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Strategies for the stabilization and preservation of prosthetic heart valves

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Strategies for stabilization and preservation of heart tissues for valve replacement

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Abstract

Every year around 60000 heart valve replacements are performed in the USA alone and more than 250000 worldwide. Valve replacement involves two major types: the mechanical valves (MHVs) and biological valves (BHVs), while MHVs are more durable they require long term anti-coagulation treatment. Biological valves possess the inherent capacity to be much more compatible with the human physiology, using crosslinking agents like glutaraldehyde (GLUT), it is possible to increase durability of the tissue by making it stiffer. This process involves the use of tissues like pericardium from animals like cows and pigs or even the autologous from the patient, after crosslinking these tissues are then mounted on stents which are inserted into the heart to replace the old valve. The problem is that through calcification processes these valves can only last at most 15 years average.

In this work, the development of an easy and fast method to crosslink pericardium using other agents besides glutaraldehyde such as sodium periodate or a carbodiimide system (EDC/NHS) is proposed. In order to optimize the process, the reactions were studied in a range of times and concentrations. Mechanical behavior was studied using a tensile tests and it is important to emphasize that was possible to obtain better samples than when using GLUT cross-linking. Chemical studies of the tissue involved FTIR-ATR, NMR and HPIC of hydrolyzed tissue to determine which amino acids reacted with the crosslinking agents. Morphological analysis involved the use of SEM in order to see the arrangement of the collagen fibres present in the pericardium tissue. Assays mimicking *in vivo* calcification were performed by immersing samples in SBF solution for 7 days. On a second phase the addition of hyaluronic acid to the tissue is studied while periodate or EDC crosslinking is taking place. Generally, it is possible to conclude a much greater resistance to calcification processes maintaining good biomechanical properties or in some cases improving.

Keywords: Pericardium, Glutaraldehyde, Glycosaminoglycans, Sodium Metaperiodate, EDC/NHS,

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Resumo

Todos os anos são realizados em média 60000 transplantes de válvulas cardíacas nos Estados Unidos apenas e mais de 250000 no mundo inteiro. A substituição de válvulas envolve dois tipos: as válvulas mecânicas e as válvulas biológicas, enquanto as mecânicas possuem uma maior durabilidade exigem que o paciente tome anticoagulantes durante o resto da sua vida. As válvulas biológicas têm a capacidade inerente de serem muito mais compatíveis com a fisiologia humana, utilizando agentes de fixação como glutaraldeído (GLUT) é possível aumentar a sua durabilidade, fazendo com que estas fiquem mais rijas. Este processo envolve o uso de tecidos como o pericárdio vindo de animais como vacas ou porcos ou até mesmo utilizando o pericárdio autógolo vindo do próprio paciente. Após o processo de reticulação o tecido é montado numa prótese que é inserida no coração de modo a substituir a válvula antiga. O problema surge quando devido a calcificações na válvula a durabilidade destas é de apenas 15 anos em média. Neste trabalho pretendemos desenvolver um método rápido e fácil de reticulação de pericárdio recorrendo a agentes de fixação para além do glutaraldeído, neste caso metaperiodato de sódio ou uma carbodiimida como por exemplo EDC/NHS. As reações foram estudadas ao longo do tempo e usando duas concentrações. Através de estudos de tração foram realizados verificou-se que algumas amostras apresentavam melhores resultados do que quando se utiliza GLUT. Estudos químicos do tecido fizeram-se através de FTIR-ATR, NMR e HPIC do tecido hidrolisado por forma a determinar que aminoácidos reagiram mais com os agentes de fixação. Análise morfológica efetuou-se através de SEM de modo a observar o rearranjo das fibras de colagénio presentes no tecido de pericárdio. Ensaios biomiméticos de calcificação *in vivo* fizeram-se imerjindo as amostras numa solução de SBF durante 7 dias. Numa segunda fase adicionou-se ácido hialurónico ao tecido ao mesmo tempo que os agentes de fixação (periodato e EDC/NHS), isto permitiu obter tecido mais resistentes à calcificação e ao mesmo tempo manter as propriedades biomecânicas ou até mesmo nalguns casos haver melhorias

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Palavras-chave: Pericárdio, Glutaraldeído, Glicosaminoglicanos, Periodato de sódio. EDC/NHS

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1.Introduction

In 2017, according to the World Health Organization (WHO) Our World in Data, cardiovascular diseases are the main cause of death in western countries, surpassing even cancer by more than double the number of deaths[1]. The use of Bioprosthetic heart valves (BHVs) began in the 50s with the first transplant surgery performed, in 1952, by Charles A. Hufnagel[2].

Since then technological advancement improved in such a way that many patients suffering from valvular heart diseases can have a better quality of life, mostly due to new prosthetic heart valves, being them either mechanical or biological, even when valve repair is not an option. Each year around 60000 valve replacements are performed in the USA alone and more than 250000 worldwide, of these approximately 55% are mechanical valves (MHVs) and 45% are biological (BHVs)[3].

1.1Anatomy of the heart valves

Heart valves allow unidirectional blood flow throughout the cardiac cycle with minimum blockage and with no return, aortic valves prevent the blood from going backwards into the ventricles during diastole and the atrioventricular and mitral valves prevent reverse flow from ventricle to the atrium during systole. The valves are tissue structures in which movement is driven by mechanical forces applied by the surrounding blood and heart. Their ability to allow blood to pass unobstructed depends on mobility, pliability and general structural integrity of the leaflets and cusps. Valves are thin enough to be nourished simply by diffusion from the blood passing, normal leaflets and cusps have only few blood vessels limited to proximal portions[4].

1.2ECM: Composition and role

To maintain unidirectional blood flow healthy native heart valves, need to possess enough strength and durability to withstand repetitive and great mechanical stress and strain throughout many years, in a single average lifetime heart valves open and close around 3 billion times. All four cardiac valves have a similar composition, they are formed by a dense collagenous layer continuous along the valvular structure, this will provide the main strength to the valve, a central part of loose connective tissue and a layer rich in elastin. In the aortic valve these are called

fibrosa, spongiosa and *ventricularis* respectively. The heart valves are also made of a small number of cells, predominantly valve endothelial cells located at the blood-contacting surfaces and the deep valve interstitial cells, as well as the extracellular matrix (ECM) which comprises collagen, elastin and glycosaminoglycans (GAGs) (**Table 1**)[4].

Table 1 Major components of the aortic valve [5]

Component	Location	Putative function
Endothelial cells	Lining inflow and outflow valve surfaces	Provide thrombo-resistance, mediate inflammation
Intersesticial cells	Deep to surface, throughout all layers	Synthesize and remodel matrix elements
Elastin	Concentrated in ventricularis layer	Extend in diastole, recoil in systole
Glycosaminoglycans	Concentrated in spongiosa layer	Absorb shear of relative movements and cushion shock between ventricularis and fibrosa during cyclical valve motion
Collagen	Concentrated in fibrosa layer	Provide strength and stiffness to maintain coaptation during diastole

1.2.1Collagen

Collagen is a protein rich in three major amino acids such as glycine, proline and hydroxyproline, which forms long chain subunits with tendency for aggregation into a triple helix. Glycine is the simplest amino acid in nature and is found approximately in every third residue thus playing a major role in the collagen triple helix structure organization. Its small size allows it to adequately fit within each helical fibre providing space for other larger amino acid residues to better accommodate. The collagen matrix in healthy aortic valves is predominantly rich in type I (around 70%) and some significant amount of type III (25%). Both are first synthesized as procollagens containing propeptide extensions at both ends of the molecule. These propeptides are post secreted into the ECM and then removed while the collagen molecules aggregate forming the fibril. The stabilization of this fibril is mainly due to intra and intermolecular cross-linking of which lysine (Lys) and hydroxylysine (Hyl) residues, located at the ends of the chain, are mostly involved[6].

Other aminoacids present in the chain are also essential for the formation of this helical structure, such as proline and hydroxyproline, by establishing strong hydrogen bonds. Collagen comprises around 55% of the dry weight from a valve cusp[7].

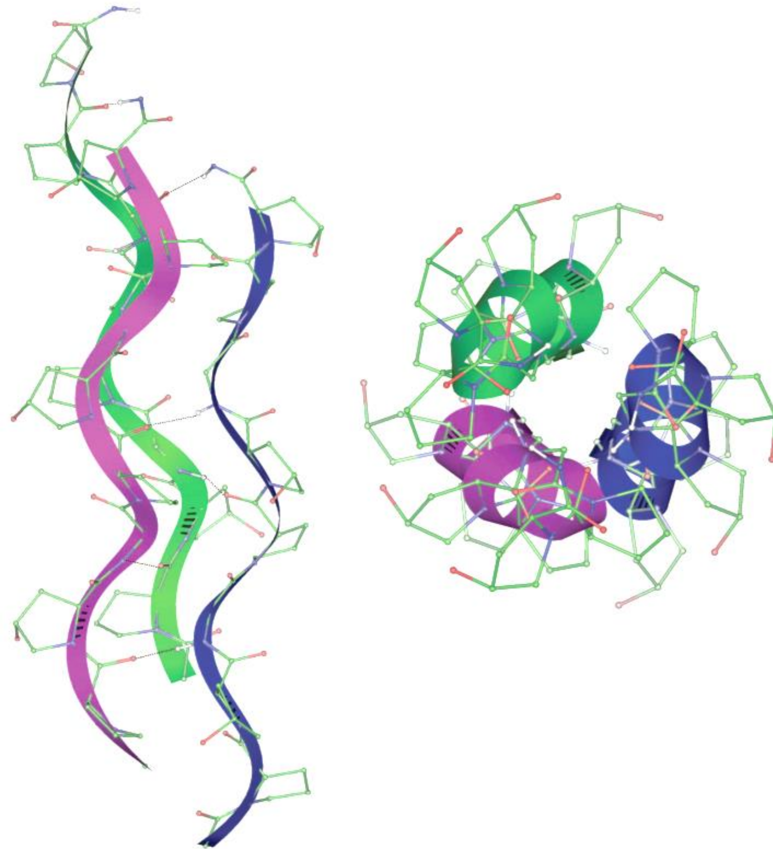

Figure 1 Triple helix collagen structure projections[8]

The fast and reversible changes are facilitated by the mechanical responses provided by the ECM components. Of these, the major stress bearing is collagen, by holding high tensile forces when stretched. However, it cannot be compressed, in contrast to elastin which can both stretch and contract. So, besides simple stretching and shortening, collagen suffers directional realignment and crimping during the cardiac cycle. The relative orientation of the collagen fibres in regions of the cusps determine the directions in which the tissue has the greatest compliance (orthogonal to the collagen fibre orientation) or can withstand the most tensile stress (parallel to collagen fibre). The glycosaminoglycan rich spongiosa layer is essential to the rearrangement of the collagen and elastin layers. There is also note that strains during mechanical movement of the aortic valve cusps are extremely anisotropic[4].

1.2.2Elastin

Elastin is a key ECM that provides resilience and elasticity to tissues and organs, it is a fibrillar protein found in connective tissue, making around 13% of the dry weight found in valves. Generally, it is formed by tropoelastin subunits and produce the microfibres that in turn form the elastin fibres. This protein provides most of the elasticity of connective tissue as well as heart valves[4].

The tropoelastin monomer possesses high amounts of hydrophobic aminoacid residues like glycine, valine, alanine, lysine and proline. Alanine and lysine parts form α-helix while glycine, valine and proline form β-sheets. Tropoelastin is formed by fibroblasts, smooth muscle cells, chondrocytes or endothelial cells, it is then processed by cleaving the signal peptide forming the final elastin. Elastin monomers are then crosslinked during the formation of desmosine molecules that are in turn synthesized by crosslinking three allysine and one lysine residues (**Figure 2**). The whole process is facilitated by the enzyme lysil oxidase which catalyses the oxidation of elastin residues into allysine (oxidative deamination - NH_2 to C=O). Several differently rich domains of elastin, from alanine rich sites, polyalanine domain (residues 97-102) and multiple hydroxyproline and allysine sites that confer elastin its high hydrophobicity and stability having a half-life up to 70 years. It is only produced during developmental periods; thus, degradation cannot be properly repaired resulting in deterioration of the ECM and progressive valvular disease[9,10].

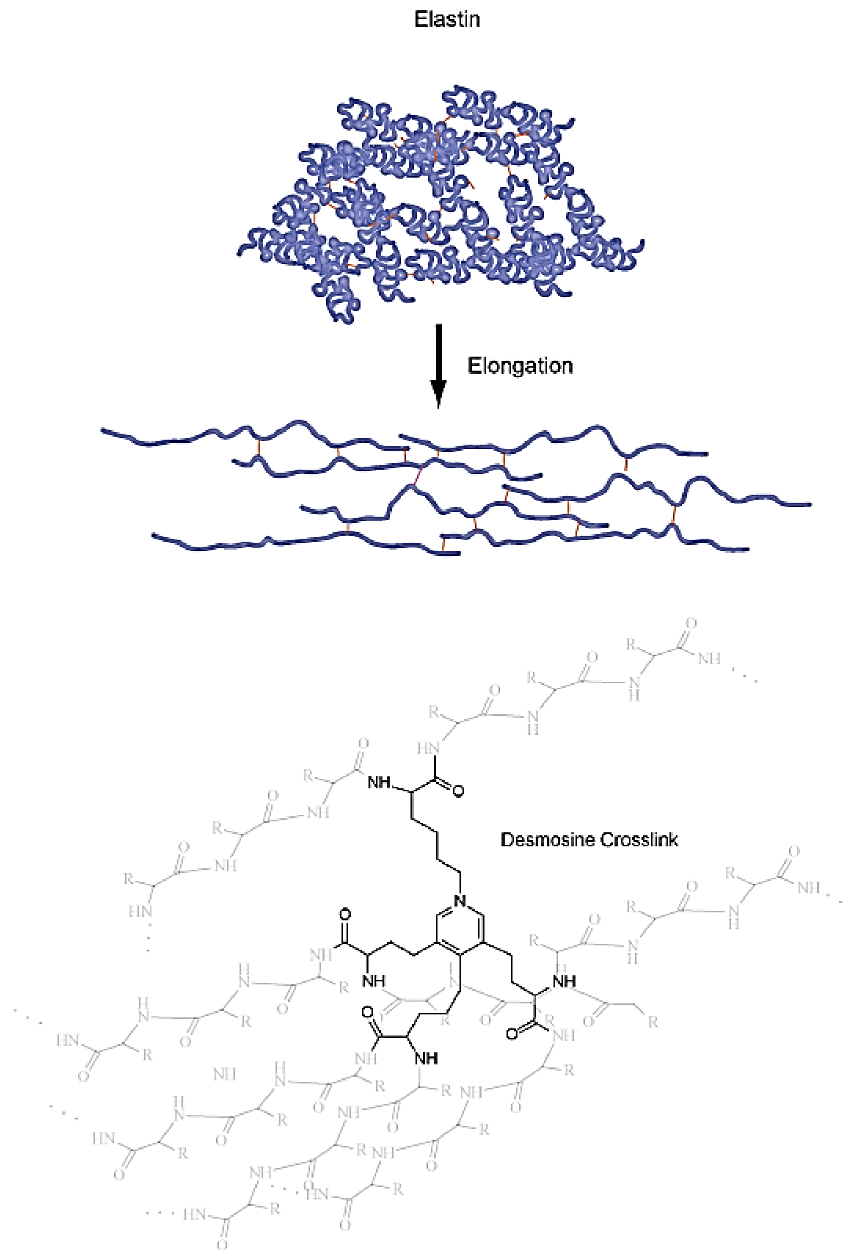

Figure 2. Elastin structure during stretching and relaxation and also desmosine crosslinking[11]

Elastin functions by providing mechanical loads at low strain, its elastic properties allow it to recoil maintaining up to 150% strain with no failure. Intrafibrillar connections link elastin and collagen allowing this to be guided when relaxation of a valve occurs. Several mechanical tests showed elastin bears tension at low strains, enabling collagen fibres to uncrimp and bear higher loads, due to the lower extensibility of collagen fibres. It is important to note that elastin is essential to the good functioning of heart valves and that its deterioration greatly affects valve cusps[11,12].

1.2.3Glycosaminoglycans

Glycosaminoglycans (GAGs) are long polysaccharides made of repeating disaccharide units composed by hexosamine and a sugar (hexuronic acid or hexose). These copolymers can be found throughout all the human body, especially in the connective tissue which includes cartilage, tendons, synovial fluid and heart valve cusps. GAGs are separated in two groups: sulfonated (e.g. chondroitin sulphate, dermatan sulphate, keratin sulphate, heparin sulphate) and non-sulfonated systems (hyaluronan). These can be found primarily aggregated in the spongiosa layer forming a brush like structure via glyosidic bonds as evidenced by electron microscopy (**Figure 3**)[7].

Proteoglycans are formed by sulfonated GAGs attached to a central protein core, forming the cell's glycocalyx; however, hyaluronan can act at the protein core[13]

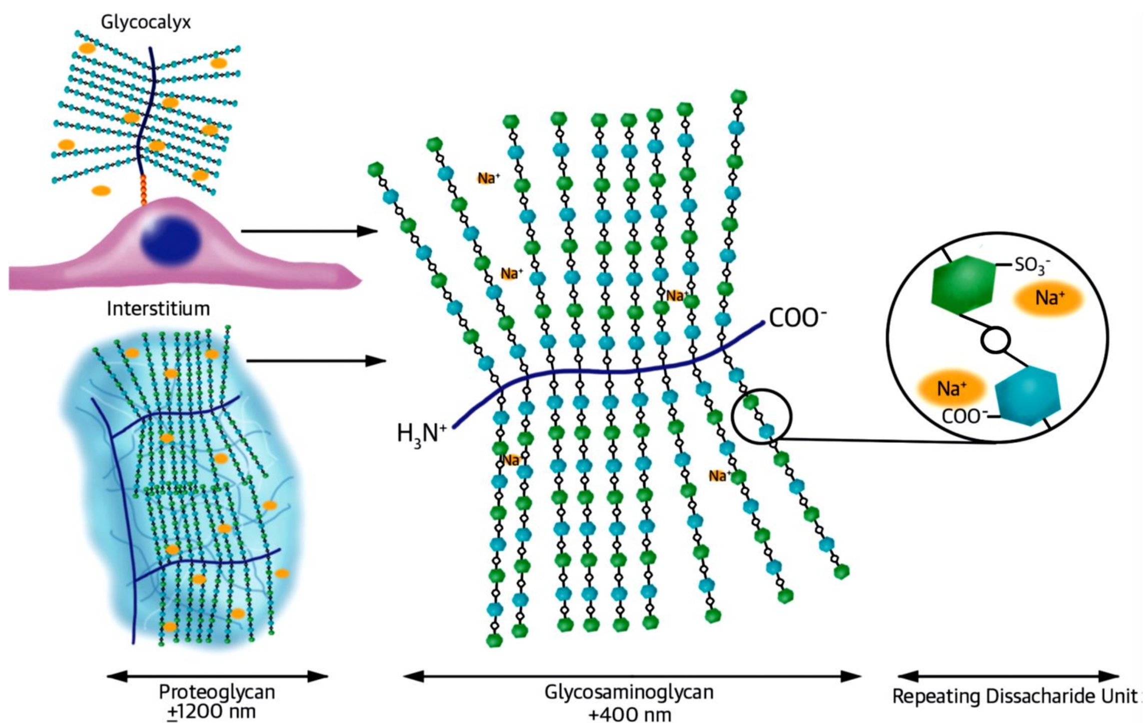

Figure 3. Diagram of glycosaminoglycans, composition and position in proteoglycan adapted from[14]

In heart valves, hyaluronan that it is formed in the protein core and chondroitin sulphate the side chain brush like structures. They possess innate high hydrophilicity, derived from the anionic properties of both hexosamine and uronic acid units, particularly carboxylate and sulfonate groups. In turn this hydrophilicity allows GAG structures to absorb a great amount of water forming a gel in the spongiosa layer contributing to stress and compressive force dissipation during native heart valve function[14].

Believed to act as an extension of the *ventricularis* and fibrosa layers, they are important when analysing heart valve clinical failure. Other functions are al-so essential to managing the structure of the proteoglycan such as binding proteins via amino acid residues using the carboxylate and sulphonate groups. They also possess other *in vivo* properties: e.g. anticoagulant in the vasculature, composing and hydrating cartilage. Additionally, they are part of all body cell's surface and extracellular matrix components. In the native heart, they have a high turnover rate, old GAGs (2 to 14 days) are digested and new GAGs are produced by the cells within the heart valve tissue. This continuous renovation of the GAG network remains an important factor when maintaining proper valve health and function[8,15]. When the adequate performance of the heart valves gets compromised, substitute prosthetic heart valves are most frequently implanted.

1.3Heart valves: mechanical vs biological

Mechanical heart valves were firstly used in 1952 by Dr. Charles Hufnagel. He performed the first surgery in a patient with aortic valve disease and a caged ball valve in the descending thoracic aorta was implanted. Seven years later, Dr.Dwight Harken used a caged ball valve in the subcoronary position in a patient with aortic stenosis. After this period, more than 70 different types of prosthetic valves and a great number of different tissue valves have been implanted in hundreds of thousands of patients around the world. During the first operations involving mechanical valves the mortality reached between 15 and 20%, but today, in most heart centres in the world, it is lower than 2% and the complications involving thromboembolism and endocarditis are extremely low. In 50 years of mechanical valves manufacturing, many types and models have been developed, although today that number is reduced because a high number of models have been discontinued. Nowadays, the most used are the Starr-Edwards ball valve, the Omniscience, Omnicarbon and Medtronic-Hall tilting disc valves, the St. Jude and Carbomedic bileaflet valves **(Figure 4)**[16].

Figure 4. Types of mechanical valves[17]

Mechanical Valves are all made of non-biological materials such as polymers, carbon alloys or metals. Typically, mechanical valves last between 20 to 30 years in adult age (much less in infancy), however life-long anticoagulant therapies are demanded to the patients with consequent immunological side effects. In contrast, bioprostheses have a much lower thromboembolic predisposition and thus there is no need for long-term anticoagulation medication[18].

1.4Bioprosthetic Valves

Biological or bioprosthetic heart valves (BHVs) are made from human or animal tissues mounted on a stent covered with fabric. These valves are different from one another mostly due to composition but also other intrinsic characteristics such as durability, thrombogenicity and haemodynamic profile. The shift from MHVs to BHVs is mostly related to their increased durability and reduced risk of haemorrhage and are suitable for implantation at any age. Bioprosthetic valves have also other more desirable properties such as structural similarity to the native tissue and ease of handling and implanting, as there is no need for an open-heart surgery. Conversely, bioprosthetic valves are derived from animal tissue, often referred to as xenograft or heterograft valves Most often these valves come from porcine hearts or made from bovine pericardial tissue. Both tissues must be previously crosslinked, typically using glutaraldehyde, in order to obtain suitable mechanical and haemodynamic properties. Then, they are incorporated on stents and sutured percutaneously[19].

Despite heart valve replacement offers a satisfactory solution to the problem at hand, it is extremely important to study the long-term problems associated with these implants. Deficient valve leaflets can be extended or re-placed, although it has been reported that an extension of a single cusp is not a feasible method. In this way, the extension of three cusps or replacement with a single bovine pericardium tissue is many times more reliable. From 1988 to 1995, Dr Zohair Halees observed 92 patients who had been subjected to valve reconstruction using bovine pericardium or glutaraldehyde treated autologous pericardium. The patients were followed from 9 to 16 years in which many aspects were evaluated including the degradation of the valves, freedom from all events and reoperation rate. It was believed that the autologous pericardium would be better than bovine and although within the first months this can be true but after 16 years was no statistical difference be-tween the two groups is mentioned. This proves that many factors besides the materials of choice should be considered when choosing a valve, age and the type of heart disease. These parameters seem to have an important impact upon the performance of the valve implant and their durability over time (**Figure 5**)[20].

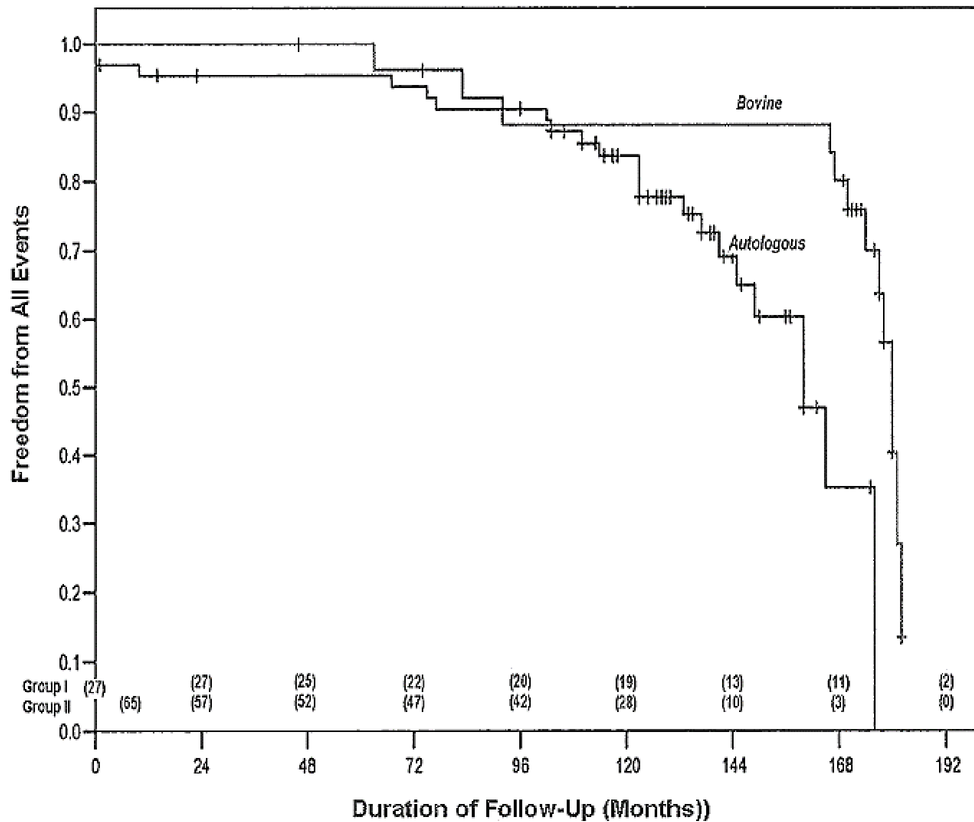

Figure 5. Freedom from all events autologous pericardium versus bovine pericardium[20]

Most strikingly there is evidence that the crosslinking methodology performed by surgeons and bioprosthetic heart valve manufacturers may be the key to better long-term success.

1.5 Valvular diseases: calcification

Calcific Aortic Valve Disease (CAVD) is the most prevalent form of aortic stenosis (AS) in the world, with an increased incidence in the developed countries, being the third most common cardiovascular disease after coronary artery disease and systemic arterial hypertension[21,22].

It is characterized by the fibro-calcific remodelling of the valve leaflets, the first stage of the disease named aortic sclerosis the valve becomes thicker and mildly calcified but with no obstruction to the blood flow. Throughout the years the disease evolves to severe valve calcification leading to a reduced leaflet motion and great blood flow obstruction, these are the main characteristics of this disease. As no pharmacotherapy proved successful in retarding the progression of the valve stenosis or the secondary effects on the left ventricular function, surgical or transcatheter (AVR) this prove to be the only options to the treatment of severe AS [23].

Upon surgical procedures to explanted valves with calcific AS it was revealed the presence of two features, fibrosis and calcification, these substantially alter the biomechanical properties of the aortic valve leaflets. Several observations point to the mineralization of the aortic valve being a response to injury, which might be triggered by lipid-derived species and inflammation processes. Some of these include the presence of osteoblast-like cells, chondrocytes and bone marrow in about 10-15% of calcific AS, the occurrence of dense inflammatory infiltrates (consisting mainly of macrophages) and that mineralization initiates within the *fibrosa* layer being often localized in the vicinity of lipid deposits[24].

Another important factor of calcific AS is fibrosis which is the excessive production of connective tissue unleashed by a regenerative process, this in turn increases the stiffness of the aortic valve and might play a considerable part in the promotion of mineralization. Upon this effect the collagen fibres produced by valvular interstitial cells (VICs) may act as a nucleation spot for calcium and phosphorous[25].

There also evidence on the increased production of many components of the extracellular matrix, such as periostin, tenascin C and proteoglycans contributing to the remodelling of the aortic valve during AS. Pathogenesis of calcific AS involves three main processes establishing a large and complex cascade of chemical and physiological events that lead to the final calcification deposits. First there is a **lipid infiltration** derived from endothelial damage, then **inflammation** processes and finally **fibro-calcific response** in all these three there is an involvement of a complex network of reactions some of which are still not well known[23,26].

Another mechanism that could potentially aggravate the calcification process is the ion pumps that regulate calcium levels, upon cell death within the living tissue the ability to regulate these pumps is lost. As such any accumulation of calcium cannot be removed increasing its concentration within the cells. Nucleation of mineralization spots can then occur in residual cells as calcium reacts with phosphorous present in organelles, nucleic acids and membrane components. Other nucleation sites may include the ECM. Elastin mediated calcification happens with no chemical crosslinking of the tissue, some authors suggest that the loss of glycoproteins and microfibrils (these protect the elastin) upon tissue preparation or the activity of the matrix metalloproteinase (MMP) potentiates the calcification to begin. This loss is believed to be important as GAGs are theorized to inherently inhibit calcium through chelation or steric hindrance, this relation as been proved to exist in *in vivo* rat subdermal implantation models where the loss of GAGs promotes calcification and GAG stabilization inhibits calcification. On the other hand, collagen crosslinking using glutaraldehyde system creates calcium nucleation sites[7].

1.6 Fixation Methods and Mechanisms

It is already proven that biomechanics and its correlation to stress play an important factor in the degradation of the heart valve tissue, as microfissures accumulate so does the probability of calcific sites appearing, as such it is relevant to improve the reticulation of the tissue without deprivation of the essential biomechanical characteristics. Fixation of xenogeneic tissue is essentially the treatment of the tissue using chemicals, this treatment aims to achieve three characteristics: **sterilization** of the tissue prior to implantation, reduce **antigenicity** and **stabilization** of the ECM. All three main components of the pericardium (collagen, elastin and ECM) must be properly stabilized to provide the necessary mechanical properties for long term application. Chemical bonds created from treatment must be irreversible and stable, these usually involve some type of bonding between amino acid side chains and extracellular matrix or intra aminoacid side chains.

The first treatment involved the use of formaldehyde however, the bonds were found to be reversible posing a problem for long-term durability. The introduction of glutaraldehyde in 1969 brought a much better option for the fixation treatment replacing in totality the use of formaldehyde. Glutaraldehyde is still the most used fixative for the tissue mainly due to its cheap price, solubility and reticulation reaction speed. Glutaraldehyde works by reacting both its aldehyde groups with the free primary amine groups from amino acid side chains (mostly lysins) in a Schiff base bond reaction forming an imine, this way connecting to collagen molecules (**Figure 6**).

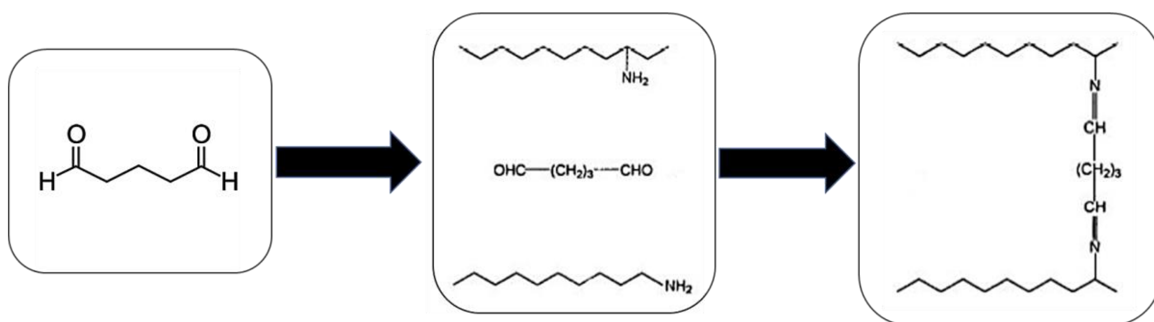

Figure 6. Schematic crosslinking reaction of glutaraldehyde with the free primary amines from lysins side chain.

Although glutaraldehyde is till the most used fixative it possesses major problems which cannot be overlooked. The first is cytotoxicity of the commercially available GLUT crosslinked pericardial patches has already been demonstrated by *in vivo* direct contact and extract assays of murine fibroblasts culture. Another aspect is the activation of macrophages associated with the release of cytokines, this results from an inflammatory response to microfissures in the pericardial patch upon mechanical stress over time[27].

GLUT-fixed autologous or xenogenic tissues are susceptible to calcification after long-term implantation. Mechanisms of calcification derived from glutaraldehyde fixation are complex, but

some evidence suggests that tissue phospholipids, free aldehyde groups of GLUT and residual antigenicity all play a role. It has been also shown that concentration and fixation time can influence the design and the biomechanics of the final tissue. Some authors reported that although tensile strength is apparently not affected by either concentration or time extensibility (elongation at break) increases with the more concentration and fixation time. However, effects of extensibility on calcification of implants remain to be determined[28,29].

As an alternative to the use of glutaraldehyde many other fixation methods have been investigated to name a few: photochemical reactions using rose Bengal[29] or Riboflavin[30], epoxies[31], polyethylene glycol[32], quercetin[33], glutamic acid[34], triglycidylamine[35]. However, our work focused solely on the use of the carbodiimide system using EDC (1-Ethyl-3-(3-dimethylaminopropyl)) and NHS (N-hydroxysuccinimide) or using sodium metaperiodate.

Periodate: Sodium metaperiodate fixation works by reacting the periodate ion with the uronic acid from hyaluronic acid to activate vicinal diols to aldehyde groups, these in turn will react with lysil amines and other molecules inside the tissue (**Figure 7**). Several studies have already shown that crosslinking using periodate when compared to glutaraldehyde further stabilizes the ECM structure providing better mechanical behaviour and a reduced calcification. Other works also demonstrated that the degradation of the GAGs by enzymes is reduced, this is an important factor in the reduction of the long-term calcification[Erro! Marcador não definido.,36 ,37].

EDC/NHS: EDC acts by activation of carboxylic groups in GAGs while NHS forms a stable intermediate, this in turn forms a complex that acts as a transfer agent, producing amide bonds with the free amine groups found in both collagen and elastin (**Figure 7**). While some studies have shown that EDC/NHS treatment used as a supplement to glutaraldehyde is proven to be effective, EDC/NHS alone fails to provide the necessary stabilization to GAGs against enzymatic degradation and biomechanical characteristics[38,39].

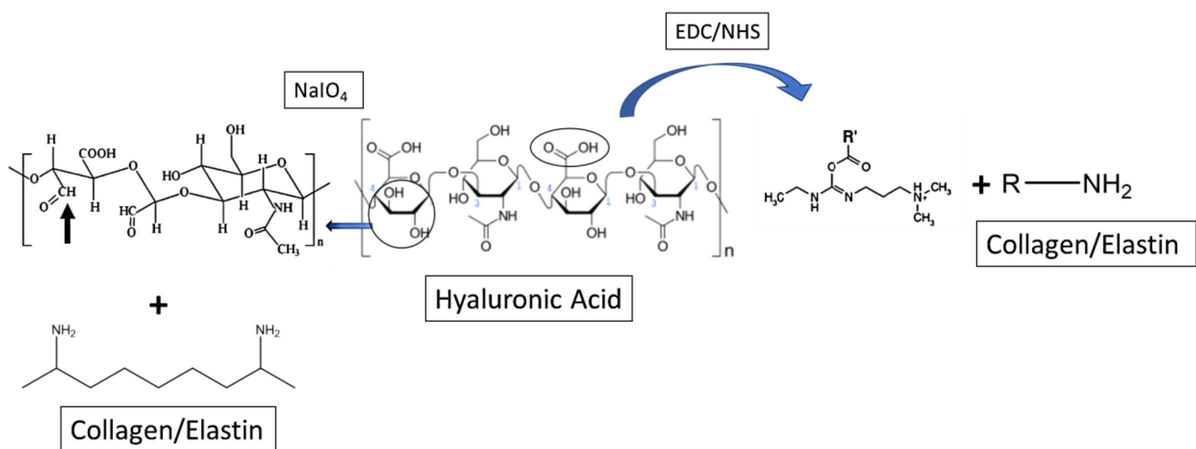

Figure 7. Proposed mechanism of reaction between modified glycosaminoglycans (in this case hyaluronic acid), using sodium metaperiodate or EDC/NHS, with the protein scaffold within pericardium tissue.

Although many different approaches have been investigated, to our knowledge there is till no work done to discover the influence of time in the range of minutes/hours and concentrations regarding the use these new compounds.

The aim of this thesis is the study of crosslinking concentrations and time, using both sodium metaperiodate and EDC/NHS, on the biomechanical and chemical changes that the tissue undergoes when these reactions take effect and if possible compare them to already existing studies or with the glutaraldehyde system already in use. It is expected to obtain an increase in biomechanical strength and/or extensibility in the tissue, but as we have already seen in some studies GAG stabilization remains one of the most decisive factors when considering long-term calcification. To circumvent this problem in a second approach during treatment with sodium metaperiodate or EDC/NHS hyaluronic acid solution was added to the tissue in hopes that this reacts with the tissue adding new GAGs.

Our focus is a readily and relatively cheap method for an operational surgery involving bioprosthetic heart valve transplant be it by using autologous or heterogenic pericardium tissue. Since glutaraldehyde achieves maximum efficacy within 30 minutes this is the main time range that is of interest. In this way the addiction of hyaluronic acid to the tissue was done during the 15 minutes and 30 minutes reaction time of the crosslinking. It is also important to notice that in many studies where post crosslinking does not involve the use of glutaraldehyde, the storage of the tissue is made using a glutaraldehyde solution and there is no way to know if this storage has influences with the GAG degradation or the mechanical characteristics. As such no glutaraldehyde storage was made, the storage of the tissue was made in saline solution at 4˚C and the necessary testing was preformed within the next 24 hours.

2 Methods

2.1 Materials

Sodium metaperiodate (99.9%, AnalitR), sodium chloride (99.9% Fluka) , sodium hydroxide (pellets 99.9% LabChem), hydrochloric acid (37% Fluka), hyaluronic acid sodium salt (20000-50000 Da, 99.9% CarboSynth), chondroitin sulfate sodium salt (10000-30000 Da, 99.9% CarboSynth), MilliQ water, *N*-hydroxysuccinimide (99.9%, CarboSynth), EDC/HCl (99.9% CarboSynth), Ethylene Glycol (99.9% Honeywell), Sodium Bicarbonate (99.9%, Fluka), Potassium Chloride (99.9%, Fluka), Magnesium Chloride Hexahydrate (99.9%, Sigma-Aldrich), Potassium Phosphate dibasic Trihydrate (99.9%, Sigma-Aldrich), Calcium Chloride (99%, Fluka), Tris(hydroxymethyl)aminomethane (99.8%, Sigma-Aldrich), Ethylene Glycol (99.8%, Sigma-Aldrich), Hydroxylamine (50% weight in water, Sigma-Aldrich), MES hydrate (99%, Alfa Aesar)

2.2 Tissue Preparation and Fixation

Fresh bovine pericardium was acquired at a slaughterhouse and transported to the lab in a cooler. The fat and any major impurity chunks like muscle tissue were removed by hand or with help of a tweezers. The pericardium was cut into 4,5 x 2,5 cm squares in 5,5 cm diameter Petri dishes and rinsed with saline solution 0,9% at a minimum of three times, these square sections were then fixed being placed in a petri dish where the solution of fixation was then dropped. To provide the best conditions for the imine formation all reagents were dissolved in 10 mL MES buffer 0.1M at pH 5.5. Various fixation times were then set from 15 to 180 minutes after which the reactions were quenched with the addition with equimolar amounts of ethylene glycol for periodate and hydroxylamine for carbodiimide crosslinking. In control and glutaraldehyde-treated samples no quenching was performed after 30 minutes in solution. The quenching reaction occurred for 1 hour, then patches of fixed pericardium were washed and rinsed with saline solution and they were stored in saline solution at 4ºC until further tests.

2.3 Mechanical Properties

Patches of fixed pericardium were dried of excessive water using paper, then 3 small stripes were cut from 3 different directions 0, 45 and 90 degrees in which the 45 and 90 angle stripes were cut according to what was considered the 0 angle pieces. The edges of the pericardium were cut to remove the influence of any border deformation like any collagen fibres loose as these would have a great impact upon the stretching of the samples. Width was measured with a calliper and thickness was measured using a digital micrometre prior to the mounting of the samples. Tissue stripes were then mounted in the pincers and stretched at a rate of 10mm/min with a 100N load. Stretching stopped after 0,9 cm from the initial position or until tissue breaking or 70% decrease from maximum tension.

2.4 Bioactivity tests

To assess the potential to prevent the formation of calcium phosphate deposits we made use of an *in vitro* method which mimics the physiological environment in order to stimulate the mineralization of bovine pericardium post crosslinking treatment. The samples were immersed in a SBF, this solution has already been reported by many authors in analogous studies[40,41].For convenience we have chosen the use of 1.5 SBF as we it allows us to reduce the time of mineralization from 14 days to 7. This *in vitro* method also avoids the use of an *in vivo* method while still enabling the study of the calcification of these biological tissues, providing a cheaper and easier approach. The SBF solution is prepared following Kokubo's protocol[42] but with a slight change to obtain a 1,5x SBF concentration. The 2,5 x 1,5 cm square samples were immersed in 1,5x SBF solution inside sealed flasks and put in a 37˚C bath to further simulate the physiological conditions. Following other authors, we changed the SBF solution every two days with fresh SBF solution, after the 7 days of immersion the samples were carefully rinsed with saline solution, lyophilized and stored at 4˚C until analysis.

2.5 Digestion of tissue for analysis

For the chemical study of the tissues it was necessary to break down the various tissue samples to their main components so that they can be analysed by HPLC and ICP. To do this we used the acidic hydrolysis method using HCl 6M at high temperatures (120˚C), this approach as already been described as effective in breaking down collagen which is the main component of pericardium tissue, and it is a cheap method. In this case we used lyophilized tissue samples for the hydrolysis. A sample was weighted in a flask and HCl 6M was added to a proportion of 1 mL for each 10mg of tissue which was then put in an already heated bath of oil and left with stirring for 6 hours under constant reflux. After the stipulated time passed a small volume of 50µL was put in a vial, this vial is then put under vacuum within a desiccator and left overnight to make sure all HCl and water content are evaporated [43].

2.6 Characterization Studies

FTIR-ATR: Attenuated total reflection, coupled with infrared spectroscopy, spectra were made using a Perkin Elmer Spectrum two with ATR module. The Attenuated total reflection (ATR) coupled with infrared spectroscopy is a technique that makes it possible to examine solid or liquid samples without further preparation, allowing the direct analysis of lyophilized tissue without any loss or damage. This technique is used to determine functional groups present in the molecules of interest as these have specific wavelength numbers.

NMR: Nuclear Magnetic Resonance ^{1}H spectra were made in a Bruker AMX400 Spectrometer. The chemical shift was presented in parts per million (ppm). The samples used were of hydrolysed tissue dissolved in DMSO. The spectra were treated using MestreNova v6. The ^{1}H-NMR is a technique that enables the identification of a molecular structure and its functional groups. In this case any change to the pericardium proteins mainly collagen and the functional groups present in GAGs.

ICP: Inductively coupled plasma mass spectrometry Horiba JobinYvon, France, Ultima model equiped with RF generator of 40.68 MHz, monocromator Czerny-Turner with 1.00 m (sequential) and automated sampler AS500. Hydrolysed tissue samples were dissolved with water and used as such. A standard solution of different concentrations of calcium and another of phosphorous were also injected as a pattern.

HPIC: High Pressure Ionic Chromatography was made using an AminoPac PA10 4x250mm where the eluent gradient was NaOH and CH3COONa 0.8ml/min. Pulsed amperometry detector. Temperature of 30˚C. Injection volume of 10 µL. The samples were dissolved in 500 µL of a solution with NaN3 and Norleucin (20 ppm each) and injected with a dilution of 10x. The quantification was made with the dilution factor applied to the prepared solutions. All data were treated using Microsoft Excel. High Pressure Ion Chromatography is a process that separates ions and polar molecules based on their affinity to the ion exchanger, allowing the amino acids and glycosaminoglycans to possess specific retention times.

SEM: The lyophilised samples were analysed using a Scanning Electron Microscope model DSM962 from Zeiss, during sample observation the tension applied to the electron beam was 5 kV. A Scanning Electron Microscopy (SEM) is a type of electron microscopy allowing the production of images by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing several signals containing the morphological information about the surface topography as well as the composition of the sample.

3 Results and Discussion

In this thesis we aimed at conducting time-based experiments on the chemical crosslink of bovine pericardium for the production of prosthetic heart valves using two distinct reticulating agents, sodium metaperiodate ($NaIO_4$) and the N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide/N-hydroxysuccinimide (EDC/NHS) system. The extension of crosslink was assessed at 15, 30, 45, 60, 90, 120, 150 and 180 minutes in the presence of one of the referred (system) reagents at two different concentrations, specifically 56 and 112 mM. In the EDC/NHS system, these reagents were used in 2:1 stoichiometry, and the reaction concentration refers to the quantity of NHS in solution, as it is the final leaving group of the multi-step reaction. The rationale for the chosen reagent concentrations lied on the optimum concentration of glutaraldehyde in the literature for use in bovine and autologous[27] pericardium crosslink, 0.6%, which corresponds to 56 mM. The reactions were carried out at room temperature, ambient light and compared with glutaraldehyde treatment for 30 minutes.

The first step consisted in cleaning the native fresh tissue from adhered fat and muscle. Roughly 4,5 x 2,5 cm squares were then cut to obtain the most uniform samples possible. To clean the blood left inside the pericardium tissue the square patches were washed and rinsed with 0.9% saline solution until all blood was dialyzed. These were then put in petri dishes where the crosslinking reactions took place by immersing the patches in the reticulating solution. After washing and storage for a day in saline 0.9% the samples were ready for further testing.

In the context of this thesis, it is important to evaluate the crosslinking effects of the two agents of interest, periodate and EDC/NHS. Previously, it is first imperative that the tissue is cleaned in order to reduce or eliminate any interferents with the crosslinks.

The preparation of the samples followed some task-specific experimental steps such as:

1) After all fat and muscle is removed, the suitable squares have been cut to obtain the most uniform samples as possible.

2) The square patches were immersed in saline solution (0.9%), washed and rinsed as better as possible.

3) If necessary, all blood can be dialyzed in order to clean the blood left inside the pericardium tissue.

4) After the complete cleaning process, the samples were placed in the petri dishes where the crosslinking occurs by immersing the patches in the reticulating solution,

5) All samples should be again washed and storage for a day in saline solution (0.9%).

The samples are ready for further characterization studies including Strain-stress assay, Scanning Electron Microscopy (SEM), Attenuated total reflection Infrared Spectroscopy (FTIR-ATR), Nuclear Magnetic Resonance (NMR), Inductively coupled plasma mass spectrometry (ICP) and High Pressure Ionic Chromatography (HPIC).

3.1 Scanning Electron Microscopy (SEM)

In our case, SEM can be a relevant technique to assess the crosslinking effects upon the bovine pericardium (BP) structure and its components. On a preliminary study, we selected the sample of BP crosslinked with 56 mM of periodate for 90 minutes. The main reason for this selection was related with the interest to evaluate one sample with a reasonable time of crosslinking. As previously described, pericardium tissue possesses two different sides with major morphological differences: the fibrous side is the outermost layer of the pericardial sac and it is composed of mainly dense and loose connective tissue (adipose tissue and reticular tissue, this last one made of a network with collagen fibres); the serous side is composed by a strong fibrous tissue giving it a more smooth like surface when compared with the fibrous side[44]. According to the differences on each side, the choice of any relevant image in order to elucidate about the crosslinking effects is a very hard task and is unexplored in the literature, to the best of our knowledge.

It was previously reported that the process of freeze drying provides a good system to store the reticulated BP without damaging or changing much of the prime structure unlike when the tissue is left to dry to natural evaporation[45].

Figures 8 and 9 contain the SEM images at 500 and 3000x magnifications of lyophilized crosslinked BP tissue The SEM images seem to correlate with this data, in particular:

a) When looking at the serous (or rough) side of both scales (A and B in both Figure 8 and 9) reduced to no change was observed. It is important to note that in this side, the collagen fibres are more compact and incorporated into the architecture of the pericardium. In general, it is more difficult to observe any significant change to the collagen bundles even after a zoom until five times more.

b) On the other hand, the fibrous side provides more clear images about the changes that occurred in the tissue as observed on both zooms (C and D, Figure 8 and 9). It is possible to observe that the loose collagen fibres presented in a non-reticulated tissue adopt a more compact and parallel conformation which might be a possible indication of a successful crosslinking. This

collagen rearrangement has already been reported by several authors using other crosslinking agents[46,47].

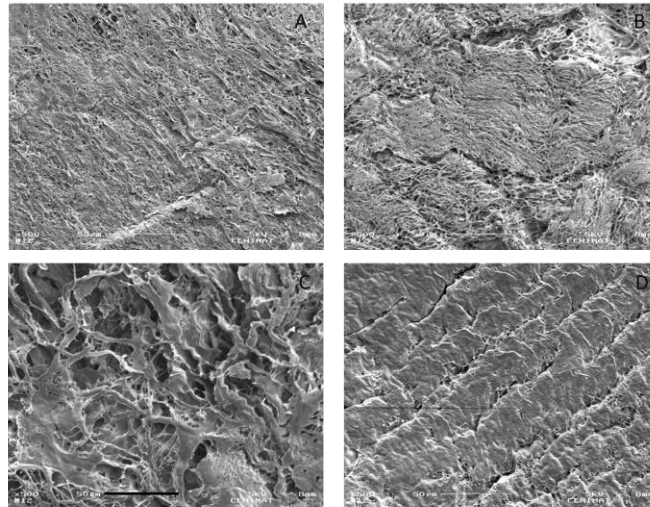

Figure 8. Scanning Electron Microscopy images of the flat surface of the serous side of the control (A) and crosslinked with periodate 56 mM for 90 minutes (B), and of the fibrous side of non-crosslinked (C) and crosslinked with periodate 56 mM for 90 minutes (D). The scale bar (black line) is 50 μm.

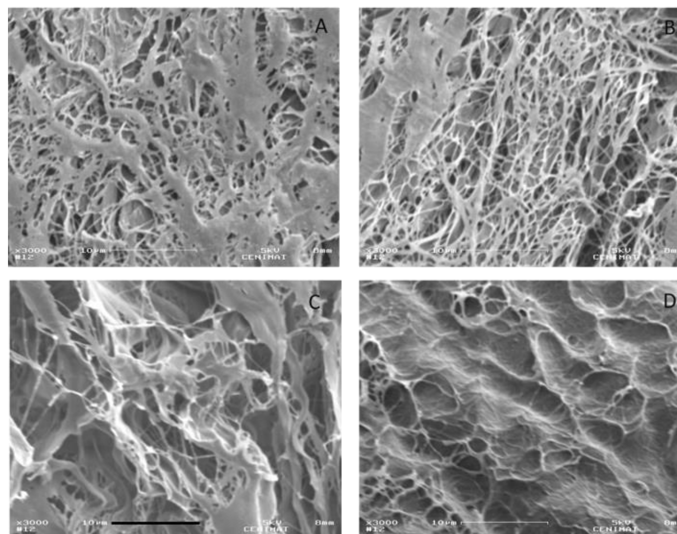

Figure 9. Scanning Electron Microscopy images of the flat surface of the serous side of the control (A) and crosslinked with periodate 56 mM for 90 minutes (B), and of the fibrous side of control (C) and crosslinked with periodate 56 mM for 90 minutes (D). The scale bar (black line) is 10 μm.

3.2 Attenuated total reflection- Fourier Transform InfraRed Spectroscopy (ATR-FTIR)

Using this technique, it is possible to obtain information about the protein conformation and in this way, determine any modifications in the structure of the main protein from pericardium tissue type I collagen[48].

Four representative samples all with the same reaction time of 90 minutes (two concentrations of periodate and two of EDC/NHS) and a control (untreated BP) were chosen to evaluate any change in the main protein structure of collagen (**Figure 10**). Generally, the difference in the peak intensity can be attributed to the precision of the technique and the device used for the analysis of these samples. It is possible to conclude that no major change has occurred to the backbone of the collagen. This observation is in agreement with the literature where amide I carbonyl stretching region between 1700 and 1600 cm^{-1} is described as sensitive to modifications in the triple helical tertiary structure of collagen. Other regions such as the amide II (1580 to 1480 cm^{-1}) and amide III (1300 to 1230 cm^{-1}) did not suffer any change in terms of position of the peaks as expected showing a solid structure in all samples. In the samples treated with $NaIO_4$, it was not possible to find a signal consistent with the presence of aldehyde and/or imine functional groups, which typically appear between 1740 and 1720, and 1690 and 1640 cm^{-1}, respectively. At first it was believed that the peak at 1740 cm^{-1} would belong to the aldehyde but other works suggest that it belongs to the esters units present in phospholipids (these can come from leftover cellular debris)[49,50,51].

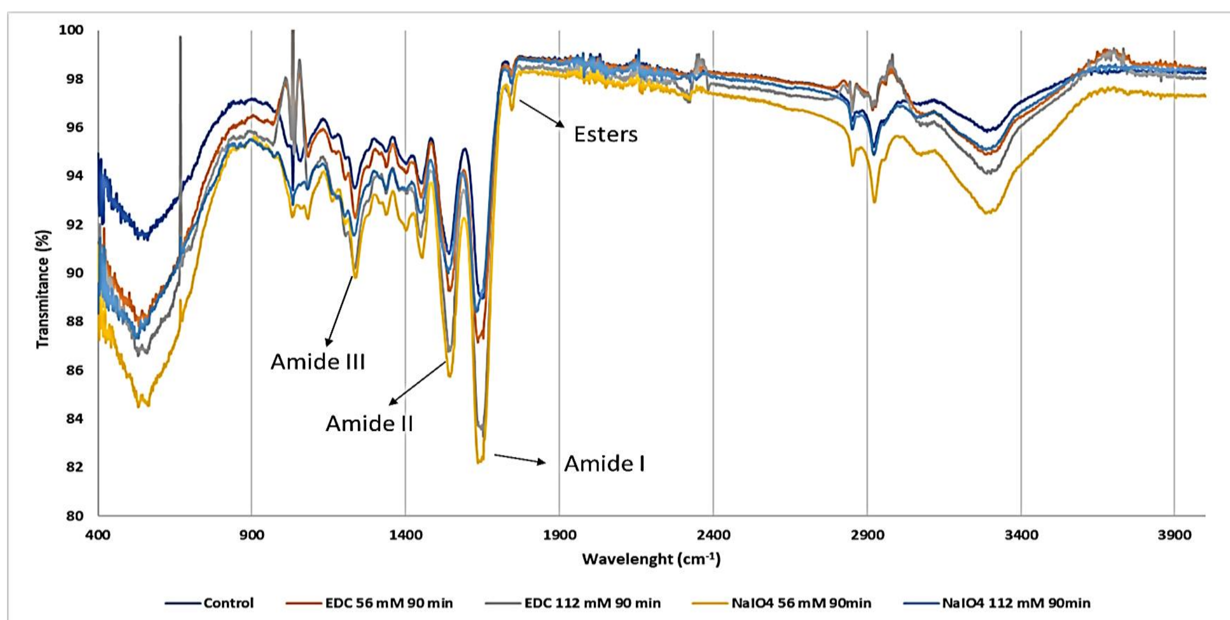

Figure 10. FTIR spectra of lyophilized pericardium control and post crosslinking at 90 minutes of reaction time using periodate (56 or 112 mM) or using EDC/NHS (56 or 112 mM).

3.3 Proton Nuclear Magnetic Resonance (NMR) spectroscopy

The samples chosen for ^{1}H-NMR analysis were collected from acid-digested treated and untreated BP dissolved in DMSO. More specifically, the treated samples correspond to 30 minutes of reaction with 56 mM and 112 mM of periodate.

In the NMR spectra presented (**Figure 11**) between reticulated and non-reticulated tissue, it is observed that the signal concordant with the presence of -NH groups around 8.24 ppm decrease in intensity almost completely. This observation could mean that the crosslinking reaction between amines and free aldehyde groups took place in the tissue. In this context, it is possible to consider that the periodate mechanism of crosslinking involves the modification of groups within the pericardium to react with existing free amine groups, increasing the reticulation of the tissue. The protein backbone appears to remain intact with a slight decrease in intensity.

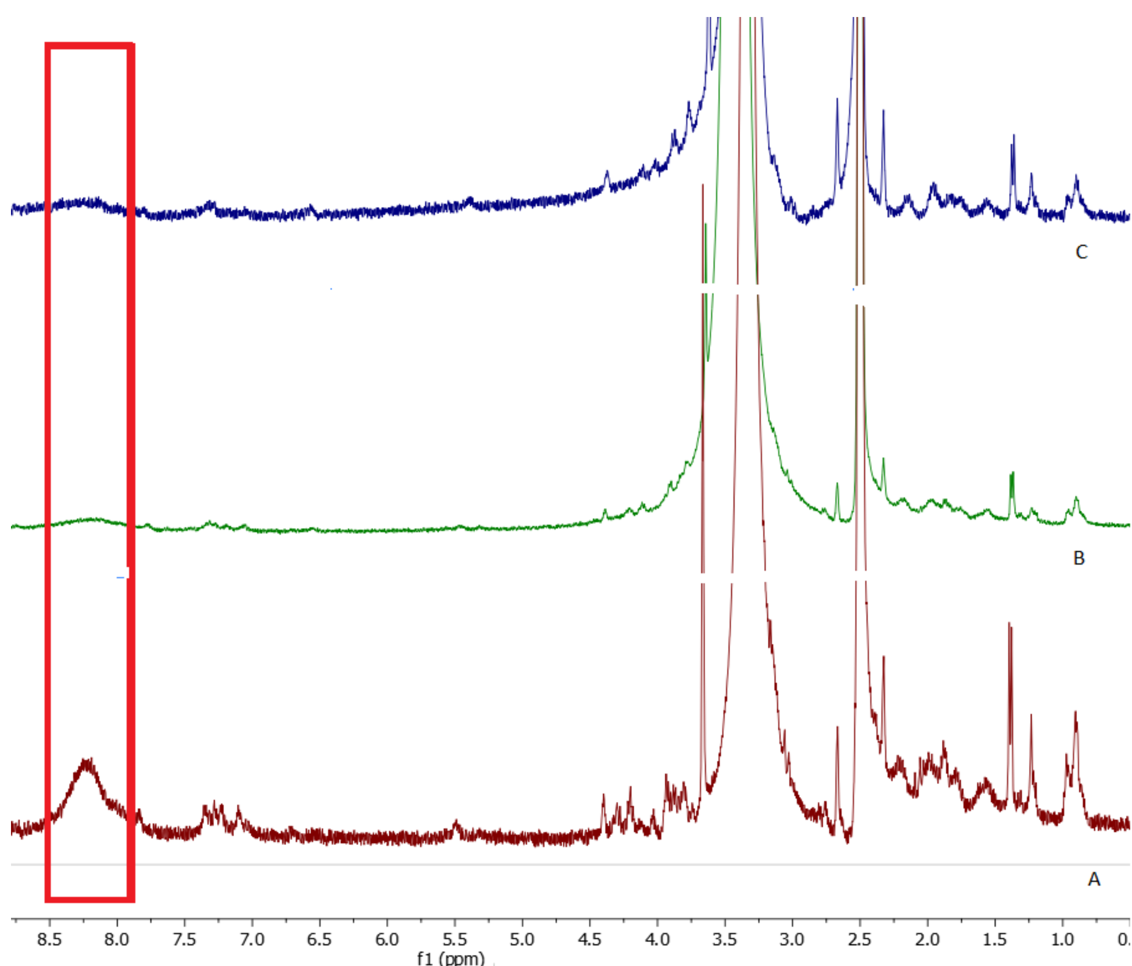

Figure 11. NMR spectra of digested non-treated pericardium (A) and treated pericardium with periodate 56 mM for 30 minutes (B) and 112 mM for 30 minutes (C), red rectangle highlights the peak concordant with the NH band around 8.2 ppm.

3.4 High Pressure Ion Chromatography (HPIC)

It is possible to determine the concentration (in the order of ppm) using the area of the individual peaks. In this case, the aim of this technique was to detect the 20 natural amino acids present in the tissue in order to detect any major change in the protein and/or ECM chemical structures after crosslinking with $NaIO_4$ 56 and 112 mM. The selected samples were from digested tissue using the acid hydrolysis methodology described in Section 3 (Methods). It is important to note that the presence of the free amino acids from pericardium allowing its complete separation in the column.

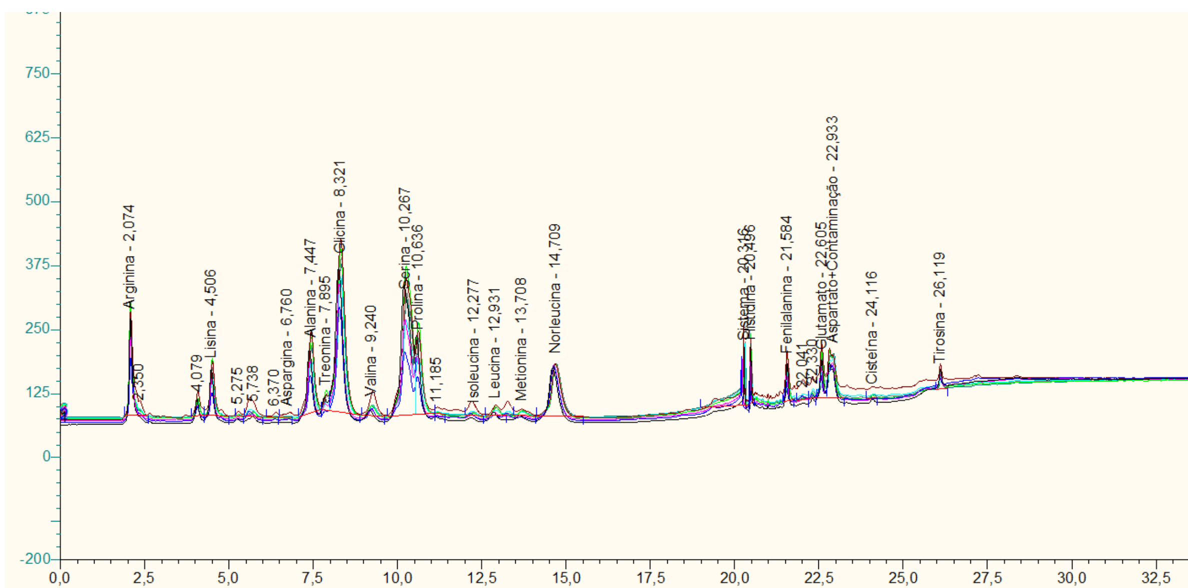

Figure 12. HPIC chromatogram of crosslinked samples: Control, Glutaraldehyde and periodate crosslinking (56mM and 112mM) with reaction times of 15, 30, 45, 60, 90, 120, 150 and 180 minutes

The chromatograms (**Figure 12**) indicates a good enough separation of the various amino acids being possible to determine the concentration of each one. Some amino acids such as methionine, tyrosine or aspartate are harder to detect and determine the correct concentration and for this reason were not included in the processed graphics. All amino acids not shown are not present in high quantity in the structure of the proteins from pericardium. As transformed amino acids like hydroxyproline and hydroxylysine possess a fundamental role in the crosslinking of the collagen structure, they are important to determine, but as the template solutions for these compounds was not in our possession, we could not determine their separation in the HPIC chromatograms.

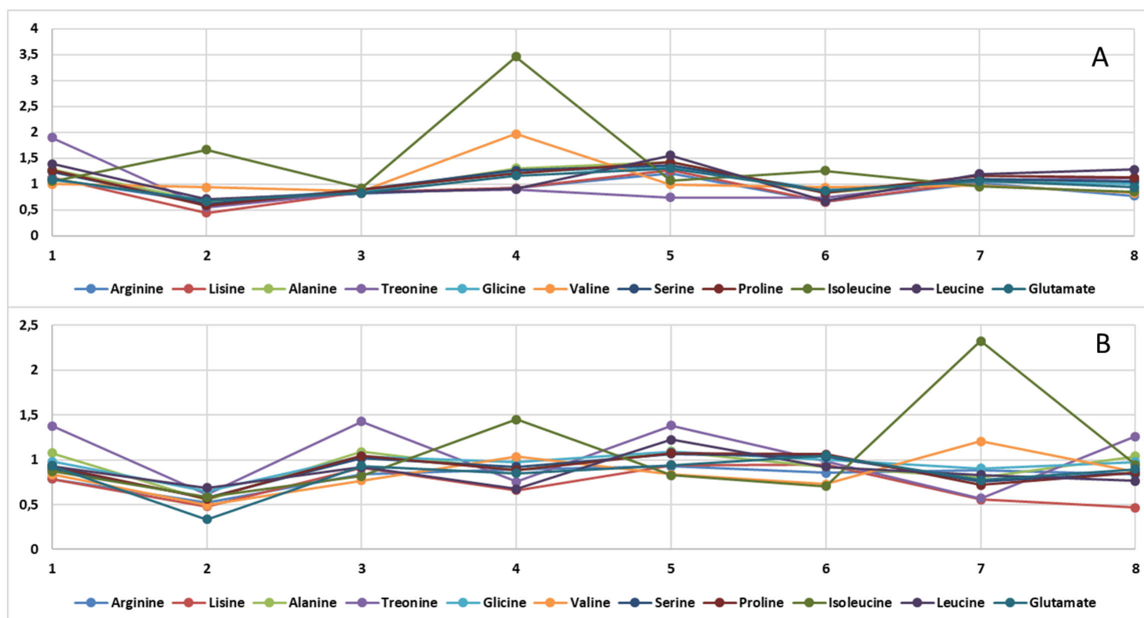

Figure 13. Data regarding the change of concentration of each amino acid throughout the samples tested, periodate 56 mM (A), periodate 112 mM (B) and GLUT. X coordinates indicate the sample as 1- 15 min, 2- 30min, 3- 45min, 4- 60min, 5- 90min, 6- 120min, 7- 150min, 8- GLUT

Figure 13 represents the normalized peak area variation for each of the amino acids. The normalized data were obtained by dividing the measured area of a specific amino acid in the sample by its area in the control sample. After analysing the data, no major correlation is found according to the decrease in concentration in the case of some amino acids that not participate in any reaction while others increase. The only plausible correlation is related to the drastic decrease in lysine in the glutaraldehyde group. This is an important observation because lysine in glutaraldehyde possess a major contribution to the reticulation of the tissue. Some research studies also reported the change in some amino acids but did not try to explain why this phenomenon is observed. For future studies, it is important to determine the concentration of modified amino-acids such as hydroxyproline, allolysine or hydroxylysine as these are known to have a great contribution to the crosslinking mechanism promoted by $NaIO_4$[52,53].

3.5 Mechanical Properties of the Tissues

Using the periodate and EDC/NHS system it was clear within some minutes the increase in stiffness of the biological material in question, when the tensile test was applied the maximum values for stress showcased these results. Biological materials and in this case pericardium tissue possess the downside of being highly anisotropic and non-homogenous, this means that different directions of the tensile test when applied to the tissues will yield different results. Our approach to this disparity in results was cutting the tissue in three directions of 0, 45 and 90 degrees three times each, then duplicates and triplicates were done using other pericardium tissues obtained in different days. This is important because if our main goal is an easy and cheap application of a crosslinking protocol in operatorial block we need to take into considerations the differences between pericardium tissues from distinct patients be it chemical or mechanical.

The periodate crosslinked tissue demonstrated rather peculiar but nonetheless interesting results. First looking at the 56mM (blue columns and line) concentration we have an increase in overall maximum strength but at two hours of reaction that strength started to decrease by a little although elongation of the tissue at these concentrations decreased by half at one hour reaction it started to increase again until two hours which then slightly decline at three hours. When we use a higher concentration of crosslinking agent, 112mM, (orange columns and line) the results change at 15 minutes we achieve a maximum strength with a minimum at one hour which then reaches a maximum again half an hour later, this may be indicative of other reactions happening that are different from those observed within the first 60 minutes. In this aspect the elongation behaves inversely when compared with absolute stress. What is interesting is that we can obtain better results when comparing with the glutaraldehyde reaction, most importantly at 15 minutes using 112mM of periodate allowing a better crosslinking at half the time which may be important when we are in an operational scenario during a valve transplant.

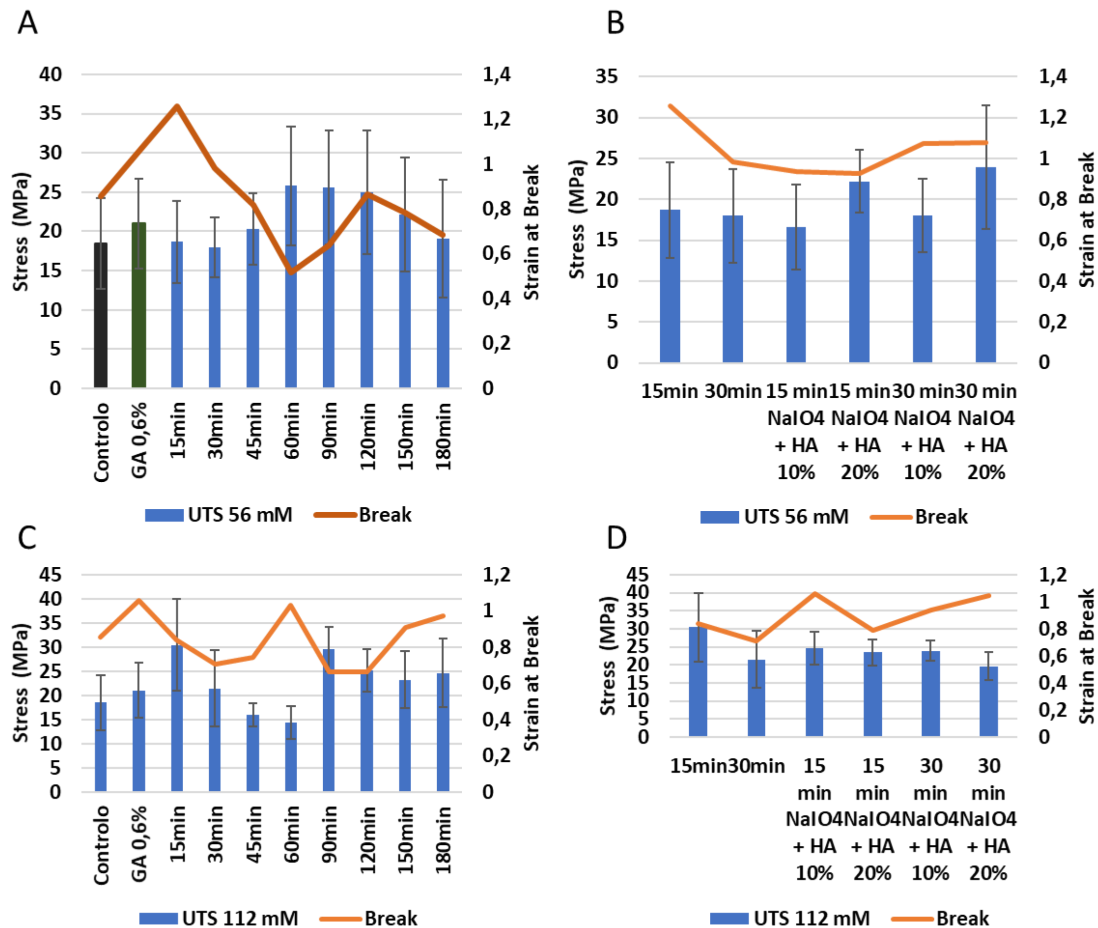

Figure 14. Stress-strain curve of crosslinked scaffolds using periodate in two concentrations 56 mM without and with hyaluronic acid (A and B), and 112 mM without and with hyaluronic acid (C and D) during different times, tested at 10mm/min at room temperature.

Looking at the carbodiimide (EDC/NHS) (**Figure 15**) reaction we could see that the stress behaviour is a little different when comparing with the periodate reaction. During both concentrations the tendency of increase and decrease of the maximum strength remained similar throughout the times of the reactions in this case we achieved a maximum between 45 and 60 minutes. In terms of elasticity or elongation what the results show is that the more time the reaction takes place the more elongation the tissue has, this means that it can be stretched to more lengths before breaking. In both concentrations after 60 minutes of reaction time every sample broke after being stretched by double or more with 120 minutes of reaction with a concentration of 56mM reaching 140% strain before breaking.

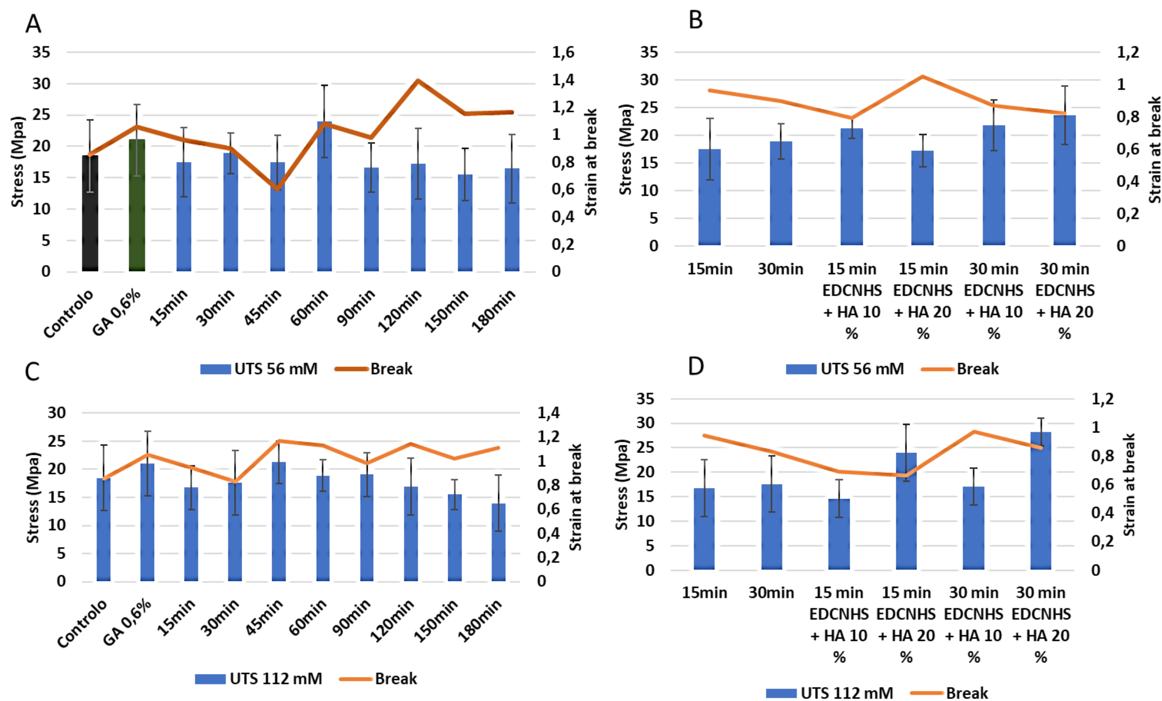

Figure 15. Stress-strain curve of crosslinked scaffolds using EDC/NHS in two concentrations 56 mM without and with hyaluronic acid (A and B), and 112 mM without and with hyaluronic acid (C and D) during different times, tested at 10mm/min at room temperature.

In next tests, the mechanical behaviour of pericardium tissue when using a low molecular hyaluronic acid as an addictive during the reactions with periodate and EDC/NHS have been evaluated. Our objective, it was to further strengthen the tissue with the extra GAG content.

Looking at the doped hyaluronic acid with the periodate, an increment in ultimate strength of the tissue is observed. For example, at 15 and 30 minutes using a concentration of 56mM of periodate with no hyaluronic acid the UTS value did not surpass 20 MPa but with the addiction of the hyaluronic acid it increased to around 23 MPa. It is a slight increase but is a good indicator of the potential when combining various crosslinking agents.

Looking at the EDC reaction with hyaluronic acid, again an increase in maximum strength especially in the 30 minutes range in almost all samples have been observed. The 30 minutes reaction with the maximum concentration of both agents surpassed the glutaraldehyde sample. It is also noteworthy that there is a slight increase in elasticity in both reaction times with a break reaching almost of the 100% mark improving once again upon the previous results comparing to the use of EDC/NHS as a crosslinking agent.

It is important to focus that good mechanical results can be obtained using other crosslinking agents besides glutaraldehyde, particularly a study about the mechanical behaviour of crosslinks throughout different times where sometimes just 15 minutes of extra reaction time can have meaningful variations. Although the carbodiimide reaction has been extensively reported, there

is not much information regarding the mechanical influences of time and concentration. This data is extremely relevant in the context of a surgery where the time is crucial. The periodate system had also never been reported to this degree, the crosslinking agent proved to be extremely interesting as better results were obtained comparing to the use of carbodiimide system or the glutaraldehyde. The described study can prove the potential of the use of this agent in the application of the operational block or not.

Comparing with other authors, merely on ultimate strength or extensibility, most of the results obtained surpass what is already reported[38,52,54]. Many of these treatments in involved the use of epoxys, neomicyn, genipin and even the ones using EDC/NHS.

It is clear that the use of a more correct buffer solution can potentially enhance the crosslinking reactions. The control of the pH is an important parameter as already described in the literature.

It is also noteworthy to mention the elongation at break of the tissues does not always follow a certain tendency, as previously reported. It was believed that the higher strength of the tissue corresponds to lower elongation. This observation is not obtained for all cases. There is a high variability of the results with some tissues including a high strength and high elasticity or vice-versa according the optimization of some parameters such as the selected concentrations, reaction time and type of agents for the crosslinking.

3.6 Inductively coupled plasma (ICP)

The ICP technique or Inductively coupled plasma is a type of spectrophotometry that allows us to determine the amount of calcium and phosphorous inside each sample, this is important to determine if the crosslinked samples possessed a better resistance to calcification when compared with a non-crosslinked sample.

Looking first at the periodate crosslinking (**Figure 16**) we can see a drastic diminution in terms of concentration of calcium concentration when comparing with control sample, in particular the sample with the highest mechanical strength (112mM at 15 minutes) was the one with the

lowest concentration of calcium indicating a possible correlation between stiffness derived from crosslinking and resistance to calcification.

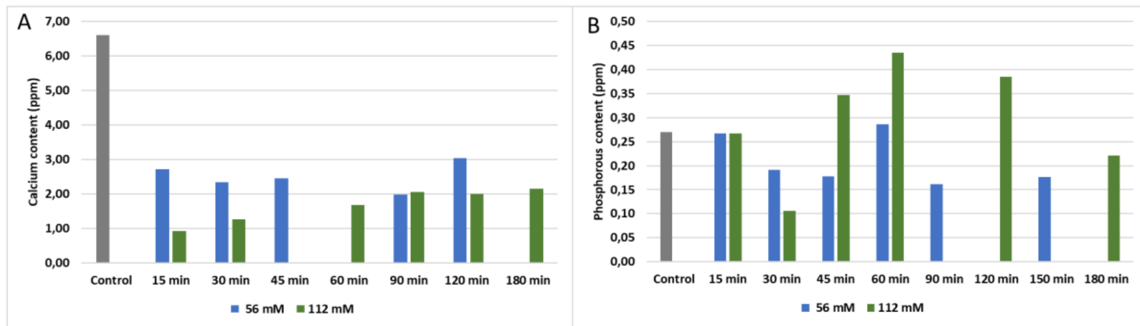

Figure 16. ICP data of concentration of calcium (A) and phosphorous (B) in crosslinked samples with periodate. Grey bars represent control sample, blue 56mM and green 112 mM

With the phosphate content the results were very different with many samples presenting higher concentrations of phosphorus, including the sample which is presented to have the highest crosslinking (112mM and 30 minutes). When comparing with the calcium concentrations these results don't seem to correlate. which is strange because what see seek to analyse is the calcium phosphate deposits. A possible explanation for this phenomenon is that the calcium analysed is not all from calcium phosphate salts but from other sources.

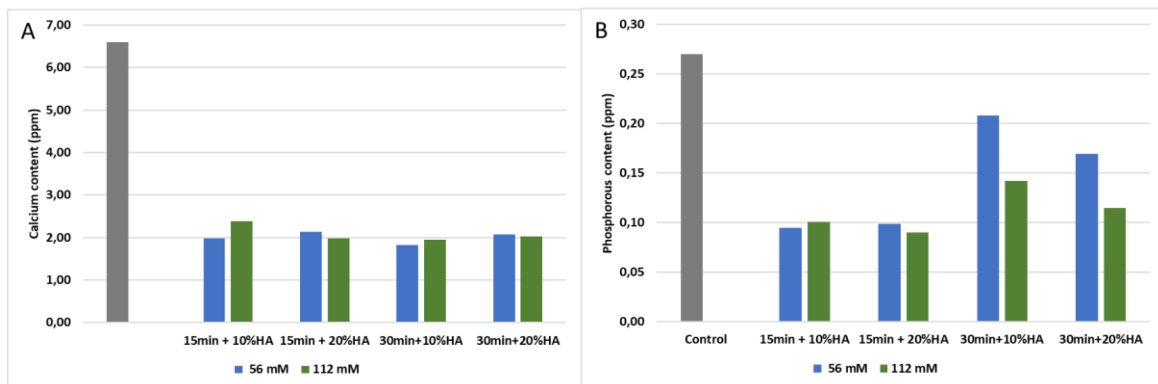

Figure 17. ICP data of concentration of calcium (A) and phosphorous (B) in crosslinked samples with periodate and hyaluronic acid added. Grey bars represent control sample, blue 56mM and green 112 mM

Looking at the hyaluronic acid added samples (**Figure 17**) of pericardium tissue there is a clear division between calcium and phosphorous concentration detected. Calcium concentration remains relativity the same in all samples with all values being 3 times lower than the control group. However, the phosphorous content clearly indicates that at 30 minutes we get an increase

in phosphorous ions detected which then lowers the higher the concentration of crosslinking agent in this case periodate. Once again, we can see that not all calcium comes from calcium phosphate molecules and that other sources are produced during the biomimetic assays.

In the EDC/NHS reaction (**Figure 18**) once again the concentration of calcium remains around 3 times lower than that of the control group with little variation between crosslinked samples. On the other hand, phosphorous concentration appears to increase the more concentration of EDC used and time the reaction takes place.

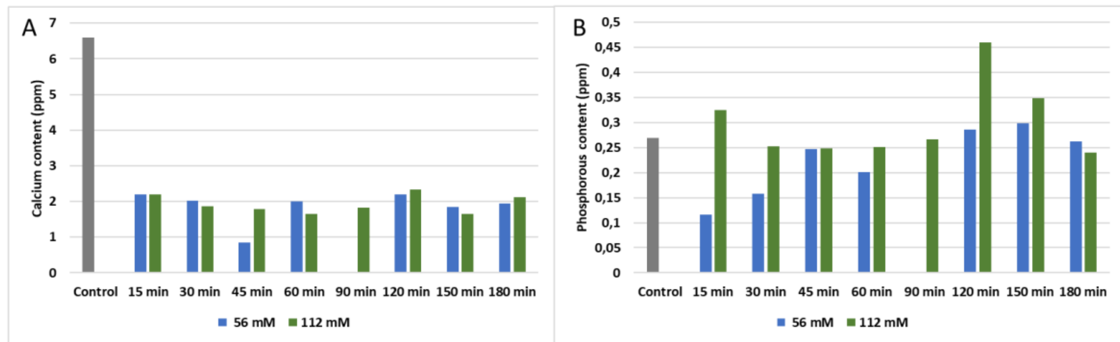

Figure 18. ICP data of concentration of calcium (A) and phosphorous (B) in crosslinked samples with EDC/NHS. Grey bars represent control sample, blue 56mM and green 112 mM

As we can see the ICP technique provides a useful way to detect the amount of ions of interest which in this case are calcium and phosphorous ions, it appears that in all crosslinked samples we determine a drastic decrease of calcium concentration, although the crosslinking degree does not seem to have an influence in the formation of calcium ions with apparently some exceptions in the periodate reactions such as 112mM of periodate with 15 and 30 minutes of reaction. Supposedly the phosphorous detection should have a correlation with the amount of calcium as it is reported that the main source of these ions is from calcium phosphate salts, this does not seem to be the case, as we can see in all types of samples tested with the SBF mineralization assay the concentration of calcium concentration is at least 1 fold higher than phosphorous, one possible explanation for this is that calcium ions deposit within the pericardium tissue without formation of new calcium phosphate salts. As we assume that all phosphate detected comes from calcium phosphate it is interesting to notice that different crosslinking times and agents produce a diverse range of results as the mechanics behind crosslinking degree and calcification still need to be more thoroughly researched. It is important to notice that the addiction of hyaluronic acid to the tissue prevents the overall formation of calcium phosphate when

comparing with the other samples without the extra GAG content, and this prevention as already been reported previously using the same SBF method[40].

This is indicative of an improvement of the crosslinking effects upon comparison with samples where just one crosslinking agent is used, it is important to determine if other GAGs could have the same potential when added to these reactions and if a combination of them could have synergetic properties in the inhibition of the formation of these calcium deposits.

4 Conclusions and Future Perspectives

The aim of this thesis is focused in the discovery of an easy and fast method for the crosslinking of pericardium tissue in order to be used in an operational block.

The bovine pericardium served as matrix model for a future application of this crosslinking method in an autologous pericardium. The use of periodate and hyaluronic acid to the tissue showed a reduction in the calcification as well as stabilized the collagen/elastin matrix by increasing the maximum tensile strength of the tissue and its extensibility. On the other hand EDC/NHS did not achieve better results than periodate. Generally, the addition of the hyaluronic acid can increase the stability matrix achieving higher strength during traction.

Biomechanical properties were measured using a tensile testing machine, where it was possible to obtain tensile strength and extensibility parameters, providing an idea of the stiffness of the material. It seems that the tensile strength did not follow a specific tendency when considering the reaction time or concentration change.

Nonetheless, it is possible to obtain good mechanical values even with just 15 minutes of reaction (in case of periodate) as opposed to the literature where reactions take several hours.

Through the SEM technique, it was possible to observe the changes in the rearrangement of the collagen fibres confirming that within the periodate crosslinked tissue there was a much more uniform organization (a possible indication of increased stiffness). FTIR-ATR spectroscopy can elucidate the collagen matrix and its stability, while in NMR spectra the decrease of a peak concordant with the -NH band could mean a possible crosslinking reaction happening inside the tissue.

Using the HPIC technique, an attempt to identify the aminoacid modifications by variation of its concentration, however it proves to be a difficult method for analysis of biological tissue. According to data, it seems that no correlation is observed between the variations in the concentration and aminoacid modification. This may indicate that the reaction mechanisms of reticulation agents within complex biological matrixes is still not clearly elucidated.

To evaluate the calcification of the reticulated tissues, an *in vitro* approach using a SBF solution was performed. All tissues presented lower calcium values than the control but the samples with hyaluronic acid resisted even better to calcification.

This observation can proof that in just 30 minutes, it is possible to reticulate the pericardium without the use of glutaraldehyde and still provide it with GAG stabilization to resist the calcification processes.

In a future perspective, it would be important to choose other techniques such as AFM in order to better elucidate the orientation of the collagen fibres after the crosslinking reactions and its influence in the mechanical behaviour. Histological studies on the crosslinked tissues before and after calcification process would also shed some light on the calcification patterns. Using this technique, it is possible to identify the collagen fibres from the elastin, the GAGs present in the tissue as well as the calcium nucleation spots.

Enzymatic activity using GAG specific enzyme in addition to collagenase would prove helpful to determine the GAG stability within the tissue after crosslinking and collagenase for the stability of the matrix of the tissue samples.

Another interesting methodology is the *in vivo* assays using rat models, the implantation of the tissues in a complex organism is still the best option to observe the degradation and the calcification of the tissue (according to the biochemical pathways and variation of enzymes present in rat model as well as other factors).

Finally, it is important to suggest the application of this methodology in autologous pericardium and its implantation in a human host. Other conditions could also be studied such as mixtures of GAGs added to crosslinking agents, or the pre-treatment of GAGs before introduction in the tissue. Also, the mixture of various crosslinking agents can be studied in order to elucidate any synergy between them and if that could provide even better results.

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