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# Decellularization, Stabilization and Functionalization of Collagenous Tissues Used as Cardiovascular Biomaterials

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## 1. Introduction

Cardiovascular diseases are a worldwide problem being a significant cause of morbidity and mortality every year. Patients requiring heart valve replacements include those exhibiting degenerative valvular diseases and rheumatic fever. The pathological processes include stenosis, fibrosis, myxoid change and calcification. The fibrosis causes a reaction to normal haemodynamic while the myxoid change reduces tensile strength of the valve due to replacement of dense collagenous tissue by loose tissue rich in glycosaminoglycans. Moreover, these pathologies can be observed in normal valves or fibrotic valves (Lindop, 2007).

Fortunately, the development of cardiovascular prostheses, either synthetic or biological, has allowed to increase life expectancy and has improved the quality of life of patient requiring either heart valves (Flanagan & Pandit, 2003; Schoen & Levi, 1999; Vesely, 2005) or vascular grafts (Matsagas et al., 2006; Monn & West, 2008; Schmidt & Baier, 2000). The implant technology for cardiovascular systems made use of raw materials of different origins. For example, metallic materials and synthetic polymers have been widely used in mechanical valves for the replacement of diseased heart valves. However, some complications such as alterations in the hemodynamical function and thrombus formation have been found (Zilla et al., 2008).

Biological prostheses provide some answers to these complications, although the bioprotheses do not fulfil their objectives satisfactorily, since they display others complications once implanted. The complications of tissue valves include calcification, remnant tissue immunogenicity, inflammatory degradation, mechanical damage and lack of repair (Zilla et al., 2008). Therefore, the need for safe, economic, physiologically acceptable and viable biomaterial has motivated the modification of collagen-rich tissues.

Collagenous tissues are alternative raw materials for the manufacture of medical devices due to their physical and biomechanical properties. These tissues promote cell interactions, exhibit good ion and macromolecular binding capacity in addition to their electrostatic, hemostatical and immunological properties (Li, 2007). Since 1960s, perichardial tissues and the porcine heart valves are two of the most widely used biological tissues in the construction of cardiovascular devices. The introduction of these biological biomaterials was

linked to the tissue crosslinking to increase durability. However, due to some complications in the stabilized tissue, several post-crosslinking protocols have been proposed to address these complications. More recently, biological scaffolds derived from acellular tissue has been used in tissue engineering and regenerative medicine.

Therefore, this chapter deals with the processing of collagenous tissue for the preparation of cardiovascular biomaterials. The processing techniques include the extraction of cellular and nuclear material by various decellularization methods, the preservation of tissue through of crosslinking reactions, hydrogen-bond interactions or interstitial space filling, and the functionalization or the blocking of free groups with various low molecular and macromolecular substances.

## 2. The biomaterial choice

The replacement of damaged organs or tissues is one of the objectives of the biomaterials science. For this, natural or synthetic materials can be used for example in the cardiovascular field in the manufacture of heart valves and vascular grafts. The success of a device depends not only on the type of biomaterials but also on a set of acceptable characteristics such as biocompatibility, biostability, haemocompatibility, anti-trombogenicity, resistance to degradation and calcification.

Among those biomaterials that can fulfil these requirements, natural tissues are good candidates and that is why they have been under investigation in the past fifty years.

### 2.1 Composition and sources of natural tissue

Natural tissue biomaterial can be obtained from either animal-derived tissue (xenograft) or human-derived tissue (homograft). However, due to the limited availability of autografts, animal-derived tissues are, in many cases, the first choice for cardiovascular biomaterials. Animal derived tissues widely used as biological biomaterials include perichardial tissue from various sources such as cows, calves and ostrich in addition to pig aortas.

Tissue-derived biomaterials are mainly comprised of collagen in addition to the tissue extracellular matrix (ECM) which is a complex mixture of structural and functional proteins such as collagen, proteoglycans, glycoproteins, elastin, metalloproteins, etc. Collagen, being the main structural protein, is a polypeptide that contain amino (-NH<sub>2</sub>), carboxylic acid (-COOH) and hydroxyl (-OH) functional groups as substituents, and together with the amide bonds in the polymer backbone form the reactive centers. The repetitive unit in the polymer backbone of collagen and the amino acid residues as side group are depicted in figure 1.

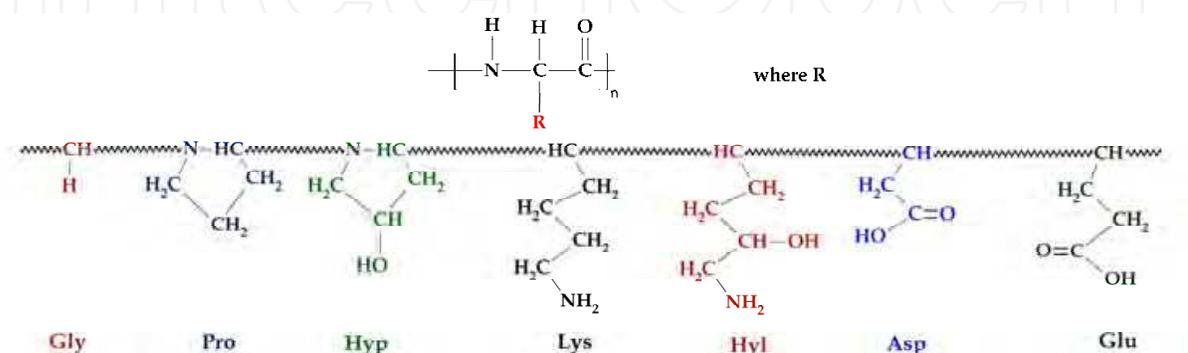


Fig. 1. Representation of the repetitive unit of collagen and some side group R of amino acid residue

The crystal lattice of collagen fibers are embedded in an amorphous matrix. The amorphous matrix is composed mainly by glycosaminoglycans as proteoglycans (sulphated glycosaminoglycans bound to proteins). In this matrix, in addition to the fibers, tissue cells and interstitial fluid (water or electrolytes) are embedded. The glycosaminoglycans are negatively charged polysaccharides of varying degrees of complexity. The glycosaminoglycan polymers consist of repeating disaccharide units, usually consisting of a hexosamine and an uronic acid (Yeung et al., 2001). The charged negatively units contribute to the elasticity and hydration of the tissues (Mavrilas et al., 2005), but may attract counter-ions, which could intervene in the processes of calcification of bioprostheses. The repetitive disaccharide unit of glycosaminoglycans mainly presents in native bovine perichardium is shown in figure 2.

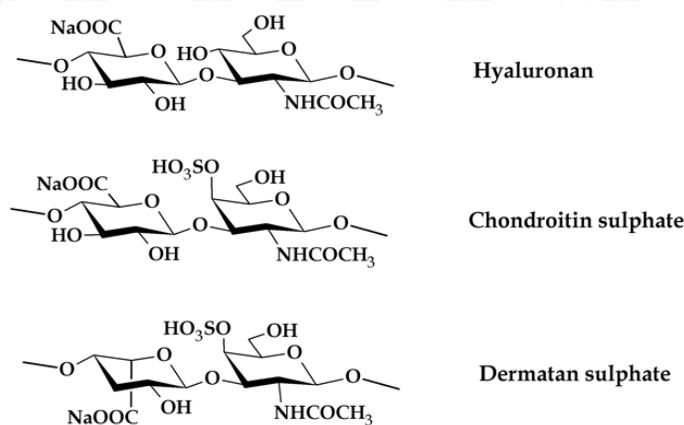


Fig. 2. Repetitive disaccharide units of common glycosaminoglycans in bovine perichardial tissue

The different soft tissues including cartilage, tendons, ligaments, skin and perichardium have the capacity of support mechanical load of variable magnitude. Therefore, the properties of the tissue depend on the number and the arrangement of collagen fibers, which can be parallel or perpendicular to the surface or randomly distributed in the matrix. The hierarchical nature of collagen confers to the tissue its structural complexity. The fibrous nature of bovine perichardial tissue is revealed in figure 3. In perichardial tissue, a multi-laminate structure is observed with difference in both serosa (Fig. 3b) and rugosa surface (Fig. 3a).

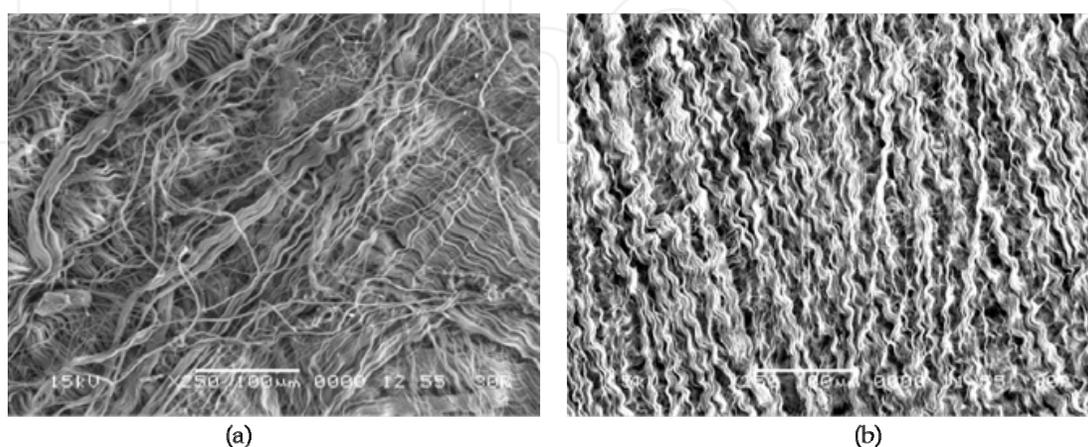


Fig. 3. SEM micrographs for the fibrosa (a) and the serosa surface (b) of native bovine perichardium

## 2.2 Properties of collagenous tissues

Collagen-rich tissues are composed of 75% of collagen, 20% of mucopolysaccharides and water, although elastin can be found in less than 5% (Cauch-Rodríguez, 2008). All these tissue components maintain the structural and functional integrity of the composite tissue. Some mechanical properties of collagenous tissues are shown in table 1.

Tissue	Property	Value	Reference
Bovine perichardium	Tensile strength	10.9 MPa	Lee at el., 1989
	Strain at rupture	33.0 %	
	Tissue modulus	58.2 MPa	
Canine perichardium	Tensile strength	18.4 MPa	Lee & Boughner, 1981; Wiegner & Bing, 1981
	Strain at rupture	21.4 %	
	Tissue modulus	198 MPa	
Human perichardium	Tensile strength	2.51 MPa	Lee & Boughner, 1985
	Strain at rupture	34.9 %	
	Tissue modulus	20.4 MPa	
Porcine aortic valve leaflet	Tensile strength	6.25 MPa	Lee at el., 1984
	Strain at rupture	30.8 %	
	Tissue modulus	54.6 MPa	

Table 1. Mechanical properties of some collagenous tissue

The thermal transitions experienced by materials with amorphous and/or crystalline regions are also observed in the collagenous tissue. When the biomaterial is heated, its specific volume increases, exhibiting the glass transition of amorphous regions and the fusion of crystalline collagen fibers to a temperature higher than the glass transition temperature (Li, 2007). The melting temperature of collagen fibers is an irreversible process and is often referred in the literature as the denaturation temperature ( $T_d$ ) or shrinkage temperature ( $T_s$ ). In fact, the denaturation temperature is widely used as an indicator of the tissue stabilization.

The collagenous tissues require chemical or physical treatments in order to be preserved or stabilized. In fact, the introduction of cardiovascular bioprotheses in 1960s was linked to the chemical fixation of porcine aortic valves or bovine perichardial tissue with glutaraldehyde. This process produces a non-living material without the capability of intrinsic repair as native tissue does after some structural injury (Flanagan & Pandit, 2003). The processed tissue tends to fail in modes related to the remnant immunogenicity,

inflammatory degradation, mechanical damage and pannus overgrowth (Zilla et al., 2008). In general, the stabilization of collagen-rich tissue is achieved by direct binding of functional groups to amino acid residues from collagen by coupling agents or by the linkage between the functional groups on collagen and various chemical agents. Both processes are referred in literature as the fixation or crosslinking processes. While the crosslinking agents make durable, stable and resistant tissues, the crosslinking density and the chemical process seems to have an effect on some of the major disadvantages of bioprotheses, such as calcification (Zilla et al., 2008). For this reason, a large number of crosslinking agents have been suggested with the aim of obtaining bioprosthesis that fulfill successfully its function. In addition to this treatment, there are reports on the post-crosslinking and pre-crosslinking treatments in order to reduce the calcification of biomaterial and in order to prepare porous biomaterials as scaffolds for tissue engineering.

### 3. Decellularization of tissues

The concept of decellularization is referred as the extraction of cellular components from natural tissues of human or animal origin. Different approaches have been reported as effective procedures to remove cells from xenogeneic and allogeneic collagenous tissue with the aim of removing cellular antigens and procalcifying remnants while the extracellular matrix (ECM) integrity is preserved as much as possible (Schmidt & Baier, 2000). The combination of chemical, physical and enzymatic methods destroys the cell membrane and removes nuclear and cellular material (Gilbert et al., 2006). The remaining acellular ECM will be a complex mixture of structural and functional proteins, glycoproteins and glycosaminoglycans arranged in a three-dimensional architecture. However, some mechanical and structural alterations on the ECM can be induced during the decellularization process.

#### 3.1 Effect of decellularization treatment on tissue properties

A biomaterial or scaffold for tissue engineering should provide not only mechanical support for the cell proliferation but also they must be versatile to give the required anatomical shape (Kidane et al., 2009). The decellularization of collagenous tissues has been explored as the ECM may serve as appropriate biological scaffold for cell attachment and proliferation. However, alterations both in the structural composition and in the mechanical properties of the remaining ECM can be induced during the decellularization protocols. The mechanical integrity can be affected and it may be associated either to the denaturation of the collagen triple helix or to the loss of macromolecular substances such as glycoproteins.

The efficiency of a given decellularization method and their effects on the properties of animal tissues must be studied in a specific manner due to compositional and structural differences (Gilbert et al., 2006). For example, the decellularization of porcine heart valve with sodium dodecyl sulphate, an anionic detergent, appeared to maintain the critical mechanical and structural properties of the valves leaflets (Liao et al., 2008) while decellularization of bovine pericardium with sodium dodecyl sulphate caused irreversible swelling, resulted in a reduction of the denaturation temperature (Courtman et al., 1994; García-Páez et al., 2000) and caused a reduction of almost 50% on tensile strength when compared to native tissue and tissue treated with Triton X100, a non-ionic detergent (Mendoza-Novelo & Cauch-Rodríguez, 2009).

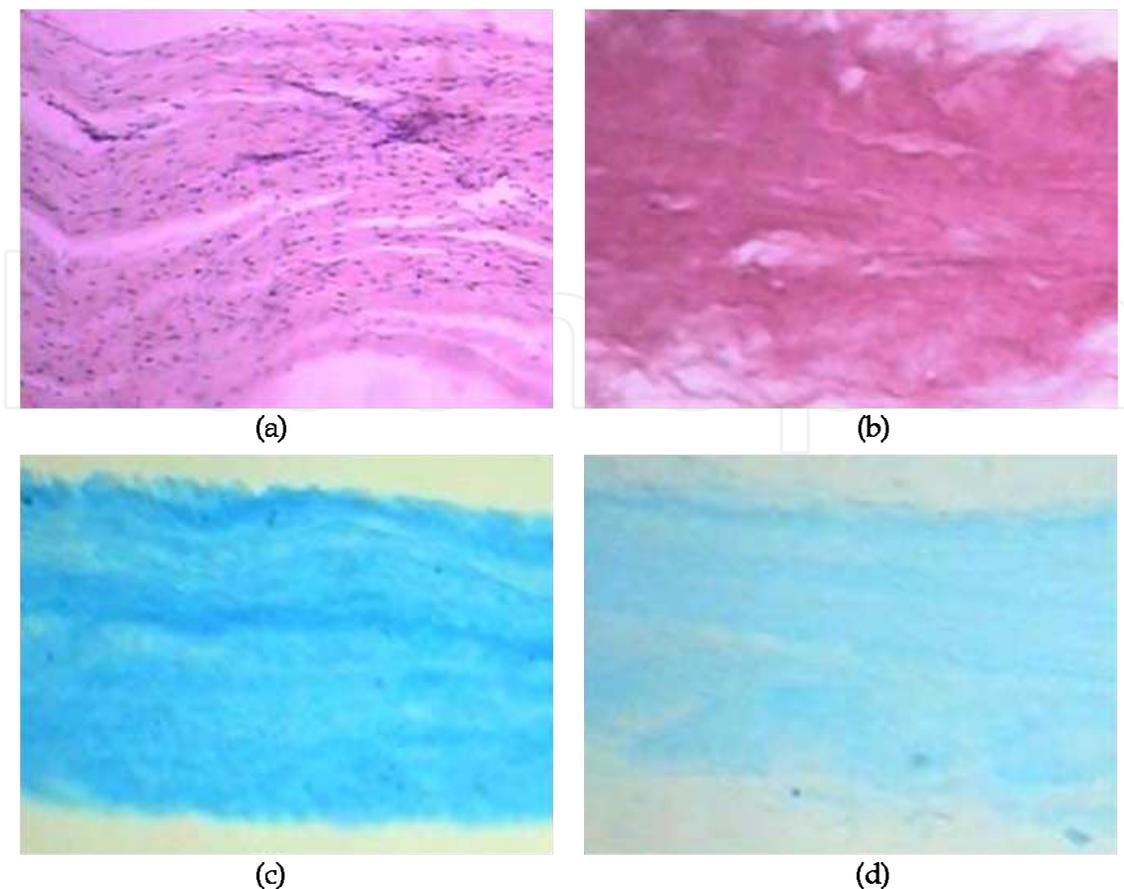


Fig. 4. Histological micrographs for native (a),(c) and decellurized (b),(d) perichardial tissue in H&E (top) and alcian blue (bottom) staining

It has been proposed that an anionic detergent binds to proteins, increases negative charges and results in tissue irreversible swelling (Courtman et al., 1994). In addition, a highly negative charged perichardial tissue has been associated to a higher tendency to tissue calcification (Jorge-Herrero et al., 2010). Due to these adverse effects, non-ionic detergents are preferred over ionic surfactants in the decellularization process of perichardial tissue. However, there are some issues related to the use of aromatic (phenolic) or non-aromatic (non-phenolic) non-ionic detergents used in the decellularization process. For example, the biodegradation products of derivatives of non-ionic detergents such as alkylphenol ethoxylates have been associated to toxicity (Argese et al., 1994) and estrogenic effects (Soto et al., 1991; Jobling & Sumpter et al., 1993). Figure 4 shows the histological results for bovine perichardial tissue decellularized with a non-aromatic non-ionic detergents. In this case, a reduction in the cell nuclei present in bovine perichardial tissue and a decrease in the glycosaminoglycan content after decellularization treatment were observed (Mendoza-Novelo et al., 2011).

In addition to tissue decellularization with nonionic surfactants, reversible swelling has also been studied. In this case, the reversible alkaline swelling did not change the three-dimensional architecture of native bovine perichardium. This means that the laminar structure and fibrous nature of the native perichardial tissue were maintained after decellularization although the opening of the interfibrillar spaces was observed. The reversible alkaline swelling cause a reversible change in the tissue thickness i.e. increased 45% after swelling step, but the tissue original thickness was regained after deswelling step.

However, the alkaline treatment altered the perichardial tissue stress relaxation behaviour (Mendoza-Novelo et al., 2011).

### 3.2 Pre-treatments (pre-crosslinking) methods to reduce tissue calcification

Bovine perichardium undergoes several treatments prior to crosslinking with the aim to improve its biocompatibility, to reduce immunogenicity, to decrease its tendency to calcification, to promote neo-vascularization and infiltration, and to increase cell adhesion and proliferation. Some of the pre-treatments proposed in the literature to reduce calcification of cardiovascular bioprostheses are showed in the table 2. It has been reported that with the treatment of bioprostheses with sodium dodecyl sulphate and Triton™X-100 most of the acidic phospholipids are extracted resulting in the initial suppression of calcification in the cell membrane (Schmidt & Baier, 2000).

Pre-treatment	Anti-calcification action mode	Reference
Surfactants	Removal of acidic phospholipids	Schmidt & Baier, 2000; Chang et al., 2004
Alcohols	Removal of phospholipids and cholesterol Alteration in the collagen conformation Cellular death Removal of Cardiolipin	Vyavahare et al. 1997; Pfau et al. 2000; Pathak et al., 2002

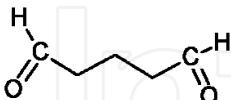
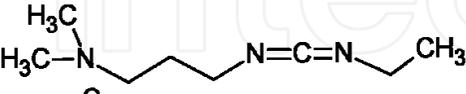
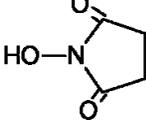
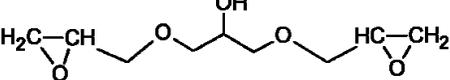
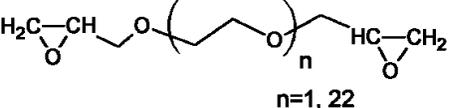
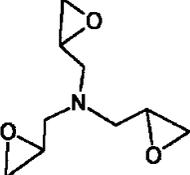
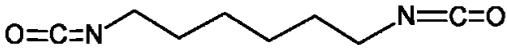
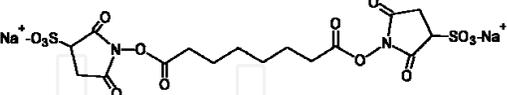
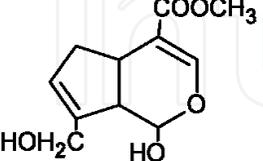
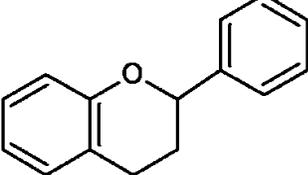
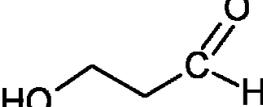
Table 2. Tissue pretreatment in order to reduce the bioprostheses calcification

The pretreatment of collagen-rich biomaterials with different concentrations of ethanol may prevent calcification through the extraction of phospholipids and cholesterol but causes a permanent alteration in the collagen conformation (Schmidt & Baier, 2000). Additionally, this treatment affected the interaction of the tissue with water and lipids and increased the resistance of the tissue to the action of collagenase. Several high molecular weight alcohols have been used in order to remove cellular components that contain elements responsible for the calcification (Pathak et al., 2002). The pretreatment with 50% ethanol for 5 min reduces fibrosis of bovine perichardium implanted in the aorta of sheep as a result of cell death and cardiolipin removal more than the phospholipids extraction (Vyavahare et al., 1997). Mixtures of chloroform/methanol have also been effective in reducing tissue calcification (Jorge-Herrero et al., 1994).

## 4. Stabilization of tissues

The stability of tissues is increased by physical or chemical crosslinking. The fixation enhances tissue stability, inhibits autolysis, allows a prolonged shelf-life, and allows a surgeon to have medical devices of various sizes readily available for implantation (Schoen & Levy, 1999). The chemical treatments also mitigate immunogenicity while maintaining both thromboresistance and antimicrobial sterility but greatly influence their degradation and calcification. However, tissue calcification is multifactorial phenomenon where chemical crosslinking is considered just one of these factors. In fact, the alteration in the electrical charge that exists in the perichardial tissue surface has been associated to the

calcification (Jorge-Herrero et al., 2010). Several crosslinking techniques have been suggested as the ideal procedure to stabilize the collagen structure while maintaining their physical and natural shaping. The structure and name of some chemicals used as crosslinking agents for collagenous tissue are shown in table 3.

Structure of stabilization agents	Name	Reference
	Glutaraldehyde	Olde Damink et al., 1995; Duncan & Boughner, 1998; Langdon et al., 1999
	Ethyl-aminopropyl carbodiimide (EDAC)	Lee et al., 1996; Everaerts et al., 2004; Mendoza-Novelo & Cauch-Rodríguez, 2009
	hydroxysuccinimide (NHS)	
	Glycerol diglycidyl ether	Lee et al., 1994
	Ethylene glycol diglycidyl ether, n=1; Poly(ethylene oxide) diglycidyl ether, n=22	Tu et al., 1993; Sung et al., 1996; Zeeman et al., 2000
	Triglycidylamine	Conolly et al., 2005; Sack et al., 2007; Rapoport et al., 2007
	Hexamethylene diisocyanate	Naimark et al., 1995; Olde Damink et al., 1995; Nowatzki & Tirrel, 2004
	Disuccinimidyl suberate	Pathak et al., 2001
	Genipin	Sung et al. 1999; Sung et al., 2000
	Proanthocyanidin, Procyanidins	Han et al., 2003; Zhai et al., 2009
	Reuterin	Sung et al., 2002; Sung et al., 2003

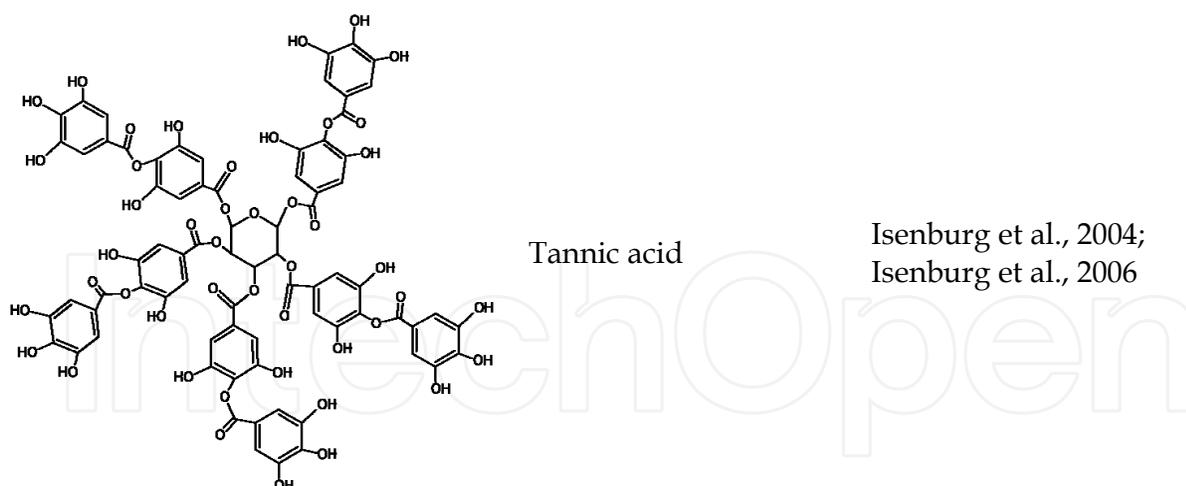


Table 3. Some chemical agents used for the stabilization and fixation of biological tissues

#### 4.1 Tissue crosslinking with glutaraldehyde

The procedure most studied and exploited in the manufacture of tissue valve includes the crosslinking with glutaraldehyde, which is also widely used as tanning agent in the leather industry. Glutaraldehyde is an important reagent in the biomedical field and has been used as crosslinking agent in the preparation of collagen-rich biomaterials or for the immobilization of enzymes or cell fixation.

Glutaraldehyde is an efficient agent for the crosslinking of collagen matrix because it reacts relatively quickly and because it is able to join separate protein molecules by means of the amino groups abundantly present in collagen. Glutaraldehyde is a cheap and water soluble five-carbon bifunctional aldehyde that in aqueous solution consists of a mixture of free aldehyde, mono and dihydrated monomeric glutaraldehyde, monomeric and polymeric cyclic hemiacetals and various  $\alpha$ ,  $\beta$  unsaturated polymers (Whipple & Ruta, 1974). This means that glutaraldehyde itself forms a number of different reactive species and that these species may also react in different ways, rendering a highly crosslinked network. Glutaraldehyde crosslinking has been and is still applied to most of the experimental and clinical bioprostheses. This process consists in blocking the  $\epsilon$ -amino groups of lysine in the protein through imino bond formation. The contribution of the glutaraldehyde as sterilization and crosslinking agent is partly due to its hydrophobicity and hydrophilicity, allowing it to penetrate both aqueous media and in the cell membrane. However, in the manufacture of bioprostheses, the use of glutaraldehyde has led to many disadvantages associated with the residual free aldehyde groups. Table 4 shows some of the problems associated with glutaraldehyde tissue crosslinking and some solutions that have been suggested to solve them.

In aqueous solution, the glutaraldehyde is presented as a mixture of free aldehyde, mono and dihydrate glutaraldehyde monomer, monomeric and polymeric cyclic hemiacetals, and several alpha or beta unsaturated polymers (Monsan et al., 1975). In turn, this heterogeneity of chemical species leads to a heterogeneous crosslinking. In addition, high concentration of glutaraldehyde promotes rapid surface crosslinking in the tissue (Olde-Damink et al., 1995), creating a barrier that impedes or prevents the diffusion of more glutaraldehyde within the biomaterial. In order to avoid this, the use of low concentrations has been suggested (Khor, 1997). It has also been proposed glutaraldehyde protection as a monomer by the formation of di-acetals, between glutaraldehyde and alcohols in acidic medium (Giossis et al., 1998).

The fixation reaction was carried out by the exposure of the tissue balanced with glutaraldehyde acetals solutions to triethylamine vapours. This process allowed the diffusion of the non-reactive glutaraldehyde into the tissue, minimized the formation of polymeric glutaraldehyde and reduced the waterproofing (hydrophobicity) at the tissue surface (Yoshioka & Giossis, 2008).

The conditions of the crosslinking reaction (pressure for instance) have been varied with the aim of improving the biomechanical properties of bovine perichardium. The crosslinking of bovine perichardium with glutaraldehyde at a pressure of 4 mm Hg (low pressure) both statically and dynamically (1.2 Hz) has been reported. By comparing the properties of crosslinked bovine perichardium, the dynamically crosslinked tissue showed a very similar extensibility to native biomaterial (non-crosslinked) in contrast to statically crosslinked tissue, which showed a higher extensibility, while no differences were reported in other mechanical properties (Duncan & Boughner, 1998). The bovine perichardial fixation with glutaraldehyde under biaxial static pressures (~225 and ~1875 mmHg) has been proposed. The bovine perichardium treated at high pressure showed an increase in stiffness and almost isotropic behaviour, while low pressure-treated bovine perichardium preserved the anisotropy exhibited by the native tissue (Langdon, et al., 1999). Porcine valves have also been subjected to crosslinking at high pressure (80 mm Hg), low and zero pressure. In this case, it was reported an increase in the rigidity of the leaflets fixed under low pressure and the preservation of geometric corrugations and undulations of the native tissue when the leaflet were fixed without pressure (Lee et al., 1984).

Heat treatment during glutaraldehyde fixation has also been reported. The thermal treatment at 50°C showed an anti-calcifying effect which was attributed to structural changes in collagen or lipid extraction by heat treatment (Carpentier et al., 2001).

#### **4.1.1 Post-treatments after glutaraldehyde fixation**

The residual unbounded aldehyde groups that remain in the tissue after glutaraldehyde fixation process have been associated with degenerative phenomenon on different bioprosthesis. The grafting of different molecules on collagenous tissues treated with glutaraldehyde has been an answer to these disadvantages.

The grafted molecules are incorporated in order to block free aldehyde groups and thus to reduce or to neutralize both cytotoxicity and calcification. Some surface modification procedures of crosslinked collagenous tissues are described in table 5.

It is known that nitric oxide releasing compounds can improve the biocompatibility of blood-contacting medical devices (Frost et al., 2005; Masters et al., 2005). Two common nitric oxide generating substances immobilized on synthetic polymers are diazeniumdiolates and S-nitrosothiols (Frost et al., 2005). In the same line of thought, surface modification of polymeric materials, such as PET or PU, with thiol compounds is interesting as it might exchange nitric oxide with endogenous donors such as S-nitrosothiols that already circulate in blood (Gappa-Fahlenkamp et al., 2004; Gappa-Fahlenkamp & Lewis, 2005).

The thiol groups on the polymer allowed the exchange reaction with S-nitroso serum albumin and then, the release of nitric oxide to inhibit platelet adhesion on the polymeric surfaces (Duan & Lewis, 2002). This approach has been proposed in perichardial tissue biomaterial by using L-cysteine as thiol compound (Mendoza-Novelo & Cauich-Rodríguez, 2009). One additional advantage of L-cysteine grafting on glutaraldehyde-crosslinked perichardial tissue is that free aldehyde groups will be diminished or even eliminated on the tissue allowing its detoxification. A schematic representation of grafting of collagenous tissue with L-cysteine is described in the figure 5. Similar approaches with other amino

acids have been suggested in order to provide non-cytotoxic tissue biomaterial and biomaterial with reduced calcification, as it is shown in table 5. In this table, it is also included molecules of biological importance as well as peptide sequences used to improve cell adhesion after the fixation treatment.

Type of molecule		Effects on biomaterial	References
Macromolecules	Hyaluronic acid -	Reduce the calcification of glutaraldehyde-treated tissue	Ohri et al., 2004
	Heparin-	Reduce the calcium deposition and the cytotoxicity of glutaraldehyde-treated	Lee et al., 2000
	Poly(ethylene glycol)-	Inhibit the platelet surface attachment and spreading and decrease the calcification of glutaraldehyde-treated tissue	Vasudev & Chandy, 1999; Park et al., 1997
	RGD peptides-	Enhance the adhesion and proliferation of human mesenchymal stem cells on acellular tissue	Dong et al., 2009
Amino acids	L-arginine, L-glutamine, L-lysine, L-glutamic acid, L-cysteine -	Reduce the protein adsorption and platelet adhesion of glutaraldehyde treated tissue. However, BP treatment with amino acids does not effectively prevent calcification. Incorporation of thiol moieties to the tissue	Jorge-Herrero et al., 1996; Jee et al., 2003; Mendoza-Novelo & Cauch-Rodríguez, 2009
Acids	Homocysteic acid -	Reduce toxicity but does not affect the stability of glutaraldehyde-treated tissue	Stacchino et al., 1998
	Amino oleic acid -	Inhibit the calcification of glutaraldehyde-treated tissue	Chen et al., 1994

Table 5. Molecules grafted on crosslinked bovine perichardial tissue

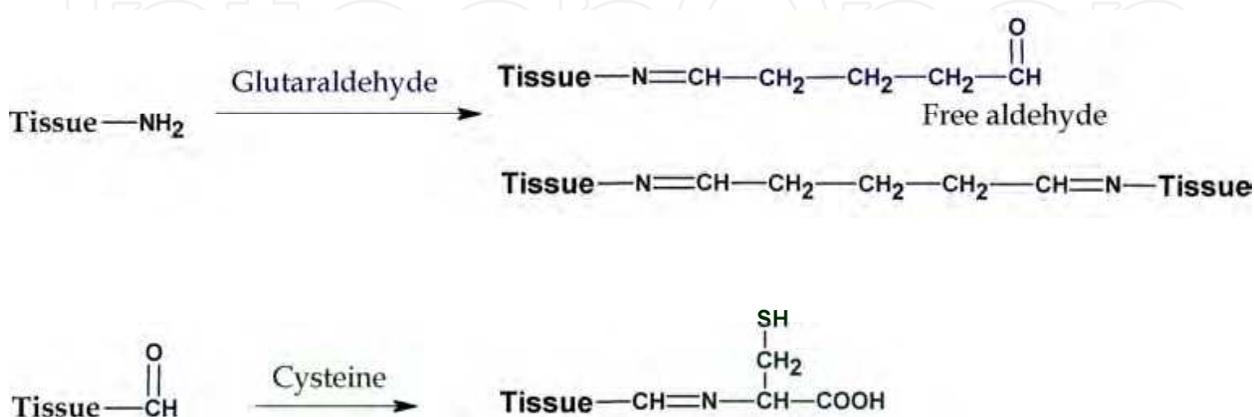


Fig. 5. Schematic representation of tissue crosslinking with glutaraldehyde and chemical coupling of L-cysteine

#### 4.2 Tissue crosslinking after carboxylic group activation

Due to the problems associated with the use of glutaraldehyde, various non-aldehyde alternative methods have been developed to stabilize and post-treat tissues. The crosslinking agents used in collagen-rich biomaterials can use both primary amino groups and acid groups of polypeptide chains. Historically, a water soluble carbodiimide (1-ethyl-3-(3-dimethyl amino propyl) carbodiimide / EDAC) was first used for the modification of carboxylic groups in proteins for peptide synthesis (Sheehan & Hlavka, 1956) and to promote crosslinking in gelatin (Sheehan & Hlavka, 1957).

The mechanism for the reaction between carboxylic groups and EDAC leading to amide bond formation is as follows: The addition of a carboxylic acid diimide produces an isourea ester, an O-acyl isourea. The intermediate O-acyl isourea is an activated carboxylic acid derivative with similar reactivity to an anhydride or acyl halide, and can be subjected to a subsequent nucleophilic substitution by an amine yielding a dialkyl amide and urea (Carraway & Khosland, 1972). Because carbodiimide is just a coupling agent, when used to crosslink collagen in the absence of agents with dual functionality, only promotes the formation of an amide bond between carboxylic acid and amino reactive groups present in the tissue, as depicted in figure 6.

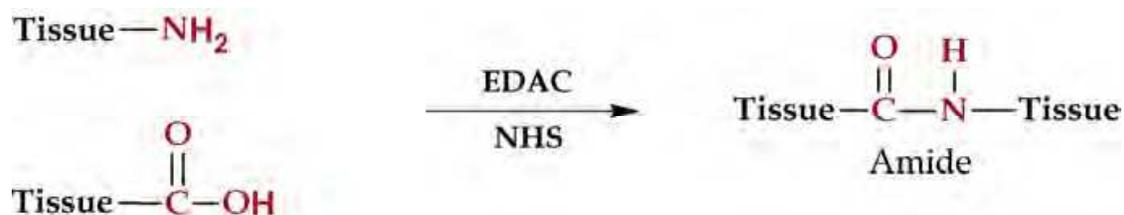


Fig. 6. Schematic representation of tissue crosslinking with EDAC and NHS

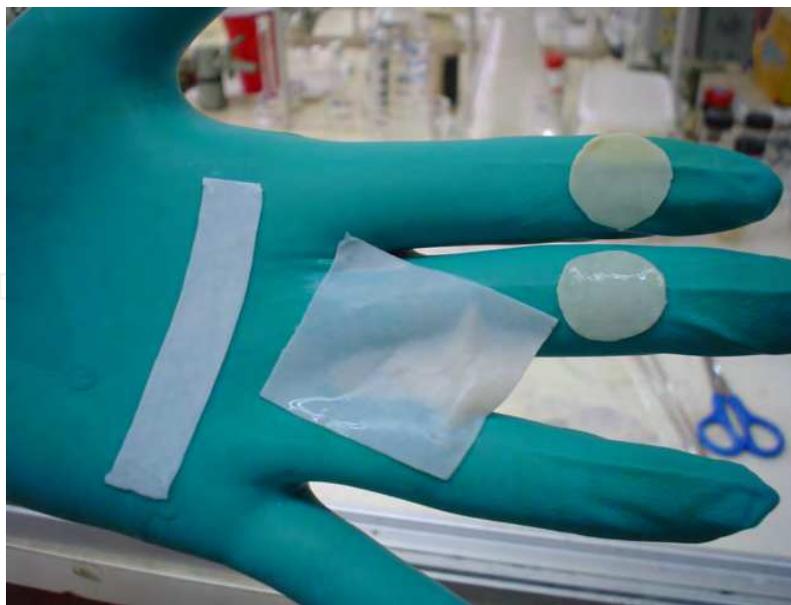


Fig. 7. Bovine perichardial tissue crosslinked with EDAC (rectangles) or glutaraldehyde (circles)

This requires that the activated carboxylic groups be close enough to the amino groups to achieve direct bonding (amide bond formation). The carbodiimides are hydrolyzed rapidly

in aqueous solution and the intermediate O-acyl isourea is extremely unstable producing a low crosslinking.

The crosslinking density and the shrinkage temperature of bovine perichardium treated with EDAC had values lower than a control of bovine perichardium fixed with glutaraldehyde (Mendoza-Novelo & Cauich-Rodríguez, 2009). However, the use of the N-hydroxysuccinimide (NHS) during crosslinking with EDAC improved the stabilization of tissue due to the formation of a stable intermediate compound after reaction of the NHS with carboxylic groups or isourea O-acyl intermediate (Lee et al., 1996). Such is the case reported for porcine aortic valves crosslinked by a two-step method. These steps included the blocking of the free primary amino groups of collagen with butanal and the crosslinking with Jeffamines™ of different molecular weights by activating the carboxylic acid groups with EDAC and NHS. This process led to a decrease in calcification (subcutaneous implantation in rats) of engineered tissue (Everaerts et al., 2004).

The appearance of bovine perichardial tissue crosslinked with glutaraldehyde and EDAC is shown in figure 7.

#### 4.3 Tissue crosslinking with epoxy compounds

The chemistry of epoxy groups, cyclic ethers of three members, has also been explored and applied in the fixation of tissue. Polyepoxide compounds or epoxy bifunctional polyether react with amino groups from collagen opening the terminal epoxide ring (Tu et al., 1993; Lee et al., 1994; Khorn, 1997). This reaction is nucleophilic and can be carried out under acidic conditions (highly reactive protonated epoxy) or alkaline (amine at its most nucleophilic). In this case, the modification of swine tendons with ethylene glycol diglycidyl ether has been reported for the repair of cruciate ligaments (Sung et al., 1996). The 1,4-butanediol diglycidyl ether (BD) has been reported as a crosslinking agent in the preparation of bioprosthetic valves (Zeeman et al., 2000). However, the fixation of porcine valves with BD caused immune response, foreign body reaction (proliferation of lymphocytes and macrophages) and calcification of implanted tissue using rats as animal model to levels similar to glutaraldehyde-fixed tissue, although low levels of cytotoxicity were reported (van Wachem et al., 2000). The combined treatment of BD and EDAC-dicarboxylic acid or detergents led to a reduction in calcification (implantation in rats) but not at significant levels (van Wachem et al., 1994). Therefore, it was concluded that the treatment with BD did not represent an alternative to glutaraldehyde to reduce the calcification of bioprosthetic valves (van Wachem et al., 1994). However, in another report the crosslinking of bovine perichardium and porcine aortic valves with triglycidylamine, a molecule of high polarity and solubility in water, resulted in an improvement in biocompatibility (assessed using bovine aortic valve interstitial cells, human umbilical endothelial cells and rats artery smooth muscle cells) and resistance to calcification (subcutaneous implantation in rats) compared with glutaraldehyde-fixed tissues (Connolly et al., 2005). Furthermore, triglycidylamine-fixed tissues showed stable mechanical properties (Sacks et al., 2007) and optimal reduction of calcification when treatments included mercapto-aminobisphosphonate (Rapoport et al., 2007). It was hypothesized that the difference between these two results, which explored the chemistry of epoxy in the crosslinking of tissue, may be due to differences in water solubility, chemical heterogeneity and contamination with used epoxy residual reactants (Connolly et al., 2005).

#### 4.4 Tissue crosslinking with diisocyanate

Bifunctional molecules capable of crosslinking proteins by urea bond formation after reaction between terminal isocyanate groups and  $\epsilon$ -amino group of lysine residue have been explored. Such is the case of crosslinking of extracellular matrix proteins (elastin and fibronectin) with hexamethylene diisocyanate in dimethyl sulfoxide (Tirrell and Nowatzki, 2004). Similarly, the crosslinking of ovine skin collagen with hexamethylene diisocyanate has been reported. This crosslinking procedure was carried out in an aqueous medium including surfactants to increase solubility and promote the penetration of diisocyanate into the tissue (Olde Damink et al., 1995). Furthermore, the effects of the tissue crosslinking with hexamethylene diisocyanate and the effects of mixtures of water/isopropanol (50/50 and 0/100) as solvent on the thermal and biomechanical properties of bovine perichardium have been reported (Naimark et al., 1995). On the other hand, the stabilization of porcine perichardium has been achieved by the interaction of polyurethane oligomers containing isocyanate end groups (Loke et al., 1996). The interaction in organic media between perichardial tissue and polyurethane oligomers resulted in the increase of the denaturation temperature, a reduction in the content of lysine and a poor diffusion of polyurethane oligomers into the tissue (H&E staining). The crosslinking of bovine perichardium with polyurethane oligomers, EDAC and diphenyl phosphoric azide showed less cytotoxicity (assessed by a direct cytotoxicity test or Homsy test) than the tissue crosslinking with glutaraldehyde (Jorge-Herrero et al., 2005).

After these results, it is clear that diisocyanates are an alternative to glutaraldehyde in the preparation of bioprostheses. However, protein fixation with isocyanates has the disadvantage of using organic solvents. In addition, during the fixation in aqueous media, the crosslinking degree can be reduced due to competition of hydrolysis reactions. Therefore, the blocking reaction of isocyanate with bisulphite salts is an alternative in the preparation of water soluble isocyanates (Petersen, 1949). The protein crosslinking process with blocking isocyanates has the advantages of the use of aqueous media and reduced isocyanate toxicity (Mata-Mata et al., 2008). In this regard, the treatment of perichardial tissue with the carbamoylsulphonate blocked polyurethane prepolymers resulted in an increase of the *in vitro* tissue biostability (Mendoza-Novelo, 2011). The coating of collagen fiber network of perichardial tissue with polyurethane is shown in figure 8.

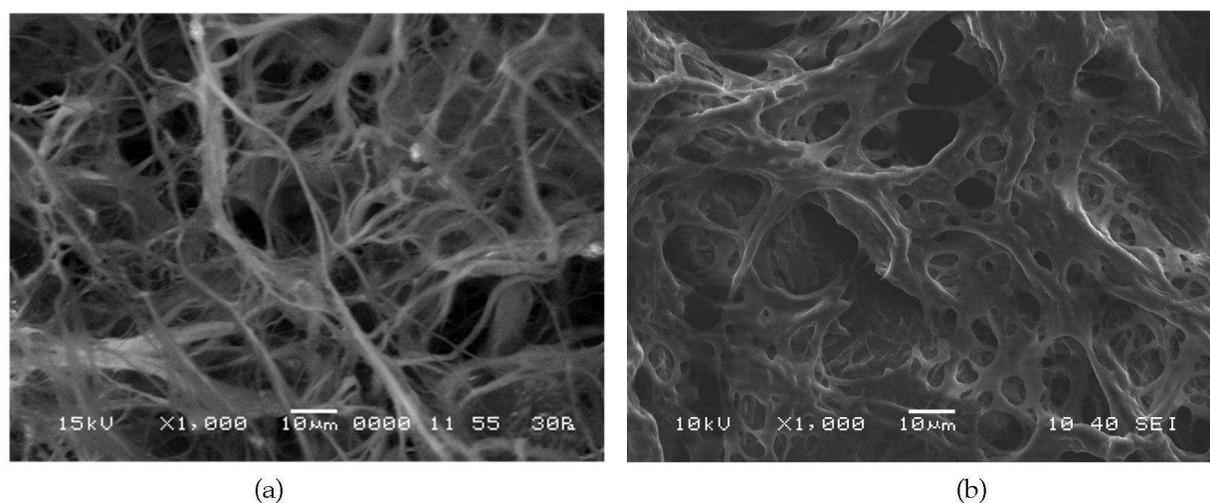


Fig. 8. SEM micrographs for bovine perichardium (a) native and treated with polyurethane prepolymers

#### 4.5 Tissue crosslinking with naturally-derived compounds

Crosslinking agents of natural origin have also been explored in the tissue crosslinking. Such is the case of genipin, an aglycone or an iridoid glycoside, which can be obtained by enzymatic hydrolysis of the glucoside previously isolated from gardenia fruit. The stabilization of porcine perichardium (Sung et al., 1999) and acellular bovine perichardium (Sung et al., 2000) with genipin probably was achieved through cyclic structures. The crosslinking density for genipin-fixed tissue was similar to glutaraldehyde and ethylene glycol diglycidyl ether -crosslinked tissues. Moreover, the genipin-crosslinked porcine perichardium was less cytotoxic (fibroblasts) than glutaraldehyde-crosslinked tissue, whereas exhibiting the same tensile strength and resistance to enzymatic degradation (Chang et al., 2002). Furthermore, acellular bovine perichardium fixed with genipin showed capacity of angiogenesis (microvessel infiltration) after implantation in rats (Liang et al., 2004). Moreover, cell extraction with solutions of Triton™X-100 and the crosslinking with different concentrations of genipin were used to establish a relationship between the crosslinking degree and the degradation rate or the model of acellular tissue regeneration (Chang et al., 2004).

Polyphenolic compounds have also been investigated as natural agents of tissue stabilization, such as the proanthocyanidins from the family known as condensed tannins, which are essentially oligomers of flavonoids available in several fruits and vegetables. The stabilization of collagen with proanthocyanidins may involve the formation of hydrogen bond type interactions between the phenolic hydroxyl and amide carbonyls of the polypeptide chains.

The proanthocyanidin has a high affinity for proline-rich proteins, because this amino acid is a good hydrogen bond acceptor (Zhai et al., 2006). The proanthocyanidins can be used to crosslink collagen sponges with similar density and efficiency to glutaraldehyde but with reduced calcification after 6 weeks implantation in rats and it was reported to be 120 times less toxic to fibroblasts direct contact (Han et al., 2003). The proanthocyanidin crosslinking procedure was repeated in decellularized porcine aortic valves resulting in low toxicity to bovine aortic valve interstitial cells and in the stimulation of cell proliferation to low concentrations of this stabilization agent in the culture media (Zhai et al., 2009).

The stabilization of elastin in porcine aortas has been achieved by treatment with polyphenolic tannins, which is composed of a central molecule of glucose (hydrophobic core) and one or more galloil residues (hydrophilic shell) (Isenburg et al., 2006). Polyphenolic compounds were acetylated tannic acid, pentagalol glucose, gallic acid and glucose. In this study, pentagalol glucose treatment was the least toxic to fibroblasts (Isenburg et al., 2004). Also, the study revealed that polyphenolic hydroxyl groups are essential for the interaction between the tannic acid and elastin. The combination of tannic acid and glutaraldehyde rendered a biostable tissue with high resistance toward elastase and collagenase and low tendency to calcify (Isenburg et al., 2006).

The reuterin ( $\beta$ -hydroxypropionic acid) produced by *Lactobacillus reuteri* has been used in the fixation of porcine perichardium (Sung et al., 2002). The reuterin is soluble in water, with antimicrobial and antifungal activity. The properties of reuterin-fixed tissue are comparable to glutaraldehyde-fixed tissue in terms of amino group content, denaturation temperature, tensile strength and collagenase digestion resistance (Sung et al., 2003).

Microbial (mTG; *Streptoverticillium mobaraense*) and tissue (TG2; tTG) transglutaminases (protein-glutamine  $\gamma$ -glutamyltransferase, EC 2.3.2.13) have been explored in the

crosslinking of collagen type I due to their ability to crosslink proteins through the  $\epsilon$ -amino group of lysine and  $\gamma$ -carboxamide group of glutamine residue (Chen et al., 2005; Chau et al., 2005). The results indicated the efficiency of this crosslinking agents in terms of denaturation temperature, mechanical strength, low toxicity to fibroblasts (Chen et al., 2005) and an increase in osteoblasts and fibroblasts adhesion and proliferation compared to native collagen (Chau et al., 2005).

#### 4.6 Other methods for the tissue stabilization

Others non-aldehydic crosslinking procedures have been proposed with the aims of prevent or mitigate tissue calcification. The disuccinimidyl glutarate (DSG) is another non- aldehyde alternative to tissue crosslinking. The process is carried out by the reaction between primary amino groups of tissue and NHS ester groups of DSG forming amide bonds with a length of five-carbon crosslinking and releasing NHS. The DSG crosslinked tissue was resistant to enzymatic degradation, exhibited low tendency to calcify and high temperature of denaturation. However, it was necessary to use dimethyl sulfoxide due to the insolubility of crosslinking agent in water (Pathak et al., 2000). In response to this drawback, a water soluble crosslinking agent has been used, i. e., the disuccinimidyl suberate. The presence of sulfonyl groups at the ends of the molecule conferred water solubility while retaining reactivity with amino groups by crosslinking chemistry similar to DSG, but with a length of 8 carbon intermediates. The tissue crosslinked under these conditions showed very low levels of calcium (0.2 mg/g of tissue) after 90 days of implantation in rats (Pathak et al., 2001). The crosslinking of collagen type I proposed for cartilage regeneration has also been achieved by the diimidoesters – dimethyl suberimidate (DMS). In this procedure, collagen amino groups react with DMS imidoester groups to form amidine groups and a length crosslinking of 8 carbons (Charulatha & Rajaram, 2003).

The stabilization of bioprosthetic tissue by filling the tissue interstitial spaces with polyacrylamide hydrogel resulted in the mitigation of tissue calcification in a rat study (Oosthuysen et al., 2006). Physical methods such as photo-oxidation (Khorn et al., 1997) or the use of ultraviolet radiation (Pfau et al., 2000) have also been proposed for the crosslinking of collagen-rich biomaterials. However, despite the increase in tissue shrinkage temperature, in some case the treated tissue did not show resistance to the proteins extraction (Moore et al., 1996).

#### 4.7 Masking reactions

At this point it is important to distinguish between the effective formation of crosslinking sites, i. e., two reactive sites in collagen linked by a same molecule of crosslinking agent, and the masking of crosslinking, i.e., the reaction between a single end of bifunctional crosslinking agent and one reactive site of collagen. Table 6 shows the possible reactions of crosslinking and masking between collagen and difunctional crosslinking agents.

#### 4.8 Glycosaminoglycans stabilization

Glycosaminoglycans present in both aortic valves and perichardium have been fixed to prevent the loss of these polysaccharides during the fixation of bioprosthetic valves. The sodium metaperiodate has been used for the stabilization of glycosaminoglycans in porcine aortic valves with the subsequent glutaraldehyde crosslinking (Vyavahare & Lovekamp, 2001). The stabilized porcine aortic valve showed compatibility and reduced calcification

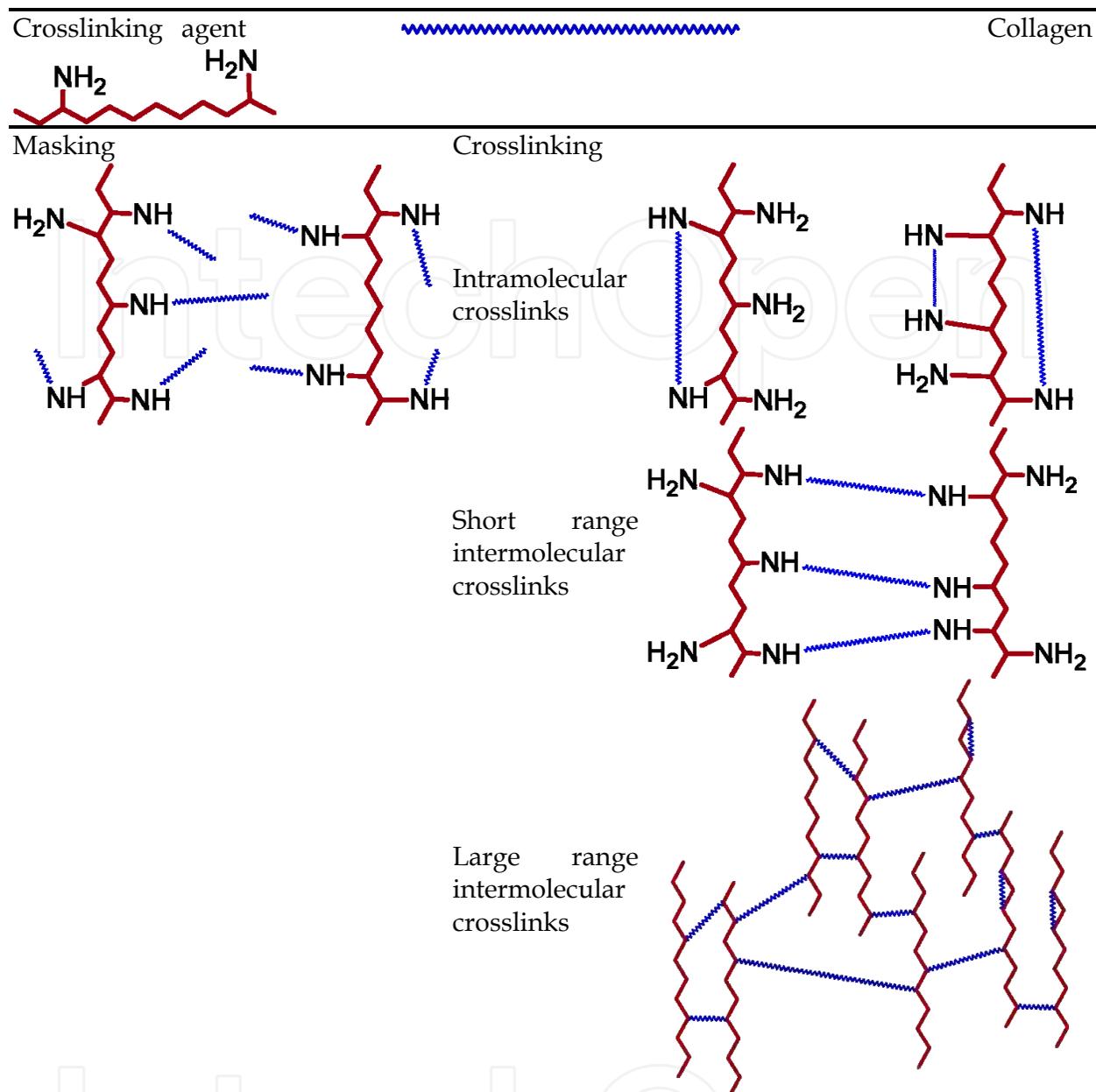


Table 6. Schematic representation of presumable masking and intra- or inter-molecular crosslinking structures

rates. Also it was reported the effectiveness of EDAC and neomycin (an inhibitor of the enzyme hyaluronidase) for the prevention of glycosaminoglycans loss (Ragharan et al., 2007; Shah & Vyavahare, 2008). The addition of exogenous glycosaminoglycans and the stabilization of endogenous glycosaminoglycans in ostrich perichardium reduced tissue calcification after implantation in rats, but slightly increased the presence of matrix-metalloproteinase at the implantation site (Arenaz et al., 2004).

## 5. Conclusions

Natural tissues from various sources can be used as biomaterials in the cardiovascular field after decellularization and fixation with various crosslinking agents. However, the current

approaches based on surfactants and difunctional crosslinking agents can affect the structure, GAG content, biocompatibility and calcification potential of these tissues. Because of this, different posttreatment methods have been suggested showing some improvements but until today, the vascular graft or heart valve obtained do not fulfill all the requirements for a long term use. In addition to this safety concerns, these treatments are not yet cost effective. Therefore, methods that preserve simultaneously the mechanical properties of collagen and the properties of the GAG matrix are desirable. Finally, those approaches that made use of enzymatic methods and low toxicity chemical such natural products and aminoacids as crosslinking agents seem promising alternatives.

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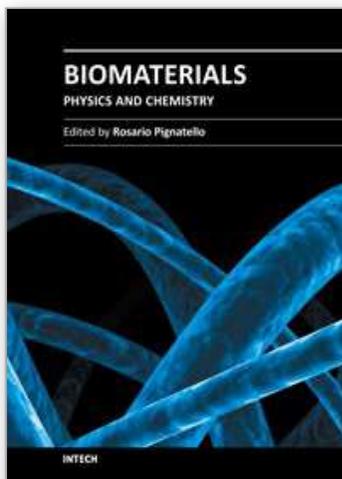
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## **Biomaterials - Physics and Chemistry**

Edited by Prof. Rosario Pignatello

ISBN 978-953-307-418-4

Hard cover, 490 pages

**Publisher** InTech

**Published online** 14, November, 2011

**Published in print edition** November, 2011

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Birzabith Mendoza-Novelo and Juan Valerio Cauich-Rodríguez (2011). Decellularization, Stabilization and Functionalization of Collagenous Tissues Used as Cardiovascular Biomaterials, *Biomaterials - Physics and Chemistry*, Prof. Rosario Pignatello (Ed.), ISBN: 978-953-307-418-4, InTech, Available from: <http://www.intechopen.com/books/biomaterials-physics-and-chemistry/decellularization-stabilization-and-functionalization-of-collagenous-tissues-used-as-cardiovascular->

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