a significantly higher UTS in the axial direction than a decellularised sample treated in both Glut and Genp that measured values of 5.0 ± 0.83 MPa and 4.50 ± 0.79 MPa, respectively. A similar measure of UTS for native tissue, Glut and Decell treatments has been recorded by Hulsmann et al. [32] where there no significant changes compared to BP_Non-Treat. Also of significance was the reduction in the BP_Decell_Glut group that was
significantly lower than that of BP_Glut in the axial direction. Fixation of BP with Genp showed a reduction in UTS compared to the BP_Non-Treat, and while lower than BP_Glut it was not of significance. Interestingly both the axial decellularised Glut and Genp fixed BP samples displayed similar values but the reduction in the UTS was significant when compared to the standard BP treatment with Glut. This result was also reported also by Hulsmann et al.[32]. This would suggest that the decellularisation process removes elements of BP that influence the mechanical properties of the tissue. The role of GAGs has been researched on native aortic leaflets with Ekert et al. determining that they do not have a direct impact on the tensile mechanical behaviour of the tissue [33]. The decellularisation process reduced the tensile strength on the tissue, but it was not as high and significant as that previously reported by Mendoza_Novelo et al. which showed an almost 50% drop in tensile strength of SDS decellularised tissue when compared to other Decell methods and native tissue [34]. Both the BP_Glut and BP_Genp groups showed no statistical difference, with a similar trend reported by Lynn et al. for porcine pericardia treated with Glut and Genp [35].
The E-modulus showed that the BP_Non-Treat in the axial direction was significantly higher than all other groups, **Fig. 5-8.** The BP_Decell axial sample was significantly higher after additional fixation with Glut and Genp in the same direction. All the single treatments of BP_Decell, BP_Glut and BP_Genp produced similar values of 24.91 ± 6.07 MPa, 17.53 ± 1.65 MPa and 19.68 ± 3.76 MPa respectively.

This decrease in stiffness from BP_Non-Treat and the treated groups was evident in a study by Hulsmann et al. who also reported a significant decrease in the stiffness after the combination treatments [32]. The variation in test protocols has been detailed previously in Chapter 3 and may be the reason for such contradiction in results that suggest the mechanical integrity of decellularised matrices are retained. For example Jang et al., described findings where BP double treatment of the Decell process and Glut and Genp does not significantly change E-modulus [36]. From this study the BP_Decell group produced equivalent E-moduli as that of Glut and Genp treatment, suggesting a similar mechanical performance to the current treatment of BP using 0.6% Glut. The biggest changes in mechanical properties were evident in the percentage strain at 1 MPa and strain at failure, **Fig. 5-9.**
Figure 5-9: Comparison of BP and treated samples at % Strain at 1MPa (A) and % Strain at Failure (B). The additionally treated Decell BP treated with Glut and Genp have demonstrated a significant difference higher values than their respective singular treatments in both strain at 1 MPa and strain at failure. Error bars represent the standard error of mean and (*) denotes a statistical difference (p < 0.05).
There was a definite increase in percentage strain at 1 MPa for the BP that were processed through the Decell protocol and followed with the treatments of Glut and Genp, when compared to their respective singular treatments and the BP_Non-Treat group. The BP_Decell_Glut group in axial and circumferential directions 32.46 ± 2.09%, 30.94 ± 3.1% respectively, produced an increase of over double than BP in its native state 6.05 ± 0.75%, 8.99 ± 0.89% in axial and circumferential directions, respectively. Comparison of BP_Decell_Glut with BP_Decell_Genp also showed statistically higher values across both directions, Fig. 5-9 (A). The BP_Glut was statistically greater than both non-treated and BP_Genp. A similar trend was evident in the percentage strain at failure, Fig. 5.9 (B), with both the Decell and additional Glut (64.22 ± 4.6%, 65.52 ± 6.85%) and Genp (45.76 ± 8.43%, 38.95 ± 3.8%) treatment groups significantly higher than those of the non-treated and individual groups. Also, BP_Glut (37.19 ± 2.53%, 42.28 ± 2.28%) was significantly greater than the BP_Non-Treat (19.54 ± 1.18%, 22.27 ± 2.03%) and the BP_Genp (25.32 ± 2.36%, 20.79 ± 0.52%) with no difference evident between the BP_Genp and the BP_Non-Treat. The extra Glut treatment after the Decell protocol also produced the increase in elongation at failure in the Hulsmann et al. study [32].
5.3.4 Histology

Histological examination was performed on non-treated BP and BP samples treated with 0.3 mM, 3 mM Genp and 0.6% Glut. H&E staining was used to provide an overview of the extracellular matrix and the presence of cell nuclei, with images presented in Fig. 5-10. Sections of samples in both axial and circumferential orientations were imaged after treatment and post mechanical testing. The extracellular matrix appeared intact and denser in the Genp and Glut samples compared to the non-treated BP. After mechanical testing, the treated samples were organised with reduction in fragmentation of the collagen fibres of the 3 mM and more so with Glut treated samples compared to the other groups. Cell nuclei were evident across all samples. H&E staining images in Fig. 5-11, display the removal of cell nuclei from BP after the decellularisation process. The BP_Decell matrix appears to have of more gaps between collagen bundles than the non-treated sample. This can be attributed to the use of SDS on the collagen and protein to protein interactions within the fibres. While SDS is proven to remove cellular activity from tissue it can denature proteins and reduce collagen integrity [11]. Masson’s Trichrome stained images are presented in Fig 5-12, with the collagenous fibres stained blue. All samples, non-mechanically tested displayed a similar intact structure of a wavy collagen arrangement. After mechanical testing there was a loss in integrity in this structure, more so in the axial direction of the non-treated and Genp treated BP samples.
Figure 5-10: H&E stained 0.3 mM and 3 mM Genp and 0.6% Glut BP with non-treated BP used as the control. Images taken on samples directly after fixation and other samples after mechanical testing. Both directions of axial and circumferential included. Scale bar 50 µm.

Figure 5-11: H&E staining of Non-treated (A) and BP_Decell (B). Nuclei evident (black arrows) in non-treated BP with no cell remnants present after decellularisation. Scale bar 50 µm.
Figure 5-12: Masson trichrome stained 0.3 mM and 3 mM Genp, 0.6% Glut BP and non-treated BP samples. Images taken on samples directly after fixation and other samples after mechanical testing. Both directions of axial and circumferential included. Scale bar 50 µm.
5.3.5 *alamarBlue®* assay

Initial assessment of the metabolic activity of cells was performed with *alamarBlue®* and MS-5 and hMSC cell lines, after 24 h interaction with non-treated and treated BP, using both the contact and elution methods. Following the contact method Fig. 5-13 (A), treated samples were compared to non-treated BP tissue and all showed significantly reduced metabolic activity, except for BP_Genp using the MS-5 cells. The decellularisation process produced a slight reduction in the activity and was comparable to BP_Genp and hMSC but statistically lower in the MS-5 group. Along with comparable results to the non-treated tissue, the Genp treatment displayed no statistical difference to the BP_Decell and the combination treatment of BP_Decell_Genp using hMSC. The elution method, Fig. 5-13 (B) also showed that both the BP_Decell and BP_Genp had statistically higher metabolically activity than the BP_Glut and BP_Decell_Glut groups. The decellularisation protocol uses an ionic detergent sodium dodecyl sulfate (SDS), where Liu et al. has suggested that it produces a toxic effect to a cell population [37]. However, this study produced a metabolically active response from the cellular interaction with the BP_Decell group. This was evident across both the single Decell treatment and the double treatment with Genp. This initial study had a limitation in its short period of 24 h, it was followed by another more detailed protocol using one cell type of hMSC with the contact method over 48 h and 7 d and an elution method for 48 h using three concentrations of eluate (diluted with DMEM) 100%, 50% and 25%. Treated and non-treated BP were compared against live and dead controls of hMSC in DMEM and DMSO respectively. Effect of Glut treatment on the BP was the focus and how it compared to the Decell protocol and in combination with decellularisation. Metabolic activity was expressed as a percentage with respect to live cells Fig. 5-14. For both time periods the Decell protocol (60.14% ± 2.31% for 48 h 69.86 ± 3.66% for 7 d) allowed for a significantly greater metabolic activity in the cells when compared to both non-treated BP (44.56% ± 2.63% for 48 h, 50.64% ± 6.96%) and Glut treated BP (39.23% ± 35.25% ± 1.94%) respectively. After 7 d the cells in contact with the BP_Decell group had greater activity than the BP_Decell_Glut group after the
same time period. While there was an increase in the activity of cells after 7 d for the BP_Non-Treat, BP_Decell and BP_Glut, it was not significant.

The elution method, Fig. 5-15, using 100% eluate produced the highest metabolically active cells, with respect to the live control, in BP_Non-Treat (88.82% ± 9.52%) compared to all other groups. Both the BP_Glut and BP_Decell_Glut groups produced the lowest at 27.49% ± 1.18 and 11.81% ± 0.751% respectively. Reduction of the concentration of the eluate to 50% yielded a significant increase in the activity of all cell types, with the BP_Non-Treat (122.57% ± 7.79%), BP_Decell (107.03% ± 5.49%) groups greater than the BP_Glut (100.3% ± 3.32%) and BP_Decell_Glut (98.94% ± 1.68%) groups. Reducing the concentration further to 25% produced a similar pattern across the groups but only a significant increase was produced in the reduction of 50% to 25% in the BP_Decell group from 107.03% ± 5.49% to 129.48% ± 3.81%.

The BP_Decell, performed significantly greater than the BP_Glut treatment across all toxicity tests. Treatment of BP with Genp also produced a higher degree of metabolically active cells when compared to the Glut treatments. Combination treatment of BP after Decell using Glut or Genp behaved similarly to their respective singular treatments. To gain further understanding of the toxicity of each treatment a viability assay was used to quantify the number of live and dead cells across each group.
Figure 5-13: alamarBlue® assessment using the contact method (A) with two separate cell types. For both cell types all groups, except for the BP_Genp using the MS-5 cells, showed statistically lower metabolic activity. The BP_Decell group was statistically greater than both Glut and Decell_Glut groups. The BP_Genp displayed no statistical difference with the BP_Decell_Genp group. All groups are statistically compared for each cell type only. Elution method (B) displays the Decell and Genp groups have a higher activity than both Glut and Decell_Glut groups. Symbols (*) and (#) indicate a statistical difference (p <0.05) for the hMSCs and the MS-5 cell types respectively. Error bars represent the standard error of mean.
Figure S-14: Contact method after 48 h and 7 d for non-treated BP, Decell, Glut and Decell_Glut treatments of BP. hMSC cell type were chosen and their metabolic activity was measured using alamarBlue®. All samples were expressed as a percentage of live control (cells in DMEM), with a negative control of cells in DMSO also used. Symbols (*) and (#) indicate a statistical difference (p < 0.05) after 48 h and 7 d respectively. Bars represent the mean while the error bars represent the standard error of mean (± SEM).
Figure 5-15: Elution method using alamarBlue® to measure the metabolic activity of hMSC cells when in concentrations of 100%, 50% and 25% of extracted solutions from non-treated BP, Decell BP, Glut treated BP and Decell BP in combination with Glut treatment. All samples were expressed as a percentage in relation to live and dead cells in DMEM and DMSO respectively. Symbols (*), (#) and (+) indicate a statistical difference (p <0.05) with 100%, 50% and 25% extracted solutions respectively. Bars represent the mean while the error bars represent the standard error of mean (± SEM).
5.3.6 Live/Dead assay

Viability of cells were measured using a Live/Dead assay following both the elution and contact with BP treated groups. The elution method was assessed using 100% eluate from the treated groups and reduced concentrations 50% and 25% eluate combined with DMEM after 48 h. Comparison of each group in relation to a live and dead cell population are presented in Fig. 5-16 (A) and (B) respectively. All groups performed similarly with 100% eluate, with no significant difference detected. For lower concentrations however there was a significant increase in the percentage of viable cells for all groups, while both BP_Non-Treat (30.61% ± 4.67%) and BP_Decell (30.79% ± 5.94%) groups had significantly fewer viable cells compared to both BP_Glut (69.17% ± 4.81%) and BP_Decell_Glut (89.07% ± 4.18%). The BP_Decell_Glut treatment produced the highest number of viable cells at both 50% (89.07% ± 4.18%) and 25% (107.33% ± 2.59%) eluate compared to all other treatment groups and was not significantly different to the live control. Analysis of the number of dead cells in each group showed that the BP_Non-Treat (7.75% ± 1.1%) and BP_Decell (10.25% ± 2.9%) groups were comparable and had statistically fewer dead cells than those of the BP_Glut (33.19% ± 1.74%) and BP_Decell_Glut (28.04% ± 3.53%) treatment groups at 100% eluate. The effect of decreasing the concentration of the eluate was evident only in the BP_Glut and BP_Decell_Glut groups where the reduction to 25% was mirrored in the decrease of viable cells to 16.11% ± 2.31% and 14.13% ± 2.52% respectively. The cytotoxic effect of Glut treatment on cellular viability is not as clear as expected, as the Glut groups performed the same as the other groups after treatment with 100% eluate for the number of live cells, while producing an increase in the number of dead cells. However, on reduction of the concentration of the eluate the number of viable cells increased significantly for the Glut treated groups compared to the BP_Non-Treat and BP_Decell groups. This indicates that the dilution of Glut can decrease its cytotoxic effect on cells.
Figure 5-16: Fluorescent analysis of Live/Dead assay across all treatment groups. Measurement of viable live cells at fluorescence of 530 nm (A) and dead cells at 645 nm (B) when cultured in eluate of 100%, 50% and 25%. Live and dead cells in DMEM and DMSO were used as controls. Symbols (*), (#) and (+) indicate a statistical difference ($p < 0.05$) with 100%, 50% and 25% extracted solutions respectively. Bars represent the mean while the error bars represent the standard error of mean (± SEM).
Another method using the Live/Dead assay involves the visual analysis of cells while in contact with the groups or the groups treatment solution. The technique can provide visual information about the cells like confluency and their interaction with a sample, with the ability to also measure the number of live and dead cells within a specific area. Images presented in Fig. 5-17 are a representative sample of hMSC cells response to the interaction with an eluate of various concentrations. The BP_Non-Treat 100% group displays a higher number of live cells compared to the treatment types, while both the BP_Glut and BP_Decell_Glut group display a greater level of confluency at 25% compared to the other groups. Manual counting of cells was carried out on images (n=5) for each sample group and concentration level.
Figure 5-17: Live/Dead assay of cells in culture with 100%, 50% and 25% concentrations of eluent from non-treated and treated BP groups. Green and red indicate live or dead cells, respectively, with the controls behaving as expected. Scale bar is 100 µm.
Both live and dead cells were counted with the results displayed in Fig. 5-18 (A) and (B) respectively. There was no significant difference in the number of live cells present after treatment with 100% solution. The most significant differences appear in the lower concentrations of 50% and 25% where the BP_Decell_Glut group had the highest number of live cells, 73.0 ± 1.39 and 78.25 ± 4.35, respectively, when compared to all other groups. BP_Glut also had significantly higher number of live cells 53.4 ± 5.64 and 46 ± 12.23 than both the BP_Non-Treat, 16.0 ± 5.57, 25.4 ± 10.29 and BP_Decell 11.8 ± 2.39 and 16.6 ± 4.77 for 50% and 25% respectively. Dead cells displayed similar numbers across all groups with BP_Decell_Glut 10.25 ± 1.26 and BP_Glut 6.8 ± 3.27 displaying a higher number of dead cells than the BP_Decell 1.6 ± 0.55 group alone for 100% eluate. What was interesting was that the BP_Decell_Glut again produced the significantly higher number of dead cells 21.0 ± 8.04 and 24.75 ± 9.46 at the lower concentrations of 50% and 25% respectively than all other groups. This in combination with the highest number of live cells and the images demonstrate that this group’s environment promoted the most confluent network of cells.
Figure 5-18: Manual counting of live (A) and dead (B) cells from images (n=5) of hMSC cells treated with 100%, 50% and 25% concentration eluate from BP_Non-Treat, BP_Decell, BP_Glut and BP_Decell_Glut groups. Bars represent the mean while the error bars represent the standard deviation (± SD). Symbols (*), (#) and (+) indicate a statistical difference (p <0.05) with 100%, 50% and 25% extracted solutions respectively.
5.4 Conclusion

The analysis of $T_d$ using DSC was used as a gauge for the stability of BP after crosslinking demonstrated that Glut fixation was not as concentration dependent as that of Genp. The ideal concentration for Genp use was determined to be 3 mM as it provided the required high $T_d$ with the minimum amount of solution required. Mechanically the Glut treated BP provided the highest UTS and modulus than the Decell treated samples and Genp. However, the strain values were considerably increased for the Decell combination treatment with Glut and Genp. The Decell protocol in combination with Glut or Genp treatment improved both its mechanical and cytotoxic properties. Treatment of BP with Genp produced mechanical properties similar to Glut fixation and would be recommended as an alternative option. Further analysis of it in combination with the Decell process warrants additional assessment.
5.5 References


Chapter 6: Conclusions and Future Research

6.1 Conclusion

Bovine pericardium is an extensively used biomaterial, widely chosen for use as the valve leaflet material in bioprosthetic heart valves. Its collagen-based structure has similarities to those of the native heart valve lending itself to mechanical properties suitable for this application. As a natural biomaterial there is a requirement to process the tissue so that it is suitable for heterogeneous use, by removing excess remnants and reducing the material to its base structure. The treatment of bovine pericardium typically involves the addition of a crosslinker glutaraldehyde, to preserve the mechanical integrity and sterilise the tissue. The characterisation of bovine pericardium is necessary on several levels, from a processing quality perspective, through the determination of the crosslinking degree, to the meeting of mechanical standards for its functionality \textit{in vivo} and the overall structural analysis and its cytotoxicity. The mechanics, physical/chemical characterisation and the biological properties of the tissue, were the areas focused on in this research.

The mechanical assessment of bovine pericardium is varied with no consistent technique, method or protocols utilised across both industry and academic research. The two most common techniques for the mechanical analysis include uniaxial and biaxial tensile testing. This research focused on uniaxial testing and two key parameters of preconditioning cycles and extension rate. The goal was to issue a recommendation for the settings of these parameters that could be applied for the standardisation of tensile testing of bovine pericardium. The analysis demonstrated that 5 load-unload cycles would be adequate for bovine pericardium and would be a recommended standard number of preconditioning cycles. Extension rate displayed minor variations but not of a significant difference. However, the 10 mm/min extension rate was recommended as a standardised rate for multiple reasons. Mechanical values generated using this rate compared favourably to literature data and from an industrial viewpoint this
rate would satisfy the time requirements for large scale sampling. The visual analysis of the bovine pericardium samples post mechanical testing displayed the viscoelastic behaviour of the collagen fibrils and how the structure returns to its original form after removal of the tensile stress.

The physical and chemical characterisation of bovine pericardium, demonstrated that the quasi-isothermal modulated differential scanning calorimetry method is a technique that can be applied, to the analysis of the rate of crosslinking between a natural biomaterial and a fixative, such as glutaraldehyde. The method showed how the volume of glutaraldehyde has an impact on the crosslinking reaction. The rate of reaction for a bovine pericardium sample to reach the required denaturation temperature for a crosslinked sample was quick and in the region of minutes rather than the days and hours that the tissue is typically subjected to as per industry and literature. Also, the change in heat capacity suggests that the reaction of the glutaraldehyde is continuous and adds further evidence of its continuously polymerising structure. The use of a Ninhydrin assay to determine if a bovine pericardium section was fixed or not proved to be an effective technique that could be utilised as an efficient quality verification. The microscopy techniques of scanning, transmission electron microscopy and atomic force microscopy all provided effective ways to analyse the microstructure of bovine pericardium and its extracellular matrix components of collagen and elastin.

The comparison of alternative treatments of genipin and decellularisation with glutaraldehyde demonstrated that these are viable methods for the treatment of bovine pericardium. Comparisons between the mechanical properties of non-treated, decellularised and after combination treatments of decellularised with glutaraldehyde and genipin, showed that genipin treated bovine pericardium behaved similarly to those of glutaraldehyde fixed bovine pericardium. The interaction of treated bovine pericardium with cells demonstrated that from a metabolic activity standpoint genipin behaved similarly if not better than glutaraldehyde on several of the tests. Viability analysis, using Live/Dead assay
showed that there was no significant difference between the non-treated and the treated groups of glutaraldehyde, decellularised and the combination of both. Both the glutaraldehyde with its low concentration of 0.6% and the decellularised protocol showed that they were equivalent to the non-treated bovine pericardium. This is of importance as in parallel research the decellularised bovine pericardium was shown to be a viable scaffold for the seeding of a hMSC cell sheet.
6.2 Future Research

The characterisation of natural biomaterials is of critical importance for effective application. In the extensive area of bioprosthetic heart valves, the characterisation techniques are established for quality control and R&D aspects however there is room for improvement whether it be consistency of mechanical testing or to the efficiency of crosslinking. This research looked at three areas for characterisation of BP, with recommendations for standardisation of a mechanical test, the application of a new calorimetry technique to the fixation assessment of glutaraldehyde and bovine pericardium and also the comparison of alternative methods for the fixation of bovine pericardium. Based on this research, future project suggestions are detailed in this chapter.

6.2.1 Further validation of uniaxial methods and standardisation of biaxial testing

Progress was made in this research for the standardisation of a uniaxial test method for the testing of bovine pericardium. Further developments could be achieved through the focus on additional parameters such as sample dimensions. A similar approach could be employed for biaxial testing where variances in test methods could be assessed with a goal of determining equal or different strain rates that could be employed. Incorporation of finite element analysis may be of additional benefit and could evaluate multiple factors related to this test method.

6.2.2 Development of QiMTDSC

The QiMTDSC technique is an established method for the pharmaceutical and food industry and has been demonstrated in this research that there are applications for it in the biological biomaterial field that warrant further exploration for development. Recommended areas of research include investigating other crosslinkers and their interactions with bovine pericardium. Also, hydrogels and their crosslinking reaction times and effects of concentrations would be of benefit.
6.2.3 Cell sheet application

Both genipin and decellularised protocols proved they are viable methods for the interaction with cells. Both methods could be utilised to treat bovine pericardium for the application of a cell sheet. Previous work demonstrated that decellularised bovine pericardium could be used, however it was over a short duration, therefore further testing of the process over an extended period would be of interest. Also, additional elements of the study could be applied such as the analysis of the effect of pore size of the decellularised bovine pericardium scaffold on the infiltration and ingrowth of the cell sheet. Another area of interest would be the examination of the differentiation of hMSC cells post seeding onto the decellularised bovine pericardium on both a gene and protein -level. This combined with testing of other cell types such as fibroblast or endothelial cells would further validate the technique.

6.2.4 Sterilisation of Genipin fixed bovine pericardium

One of the key reasons for using Glut is the sterilisation qualities of the solution. The benefits of fixing bovine pericardium with genipin have been documented in this research however, it lacks the assessment of sterilant capabilities compared to glutaraldehyde. Other sterilant methods would have to be investigated for genipin fixed bovine pericardium, focusing on the mechanical integrity of the tissue, toxicity effects and the sterilisation process itself such as time and complexity.
Appendix A: Publications and Conference Participation

List of Publications

- K. Joyce, N. Dzhoyashvili, S. Rahmani and Y. Rochev. “Decellularised bovine pericardium scaffold for the application of hMSC cell sheet”. In Progress

List of Conferences

Assessment of the uniaxial experimental parameters utilised for the mechanical testing of bovine pericardium

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ABSTRACT

Bovine pericardium (BP) is an extensively used biomaterial utilised in a wide range of biomedical devices such as bioprosthetic heart valves. However, the mechanical testing techniques that assess soft biomaterial tissue like BP are varied with no common method utilised across the literature, producing variations and contradictions in reported values. Uniaxial testing is a common technique used to measure traditional mechanical characteristics of ultimate tensile strength (UTS), modulus and the percentage strain at failure. The aim of this study was to take two standard uniaxial test parameters, strain rate and the number of preconditioning cycles and to elucidate recommendations for the standardisation of a uniaxial method, while also measuring not so common parameters of low modulus and hysteresis. Samples post uniaxial testing were treated and analysed with scanning electron microscopy (SEM) and an imaging software (ImageJ) to measure the effect of the parameters on the crimping structure and orientation of the collagen fibres. The study recommends an extension rate of 10 mm/min and 5 preconditioning load-unload cycles as a starting point for the standardisation of a uniaxial testing method. The image analysis of the collagen structure carried out provides a quantitative assessment of the BP post mechanical testing and allows for a better understanding of the behaviour of BP under stress.

1. Introduction

Soft tissue biomaterials such as bovine pericardium (BP), porcine pericardium and porcine aortic valves are utilised in bioprosthetic heart valve manufacturing. Number of heart valves replaced worldwide each year have been reported as over 250,000 with 45% of these bioprosthetic heart valves (Siddiqui et al., 2009) and projection of heart valve failure and modulus values are quoted yet variations in method or technique mean that they cannot be fairly compared across the literature, Table 1. Taking a uniaxial tested BP sample modulus of 10 MPa reported as 8.3%–14.5% and 0.71–1.22 MPa for bending stress and strain respectively (Corden et al., 1995). However, the most common techniques used in the literature include uniaxial (Hulsmann et al., 2012; Gauvin et al., 2013; Sanchez-Arevalo et al., 2010; Páez et al., 2003), and biaxial (Caballero et al., 2017; Sacks and Chuong, 1998; García Páez et al., 2000; O’Leary et al., 2014) testing. Also from an industrial perspective the standards around cardiovascular implants have been purposely not specified to allow for development and innovation (5840-1:2015, 2015). The mechanical specifications reported for the raw materials of tissue engineering applications, include more characteristic properties of polymer materials such as elastic modulus and ultimate tensile strength (UTS) (F2027-16 et al., 2016). Inconsistencies arise when the standard parameters such as UTS, strain at failure and modulus values are quoted yet variations in method or technique that they cannot be fairly compared across the literature, Table 1. Taking a uniaxial tested BP sample modulus of 10 MPa reported by Hulsmann et al. (2012), while for a biaxial test 250 ± 100 MPa was a reported value from Langdon et al. (1999). Not only can the methods vary but also the conditions of that test. Taking one of the most accessible techniques, uniaxial testing, for mechanical assessment of heart valves (Liao et al., 2005) extension rates can vary from 2 mm/min to 25 mm/min (Arcidiacono et al., 2005; Chen et al.,...
The number of these cycles vary from three (Gauvin et al., 2013), ten (Caballero et al., 2017; Sacks and Chuong, 1998; Claramunt et al., 2013; Lally et al., 2004) or when a stable response is evident (Sung et al., 1999b; Fratzl et al., 1998; Oswal et al., 2007; #775; Oswal et al., 2007 #952; Sung, 1999 #782). The complete stress-strain process represents the rearrangement and movement of the collagen molecules without the breaking of bonds. Elastin’s function within the native heart valve is to support collagen when the external forces are removed restoring the valve to its original configuration (Vesely, 1997).

Most modulus values are taken from the linear section of the graph and will provide information on the stiffness of the tissue, and while values can vary from 10 MPa (Hulsman et al., 2012) to 77 ± 23 MPa (Oswal et al., 2007) they are typically in the region beyond which the peak stresses of 1.0 MPa associated with a heart valve in vivo (Zioupos and Barbenel, 1994). Therefore, assessment of the low modulus would provide more applicable data regarding the mechanics of the tissue in vivo.

Extension rate effects on polymers during uniaxial testing is that at slower rates the polymer chains can extend gradually favoring the viscous properties with elastic properties preferred at higher rates. Another factor in selecting an extension rate is the length of time, while a slow rate of 2 mm/min may be ideal, as number of samples increase the practicality would require a faster rate of 20 mm/min.

Assessing the extension rate impact on the mechanical properties of a soft biological tissue, BP, this paper will compare three extension rates of 5, 10 and 20 mm/min, while in tandem measuring the effect of three different preconditioning cycles, one, two and five. The standard parameters of UTS, modulus and strain at failure along with the lesser measured mechanical values for low modulus and hysteresis. Another feature of the report will be to provide quantitative analysis of the mechanically tested samples post testing using SEM and an imaging software, where the orientation and crimping structure of the collagen fibers will be assessed.
This paper will investigate the effect of preconditioning cycles and the extension rates on the uniaxial testing of BP. The BP will be treated with an industrial standard fixative and sterilant, Glutaraldehyde (GLUT) that maintains the structural integrity of the pericardium and provides resistance to enzymatic degradation (Jayakrishnan and Jameela, 1996; Sung et al., 1998). Phosphate buffer solution (PBS) treated BP will act as the control. The standardizing of parameters for the testing and analysis of soft biological tissues such as BP are relevant from both a research and an industrial perspective for the design and production of medical devices like bioprosthetic heart valves. It would provide a more consistent platform for the comparison and quality assessment of mechanical properties across soft biological tissue biomaterials. The quantitative analysis of the samples post testing will further the knowledge on the behavior of these tissues under mechanical loads.

1.1. Tissue fixation

Bovine pericardium (BP) tissue of an age less than 24 months was acquired from a proprietary vendor defatted and stored in EDTA/PBS solution at 2–8 °C. Samples were washed prior to use twice with saline. Sections of BP were placed in 0.6% GLUT prepared from an electron microscopy grade solution of 25% GLUT (Merck, Darmstadt, Germany) in 0.1 M phosphate buffered solution (PBS) (Sigma Aldrich) (García Páez et al., 2002; Sung et al., 1997) for 3 d at room temperature. The same method was applied to the PBS group but were stored in a solution of 0.1 M PBS.

1.2. Uniaxial testing

Tissue samples of 5 mm × 50 mm were cut using a scalpel in axial and circumferential (Circum) directions (Fig. 2.) and were fixed in 0.6% GLUT or PBS as detailed earlier in tissue preparation. Thickness measurements were taken on three locations across each sample using a digital indicator (Mitutoyo Absolute ID-S, Toronto, ON, Canada). The average thickness value was used for the calculation of the cross-sectional area for each sample. Uniaxial testing was performed on a Zwick Roell tensile tester using a 100N load cell. Each sample (n = 5) was held in position with an air pressurized roughened surface clamp, gripped at 3 Bar with a gauge length of 15 mm. Samples were tested under a range of pre-conditioning cycles, 1, 2 and 5 in a loading range of 0 N–1 N. Extension rates of 5, 10 and 20 mm/min were utilized with each sample was extended to failure after the last preconditioning cycle. Failure was set at 80% drop in the peak load. The force extension curves produced were converted into stress strain curves using the cross-sectional area of each sample. The parameters calculated include, 1) the low modulus of the sample taken as the slope of last preconditioning cycle, 2) the high modulus as characterized by the slope of the linear portion of the stress/strain curve 3) the ultimate tensile stress (UTS) calculated as the peak stress of the curve before failure, 4) the percentage strain of failure is the strain at the point of UTS and 5) the hysteresis of the first and 6) final preconditioning cycle.

1.3. SEM imaging and analysis

Post uniaxial testing samples were dried in increasing concentrations of ethanol from 50% to 100% where they were then treated with Hexamethyldisilazane (HMDS) for 30 mins and then allowed to air dry. Samples were then gold sputtered. SEM (Hitachi, S-4700) imaging was carried out at accelerating voltages of between 5 kV and 10 kV.

Analysis of the SEM images was carried out using ImageJ software (NIH, Bestheda, Maryland, USA) (Schneider et al., 2012). Measurements of the collagen fibre bundles included the straight-line distance between three crimps of a collagen bundle (L0), the actual length of this bundle (Lb), the thickness (t) averaged across three areas of the fibre bundle and the angle of the crimp (θ), as shown in a representative image Fig. 3. These measurements were used to calculate two parameters, waviness, and the orientation angle of the fibres (Rezakhaniha et al., 2012). The waviness or straightness parameter (Pw) is calculated from the actual distance and the straight-line distance of three crimps.
on a collagen bundle (Eq. 2). The straighter the fibre bundle the closer to a value of 1 while a wavier sample would measure 0.

\[ PL = \frac{L_0}{L_f} \] (2)

The global angle (Θ) is measured in respect to the axial (0°) and circumferential (90°) direction of the sample.

1.4. Differential scanning calorimetry

The denaturation temperature (T\(_d\)), also known as the shrinkage temperature, provides a gauge to the degree of crosslinking within a fixed tissue. The T\(_d\) for both GLUT and PBS samples (n = 3) were obtained using a differential scanning calorimeter (DSC) (Model Q1000, TA Instruments, Delaware, US). Circular samples of 3 mm diameter were cut using a biopsy punch, patted dry and hermetically sealed into aluminium pans. The test method followed a heating rate of 5 °C/min as suggested by Loke et al. (Loke and Khor, 1995) in a temperature range of 30 °C–110 °C. An empty pan was used as the reference. Heating was carried out in a nitrogen atmosphere of 50 ml/min. The T\(_d\) can be calculated from the extrapolated onset or the peak temperature of the endothermic phenomena, here the peak temperature was used to determine the T\(_d\) (Oswal et al., 2007; Loke and Khor, 1995; Tattini et al., 2007). Calibration of the instrument was carried out using an Indium standard (Melting point, T\(_m\) 156.6 °C).

1.5. Statistical analysis

Measurements across all groups were averaged and presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to compare the differences across all samples. A p-value of less than 0.05 was deemed statistically significant. Statistical analysis was carried out using Minitab (Minitab 17) statistical software.

Fig. 3. Representative SEM image with the key measurements analysed using ImageJ. The measurements include the crimped fiber length (L\(_f\)) calculated along the collagen fiber including three peaks of the fiber, and the straight fiber length (L\(_0\)) between the first and third peaks of the collagen fiber. The other parameter is the angle (Θ) based of the orientation of the sample.

Fig. 4. Mean and SD of the UTS (a), (b) and percentage strain at failure (c) and (d) for both Glut and non-fixed PBS samples in the axial and circumferential directions. The axial direction values for UTS showed a statistically higher value for the PBS BP compared to the Glut BP. Circumferential direction, PBS 5 mm/min at 5 Cycles had statistically higher values than 10 and 20 mm/min 5 cycle PBS samples. The strain at failure values were statistically higher for the Glut against PBS samples over all strain rates at 1 cycle in both the axial and circumferential direction. There were also statistical differences over the 10 mm/min and 20 mm/min at 5 cycles in the axial direction and in the circumferential direction at 1 cycle. (∗) indicates a statistical difference (p < 0.05).
1.6. Thickness and DSC results

Pericardium thickness measurements after treatment in PBS and GLUT resulted in statistically significant higher values of 0.62 ± 0.01 mm and 0.55 ± 0.01 mm respectively, compared to the native tissue 0.44 ± 0.01 mm. The PBS had a statistically greater thickness than the Glut BP.

The denaturation temperature as analysed with DSC, showed that a non-treated sample displayed a temperature of 64.5°C ± 1.2°C while the PBS and GLUT samples had significantly higher values of 75.2°C ± 2.9°C and 84.7°C ± 0.2°C respectively. The GLUT prepared samples were also statistically higher than those of the PBS treated group.

1.7. Mechanical properties

The UTS samples displayed a statistical difference for the 5 mm/min extension rate at 5 cycles for the higher PBS BP compared to the GLUT BP with the trend of higher PBS values evident across both directions Fig. 4 (a) and (b). Also in the circumferential direction there is significant differences across the extension rates at 5 cycles with 5 mm/min displaying higher values than those of both 10 and 20 mm/min. Fig. 4 (c) and (d) display GLUT BP with higher values than PBS samples for strain at failure, statistically greater at the higher strain rates and cycling numbers. The GLUT BP had higher values for 5 cycles at 10 mm/min than that of its respective settings of 20 mm/min and 5 mm/min in the axial direction. Low modulus, Fig. 5 (a) and (b) for the GLUT BP did not display a significant difference across extension rates or pre-conditioning cycling number. The PBS BP did however display significantly greater low modulus values than the GLUT BP across all settings. Also, PBS BP had a statistically lower value in 20 mm/min 2 cycles compared to the 5 cycles at 20 mm/min, and 2 cycles at 5 mm/min in the axial direction. In the circumferential direction PBS BP at 5 cycles 5 mm/min was statistically greater than that of the same cycle number at 10 mm/min. Comparing the GLUT and PBS samples, the PBS BP samples displayed slightly higher values than the GLUT BP samples with statistically different values evident across the 5 mm/min extension rate for 2 and 5 cycles, the 10 and 20 mm/min extension rates at the 1 cycle for the high modulus in the axial direction Fig. 5 (c). The circumferential direction, Fig. 5 (d) displayed the same significantly higher differences at 5 mm/min 2 and 5 cycles along with the 10 mm/min 5 cycle between the PBS and GLUT samples. There were significantly higher values across the extension rates for PBS with 5 mm/min 5 cycle group showing higher values than those of both the 10 and 20 mm/min groups.

Fig. 7 (a) – (c) displays the hysteresis values across all the extension rates for both the GLUT and PBS samples in the axial directions. The increase in extension rate shows an increase in variation between the GLUT and PBS samples with values overlaying at 5 mm/min compared to the statistical significant differences at 20 mm/min. The trend across all rates and sample types is that the hysteresis for the first cycle is significantly greater than that of the 2nd cycle were the values begin to stabilise. When the data of all the extension rates for the GLUT BP are combined, Fig. 8 for each cycle number the 1st cycle is over two times that of the 2nd cycle, 44.63% compared to 19.31% respectively for the axial direction. The circumferential direction displays similar results of to 44.46% and 19.63% for the 1st versus 2nd cycle respectively. The 2nd cycle of both the axial and circumferential directions shows a statistical difference with the 5th cycle of each direction.

Combining the results of each cycle number for each extension rate displayed statistical differences across the directionality of the BP but not within each direction group. The axial direction showed statistically higher values for the UTS and High modulus parameters for each extension rate. While the axial direction showed higher values for the
percentage strain at failure, there was no statistical difference and both directions displayed similar results at the low modulus.

1.8. Image analysis results

Key values from the SEM images are displayed in Table 2, with the representative images for each of the parameters and circumferential and axial directions displayed in Figs. 10 and 11 respectively. The straightness parameter of Ps consisted of a scale of 0–1 representing a highly crimped and straight collagen fibre respectively. The Ps values ranged from a low of 0.62 ± 0.05 for 5 mm/min 5 cycles in the circumferential direction compared to the highest value of 0.89 at 10 mm/min for 2 cycles. The axial direction samples values did not fluctuate as much with a lowest value of 0.69 ± 0.01 at 10 mm/min 5 cycles to a maximum of 0.82 ± 0.1 on average for 10 mm/min 2 Cycles and 20 mm/min 5 cycles. With a statistical significant difference at 10 mm/min 5 cycles when compared to 1 and 2 cycles at the same extension rate and the 5 cycles of the other extension rates. The circumferential fibre had on average lower values than the axial direction at rates and cycle numbers of 5 mm/min 5 cycles and 20 mm/min 5 cycles.

The orientation angles of the fibres showed variation in both directions. The axial displayed fibres at a low of 22.54° ± 0.82° and a high of 148.82° ± 15.32° for 5 mm/min 2 cycle and 20 mm/min 2 cycle respectively. Circumferential fibres ranged from a low of 34.46° to a high of 135.24° ± 23.96°.

2. Discussion

This report investigated the mechanical properties of GLUT BP and how the effect extension rate and preconditioning cycles had on mechanical parameters. The mechanical parameters measured included the standard properties UTS, Modulus and % strain measured. The effect of extension rate and preconditioning cycle did not have a significant effect on UTS and the high modulus results, (Fig. 4), (a-b) and Fig. 5(c and d) respectively for the GLUT fixed group. However, this was not the case with the PBS group, where statistically higher values of modulus in the axial direction for the 10 and 20 mm/min rates at 1 preconditioning cycle, and higher values at 5 mm/min for the 2 and 5 cycles were evident. The circumferential direction at 5 mm/min also displayed significant differences in the 5 mm/min group at 5 cycles compared to the 10 and 20 mm/min rates. Fixation with GLUT did not significantly alter the UTS of the tissue compared to the PBS group except for the axial and circumferential direction at 5 mm/min 5 cycles and across both 10 and 20 mm/min respectively. This too was reported by Hulsmann et al. (2012), who observed a reduction in the high modulus between fixed and native BP tissue, also evident in this study. The author suggested that the fixation without any loading reduces the elasticity of the tissue (Hulsmann et al., 2012). Another reason for the reduction in modulus is that native non-fixed tissue is thinner than GLUT BP and therefore the cross-sectional area is smaller yielding higher values after calculating (Sung et al., 1999a). This however is not the case here where the PBS samples tissue were also statistically higher than those of the GLUT BP.

Low modulus values for PBS tissue were significantly higher than the GLUT tissue across all extension rates and cycle numbers for both directions. As elastin is the primary load bearer during this toe elastic region due to the uncrimping of the collagen fibers (Caballero et al., 2017), the GLUT appears to have a significant effect on the elasticity of the elastin. The biomechanics of elastin are like those of a rubber with a low Young’s Modulus, high extensibility and a sharp rise in stress-strain curve at high elongations (Hoeve and Flory, 1958). The extension rate and cycle number did not have a significant difference across all the low modulus samples. The effect of crosslinking was also evident with the % strain at failure, where across all the extension rates the strain at failure was greater than the PBS samples in both directions. The cycle number also reported higher values with only 5 cycles at 20 mm/min displaying
lower values, but this was not significant. The increase in extension after crosslinking is similar to what occurs in the vulcanisation of a rubber but depending on the crosslinking density there can also be a significant increase in the stiffness and UTS of the rubber. Crosslinking of collagen fibres with GLUT, involves the formation of both inter- and intra-molecular crosslinks that form a network creating a stable tissue with an increase in extensibility (Sung et al., 1999a). This also is evident with the PBS samples where the H2O bound within the tissue...
stabilizes the collagen and elastin (Chen et al., 2008) which accounts for the increase in the Td temperature and the comparable UTS and high modulus values, but is limited in its extension affect. It is suggested that the elastic limit is a much more reliable mechanical reference than the UTS as the valve should never approach this value (Páez and Jorge-Herrero, 1999).

The anisotropic effect was present on the UTS and high modulus with the axial direction displaying statistically higher values than circumferentially. Reported retention or removal of the anisotropic effect varies with Paez et al., suggesting that the tissue directionality differs for both strength and stiffness (Páez and Jorge-Herrero, 1999) with others suggesting that the anisotropy is removed (Sung et al., 1999a).

The directionality effect (Fig. 6.) across all the extension rates after the combination of all cycles values showed that axial direction is statistically higher for the UTS and high modulus by two-fold and between 10 and 20% respectively. This is typically the scenario in the literature where the axial direction has reported higher values (Sung et al., 1999b; Oswal et al., 2007) contrary to the anisotropic effects of glut fixation where the circumferential direction is stiffer (Páez and Jorge-Herrero, 1999; Zioupos et al., 1994). There was no statistical difference in the hysteresis, low modulus or strain at failure between both directions in this study. There was no statistical difference across the extension rates.

Analysis of the hysteresis curves showed a wider variation for the PBS samples compared to the GLUT BP, (Fig. 8.), in the 5th load-unload cycle. The effect of extension rate is evident at that higher rates of 10 and 20 mm/min, where there are statistically different after the 1st and 2nd cycle when compared to the PBS samples, with the GLUT samples displaying lower hysteresis of around 10%. The 5 mm/min rate did not show a significant difference across each cycle. The GLUT samples also displayed less variation per each cycle compared to the much wider spread of the PBS samples, this is most evident at the 20 mm/min rate, Fig. 7. Comparison of hysteresis cycle number for each direction regardless of extension rate, Fig. 9 showed that the directionality of the tissue did not influence the hysteresis and that both directions had no significant difference after the 2nd cycle. It was also noted by Daar et al., that hysteresis values tended to stabilise after approximately 5 cycles (Daar et al., 2014). From this examination the 1st and 2nd cycles are critical in the conditioning of the tissue. Also, the GLUT samples did not have significant differences across the extension rates as too was noted by Trowbridge et al. (Trowbridge and Crofts, 1987) who reported that hysteresis was extension rate independent.

Analysis of the samples post mechanical testing assessed the crimping structure of the collagen fibres and their respective
orientation to the axial and circumferential direction. The crimping structure of the fibres ranged from 0.62 to a highest of 0.89 for the circumferential direction at 10 mm/min. This shows that the fibres are not fully extended after the tensile test but still retain elements of their crimp structure. The extension rate variation did not have a significant effect which was due to the viscous properties of the tissue that allow it to return to a crimped structure while retaining elements of the applied stress exertions after the stress has been removed (Páez and Jorge-Herrero, 1999). Therefore while the samples were treated for SEM analysis in a timely manner, they had the opportunity to recoil on removal of the extension load.

Pericardium is known as an anisotropic material, the analysis of the fiber orientation here confirms this with variation across both directions. Given that the analysis is at a microlevel and looking at individual fibre bundles, the variation is evident and a possible cause for the variation in both here and across the literature. Uniaxial testing exert an extension in one direction so if the tissue in the axial direction is misaligned then inconsistencies will occur.

This report assessed the effect of different extension rate and preconditioning number of cycles on GLUT BP. Across the literature the
characteristic mechanical parameters of UTS, high modulus and strain at failure are assessed. However the range of methods and in turn the reported results are varied (Aguiari et al., 2016). The extension rate of 10 mm/min in this report produced similar UTS, modulus and strain values compared to other studies (Sung et al., 1999a, 1999b; Oswal et al., 2007) and would be recommended for the application as a standardised extension rate. The preconditioning number of cycles showed that after the first two there is a plateau effect suggesting that 5 cycles would be an adequate number for the preconditioning of BP tissue prior to the uniaxial test.

3. Conclusion

Comparing the mechanical properties of GLUT BP through a range of parameters provided insight into the importance of the number of preconditioning cycles and a suggestion for a standardised extension rate. The effect of the number of preconditioning cycles on the hysteresis of GLUT BP was shown with a significant drop from the first cycle to the 2nd cycle, with a plateau in hysteresis values after the 3rd cycle, indicating that 5 load-unload cycles is a required standard for the preconditioning of BP. The directionality of the fibres was not a factor in hysteresis with no significant difference between the GLUT BP Axial and Circum samples, while a significant difference is evident between the GLUT and PBS samples at the higher extension rates of 10 and 20 mm/min. The effect of extension rate showed minor variations but no statistically significant differences across the mechanical properties of BP. However, the 10 mm/min rate produced values close to those in the literature at the same rate and would satisfy the time requirements for large scale sampling and is recommended as a standardised rate. Orientation effects and crimping of the collagen fibres as assessed by SEM post mechanical testing provides quantitative data on the orientation of the fibres and its correlation to the mechanical properties of uniaxial tested BP. The similar crimped pattern across all extension rates provide evidence of the viscous behaviour of the BP and how the structure remains upon removal of the stress. This quantitative method could be employed exclusively or used to complement other techniques such as small angle light scattering or second-harmonic imaging microscopy for the assessment of BP.

Conflict of interest

The author(s) confirm that this article content has no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmbbm.2019.04.025.

References


Quasi-isothermal modulated DSC as a valuable characterisation method for soft tissue biomaterial crosslinking reactions

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\textbf{A R T I C L E   I N F O}

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\textbf{ABSTRACT}

Glutaraldehyde (Glut) is an extensively used sterilant and fixative for the crosslinking of natural soft tissue biomaterials like bovine pericardium (BP) to provide stability and is required for its application in vivo. There is plenty of debate around the reaction mechanism of Glut with natural biomaterials. Differential scanning calorimetry (DSC) is a commonly used technique that is typically used to measure the thermal profile of polymers. However, a variation known as quasi-isothermal modulated differential scanning calorimetry (QiMDSC) has been utilised for the analysis of polymorphic transformations in both the pharmaceutical and food industries. This communication will address QiMDSC as a method for analysing soft tissue biomaterials and their crosslinking mechanisms and how it can be applied to other biomaterial applications.

1. Introduction

Soft tissue biomaterials like bovine pericardium (BP) are extensively used biomaterials with an array of applications [1–3]. Treatment of tissue is required for it to be used in vivo. These treatments involve the fixation of the tissue using a popular crosslinker and sterilant, glutaraldehyde (Glut) [4,5]. Glutaraldehyde is a five-carbon dialdehyde, with multiple functionalities of sterilant, crosslinker and fixative [6] that make it the “gold standard” [7] over other alternative crosslinkers such as carbodiimides [5], Genipin [8,9] and epoxies [10]. The process for crosslinking involves the immersion of the tissue into a Glut solution of a known concentration, typically 0.6% in phosphate buffer solution (PBS).

The stability of Glut is well documented with its ability to polymerise freely in an aqueous solution [11,12]. The variation of Glut in solution leads to debate over its possible crosslinking mechanisms [13]. Reaction with the primary amine groups in lysyl residues of proteins leads to inter- and intra-molecular crosslinking via covalent bonding, stabilising the tissue against enzymatic degradation [4,14,15]. Numerous crosslinking mechanisms have been reported between Glut and collagen-based materials, with temperature and pH effects of critical importance [11,16–18]. The formation of Schiff bases is the initial mechanism occurring between the ε-amino group of a lysine or hydroxylysine [17,19] present in solution available for reaction with lysine residues [11]. Mechanism of crosslinking post this Schiff base formation is contentious, with the stability of the base key to the debate. Stable Schiff bases from Glut polymers through aldol condensation, to intermediate Schiff bases forming additional crosslinks [5,20] have been reported. The unstable nature under acidic conditions of Schiff bases is known with suggestions that they undergo further reactions during the crosslinking process, Okuda et al. [21] suggested that the Schiff base is the central intermediate where reactions occur before a crosslink is formed. This was also proven by Daminik et al. [5] by monitoring the primary amine groups present with respect to the duration of crosslinking. It was found that only after the hydrolysable Schiff bases were stable that crosslinking of the collagen could occur. The overall effect of the crosslinking is to provide a structure that is biocompatible, non-thrombogenic, prevent against structural deterioration while also maintain the mechanical properties similar to that of a native aortic valve and its hemodynamic properties.

The sterilant properties of Glut for medical devices can be the justified reasoning for the longer time periods and variation in this fixation process [4]. However, for general investigative research purposes, where sterilisation is not of a key priority, there is still a wide ranging of...
times used for the crosslinking of BP using Glut with some fixing between 5 min and 3 h \([5,22,23]\), 24 h \([24]\) to 14 d \([25]\). A typical method to confirm that the tissue is fully crosslinked is the measurement of its shrinkage temperature using a water or saline bath of increasing temperature of approximately 1 °C, with the sample held in a load cell of a tensometer \([26,27]\). Another method of assessing the crosslinking efficacy, or denaturation temperature \(T_d\) introduced is that of Differential Scanning Calorimetry (DSC) \([28]\). This is a common technique that allows the quick assessment of the degree of crosslinking based on an increase of the endothermic peak by approximately 20 °C from the untreated state to the fixed state \([5]\). Denaturation of tropocollagen occurs as the tissue is heated beyond 60 °C and the helical structure unfolds to produce random chains of gelatin \([29,30]\). The DSC technique is typically used for the analysis of a polymers thermal profile, providing information on first and second order transitions of polymeric chains like glass transitions \(T_g\), melting temperatures \(T_m\) and crystallisation. Further development of the technique in 1993 by Gill et al. established a variation on DSC with the introduction of modulated DSC (mDSC) \([31]\). The development allowed mDSC to superimpose a sinusoidal modulation over the DSC’s standard linear rate \([32]\). The mDSC model adds the additional parameters of the amplitude of the modulating temperature and the linear rate lets the overlay of the modulating temperature and the linear rate lets the total heat flow signal be deconvoluted into its reversing element of the specific heat from the Cp and heating rate and its non-reversing element due to the kinetic component of temperature and time \([33]\).

The equation that describes the components of both DSC and mDSC is shown in Eq. (1)

\[
\frac{dH}{dt} = C_P \frac{dT}{dt} + f(T, t)
\]

The total heat flow \(dH/dt\) as measured by conventional DSC, is equal to the heat capacity \(C_P\) \((J/C)\) and its heating rate \(dT/dt\) \((C/min)\) and the kinetic component of the heat flow that is a function of \(T\), temperature and \(t\), time.

The overlay of the modulating temperature and the linear rate lets the total heat flow signal be deconvoluted into its reversing element of the specific heat from the Cp and heating rate and its non-reversing element due to the kinetic component of temperature and time \([33]\).

\[
Q_{(Total)} = Q_{Reversing} + Q_{(Non - Reversing)}
\]

This method produces an increase in both resolution and sensitivity, through the separation of overlapping signals from the baseline \([34]\). For example, hard to detect \(T_g\) in polymers can be separated to clearly identify other features such as enthalpic recovery beside the \(T_g\) that would typically give the inaccurate impression of a melting peak.

The mDSC model adds the additional parameters of the amplitude of the temperature modulation \(A(T)\), angular frequency \(\omega\) and phase shift \(\theta\), to those of temperature \(T\), time \(t\), initial temperature \(T_0\) and underlying heating rate \(\beta\), to produce Eq. (3).

\[
T = T_0 + \beta t + Ar \sin(\omega t - \theta)
\]

Further evolution of mDSC has seen the development of quasi-isothermal modulated temperature differential scanning calorimetry (QIMDSC). Like mDSC a modulated temperature profile is applied but across a constant underlying temperature or by collecting a series of quasi-isothermal data points by increasing the underlying temperature incrementally \([32]\). This has found applications in pharmaceutical industry for the analysis of polymorphic transformations through the changes in heat capacity \(Cp\) \([35,36]\). The removal of a heating rate creates an increase in the sensitivity to the reverse \(Cp\) (Rev \(Cp\)) and the removal of melting effects due to the large number of modulations \([37]\).

The measurement of heat capacity in situ is of great benefit in the analysis of the molecular mobility within the crosslinking or curing reactions of a sample \([38]\). An example of this would be the curing of an epoxy, as it cures the heat capacity will decrease because the crosslinking of molecules reduces their mobility \([39]\). Also in the crosslinking reaction between biological tissues like BP and Glut there is an increase in stiffness and strength of the tissue due to the bond between the amino acids.

The goal of this paper is to introduce the QIMDSC technique for the analysis of the crosslinking reaction between a biological biomaterial, BP and Glut. The focus will be on the reaction over time at an isothermal temperature, with the degree of crosslinking measured using conventional DSC. The proof of concept will employ a similar model of crosslinking using an epoxy reacting with a hardener.

2. Materials and methods

2.1. Materials

Bovine pericardium (BP) tissue of an age less than 24 months was acquired from a proprietary vendor defatted and stored in EDTA/PBS solution at 2-8 °C. Samples were washed prior to use twice with saline. Samples of BP were cut using a 3 mm diameter biopsy punch and consumed in the protocols detailed in the methods. A fixation solution of 0.6% GLUT prepared from an electron microscopy grade solution of 25% GLUT (Merck, Darmstadt, Germany) in 0.1 M phosphate buffered solution (PBS) (Sigma Aldrich) was used.

A Loctite M = 31 Cl epoxy (Henkel Technologies) consisted of a Part A and Part B components of epoxy and hardener respectively. The ratio of the mix is 2:1 epoxy to hardener. The mix of each sample was tested at different temperatures for 12 h period. Curing was determined when a drop, in heat capacity created a flat baseline. Curing was confirmed using conventional DSC at 10 °C/min from 30 °C to 100 °C of the sample post QIMDSC method with a \(T_g\) of 70 °C indicative of a fully cured epoxy.

2.2. Methods

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2.2. Methods

2.2.1. QiMDSC

The 3 mm diameter BP sections were patted dry, and followed one of two sample preparation methods for QiMDSC, Fig. 1. Method 1 applied a 10 μl volume of 0.6% Glut to the 3 mm BP sample in an aluminium pan and was then hermetically sealed. Method 2 involved immersing the 3 mm BP sample in 10 μl of 0.6% Glut for 30 s. Both timelines for Method 1 and 2 are displayed in Fig. 1. The sample was patted dry and hermetically sealed in an aluminium pan. All QiMDSC and conventional DSC testing were performed using a DSC, (Model Q2000, TA Instruments, Delaware, US). Both method 1 and 2 followed the same protocol of equilibrating the sample at 25 °C and holding isothermally for a selection of isothermal period’s from 10 min, 120 min, 240 min, 360 min and 720 min. The epoxy resin samples were also tested over a range of isothermal temperatures consisting of 25 °C, 50 °C, 100 °C and 200 °C for a period of 720 min. All samples were run under a modulation of ± 1 °C every 100 s. The thermograms of the heat capacity (mJ/°C) as a function of temperature were used to measure the total change in heat capacity after 30 min to the end of the time periods.

2.2.2. Conventional DSC

Directly after each QiMDSC test the samples were tested using a conventional DSC to determine the denaturation temperature (T_d). This provides a gauge to the degree of crosslinking within a fixed tissue. The test method followed a heating rate of 5 °C/min as suggested by Loke et al. [28] in a temperature range of 30 °C–100 °C. An empty aluminium pan was used as the reference. Heating was carried out in a nitrogen atmosphere of 50 ml/min. The T_d can be calculated from the

Fig. 2. Comparison of curing behaviour at different isothermal temperatures for an epoxy adhesive. The Rev Cp was taken as the drop in the baseline from 10 min to 720 min. Also extrapolated onset was used to determine the drop in the baseline as an indicator of crosslinking reaction occurring between epoxy and hardener.

Fig. 3. Measurement of T_g for each epoxy sample after their respective isothermal curing temperature.
extrapolated onset or the peak temperature of the endothermic phenomena, here the peak temperature was used to determine the $T_d$ \cite{28,40,41}. Calibration of the instrument was carried out using an Indium standard (Melting point, $T_m$ 156.6 °C, Enthalpy 28.74 J/g).

3. Results and discussion

Demonstration of the QiMDSC technique and the proof of concept for its application to the analysis of a crosslinking reaction is illustrated by the thermogram of the epoxy across various isothermal curing temperatures, Fig. 2. The extrapolated onset of the Rev Cp curve was used as an indicator for the start of the epoxy hardener crosslinking reaction. The epoxy samples held isothermally at 25 °C and 50 °C displayed a drop in the Rev Cp after 361.97 min and 251.55 min respectively. While the higher temperature curve of 100 °C produced a drop at 15.96 min. The drop could not be calculated at 200 °C as the baseline appeared flat almost immediately. The total drop in the Rev Cp baseline was also measured between 10 min and 720 min. There was a difference evident across all the cure rates with both the 25 °C and 50 °C cured samples displaying a similar drop of 3.310 and 3.509 mJ/°C.

Fig. 4. QiMDSC thermograms after 10, 360 and 720 min for BP and 0.6% Glut following method 1.

Fig. 5. Measurement of $T_d$ post QiMDSC method that suggests BP and Glut reaction produced a fully crosslinked material, even after 10 min.
respectively. However, these were considerably greater than the values measured at the higher isothermal temperatures with 0.4335 and 0.05322 mJ/°C measured for 100 °C and 200 °C respectively. This would indicate that at the lower curing temperatures there was a greater degree of molecular mobility during curing, resulting in a longer curing time.

The measurement of the Tg of each epoxy post curing are shown in Fig. 3. The lowest curing temperature of 25 °C displayed a Tg value of 37.15 °C, while also displaying an exothermic peak at 83.73 °C suggesting some unreacted components present within the epoxy. The epoxy cured at 50 °C showed an increase in the Tg to 57.66 °C. However, this temperature is significantly lower than those for the higher cure temperatures of 100 °C and 200 °C with both displaying temperatures above 74 °C. Both these temperatures are above the reported value of the typical physical properties of the cured epoxy with a Tg of 70 °C.

The curves in Fig. 4, display the BP and 0.6% Glut reaction of respective samples after 10 min, 360 min and 720 min following method 1. A continuous drop in the curves and Rev Cp is evident after 360 min and is also present with a sample after 720 min. Applying the same principle, from the epoxy model, this continuous drop in the curve indicates that the reaction is an ongoing process.

However, on testing the samples once the QiMDSC method is complete using conventional DSC and analysing the Td, it is evident that all samples are fully crosslinked even after 10 min, Fig. 5. This suggests that while the Glut reaction with the amide bonds is swift and occurs before 10 min, there is still a continuing reaction taking place. This quick reaction was also reported by Damink et al. who reported that free amine groups in collagen of dermal sheep react quickly with Glut to form Schiff bases [5]. When looking at the control samples of Glut only, the continuous drop in the baseline is present Fig. 6.

This suggests that the Glut polymerisation is a continuous process over time. When the BP was dipped into 0.6% Glut the similar drop in the Rev Cp was again present, however the Td analysis post-test showed a gradually increasing Td of value with increase in time with a levelling off at 720 min Fig. 7.

The overlay of the 0.6% Glut only and BP + PBS controls were
overlaid with BP + Glut, Fig. 8. There is a difference across the sample’s slopes. The 0.6% Glut slope is dropping, suggesting that there is a continuous reaction occurring with the Glut solution itself, which is indicative of its reported behaviour of continuous monomer transformations in aqueous solutions [4,18]. The drop-in slope of Rev Cp in BP + PBS, 2.114 mJ/°C is greater than that of the Glut solution alone 1.239 mJ/°C but half that of the BP + Glut 5.406 mJ/°C. This change in BP + PBS indicates that the reaction mechanism between the Hydrogen bonds while continuous is not as great as the molecular mobility and the continuous reaction between BP + Glut.

For lower and higher concentrations, a follow-on study is warranted. The authors believe that a lower crosslinking density would be at the lower tolerances of the method. However, at higher concentrations such as the 2.5% Glut traditionally used for the crosslinking and preparation of biological samples electron microscopy samples, the continuous and ongoing crosslinking reaction would be present.

The technique has been utilised in pharmaceuticals and the food industry, and this paper demonstrates its benefits when applied to soft tissue biomaterials and their crosslinking mechanisms with further development into other biomaterials such as hydrogels and polymers worth exploring.

CRediT authorship contribution statement


Declaration of competing interest

The author(s) confirm that this article content has no conflicts of interest.

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Decellularised bovine pericardium scaffold for the application of a hMSC cell sheet

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ABSTRACT: Bovine pericardium (BP) is an extensively used biomaterial particularly in the cardiovascular application of bioprosthetic heart valves. This study uses a tissue engineering approach to increase the regenerative capabilities of BP using a new cell sheet technique. Decellularised bovine pericardium (Decell BP) extracellular matrix was used as a scaffold for the seed of a cell sheet of human mesenchymal stem cells (hMSCs) onto its surface. Analysis of the ECM structure, the attachment, interaction at the interface and viability of the cell sheet was performed. Histological observations through hematoxylin and eosin (H&E) staining and scanning electron microscopy (SEM) imaging provided qualitative information, while quantitatively the viability and metabolic activity of live/dead and Alamar Blue assays respectively were assessed. Results demonstrated that the Decell BP was a viable scaffold for a hMSC cell sheet. With the cell sheet method providing comparable metabolic and viability to cell suspensions while also avoiding some of the pitfalls related to cell suspension seeding, Overall this research displayed Decell BP as a viable scaffold material for the implantation of a cell sheet and demonstrated positive results warranting further research into its application for bioprosthetic heart valves.

Keywords: Decellularised bovine pericardium; poly(N-isopropylacrylamide), cell sheet

1. INTRODUCTION

The fields of tissue engineering and regenerative medicine have seen increasing development in cardiac applications, from heart valves to coronary grafts and myocardium (1). Heart valve disease is a growing area of concern with over 250,000 replaced each year (2) and estimates tripling this number by 2050 (3). Prosthetic heart valves fall into two main categories of mechanical (4-6) and xenogenic natural tissue of porcine heart valve or bovine pericardium (BP) (7). These bioprosthetic heart valves account for approximately 45% of implants (2). Mechanical heart valves while robust and long lasting require the lifelong supplementation of anti-coagulant medication (8). Bioprosthetic heart valves have a limitation in their age-related lifespan of < 15 yrs (9). This limited lifespan can be attributed to failure modes such as mechanical or calcification due to the treatment of the tissue with glutaraldehyde (2, 10, 11) and the loss of interstitial cell viability (9). The key factor in these failure modes is that the bioprosthetic valve leaflet is not a viable tissue for new cell growth to allow for the co-adaptation of the valve to the in vivo environment.

A tissue engineering approach can be employed in numerous guises from 3D scaffold (12, 13), decellularized tissue (14, 15), spheroids (16), 3D printing (17), and cell sheet (18). Choosing a platform or a combination of these techniques is much dependent on the application. The decellularized extracellular matrix scaffold can provide reinforcement and provide remodelling to tissues (19, 20) while the application and growing of cells on a biological or synthetic scaffold have shown promise (21, 22). Also the endothelialisation of bovine pericardium has been researched previously (23) and provides the opportunity for adaptation of the tissue to the in vivo environment.

Biomaterial scaffolds derived from natural sources consist of an extracellular matrix (ECM) that is composed of the structural polymeric proteins collagen and elastin and glycosaminoglycans
(GAGs). Collagen and elastin of the ECM are the chief structural framework for a biological scaffold and allow for the material to function biologically on both the molecular and macroscopic level (24), compared to the macroscopic level of synthetic materials. The elimination of any possible immunogenic response of the biomaterial to a host or application of cells can be made possible through the removal of all cellular and nuclear remnants via decellularization (25). Decellularisation can be completed through physical, chemical or enzymatic methods, with the aim to remove all cellular material, a focus on reducing any possible negative effects on the composition, biological activity and mechanical integrity of the ECM (26). Physical methods include temperature (27) and pressure (28) changes that interrupt and burst the cell membrane respectively. An enzymatic approach with the likes of nucleases, Trypsin and chelating agents such as ethylenediaminetetraacetic acid (EDTA) are frequently used in several different combinations (29, 30) with varying degrees of success. Chemical methods of decellularization include non-ionic and ionic detergents like Triton X-100 (31) and sodium dodecyl sulphate (SDS) (32) respectively. All these methods are generally not used individually but rather in combinations, where initial ionic solutions or physical methods proceed and enzymatic agent to separate any cellular component from the ECM, then followed by a detergent process to solubilise cellular components and to finish the decellularization procedure a final washing cycle to remove all residual chemicals from the tissue (26). Decellularised bovine pericardium (Decell BP) has been researched (33) and utilised for numerous applications (15, 34).

Seeding of cells onto a scaffold is commonly used technique. The use of cell-sheets in tissue engineering have been rapidly developed and applied in different areas including regenerative medicine (35-37), cell based drug screening assays (38, 39) and tissue and disease modelling (40-43). High density cell-sheets prevent an uneven distribution and increase the number of attached cells that is not possible with the traditional seeding of a concentrated cell solution where there is a risk of cell loss and low cell attachment. Demonstrations of the regenerative potential of bioscaffold-assisted mesenchymal stromal cell sheets for heart regeneration have been reported (44, 45). Currently the most effective method for cell-sheet production involves the use of poly (N-isopropylacrylamide) (pNIPAm) based substrates (46-49). The thermo-responsive polymer pNIPAm dissolves in cell culture media when the temperature drops below its low critical solution temperature of about 32 °C. Cultured cells grown on pNIPAm coated culture dishes can be detached in the form of an intact cell sheet by reducing the incubation temperature with the conversion of the pNIPAm from hydrophobic to hydrophilic states (50, 51). This thermo-responsive polymer, pNIPAm, has proven to be biocompatible with cell culture and shown that cells are viable after both cell detachment and reattachment (46). Human mesenchymal stem cells (hMSCs) have shown promise in cell therapy applications and used in numerous clinical trials for cardiovascular applications due to their immunomodulatory and regenerative properties (52-54)

For this study hMSCs were produced into a cell sheet and seeded onto a biological scaffold of decellularized bovine pericardium. Evaluation of the scaffold was assessed over the parameters of cell viability and behaviour after seeding along with cell-to-cell and cell-to-scaffold interactions. Cell sheets seeded onto native non-treated BP will act as the control.

2. MATERIALS AND METHODS

2.1. CELL SHEET PREPARATION

2.1.1. Thermoresponsive poly (N-isopropylacrylamide) film substrate preparation

A thermo-responsive polymer poly(N-isopropylacrylamide) (pNIPAm) of Mn = 20,000 – 40,000 (Sigma-Aldrich, St. Louis, USA) was prepared into a 50 nm thick film by a spin-coating technique (51). Both tissue culture polystyrene (TCPS) substrates of 35 mm or borosilicate glass slides were used as the pNIPAm spin coated substrate. The TCPS substrate required for the cell morphology, cell assays (alamarBlue) and cell detachment analysis. While the borosilicate glass was required for the fluorescence microscopy analysis and DAPI staining. In brief the film deposition process involved a 5 mg/ml polymer ethanol solution deposited onto a substrate spinning at a rate of 150 RPM for 9 s
followed by 30 s at 4000 PRM on a Laurell Technologies WS-400B-256 spin coater. Samples were then dried overnight in an ethanol soaked atmosphere before leaving in a vacuum at 40 C and 600 mBar for over 4 h.

2.1.2. Cell Propagation

Human mesenchymal stem cells (hMSCs) cell line were cultivated in Dulbecco’s Modified Eagles Medium (DMEM) (Lonza, Basel, Switzerland), supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin antibiotics, both purchased from HyClone (Logan, USA). Cultures were maintained in a humidified incubator at 37 °C and 5% CO2. Culture media was changed every two-days. When cells reached 80% confluency they were sub-cultured.

2.1.3. Cell Detachment

Cells grown on the pNIPAm film were rinsed with HBSS to remove any excess serum. This was then followed by the addition of a cold serum-free DMEM where the cells were allowed to cool down to 4 °C as monitored by a digitally controlled heating/cooling plate set. Micrograph of cells were taken every 10 min on a phase contrast microscope to monitor the cell detachment.

2.2. BOVINE PERICARDIUM DECELLULARISATION

Bovine pericardia (< 24 months) procured from a local slaughterhouse (Bradys, Athenry) were used as the raw materials. The decellularization of the BP followed a previous method developed by Oswal et al. with some modifications, that displayed the collagen elastin matrix to be intact. The sample was washed in PBS supplemented with 1% penicillin-streptomycin antibiotics and left in sodium dodecyl sulfate (SDS, 0.1% w/v) for 24 h, both in the presence of protease inhibitors. The tissue was finally treated with a nuclease solution (RNase/DNase) and then washed in sterile PBS supplemented with 1% penicillin-streptomycin antibiotics for 24 h with agitation. The decellularized tissue was sterilised under UV condition in a laminar flow hood for 4 h before cell seeding.

2.2.1. Static cell seeding of acellular pericardial scaffolds

Decellularized BP scaffolds of 10 mm x 10 mm were placed into a 12-well plate and used for cell attachment. Single cell suspension (1 x 10^5 cells/cm^2) or cell sheets were first seeded onto fibrous side of BP scaffolds in a culture volume of 200 μL. After 4 h of incubation in a humified incubator at 37 °C and 5% CO2, a final culture medium volume of 1.5 ml was added to each well of the plate. Seeded scaffolds were maintained in DMEM, supplemented with 10% FBS, 1% penicillin-streptomycin antibiotics at 37°C, 5% CO2 with culture medium replaced daily. Tissue samples were removed for analysis after 7 days.

2.3. ASSESSMENT OF CELL PROLIFERATION, VIABILITY AND METABOLIC ACTIVITY

2.3.1. DAPI Staining

The 4, 6-Diamino-2-phenylindole (DAPI) staining was performed to confirm the removal of cell nuclei. Procedure was performed as per manufacturers guidelines (Thermo Fischer Scientific, Waltham, MA, USA). Tissue samples pre- and post-decellularisation were placed onto slides, rinsed in HBSS and then mounted with Ultra Cruz TM mounting medium containing 1.5 μg/ml DAPI for nuclear counter staining. The slides were analysed using Andor spinning disc confocal microscopy.

2.3.2. AlamarBlue Assay

The metabolic activity of cells was assessed using an AlamarBlue assay according to the manufacturer’s instructions. The AlamarBlue reagent, resazurin, is an oxidized form of redox indicator that is blue in color and non-fluorescent. After incubation with viable cells, the reagent becomes fluorescent and changes color converting to its highly fluorescent pink form, resorufin. The
fluorescence was measured using a Thermo Scientific Varioscan FlashMultimode plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

2.3.3. Live/Dead Cell Viability Assay

Viability of cells were measured using a Live/Dead cell viability assay. Titration was performed for both calcein-AM and ethidium homodimer-1 to define optimal dye concentration according to the manufacture protocol. A solution of 1 μM Calcein-AM and 2 μM ethidium homodimer-1 in HBSS was mixed thoroughly and then added to the cells for 20 min at 37 °C. The fluorescent staining was observed using an Olympus IX81 fluorescence microscope (Olympus) and images captured using a DP72 CCD camera (Olympus) linked to CellSens Dimension software (Olympus).

2.4. MICROSCOPY AND HISTOLOGICAL ANALYSIS

2.4.1. SEM Microscopy

Samples were dehydrated using increasing concentrations of ethanol from 50% to 100% where they were then treated with Hexamethyldisilazane (HMDS) for 30 mins and then allowed to air dry. Samples were then gold sputtered. SEM (Hitachi, S-4700) imaging was carried out at accelerating voltages of between 5 kV to 10 kV.

2.4.2. Histological Analysis

Cell sheets were removed and fixed in 4% paraformaldehyde solution for 24 h. The samples were then embedded in paraffin (Leica ASP300) and sectioned using a microtome (Leica RM2235). Samples were stained with Hematoxylin and Eosin (H&E) (Sigma Aldrich, St. Louis, USA) and imaged using an Olympus IX-81 inverted microscope.

2.5. STATISTICAL ANALYSIS

Statistical analysis was performed using Statistica 8.0 software. Values are expresses as mean ± standard deviation (SD) (n=3 for each group). If normal distribution was confirmed using Kolmogorov-Smirnov test and Shapiro-Wilk’s W test, student t-test were conducted to compare independent groups and multiple comparisons were made using one-way ANOVA test. Statistical significance was defined as p-value <0.05.

3. RESULTS

3.1. Microscopy Analysis of Cell Sheet and Decellularised BP.

Analysis of the BP samples using SEM revealed collagen fibres in native and the decellularized tissue on the fibrosa surface. The collagen fibres were arranged in an orderly structure in the native tissue compared to the more disorganised, porous microstructure and delamination of multiple layers of collagen bundles in the decellularized tissue (Fig.1 (A)).

Histological imaging, through H&E staining, revealed cells scattered among the collagen fibres in the native tissue. The decellularized tissue showed no cells and the mesh of collagen fibres looser than in the native tissue. (Fig. 1 (B)) Also the lamellar arrangements of collagen displayed gaps between the fibres. Histological results were similar to those of transfer of the cell sheet to the Decell BP showed after 7 d in cell culture revealed no obvious gaps in SEM and histological images Fig. 2 A and B, respectively.

3.2. Cytotoxicity Evaluation of Cell sheet and Decellularised BP.

Similar to the histological results, DAPI staining in Fig. 3 (A), presented residual cell remnants to a higher degree than the Decell BP. The contact cytotoxicity of hMSCs grown adjacent to the native and Decell BP revealed a strong viability on both tissue types, as identified using Live/Dead assay
While the morphology of the Decell BP changed there was similar viability present. Also, the morphology of hMSCs and viability of the cells remained unchanged after 7 d culture period. The images of the cell suspension samples compared to the cell sheet Fig. 3 (B – C) display a significantly lower coverage of the BP. AlamarBlue assay confirmed a high metabolic activity of cells grown on decellularized tissue with no significant difference compared to the untreated control (native untreated tissue), Fig. 4 (A). The comparison of metabolic activity between the hMSC single cell-suspension and the cell sheets grown on decellularized BP matrices for 7 d did not reveal any statistically significant differences Fig. 4 (B).

![Native BP and Decell BP](image)

**Figure 1.** Comparison of native (untreated) BP and Decell BP. The SEM Images (A) display the orderly arrangement of the collagen fibres while the Decell BP have a more porous structure. Scale Bar 50 μm. The H&E staining (B) display cell nuclei (white arrows) dispersed among the collagen fibres while these cells have been removed in the decellularization process. Scale Bar: SEM Images 50 μm, H&E, DAPI and Live/Dead Assay Images 100 μm.
Figure 2. Microscopy analysis of a multi-layered hMSC sheet (white arrows) onto decellularized BP (black arrows). (A) SEM image of hMSC sheet cultured on decellularized BP and (B) H&E staining cross-section of decellularized BP and the cell sheet on its surface.
Figure 3. Cytotoxicity comparison of native (untreated) BP and Decell BP. The DAPI staining (A) displayed all residual cell remnants (white arrow) that are present in the native tissue removed following the decellularization process. Cytotoxic evaluation of the BP with Live/Dead assay (B) demonstrated strong viability for the hMSCs grown next to both tissue sample types, despite the morphological tissue changes in the tissue. Live/Dead assay (C) images of cell suspension samples seeded onto both native and decell BP display a significantly lower coverage of the BP than the cell sheet images. Bar: SEM Images 50 μm, H&E, DAPI and Live/Dead Assay Images 100 μm.
Figure 4. AlamarBlue assay results for (A) hMSCs grown on native untreated and decellularized BP after 7 d in culture, (B) hMSC single cell suspension and cell sheets grown on decellularized BP matrices for 7 d. Data expressed as the mean ± SD, p <0.05.

4. DISCUSSION

Decellularized biological scaffolds are widely used for numerous applications such as tendon repair, skin grafts and in bioprosthetic heart valves. The primary goal of decellularization is the removal of cellular components while preserving the mechanical integrity of the ECM. Elimination of endogenous cells and the presence of their antigens reduces if not removes the possibility of an immune response on transplantation. The decellularization technique in this paper, adopted from Oswal et al. (33) with some modifications using SDS, removed the endogenous cells, cell debris while keeping the ECM intact. Bovine pericardium is a bulky tissue so mechanical agitation was required to ensure full exposure of the washing solution. Microscopy analysis of the tissue revealed the presence of collagen fibres in both untreated and decellularized tissue. The Decell BP also displayed
a porous structure, delamination of the multiple layers of the collagen bundles along with a more disorganised arrangement of collagen compared to the untreated tissue. The histological examination of the tissue also displayed a looser architecture of the decellularised collagen fibres with no cells present compared to the numerous cells on the untreated tissue and its ordered arrangement. The DAPI staining results echoed those of the histological analysis with many fluorescent dots representing cellular DNA evenly distributed in the untreated tissue with none present in the Decell BP (Fig. 3 (A)). The presence of residual cell remnants, including DNA fragments and antigens may induce negative effects, however their existence should not cause severe immunoreactions.

The Decell BP displayed high metabolic activity and viability with cells. Both the live/dead assay and the alamarBlue assessments of the hMSCs over 7 d cultures showed no significant difference with the control groups, contrary to other research using SDS that produced a toxic effect to a cell population (55). The integration of the cell sheets with the decellularized scaffold was also assessed. The cell sheets of hMSCs were found to adhere successfully to the surface of the tissue revealing no gaps at the interface of the sheet and tissue. Viability of the hMSC single-cell suspension and cell sheets grown on the acellular pericardial matrices for 7 d did not reveal any significant differences. These findings demonstrate the effective decellularization of bovine pericardium while also preserving its biological properties. Decellularisation removed majority of cells while those remaining were metabolically inactive. These endogenous cell remnants possibly encouraged the growth and survival of newly introduced cells.

Penetration of a cellular network through a scaffold is challenging for tissue engineering. Difficulties arise in cultured cells penetrating, migrating and distributing in natural tissue scaffolds. While this study demonstrated similar difficulties with cells migrating to the scaffold with limited penetration, it is mainly due to the short time scale of the study with cells seeded in vitro conditions for only 7 d. Other contributory factors may be due to decellularization process removing growth factors that could promote cell migration and the distortion of the collagen fiber meshwork as caused by the SDS in the decellularisation process. Further in vivo studies would be required to investigate the long-term interaction of the cell sheet on the tissue scaffold.

The findings suggest that decellularized pericardial tissue can act as a support for hMSC cell sheet and serve as a functional framework for tissue engineering. It may also provide a microenvironmnet for the attachment and proliferation of stem cells as well as heart-specific cells for further implantation and tissue regeneration.

5. CONCLUSION

This research demonstrated that Decell BP has the ultrastructure that is suitable for the attachment and growth of cell sheets. A monolayer of hMSCs was shown to be both viable and metabolically active when seeded onto the surface of the decellularized tissue. There is scope for further development of this technology with investigation of different decellularisation protocols and their effects on the interaction of cell sheets. Also, a longer-term study using different cell type(s) with Decell BP and investigating further their compatibility and interaction is warranted.

CONFLICT OF INTEREST: The author(s) confirm that this article content has no conflicts of interest.

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Fabrication of Functional High Density Cell Sheets for Tissue Engineering Using Spin-Coated Poly(N-isopropylacrylamide) Thin Films

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Abstract
The current goal of cell sheet engineering technology is to provide an improved approach for more predictive and effective generation of viable tissue-like constructs. Success requires validation and characterization of cell sheet growth and detachment. In this study, we perform qualitative and quantitative assessments of cell sheets lifted from thin spin-coated poly(N-isopropylacrylamide) films.

Cell morphology, detachment time, metabolic activity and viability of mouse stromal and human corneal epithelial cell sheets were examined before and after lifting and after reattachment. The histological analysis of cell sheets and the immunofluorescence analysis of acting cytoskeleton, paxillin and cadherins were performed. The structure of extracellular matrix and the content of type I collagen in stromal and epithelial cell sheets were assessed by scanning electron microscopy and SDS-PAGE analysis, respectively.

Results demonstrate that lifted cell sheets remain viable and maintain tissue-like integrity that strongly depends on reinforcement of acting cytoskeleton and cadherins at cell-cell junctions. Strong cell-cell adhesions and a high density of confluent cell sheets promote collective cell sheet detachment. Preserved extracellular matrix and focal adhesion complexes support cell sheet reattachment.

This study demonstrates the potential of spin-coated poly(N-isopropylacrylamide) films in producing viable cell sheets that might be considered as a building block to create large biological tissues. Our results also describe the central cellular mechanisms involved in temperature-controlled collective cell migration. This study should promote further research in engineering three-dimensional tissues with complex organizational architecture.

Keywords
Spin-coating, Poly(N-isopropylacrylamide), Cell sheets, Cytoskeletal reorganization, Collective cell migration, Tissue engineering

Abbreviations
3D: Three-Dimensional; DAPI: 4',6-Diamidino-2-Phenylindole; DMEM: Dulbecco’s Modified Eagle Medium; DMSO: Dimethyl Sulfoxide; ECM: Extracellular Matrix; FAs: Focal Adhesions; FBS: Fetal Bovine Serum; HBSS: Hank’s Balanced Salts Solution; HCEC: Human Corneal Epithelial Cells; H&E: Hematoxylin and Eosin; LCST: Low Critical Solution Temperature; MS-5: Mouse Bone Marrow-derived Stromal Cell Line; pNIPAm: Poly(N-isopropylacrylamide); SD: Standard Deviations; SDS: Sodium Dodecyl Sulfate; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; SEM: Scanning Electron Microscopy; TCPS: Tissue Culture Polystyrene

Introduction
Cell sheet tissue engineering technologies have been rapidly developed and applied in different areas of biomedical research, including regenerative medicine [1-3], cell-based drug screening assays [4,5], and tissue and disease modelling [6-9]. Most experimental studies have used cell sheets for scaffold-free tissue engineering
Several studies have shown that cell sheet transplantation has been effective in the treatment of severe diseases such as cardiomyopathy and congestive heart failure [13], neovascular age-related macular degeneration [14], many forms of liver disease [15], diabetes mellitus [16], massive burns [17] and cartilage degeneration and defects [18]. Because cell sheets can be transplanted without sutures, this procedure saves time, reduces risk from biological materials and can avoid suture-related problems, such as inflammation and scars. This approach offers several distinctive therapeutic advantages, especially for cornea, esophageal epithelium or oral mucosa regeneration [19-21].

Currently, the most effective way of cell sheet generation involves the use of pNIPAm-based thermoresponsive substrates [22-25]. pNIPAm dissolves in cell culture media when the temperature drops below its Low Critical Solution Temperature (LCST) of about 32 °C and ultimately results in cell detachment. However, the exact mechanism of cell sheet detachment from thermoresponsive surfaces is still under investigation. Among many methods, spin-coating technique has been shown to provide an inexpensive and convenient way to prepare thermoresponsive polymer films which thickness and surface characteristics can be carefully controlled [26,27]. Proteolytic enzymes generally used to detach adherent cells can potentially damage cell surface proteins, including ion channels and growth factor receptors, destroy ECM and affect cellular signal transduction pathways [28]. In contrast, thermoresponsive polymer-based technology enables fabrication of contiguous and viable cell sheets composed of cells and functional Extracellular Matrix (ECM) avoiding the use of proteolytic reagents. In cell sheets, the cells are embedded in ECM and delivered together with secreted growth factors that might increase survival rate and the ability of cells to engraft into damaged tissue after transplantation [29,30]. Mechanosensitive properties of cell sheets, undergoing force-dependent remodeling after detachment, play an essential role in cell sheet integrity. Different components of the cytoskeleton network are important in regulation of cell sheet integrity that influences the cell sheet strength in the absence of a substrate [31,32]. In recent years, an increasing number of studies have investigated the relationship between cytoskeleton reorganization and collective cell motion in both embryonic development and normal growth of adult tissue, wound healing and metastatic invasion [33-37]. It has been suggested that cellular density may play a crucial role in these processes. As a cell sheet density increases as a result of proliferation, cells become crowded and exert a pressure on their neighbors. As the effect of pressure progressively increases, independent cellular motions become frozen, and the cells become increasingly cooperative and have to move in a collective manner [38,39]. In our previous study, it was found that 50 nm thick spin-coated poly(N-isopropylacryl-

**Materials and Methods**

**Materials**

The polymer poly(N-isopropylacrylamide) with Mn = 20000–40000, anhydrous ethanol (200 proof, > 99.5% assay), Triton-X-100, paraformaldehyde, Harris modified hematoxylin and Eosin Y, Sodium Dodecyl Sul-
fate (SDS), glutaraldehyde solution (25 wt. %), acetic acid glacial (≥ 99.8%), porcine gastric mucosa pepsin (≥ 2,500 U/mg), 0.5 M EDTA, 10 mM Tris-HCl, sodium cacodylate tri hydrate (≥ 98.0%) and DMSO (anhydrous, ≥ 99.9%) were purchased from Sigma-Aldrich (St. Louis, USA). Plastic consumables were purchased from Sarstedt (Nümbrecht, Germany). Borosilicate cover glass slides (25 mm in diameter) were purchased from VWR (Radnor, USA). Hanks Balanced Salt Solution (HBSS) and Dulbecco’s Modified Eagles Medium (DMEM) were purchased from Lonza (Basel, Switzerland). Antibiotics (penicillin-streptomycin) and Fetal Bovine Serum (FBS) were purchased from HyClone (Logan, USA). Alamar Blue® reagent, Live/Dead® viability/cytotoxicity kit, Quant-iTM Pico Green® dsDNA assay kit and Silver Quest silver staining kit were purchased from Invitrogen-Thermo Fisher Scientific (Waltham, USA). Rabbit monoclonal anti-paxillin antibody (ab32084), rabbit polyclonal anti-pan cadherin antibody (ab65292) and goat anti-rabbit secondary antibody (ab150077) were purchased from Abcam (Cambridge, UK). Phalloidin eFluor 660 (50-6559-05) was purchased from Affymetrix (Santa Clara, USA). UltraCruz™ mounting medium was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Heating dry bath from Torrey Pines Scientific (Carlsbad, USA) was used for careful temperature control.

**Thermoresponsive pNIPAm film fabrication**

Spin-coated poly(N-isopropylacrylamide) films were fabricated as described previously [26]. Briefly, a 150 μl aliquot of a 5 mg/ml ethanol polymer solution was deposited onto a slowly spinning, (150 RPM), substrate (Tissue Culture Polystyrene (TCPS) dishes (35 mm) or glass slides) for 9 s followed by rapid acceleration to 4000 RPM for 30 s, on a Laurell Technologies WS-400B-256. The spin-coated samples were slowly dried overnight in an ethanol saturated atmosphere and then left in a vacuum oven at 40 °C and 600 m Bar for a minimum of 4 h to eliminate any residual solvent. TCPS dishes were used as an under layer substrate for cell morphology analysis, qualitative cell assays (alamarBlue and PicoGreen) and cell detachment analysis. Clean and optically flat borosilicate cover glass slides were used as an under layer substrate for fluorescence microscopy analysis.

Optical profilometry analysis was used to confirm the thickness of 50 nm thick pNIPAm films as described previously [26]. Briefly, an ArF excimer laser (ATL Atlas®, Wermelskirchen, Germany) was used to ablate the selected areas on thin pNIPAm films deposited on fused silica glass discs. The thickness of pNIPAm films was measured using white light interferometry (Zygo New View 100) with an accuracy of 0.1 nm. The z-height distance between the remaining polymer and the underlying substrate was measured to accurate assess the thickness. Statistically relevant data was obtained by repeating all measurements three times, and scans of 5 randomly selected ablated windows were recorded on 3 different samples.

**Cell sheet generation and morphology observation**

For cell culture experiments, 50 nm thick pNIPAm films were sterilized under mild UV light for 2 hrs. For experimentation, MS-5 or HCEC cells were seeded on pNIPAm-coated or control uncoated TCPS dishes in triplicate at a density of 40000 cells/cm². The cells were cultivated in DMEM, supplemented with 10% FBS, 1% penicillin-streptomycin antibiotics and maintained in a humidified incubator at 37 °C and 5% CO₂. The DMEM was changed at two-day intervals. When the cells were confluent, the medium was changed daily.

The detachment of cells grown on pNIPAm films was mediated by reducing temperature. The cells were rinsed with pre-warmed HBSS to remove any traces of serum. Cold serum-free DMEM was added to cells and the samples were left on a digitally controlled thermal/cooling plate set to 4 °C until detachment.

Then the lifted cell sheets were transferred onto other control TCPS dish and incubated in a growth medium volume of 200 μl at 37 °C in a humidified incubator for 1 hr. After attachment, a final culture medium volume of 1.5 ml was added to each dish.

MS-5 and HCEC cell sheet growth and detachment were microscopically monitored, and micrographs of cells were taken using Leica inverted microscope (Leica, Solms, Germany). The experiments were repeated three times on independent days.

**Cell sheet proliferation, viability and metabolic activity analysis**

Quant-I T™ PicoGreen® dsDNA assay kit was used to assess the DNA content in lifted cells. Cell numbers in cell sheets were calculated when the cell sheets were gently lifted from pNIPAm-coated dishes, as described above. Cell numbers were obtained by calculation based on the cell DNA, using a calibration curve of total cell DNA versus known numbers of cells.

A Live/Dead cell viability assay was performed to test cell sheet viability. Titration was performed for both calcine AM and ethidium homodimer-1 to define optimal dye concentration according to the manufacture protocol. A solution of 1 μM Calcein AM and 2 μM ethidium homodimer-1 in HBSS were mixed thoroughly, and then added to the cells for 20 min at 37 °C. The staining with calcine AM resulted in bright green fluorescence in the cytoplasm of viable cells with intact cell membranes.
Ethidium homodimer stained dead cells red. The control dead cells were prepared by the treatment of cultured cells with cell death inducing agent, Dimethyl Sulfoxide (DMSO), for 20 min.

AlamarBlue\textsuperscript* assay was used to examine metabolic activity. Cell metabolism was expressed as the fluorescence per cell after the fluorescence was divided by the number of vital cells in a sample.

All assays were performed following the manufacturer's instructions before and after cell sheet detachment and after reattachment. AlamarBlue and PicoGreen fluorescence was measured using a Thermo Scientific Varioskan Flash Multi-mode plate reader. The fluorescent staining of live and dead cells was observed using an Olympus IX81 fluorescence microscope (Olympus) and images were captured using a DP72 CCD camera (Olympus) linked to cellSens Dimension software (Olympus). Each assay was repeated three times on independent days.

Immunofluorescent staining and image analysis

The MS-5 and HCEC cell sheets were fixed with 4% paraformaldehyde and then permeabilized with Triton X-100 (0.1%). Afterwards, the cell sheets were incubated with 3% goat normal serum for 1 h at 37 °C to block non-specific binding. Cell sheets were then incubated with rabbit monoclonal anti-paxillin antibody at 1: 250 dilution or rabbit polyclonal anti-pan cadherin antibody at 1: 100 dilution at 37 °C for 1 hr. The slides were washed with HBSS followed by incubation for 1 h with a 1: 1000 dilution of Alexa Fluor\textsuperscript* 488 nm goat anti-rabbit secondary antibody at 37 °C. Actin stress fibers were detected using Phalloidin eFluor 660 at 1: 500 dilutions. After rinsing with HBSS, the samples were mounted with Ultra Cruz TM mounting medium containing 1.5 µg/ml DAPI for nuclear counter staining. All stained slides were observed using Andor spinning disc confocal microscope.

To quantify actin or cadherin levels in cells, an outline of 30 × 30 pixels was drawn around each cell junction region (n > 10), and the area, integrated density along with mean fluorescence of background readings were measured using Image J software (NIH, Bethesda, USA). The calculation of Total Corrected Cellular Fluorescence (TCCF) was performed according to formula, TCCF = integrated density - (area of selected cell x mean fluorescence of background readings) \cite{46}. Relative actin or cadherin intensity levels at cell junctions in lifted cell sheets were normalized to unlifted cell sheet controls. For nuclear quantification, images from 10 independent fields per slide and 50-200 cells per image excluding those crossing the right and the bottom boundary were counted. All images were analyzed as Tiff files. The absence of nonspecific binding was confirmed by the use of appropriate primary and secondary antibody controls.

Histological examination

After being incubated for 2 weeks, the MS-5 and HCEC sheets were detached from dishes and fixed with 4% paraformaldehyde solution for 24 hrs. Specimens were then embedded in paraffin using an automated tissue processing machine (Leica ASP300), sectioned using a microtome (Leica RM2235) and stained with Hematoxylin and Eosin (H&E) according to manufacturer's instruction. Images were captured with Olympus IX-81 inverted microscope. Thicknesses of cross-sectioned cell sheets were measured using Image J software.

Extraction of ECM from cell sheets

To prepare and analyze ECM, lifted MS-5 or HCEC cell sheets cultured for 2 weeks were decellularized using low concentration of SDS as previously described \cite{47}. Briefly, the cell sheets were shaken for 30 min in decellularization solution containing 0.05 wt% SDS, 10 mM Tris, and 25 mM EDTA and then thoroughly washed with HBSS.

Scanning electron microscopy

The decellularized cell sheets were analyzed using SEM. For SEM imaging, the ECM specimens were first fixed in a 0.2 M sodium cacodylate buffer (pH 7.2) containing 2% paraformaldehyde and 2.5% glutaraldehyde for 10 min at room temperature, dehydrated in a graded ethanol series, air-dried and finally sputter-coated with gold and examined using a Hitachi S-4700 SEM. The specimens were examined at 15 kV in high vacuum mode.

SDS-PAGE analysis of type I collagen

The MS-5 and HCEC cell sheets were washed twice with HBSS and digested with porcine gastric mucosa pepsin in a final concentration of 0.1 mg/ml in 0.05 M acetic acid. Samples were incubated at 37 °C for 2 h with gentle shaking. After incubation, the samples were neutralized with 0.1 N NaOH. The samples were then analyzed by SDS-PAGE under non-reducing conditions \cite{48}. Briefly, 0.1 mg/ml of bovine type I collagen was used as standards with every gel. The 10% SDS gel made up of 3% stacking and 5% resolving components were used. Collagen bands were stained using Silver Quest kit (Invitrogen) according to manufacturer’s protocol. For this experiment, type I collagen α1 and α2 chains were quantified. Densitometric analysis was performed to quantify collagen bands in wet gels. The amount of each collagen band was calculated using a rectangular tool with background subtraction provided by Image J software (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed using Statistica 8.0 software. Values are expressed as mean ± Standard Devi-
Results

Generation and morphology of MS-5 and HCEC sheets

The morphology and size of cell sheets, cell numbers and time required for detachment of intact MS-5 and HCECs sheets were compared (Table 1). Microscopy analysis showed that the cells, when initially seeded
on pNIPAm-coated surface, attached, expanded and reached confluence after 24 hrs. MS-5 cells were fully spread and had spindle-shaped morphology and HCEC displayed normal cobblestone-like cell morphology (Figure 1A). During and immediately after low temperature treatment, the cells became less spread and flattened and started to detach (Figure 1B). The visual tracking revealed that one-week and two-week MS-5 started to detach as a contiguous cell sheet first from the dish edges only, followed by the complete detachment from the substrate within around 10 min. In contrast, one-week HCEC did not detach as an intact cell sheet, and only those HCEC cultured on pNIPAm surface for 2 weeks were able to detach as an intact sheet within around 40 min. Moreover, in order to facilitate cell sheet recovery, the partly lifted premature one-week HCEC sheets had to be gently rinsed with culture medium against the detached part of the cell sheets (Figure 1C).

There was no significant difference in a cell sheet area or in shrinkage rate between two-week MS-5 and two-week HCEC lifted cell sheets. One-week MS-5 sheets had a smaller area and a higher shrinkage rate than two-week MS-5 sheets (Figure 2).

**Viability and metabolic activity of MS-5 and HCEC sheets**

To ensure that long-term culture, cell sheet detachment and manipulation do not affect cell behavior, viability and metabolic activity assays were applied to cell sheets after 2 weeks in culture under several conditions: prior to cell sheet detachment, immediately after cell sheet lifting and 48 h after reattachment of lifted cell sheets. Importantly, the lifted cell sheets reattached faster, within around 20 min, than a single cell suspension that attached within 3-4 h. The viability test was done on detached and reattached MS-5 and HCEC sheets with

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### Figure 2: Intact MS-5 and HCEC sheets contracted after detachment from pNIPAm films. During and immediately after lifting, one-week MS-5, two-week MS-5 and two-week HCEC sheets shrunk to 11%, 40% and 44% of their original area, respectively. *p < 0.05, ***p < 0.01. Data are expressed as the mean ± Standard Deviation (SD).

### Figure 3: Live/Dead fluorescent staining of two-week MS-5 and two-week HCEC sheets before detachment, immediately after detachment and 48 h after reattachment to TCPS. Red fluorescence indicates the dead cells. Alive cells with intact cell membranes fluoresce green. The control for each cell type was treated with cell death-inducing agent DMSO. Scale bar 100 μm.
the cell membrane. The detached HCEC sheets also displayed stronger cortical fluorescent ring of filamentous actin (Figure 5A). DAPI nuclear staining showed that lifted MS-5 and HCEC cell sheets exhibited significant increase in nuclear density (Figure 5B). The cell sheet detachment resulted in cell sheet contraction and significant increase of cell-cell contact area. The quantitative analysis of fluorescence signal at cell-cell junctions confirmed that the level of cortical actin was significantly increased in around 2.8 times and 1.5 times for MS-5 and HCEC sheets, respectively (Figure 5C). The analysis of cell-cell adhesion protein cadherins revealed the preservation of adherence junctions after cell sheet detachment. Moreover, the lifted MS-5 and HCEC cells showed enhanced junctional localization of cadherins. The level of cadherins at cell-cell junctions was increased in 2.5 and 1.6 times for lifted MS-5 and HCEC sheets, respectively (Figure 5D). Results demonstrated that both actin filaments and cell adhesion proteins cadherins were reinforced at cell-cell junctions in lifted cell sheets indicating their cooperative roles during and immediately after cell sheet detachment.

**ECM deposition and focal adhesion analysis in MS-5 and HCEC cell sheets**

ECM deposition and focal adhesion analysis was performed to assess their contribution to cell sheet remodeling during cell sheet detachment and reattachment. Paxillin is a key component of focal adhesion network, found in regions closely adherent to the substratum and

![Figure 4: Results of alamarBlue assay for two-week MS-5 and two-week HCEC sheet before and after detachment and 48 h after reattachment to TCPS. Data is mean ± Standard Deviation (SD), no significant difference compared to control cells grown on TCPS.](image-url)
Figure 5: Cell sheet contraction induces the coordinated reorganization of the cytoskeleton of two-week MS-5 and two-week HCEC lifted cell sheets. A) Fluorescence images of F-actin (red), cadherins (green), DAPI nuclear staining (blue) in control and lifted cell sheets show the spatial rearrangement of actin filaments and cadherins. Scale bar: 20 μm; B) Both MS-5 and HCEC lifted cell sheets showed significant increase in nuclei density indicating cell sheet contraction. *p < 0.05 compared with unlifted control. **p < 0.05 for lifted MS-5 versus lifted HCEC; C,D) Quantitative analysis of actin and cadherins at cell-cell junctions showed the increase of both cortical actin and cadherins in lifted MS-5 and HCEC sheets. *p < 0.05 compared with unlifted control. **p < 0.05 for lifted MS-5 versus lifted HCEC. Data are expressed as the mean ± Standard Deviation (SD).
involved in attaching the act in cytoskeleton to the ECM. Paxillin was evenly distributed before MS-5 or HCEC cell sheet lifting. However, in lifted cell sheets, paxillin was enhanced and organized into cortical networks similar to the arrangement of act in bundles (Figure 6).

The histological analysis of cross-sections of lifted cell sheets revealed that two-week MS-5 sheets were composed of 4-5 well cell layers with an approximately 40 µm in thickness, and extracellular matrix was abundant. In contrast, two-week HCEC sheets were composed of approximately 2-3 layers with 10-20 µm in thickness, and the extracellular matrix was undeveloped or absent (Figure 7A).

SEM images of decellularized MS-5 sheets confirmed abundant ECM depositions with three-dimensional tightly packed and well-organized fibrous networks. In contrast, ECM fibers in HCEC sheets were loosely packed with more spaces between fibers (Figure 7B). Densitometry analysis confirmed the presence of type I collagen main chain bands, α1 and α2, only in two-week MS-5 sheets which were absent in cell extract samples from two-week HCEC sheets (Figure 7C and Figure 7D).

Discussion

Despite the wide use of pNIPAm-coated thermoresponsive surfaces, little is known about the main factors influencing the successful production of intact cell sheets. Several studies have used cell sheets as a model to investigate wound healing and to describe the mechanisms regulating cell sheet motilities during wound repair. The thought-provoking study, performed by Rosen and Misfeldt, showed that when confluent kidney epithelial (MDCK) cells were wounded, they enable to migrate into the denuded area as a cell sheet only after a threshold density was reached [49]. The authors suggested that the velocity of cells as a contiguous cell sheet could be the result of a force generated from the mutual pressures caused by the crowding cells. It was concluded that the confluent cells can be detached as a cell sheet only when its density is at or above that threshold density and they possess the property of motion as
a united cell sheet. Another study analyzed collective cell behavior using a computational model [50]. In this model, Lober and co-authors used a series of parameters including reorganization of adhesion complexes and actomyosin-induced cell contraction to decipher the mechanisms of cellular motility. The study revealed
that the collisions of neighboring individual cells cause the formation of coordinately moving high density tissue-like clusters. These studies corroborated our hypothesis that the trigger mechanism of cell sheet detachment from pNIPAm-coated substrate is closely related to the density-dependent forces. As temperature drops to 4 °C, the cells lose their contacts with a pNIPAm coated film which dissolves in cell culture medium. As a result of being restricted in their movements, detaching and highly-packed cells release the inner pressure that promotes the further detachment of cells as a contiguous cell sheet. In other words, this might indicate that intact cell sheets could be detached from pNIPAm-coated substrates only when a confluent layer reaches the critical cell density. In our study, both lifted epithelial and stromal cells were confluent and formed multilayered sheets. Stromal MS-5 sheets, which were compared to epithelial HCEC sheets, were found to be able to detach as an intact cell sheet faster (10 ± 3 min vs. 35 ± 5 min, respectively) and after a shorter period in culture (1 week vs. 2 weeks, respectively). Moreover, the number of cells needed for successful cell sheet detachment was significantly different between HCEC and MS-5 cells (0.8 ± 0.1 × 10^6 and 2.2 ± 0.3 × 10^6, respectively; p < 0.05). Taken together, this implies that the trigger mechanism of cell sheet detachment from pNIPAm films depends on cell type and requires high cell density cultivation.

High cell density results in contact inhibition and drives the cells to enter a quiescence state. In the present study, alamarBlue assay showed that lifted and reattached cell sheet’s metabolic activity was the same as in control cells. Live/Dead staining revealed that the percentage of viable cells stained with calcein AM reached almost 100% on all tested samples before and after detachment and reattachment. These data indicate that despite the culturing of cells at high density, the cell sheets remained viable and highly metabolically active. Our results corroborated the recent study showing that contact inhibited primary fibroblasts remain highly metabolically active resembling terminally differentiated cells like epithelial cells or cardio myocytes, which are characterized by very high energy consumption [51]. Another interesting study, published by Blagosklonny and co-authors, showed that contact inhibited cells did not undergo senescence and retained proliferative potential after splitting [52]. Together, these data and our present findings suggest that high density cell sheets may retain their biological activity after detachment and reattachment, indicating their potential application in cell sheet-based tissue engineering.

Cellular movements are driven by continuous and dynamic reorganization of actin cytoskeleton [53]. Huang, et al. previously revealed that lifted keratinocyte cell sheets exhibited changes in the distribution of actin cytoskeletal. It was shown that act in cytoskeleton is an essential contributor to cell sheet formation, its mechanical strength and cohesion [31]. The actin cytoskeleton of neighboring cells is connected through cell-cell junctions that seal cells together. Other major players in regulating tissue cohesion and homeostasis are cadherins. Cadherin ectodomains mediate cell-cell contacts, whereas the intracellular functionally links cadherins to the underlying actin cytoskeleton [54,55]. It was shown that E-cadherin is critical for collective epithelial cell sheet movements [56] whereas N-cadherin which is considered as a marker for the identification of bone marrow stromal cells [57] can also be involved in cell-cell contacts during the motions of high density cultured stromal cell sheets [58,59]. Shih and Yamada have shown that the up-regulation of dynamic cadherin-actin interactions in MDCK epithelial cells can be essential for collective cell movements in a three-dimensional matrix [60]. In the present study, lifted MS-5 and HCEC cell sheets significantly increased the fluorescence signal from cortical actin and cadherins at cell-cell junctions. Our results showed that lifted MS-5 and HCEC cell sheets lost cytoplasm actin but instead enhanced cortical actin fibers at cell-cell junctions. This implies that cell-cell junctions were sensitive to cell sheet detachment, and that the assembly of cadherins and actin fibers across intercellular junctions may represent a major mechanism that maintains the integrity of lifted cell sheets. ECM is another important factor that may accelerate tissue maturation in terms of mechanical properties and structural integrity. The thermoresponsive pNIPAm-coated substrates may offer significant advantage for cell sheet technology allowing for the preservation of cell-ECM contacts after detachment. Previously Cana van, et al. have shown that ECM remains preserved and attached to the cell sheets after rapid recovery from pNIPAm-coated surfaces [61,62]. The strong interactions between detaching cells and ECM might additionally facilitate rapid recovery of intact cell sheets. Microscopy analysis confirmed that MS-5 stromal sheets composed of higher number of cell layers with a dense network of ECM fibers whereas HCEC epithelial sheets revealed poor and undeveloped ECM formation. These data are in agreement with SDS-PAGE and complementary densitometric analysis which confirmed the deposition of type I collagen in MS-5 sheets. Therefore, ECM of MS-5 sheets generated a stronger contractile force facilitating a rapid lifting from a substrate by physically pulling up the shrunken cell sheet. This might result in a higher speed of MS-5 cell sheet recovery in comparison with HCEC cell sheets. In addition, one-week MS-5 cell sheets were stronger and easily manipulated. In contrast, one-week HCEC sheets were more fragile and partly broken in culture that might
Conclusions

Taken together, the data show substantial changes in structural assembly of intercellular junctions of cell sheets undergoing force-dependent remodeling during detachment. The intact cell sheets lifted from pNIPAm-coated substrate are able to preserve structural integrity possibly due to being highly-reactive towards lifting that allowed them to rapidly reinforce their cytoskeleton network and cell-cell adhesions. The cell sheets acquire mechanosensitive properties if they reach critical cell density that depends on cell type. It is unlikely that ECM deposition play a key role in cell sheet detachment. However, the cells produced higher level of ECM may facilitate a more rapid cell sheet detachment at an earlier culture time. In addition, ECM and preserved focal adhesions that serve as mechanical linkages to the ECM promote successful reattachment of lifted cell sheets despite their shrinkage. Based on our results, we speculate that the dissolution of pNIPAm substrate might be an initial factor in the mechanism where together the accumulation of internal pressure within high density cell sheets, the assembly of cadherins and actin fibers across intercellular junctions and contractile force generated by ECM contraction result in contiguous cell sheet recovery.

The cell sheet harvesting using thin spin-coated pNIPAm films represents a convenient and cost-effective technique for 3D tissue engineering. This method has advantage of creating viable, metabolically active and open for manipulation cell sheets. The control of cell sheet characteristics including cell sheet density, structural integrity and the ability of rapid reattachment can improve the safety and efficacy of regenerative therapy. Understanding the mechanism of cell sheet formation and maturation is important for standardization and optimization of cell sheet engineering which has become an important tool in tissue engineering and regenerative medicine.

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Conflict of Interest

The authors declare no competing interests.

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