A NOVEL APPROACH IN CROSS-LINKING OF BIOPROSTHETIC HEART VALVES

PROEFSCHRIFT

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Promotor: Prof. Dr. J. Feijen

“Gutta cavat lapidem, non vi sed saepe cadendo”

Oude maar immer actuele spreuk
Frans M Everaerts

Voor Inge
Milan en Jasmijn
Aan mijn ouders

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Back: morphed image depicting valve leaflets

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Voorwoord

Het is alweer een hele tijd geleden dat ik de uitdaging accepteerde om project 'IdeFix' een stapje verder te helpen. Wat er lag was een veelbelovende fixatie techniek voor weefsel en het was de bedoeling om na een aantal experimenten de processen te optimaliseren en hiermee een product te realiseren. Het hoofddoel van de eerste experimenten die ik deed was het kwijtraken van de 'nare lucht' van een van componenten in het proces. We wisten toen nog niet dat deze component een zo dramatisch effect zou hebben op de in-vivo verkalking van het behandelde weefsel en dat het project uiteindelijk zou uitmonden in een promotie onderzoek.

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Chapter 1

General introduction

There has been a dramatic improvement in healthcare and the standard of living of the general population in the industrialized countries over the last 50 years. This has resulted in a decrease in the incidence of for example rheumatic heart disease. However the number of elderly people with some form of valve disease has increased since valve calcification, stenosis and regurgitation become more prevalent with increasing age. This can lead to malfunctioning of the heart valve, thus requiring surgical correction or replacement [1].

An estimate of the American Heart Association of 2004 learns that worldwide about 250,000 patients receive valve implants, of which about 70% are mechanical and 30% are tissue prostheses. Mechanical valves are preferred, despite the fact that all mechanical valves share a common disadvantage, which is the need for permanent anticoagulant therapy for the patient. In general, mechanical valves provide better durability and better haemodynamics, especially for smaller sizes, compared to tissue valves [2]. So practically, in young patients the mechanical valve is preferred, while biological prostheses continue to be recommended for aortic valve replacement in patients over 65 years of age [3,4]. However, thrombo-embolism and thrombotic events associated with mechanical valves, as well as haemorrhagic complications associated with the anticoagulant therapy continue to trigger the desire to have a better, more durable bioprosthesis. Since the commonly used glutaraldehyde fixation technology for bioprostheses has been implicated in the calcification process many research groups are focusing on the development of alternative cross-linking techniques.

Another approach is the so-called ‘decellularization’ process, in which all non-necessary components are removed from the matrix. In theory this gives a tissue matrix that does not need to be cross-linked. After implantation the matrix structure may be replaced by patient own material. However since it is impossible to clean-up the matrix totally, much research still needs to follow and more knowledge regarding the mechanism of calcification (and associated inflammation) is required before these kind of materials, without further cross-linking, could become a commercial product.

Since the first commercial bioprosthetic valves became available around 1958, research has focused on three major topics: (1) reduction of the degradation of the tissue and reduction of inflammatory responses caused by the tissue, (2) reduction of calcification and (3) reduction of pannus overgrowth with resulting mechanical loss of the valves.

While in the first years, in which the formaldehyde and glutaraldehyde cross-linking technology was developed, the focus has been on the reduction of degradation and calcification; research in the last decade mainly is focused on reduction of calcification and pannus overgrowth.

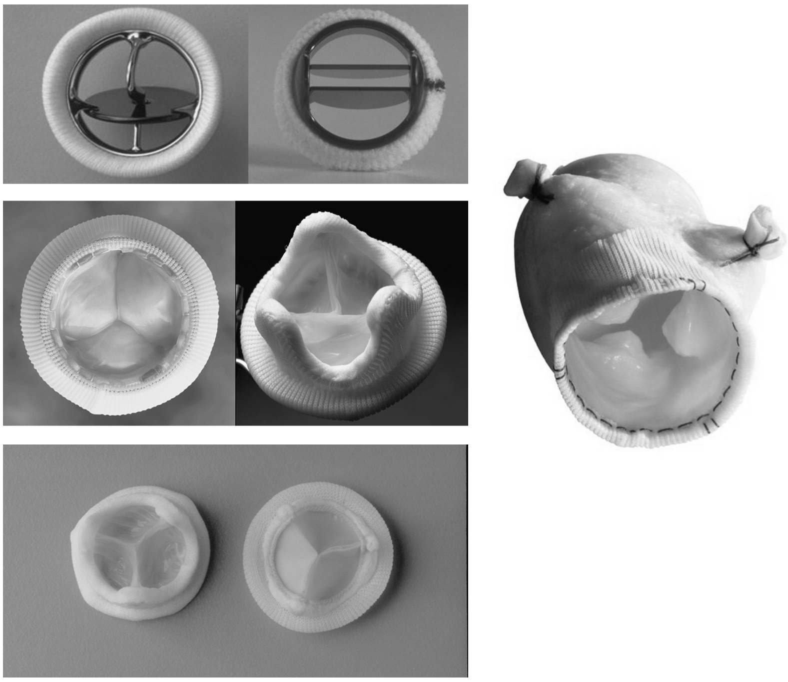

Figure 1; *Examples of commercial heart valves: mechanical (Hall™), stented tissue (Intact™, Hancock™) and stentless (Freestyle™) configuration. Reproduced with permission of Medtronic (Minneapolis USA).*

Aim of this study

The aim of the study presented in this thesis is the development of a non-glutaraldehyde based cross-linking technology for bioprosthetic material, providing the material with a low propensity for calcification, a high durability and a good biocompatibility of both wall and leaflet material. A water soluble carbodiimide (N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide, EDC) cross-linking agent was selected, based on earlier studies carried out by Olde Damink et al [5] and Zeeman et al [6].

To achieve this aim the following goals were defined:

1. Further understanding the chemical reactions that occur in the matrix during the cross-linking reaction.
2. Correlating the tissue properties, bio(chemical) observations and calcification with the chemistry applied to the matrix.

Outline of this thesis

A literature study on current cross-linking technologies for bioprosthetic heart valves and general information on its structure and composition is described in chapter 2. It is demonstrated that although several promising cross-linking technologies are found in literature there is still a need for developing new or optimizing existing technologies. In chapter 3, as we named it, an "enhanced carbodiimide" cross-linking method is presented. In valves processed via this method, prior to EDC and N-hydroxysuccinimide (NHS) activation of carboxyl groups, the available amine groups in (tissue) collagen are blocked with butanal. Cross-links are thereafter formed by reacting the matrix with poly(propylene glycol)bis 2-(aminopropyl) ether (Jeffamine™), EDC and NHS. Complete heart valves (wall and leaflet) have been cross-linked, in-vitro assessed and their propensity to calcify in an in-vivo calcification model has been studied. It is demonstrated that both wall and leaflet, cross-linked with the enhanced cross-linking method give a significantly reduced calcification compared to a reference glutaraldehyde fixed bioprosthesis. The method has been further optimized and the results of this study are presented in chapter 4. It is shown that the type of the aldehyde used for the blocking reaction is a key element in the in vivo calcification process of the prostheses. However, despite the promising results with respect to the tissue response, an unexpected increase in tissue stiffness occurs, caused by the use of EDC/NHS as compared to GA.

In the absence of a reliable method to quantify residual carboxyl groups in cross-linked tissue, unfortunately the degree of cross-linking can only be estimated by indirect measurements. In chapter 5 the development of a new method to quantify residual carboxyl groups on cross-linked collagen model materials is described. In this method matrix carboxyl groups are labeled with 5-bromomethyl fluorescein (5-BMF). Subsequently, the attached fluorescent label is released by mild hydrolysis, collected and quantified with capillary zone electrophoresis (CZE). A calibration curve relating the concentration of carboxyl groups with peak intensities is obtained using carboxyl group modified Sephadex™ standards. With this method in hand an investigation to the degree of cross-linking of collagen matrices after reaction with EDC and NHS has been made and is demonstrated that besides amide group containing cross-links, additional cross-links are formed in the matrix. Based on literature it is hypothesized that these additional cross-links are esters.

Further investigation of which the results are described in chapter 6 reveal that EDC/NHS activated carboxyl groups in collagen do not only react with residual amine groups but also with hydroxyl groups. This results in the formation of both amide and ester linkages in the matrix. It is furthermore demonstrated that these esters significantly attribute to the tissue mechanics as determined by uni-axial tensile testing. Fortunately, esters are relatively instable and a method has been developed to remove these ester groups by mild hydrolysis. It is demonstrated that by rinsing cross-linked collagen matrices at pH 10, esters are removed while amide linkages are unaffected. This results materials with improved biomechanics. In chapter 7 the results of a screening study to the effects of removal of these ester groups on the in-vivo performance of the tissue is discussed. Hereto porcine aortic tissue valves have been EDC/NHS cross-linked with Jeffamine™ (with a molecular weight of 230 or 400) after a blocking reaction of matrix amine groups with propional. From each processed sample group valves were stored at pH 7.2 (control group) and pH 10 (in order to remove the esters) for 2.5 months and thereafter evaluated in-vivo in juvenile rats. It is demonstrated that while subtle but significant changes are noted in in-vitro tests typically used to characterize bioprosthetic tissue, these changes do not significantly affect the in-vivo performance. Interestingly it has been observed that after applying the hydrolysis reaction to the matrix the calcification patterns change.

While eliminating the additional cross-links formed during the EDC and NHS process did not have direct consequences for its performance, there are indications that the removal of extraneous cross-links has an effect on the biomechanical properties of the tissue. It is suggested that the observed increase in tissue suppleness could impart a more biological character to these stabilized bioprosthetic matrices potentially increasing their long term durability.

Many research groups work on the development of a method in which tissue, after processing and implantation, will act as a natural scaffold. Bioprosthetic tissue that ultimately will be replaced in-vivo by the patient’s own material and has the ability to grow after implantation is desired for many years now. Although promising efforts have been undertaken in order to achieve this goal, it becomes clear that a multidisciplinary effort is required to reach this goal. In our view, despite successes booked in the area of decellularization of tissue matrices on one hand and creation of tissue with tissue engineering on the other hand, cross-linking of tissue will be still the key parameter in the area of tissue bioprostheses in the next coming years. As suggested in chapter 7 the combination of decellularization and subsequent cross-linking with the technique described in this thesis may result in materials with even better promising in-vivo performance. Although the technology was developed and optimized for porcine aortic tissue valves there are many other tissue derived products that could benefit.

Objective

The objective of the study described in this thesis is to develop and optimize a carbodiimide based cross-linking technology for (porcine) tissue heart valves. Cross-linked materials should be biocompatible, have a low propensity for calcification in-vivo and the mechanical properties should be at least comparable to those of current commercial valves.

The research presented in this thesis is a continuation of the work performed by Leon Olde Damink [5] and Raymond Zeeman [6]

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Chapter 2

Calcification…still an issue in bioprosthetic valve design ?[*]

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[*] Parts of this chapter are published in: *Hendriks M, Everaerts F, Verhoeven M, Alternative Fixation of Bioprostheses, J Long Term Eff Med Implants 2001; 11(3-4): 163-183*

Introduction

A 2004 estimate learns that worldwide about 250,000 patients undergo valve implants, of which about 70% are mechanical and 30% are tissue prostheses. Mechanical valves are preferred, despite the fact that all mechanical valves share a common disadvantage, which is the need for permanent anticoagulant therapy for the patient. In general, mechanical valves provide better durability and better haemodynamics, especially in smaller sizes, compared with tissue valves [1,2]. Especially in young patients the mechanical valve is preferred [3,4], while biological prostheses continue to be recommended for aortic valve replacement in patients over 65 years of age.

Thromboembolism and thrombosis events associated with mechanical valves, as well as haemorrhagic complications associated with the anticoagulant therapy continue to trigger the desire to have a better, i.e., more durable bioprosthesis. Calcification is one of the primary failure modes of bioprostheses, limiting their durability. The commonly used glutaraldehyde fixation technology has been implicated in the calcification process. It is therefore of no surprise that many efforts have gone in to the development of alternative fixation technologies not involving glutaraldehyde, as well as means of ameliorating the toxicity of these agents. In this chapter we report on newly developed tissue fixation processes as well as tissue fixation processes that are under investigation. Furthermore, as porcine aortic valve matrices were used in work described in this thesis, its morphology and composition is described in more detail

Cross-linking

In natural tissue presence of cross-links (for example in collagenous structures) is important for its stability and performance. Under normal circumstances cross-links are reversible allowing an organism to grow and replace damaged structures; in some cases formed cross-links are irreversible (such as in scar tissue) with associated issues.

Tissue with indended use to serve as bioprosthesis is mostly obtained from abattoirs. The moment the animal is sacrified the natural process of collagen turnover has stopped and without support it almost immediately starts to degrade. Since the aim is to prolong the original and structural integrity as long as possible, this degradation must be arrested and deferred as soon as possible. In addition the antigenic properties of these tissues must be removed or at least neutralized in order to make them suitable to serve as bioprostheses. Furthermore, in most cases, the structure is not allowed to degrade by the various biological processes after implantation. As of such many methods have been developed to preserve tissue.

With most methods found in literature, cross-links are made between the collagen molecules in the material, which reinforce the matrix to give a tough and strong material that maintains the original shape of the tissue [1]. As collagen molecules are made of linked amino acids, residual side groups of these amino acids (amine, carboxyl and/or hydroxyl groups) are used as anchoring locations for these bonds. A summary of the most important cross-linking technologies that have been developed and published in literature so far are discussed below.

Aldehyde-based crosslinking

Formaldehyde (formalin) has been used for many years in biological and pathological research. The reagent stops deterioration of tissue and prolongs the original structure and mechanical integrity of the tissue treated; the first commercially available tissue valves were formaldehyde fixed (Hancock, 1968) [5], but studies showed that formaldehyde treated tissue is considerably less stable compared to glutaraldehyde (GA) fixed tissue [6], and therefore nowadays almost all commercially available tissue valves are GA fixed.

Although GA is a relative simple compound, various reactions may occur during crosslinking of collagen-based materials [7,8,9,10,11,12]. Despite the relative simplicity of the GA compound, the GA fixation process is obviously much more complex than at first anticipated. This makes it very difficult to establish any correlations between the chemical modifications that have taken place and the resulting biological behavior, notably the propensity of GA fixed tissues to calcify [13]. A further disadvantage of using GA as crosslinking agent is its toxicity, even at low concentrations. Concentrations as low as 3ppm inhibit 99% of the ^{3}H-thymide uptake of cultured fibroblasts [14] The lack of endothelial cell coverage on implanted tissue valves has been ascribed to the toxic effect of slowly leached aldehydes [15]. It has been reported that during in vivo application of GA crosslinked materials upto 60% of the incorporated GA is released into the tissue [16]. The mechanism of this release and the composition of the released compounds is still not clear.

Despite about 40 years of experience in working with GA as a fixative, it is striking how little actually is known about the precise mechanisms involved in either the chemistry of the fixation process itself or the GA induced process of calcification. Zilla and co-workers showed that increasing concentrations of GA correlate with a significant decrease in calcification [17]. Improved preservation of tissue integrity was postulated as the underlying mechanism explaining these results. At such GA concentrations (upto 3%) the tissue gets very stiff, and these unfavorable mechanical properties would prohibit actual usage of this particular GA fixation process. Nevertheless, this study is exemplary in that it has been one of the few that has tried to establish a correlation between the chemical process and eventual biological performance.

Alternative fixation

Due to its superb crosslinking characteristics, GA is still the most widely used fixative for heart valve tissues. At the same time, however, GA is also considered to be, at least partly, responsible for tissue calcification and the lack of surface re-endothelialization, both of which may contribute to valve degeneration. Many efforts have therefore gone into the design and development of tissue fixation processes that have a reduced propensity to calcification and better biocompatibility. These efforts can roughly be divided into three generic approaches, i.e., [1] improvements to GA fixation processes; [2] polymer incorporation to fill empty spaces in the tissue; and [3] processes that make use of alternative, non-GA fixation reagents.

Improved GA fixation processes

Various strategies for enhancing the *in vivo* performance of GA fixed tissue have been disclosed. Most of the strategies are directed at inhibition of implant-associated calcification, but also processes have been developed to stabilize the GA crosslinks, thus reducing release of toxic GA derived compounds, and thus enhancing biocompatibility.

reductive agents

The use of reductive agents has been suggested for elimination of residual aldehydes, and stabilization of labile crosslinks formed. Reductive agents, such as borohydride (BH_4^-), cyanoborohydride ($CNBH_3^-$), and amine borane complexes, can be used to reduce aldehydes to alcohols [18,19].

Borohydride has been used for stabilization of labile crosslinks in natural tissues [20], but also for reductive treatment of GA crosslinked materials [21-24,27] . Dewanjee suggested the use of cyanoborohydride for stabilization of GA crosslinked tissues [24]. To avoid any possible problems from toxic residues, however, according to Dewanjee the preferred reducing agent is borohydride.

blocking residual aldehydes

Another suggested approach for improvement of the conventional GA fixation process involves the blocking of reactive aldehydes. Grimm et al studied the binding of excess aldehyde by treatment with L-glutamic acid in an acidic medium [29,30,31]. L-glutamic acid treatment of GA crosslinked heart valve tissue was reported to markedly improve the biocompatibility, including significant reduction of calcification and decreased inflammatory response [30,31] and enhanced cellular overgrowth, *in vitro* [32,33] as well as *in vivo* [31-33]. The precise reaction mechanism is not completely understood, but besides blocking of aldehydes by the L-glutamic acid, the acidic pH during L-glutamic acid treatment is suggested to favour depolymerization of polymeric GA, thus facilitating wash-out of excess aldehyde.

The use of glycine to improve the clean-up of the GA crosslinked matrix also was suggested, but has been shown to be less effective long-term than the L-glutamic acid treatment in enhancing the biocompatibility [29]. Zilla et al did a large study to identify the most optimal *detoxification* agent and the most optimal reaction conditions for clean-up of GA crosslinked aortic tissue. Hereto they compared 12 different amino-reagents from four chemical groups: low pKa aromatic amines, amino acids, low pKa N-heterocyclic compounds, and amino sugars [34]. While differences were observed between candidates, all 12 amino-reagents were able to satisfactorily detoxify aortic tissue under the right reaction conditions as measured by endothelial cell outgrowth.

Enhanced GA crosslinking

Introduction of additional crosslinks throughout the collagen-based material has been suggested as another approach to improve the GA fixation process.

Nimni et al suggested to enhance crosslinking through bridging of carbodiimide activated carboxyl groups with hexanediamine. In a sequential step GA is used to crosslink collagen and the unreacted amines introduced by hexanediamine [35,36]. Their proposed crosslinking mechanism includes a variety of possible modifications of the collagen matrix; however, it failed to include the amide type crosslinks that will form as a result from the reaction between the activated carboxylic acid groups present in the polypeptide chains followed by reaction with the free amine groups of other polypeptide chains.

When additional crosslinks were introduced by hexanediamine, resistance to enzymatic degradation was enhanced beyond that obtained with increased concentrations of GA. Furthermore, introduction of additional hexanediamine crosslinks lowered the antigenicity significantly.

Simionescu et al proposed the use of L-lysine to introduce a larger number of crosslinks by bridging free aldehyde groups, that remain after GA crosslinking [37]. They showed that sequential repetitive treatments with GA and lysine introduces additional crosslinks, denoted by the increase in shrink temperature. *In vitro* fatigue testing proved the material to be very durable. Raxworthy et al. found that lysine-treated GA crosslinked collagen was more elastic but mechanically weaker than GA crosslinked collagen alone [38].

Zilla and coworkers reported that enhanced fixation of bioprosthetic tissue by both increased GA concentrations and the introduction of additional crosslinks with L-lysine significantly reduces calcification.

The combination of enhanced crosslinking with subsequent detoxification even further improved tissue calcification in the rat model [30].

Coupling calcification inhibitors

Control of calcification in GA treated collagen-based materials is an option to improve the *in vivo* performance. Complete prevention of calcification is desirable, but inhibition of mineralization below certain levels may be sufficient to make an important impact on long-term performance [40]. The two most commone types of tissue antimineralization treatments that have been proposed are clean-up of the matrix by removal of cellular debris, which include phospholipids, and mitigation of calcification via agents that bind to the nucleation sites for calciumphosphate.

Several techniques have been developed for lipid extraction. One technique involves the post-fixation dialysis of the tissue with ethanol. Several groups have investigated this treatment with good to very good reduction of calcification as observed in the rat [41,42]. Lipid extraction can also be performed by treatment of GA crosslinked materials with detergent compounds, such as sodium dodecylsulfate (SDS) [43,44]. Subcutaneous implant studies suggest that this approach is efficacious, but the results in experimental circulatory models have been inconsistent [45].
The main concern with all these extraction methods is that they may worsen the structural integrity of the collagen-based material [40].

In 1966, Urist and Adams reported that treatment of non-crosslinked tendon in solutions of inorganic salts, such as $CuCl_2$, $SrCl_2$, or organic cations, such as toluidine blue or protamine sulfate, to block carboxylic acid groups, followed by implantation in hypercalcemic rats, consistently inhibited calcification [46]. However, treatment with solutions of $CaCl_2$ to effect ion exchange of Ca^{2+} for the blocking agent demonstrated the processes to be reversible.
Iron and aluminum are presumed to complex phosphorous groups in the early nucleation sites as such disrupting formation of hydroxyapatite [47]. Incubation of GA crosslinked tissue treated with Al(III) and Fe(III) salts was found to dramatically reduce calcification in the rat model [48-50].
An alternative strategy employs diphosphonate compounds, used for therapy of metabolic bone disease, to inhibit calcium phosphate crystal growth. Diphosphonates can have severe and irreversible adverse effects on bone and calcium metabolism. This implicates that localized sustained release administration is strongly preferable over systemic administration. In his US patent, Dewanjee suggested the coupling of aminodiphosphonate (ADP) compounds to GA crosslinked collagen-based materials [24]. Dramatically less bioprosthetic valve leaflet calcification has been reported by Nimni [36], and Webb et al [51]. Collagen-based materials that underwent the enhanced GA crosslinking procedure as described by Nimni et al [35], were able to couple more of the aminodiphosphonate drug, thus completely inhibiting calcification

in the animal model employed [36]. Besides the direct coupling of the diphosphonates to the tissue, the use of localized diphophonate delivery systems has been studied [52,53,54]. Another technology for inhibiting bioprosthetic calcification was suggested by Girardot et al [55] Treatment of GA-preserved bioprostheses with α-aminooleic acid (AOA), a non-toxic, biocompatible long-chain fatty amino acid, reduced calcification dramatically, both in the subcutaneous rat model [55] and in the circulatory sheep model [56]. Investigation studies have shown that the AOA post-treatment induces a significant decrease in calcification of the cusp, but not the aortic wall [57]. The postulated mechanism by which AOA reduces calcification is that a reduction of the calcium transport in the tissue is brought about through the incorporation of the long fatty chains of AOA. Less effective penetration of the AOA into the dense aortic wall structures is the likely reason for the observed discrepancy in calcification results between cusps and aortic wall. AOA may also act as a detergent but there is no evidence that this effect is truly present. The AOA process has been commercialized by Medtronic on the Freestyle$^{\text{TM}}$ stentless bioprosthesis.

Polymer incorporation

Golomb and Ezra studied the modification of glutaraldehyde crosslinked tissue with the polybasic peptide protamine sulfate to compensate for the net positive charge of the tissue after fixation [58]. This method resulted in a 70% reduction in calcification in a rat model. Modification of the tissue was also performed using sulphonated polyethylene oxide (PEO) [59] or non-functionalized PEO. Reduction in calcification of tissue treated with sulphonated PEO was investigated in subcutaneous rat model, in canine circulatory implantation model, and in in vitro tests, showing 65%, 85%, and 50% reduction in calcification respectively [59,60]. Polyethylene glycol grafting of bioprosthetic tissue after GA fixation resulted also in reduction of calcification. Subcutaneous studies in rats showed 80% reduction, while in vitro calciumphosphate solution studies showed a 50% reduction in calcification [61-63]. Grafting of tissue with several methacrylates showed mitigation of calcification when implanted subcutaneously in rats. In a circulatory sheep model 50% reduction in calcification was observed [64]. The post-treatment of GA fixed tissue with chitosan or heparin was also shown to be effective in mitigating calcification [65,66].

Other non-glutaraldehyde technologies

Non GA crosslinking reagents have also been explored for their capability of tissue fixation. In the design and development of new crosslinking or modification methods, the free carboxylic acid (primarily from aspartic acid and glutamic acid) and the free amine groups (primarily from lysine and hydroxylysine) are the most important functional groups. In next sections we will discuss alternative, non GA tissue fixation processes that have been reported on.

Dye-mediated photo-oxidation

A dye mediated photo-oxidation method to crosslink tissue was first reported on almost ten years ago [67]. The tissue to be crosslinked is soaked in a solution containing the photo-oxidative dye, and then irradiated with light at wavelenghts that are selectively absorbed by the catalyst. The amino acids histidine, tryptophan, tyrosine and methionine are particularly modified by this method. Unusually, the shrink temperature of the fixed tissue was found almost equal to that of fresh tissue while having more resistance to enzymatic digestion [68]. In a juvenile sheep model, in the descending aorta and subcoronary positions, valves prepared using this photoxidation process showed minimal calcification [69]. Carbomedics, Inc. started clinical investigation studies with this very promising new tissue fixation method (Photofix^{TM}), but early clinical failures (caused by abrasion of the inflow surface of the leaflets against the cloth-covered inner face of the outer valve frame [70] have caused these trials to be stopped, so that long-term clinical results are not available.

Epoxy compounds

Epoxy compounds have been extensively reported on in the past decade as an alternative to GA [71-78]. Generally, mixtures of bi- and tri-functional glycidyl ethers based on glycerol are used. Due to its highly strained three membered ring, epoxide groups are susceptible to a nucleophilic attack. Predominantly a reaction with the amine groups of (hydroxy)lysine residues will occur. Addionally, epoxide groups can react with the secondary amine groups of histidine, and dependent on the pH, also with the carboxylic acid groups present in the collagen matrix. It has been reported that, contrary to GA crosslinked materials, epoxy crosslinked materials maintain their natural pliability and appearance, and become more hydrophilic and hydrated. The crosslinking density of epoxy treated materials has been found to be as good as that of its

GA counterpart. In addition, it has been reported that epoxy crosslinked tissue shows a comparable reduction of the antigenicity and immunogenicity. In one study reduced calcification has been reported [71], but this result has been contradicted by several other studies [79]. In this study tissue was compared that was crosslinked with butanediol diglycidylether (BDDGE) at low (pH=4) and high (pH=9) pH-values with GA fixed tissue. It was found that the amount of calcium in the epoxy crosslinked tissue after implantation was approximately equal to the calcium detected in the GA control.

Carbodiimides

The carbodiimide process involves activation of carboxylic acid groups present in the polypeptide chains followed by reaction with free amine groups of other polypeptide chains or introduced crosslinker molecules, resulting in amide type crosslinks.

Weadock et al. reported on the use of the highly toxic cyanamide {R_2-N=C-N} for crosslinking reconstituted collagen [80], after which they suggested the use of several other carbodiimide reagents in a patent application [81]. Olde Damink thoroughly studied crosslinking of dermal sheep collagen (DSC) by using the watersoluble carbodiimide

The process can be accelerated by addition of catalysts such as N-hydroxybenzotriazole (HOBT) and N-hydroxysuccinimide (NHS), yielding activated esters. The beneficial effect of these agents is based on the suppression of two side reactions that can occur when carbodiimides are used for the activation of carboxylic acid groups [82]. First, the thus activated carboxylic acid groups are less susceptible to hydrolysis at acidic pH compared to the O-acylisourea group. Additionally, suppression of the formation of N-acylurea takes place, as has been demonstrated by Olde Damink [16].

Girardot describes a procedure where this EDC/NHS method is used in combination with di- or tri-carboxyl acid and di- or tri-amine crosslinkers for fixation of aortic tissue (Ultifix^{TM}) [83]. The tissues were evaluated for their propensity to calcification when implanted subdermally in rats. They found that the cusps of the porcine aortic root and the porcine pericardium calcified minimally, and significantly less than the control material fixed with glutaraldehyde when implanted for up to 16 weeks. The wall of the porcine aortic root, however, calcified comparable to GA fixed tissue.

Acyl azide

This crosslinking method involves the transformation of free carboxyl groups into acyl azide groups that then can react with the free amine groups present on the polypeptide chains of the collagen or introduced crosslinker molecules. The acyl azide method is very efficient as was demonstrated by Simmons and Kearney [84]. They demonstrated that a crosslinking efficacy comparable to GA could be achieved. A disadvantage of this process is that it is rather time-consuming and requires extensive washing for complete removal of all by-products [85]. If di-phenylphosphorylazide (DPPA) is used, carboxyl groups in the tissue can be directly transformed to acyl azide, after which reaction with the amino groups can take place [86]. With this alternative process the extensive washing step is not required. The DPPA crosslinking reaction, however, needs to be performed in a non-aqueous medium, preferably dimethylformamide (DMF).

There are no *in vivo* results published on bioprosthetic tissue crosslinked using the acyl azide method. With data from a screening experiment performed in our lab were heart valves cross-linked with an epoxy, an carbodiimide and acyl azide we concluded that the acyl azide cross-linked heart valve had a significant increased calcification and inflammation as compared two the other two groups. However it was demonstrated that collagen substrates have shown reduced propensity to calcification when compared to GA crosslinked collagen substrates [87]. In an *in vitro* organotypic culture method improved biocompatibility of calf pericardium was demonstrated when compared to GA fixed pericardium [88].

Other reported alternative crosslinking methods

No *in vivo* results are available on valve tissue for the following crosslinking methods, but as these have been reported to be effective in crosslinking of, mostly, pure collagen or collagenous substrates, these methods will be briefly described below.

Chvapil et al used the bifunctional reagent hexamethylene diisocyanate (HMDIC) as an alternative for GA crosslinking [89]. Similar as with GA, the isocyanate groups of HMDIC react with the amine groups of lysine or hydroxylysine residues present in the polypeptide chains, resulting in crosslinking of the collagen-based material. As a consequence of hydrolysis of pendant free isocyanate groups, (pendant) free amine groups are generated, that can further crosslink to form extended oligomeric crosslinks. All HMDIC crosslinking reactions involve the formation of aliphatic chains containing urea bonds. HMDIC has the disadvantage of being only slightly water soluble. Additional use of surfactants is needed to improve penetration of the crosslinking reagent into the matrix of the collagen-based material. However, use of water soluble diisocyanates may not at all be advantageous, since the isocyanate group is very susceptible to hydrolysis.

Chvapil compared the cellular reactions to subcutaneously implanted collagen sponges crosslinked with GA. HMDIC crosslinked collagen was demonstrated to form a proper matrix for tissue regeneration, as it was continuously and thoroughly infiltrated by cells forming granulation tissue. Contrarily, GA crosslinked collagen prevented cellular ingrowth, and showed a considerable tissue response at the outer rim of the implant. Van Luyn et al confirmed these observations [90].

Imidoesters are water-soluble and react under mild conditions with a high degree of specificity with amino groups in proteins. The formed amidine groups are quite stable to hydrolysis. Since these amidines carry a formal positive charge (pK_a of amidines is considerably higher than that of the ε-amino groups of lysine), extensive reaction with lysine residues can be carried out without any change in the net charge. The latter may be favourable as several investigators hypothesised that change in charge may effect cell-material interactions considerably.

Hey et al used the bisimidoester crosslinker dimethylsuberimidate (DMS) to crosslink fibrous dermal collagen to develop a dermal implant for repair of burns and other large cutaneous wounds [22]. DMS failed to increase the mechanical strength of the dermal collagen, indicating that crosslinking might not have occurred. The reported failure of DMS to increase resistance to enzymatic degradation of human dermis may confirm this [84].

Fujimoto and Horiuchi studied glucose-mediated crosslinking of collagen [91]. They found that free amino acids, particularly lysine, accelerate the crosslinking in collagen. A marked resistance to solubilization by cyanogen bromide was found. Crosslinking may be caused by dicarbonyl compounds formed from glucose by the reaction with amino groups. Simmons and Kearney demonstrated glucose-mediated crosslinking to be ineffective in crosslinking human dermis [84]. Collagen molecules may be hindered to orientate more freely in comparison to pure collagen. This may cause the demonstrated failure to increase resistance to enzymatic degradation in human dermis.

Kano et al reported crosslinking of collagen using an ascorbate (vitamin C)/copper ion system [92]. Evaluation led to the conclusion that in some respects the modifications by the ascorbate-copper ion systems mimicked those that occurred in human collagen with aging. Application of this process in crosslinking human dermis was not a success [84].

A method involving succinic anhydride crosslinking, previously reported by Noishiki and Myata [93], was also demonstrated to fail in crosslinking human dermis [84].

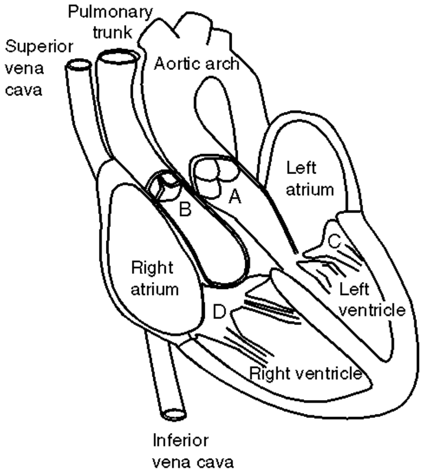
Genipin and its related compounds, extracted from gardenia fruits, have been used in traditional Chinese medicine for the treatments of jaundice and various inflammatory and hepatic diseases. Amongst others, Sung et al [94] successfully studied the effect of genepin as natural cross-linking agent for biological tissue. They report that in contradiction to carbodiimide that may form intrahelical and interhelical crosslinks within or between tropocollagen molecules and genipin may further introduce intermicrofibrillar crosslinks between adjacent collagen microfibrils. The cross-linking reaction reaction itself is time-consuming and sterility needs to be maintained during this process for a longer period of time. In vitro experiments showed that material cross-linked with genipin has a good biocompatible behavior.

General morphology of Heart Valves

The human heart contains 4 valves, needed for one-way directing blood during pumping (see Figure 1). The mitral and tricuspid valves are located between the atria and ventricles; the aortic and mitral valves are located in the outflow tracts of the heart.

Since bioprosthetic aortic tissue valves were studied in the project described in this thesis its composition will be discussed in more detail.

In general bioprosthetic tissue valves include allo- or homografts, which are taken from human donors, and hetero- or xenografts which are of animal origin. Furthermore these tissue valves can be subdivided in stented and stentless configurations. In stented valves the leaflets are mounted on a stent, often made of a polymer while in a stentless configuration the original root of the porcine bioprosthesis is used as a natural stent providing superiour heamadymanic performance [2]. See also Figure 1 in chapter 1 depicting samples of various commercial valve configurations.



A. Aortic Valve
B. Pulmonary Valve
C. Mitral Valve
D. Tricuspid Valve

Figure 1*, location of 4 valves in the human heart (http://heartlab.robarts.ca/heartlab.html)*

Shown in figure 2, the aortic valve is composed of three, endothelially invested membranous cusps or leaflets and aortic sinuses. The leaflets, which are the most mobile parts of the valve, are anchored in the aortic wall. The sites where the leaflets come together are called the commissures. Between the leaflets and the aortic wall there are dilated pockets called the aortic sinuses. From two of these sinuses the coronary arteries originate. Along the top of each leaflet is the free edge. In the middle of it, there is a collagenous-rich area, the Corpus Arantii (or Nodulus of Arantius), which supposedly aids in the valve closure and reduces regurgitation [98-99].

The only anatomical difference between the human and the porcine aortic heart valve is the presence of a muscular shelf on the right coronary leaflet. The presence of this muscle shelf results in a delayed opening of the right coronary leaflet relative to that of the left and the non-coronary [100-103].

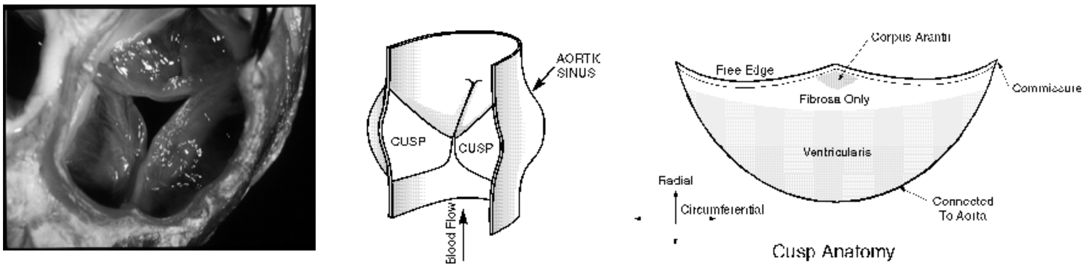


Figure 2, *Anatomy of a heart valve ((http://heartlab.robarts.ca/heartlab.html)*

As indicated in table 1, leaflets and wall contain primarily the structural proteins collagen and elastin.

Collagen is a polymer consisting of amino acid building blocks. It is synthesized by fibroblast; procollagen is secreted and thereafter converted to collagen. One molecule of collagen consists of two $\alpha 1$ and one $\alpha 2$ polypeptide chains each coiled in a left handed helix. The three chains coil together in a right handed triple helix to form a molecule of collagen. 5 molecules together are called tropocollagen and 5 of these molecules are called a microfibril. A fibril or fascicle

Table 1; *Tissue composition of the porcine leaflet and aortic wall (Reproduced with permission) [111]*

Component	Leaflet (%)[2]	Aortic Wall (%)[2]
Collagen	58±2	19±4
Elastin	13±4	34±4
Glycosaminoglycans (GAG)	14±3	7±2
Others[1]	15±1	40±8

[1] Others include lipids, proteoglycans and cellular components (fibroblasts for leaflets and fibroblasts and smooth muscle cells for aortic wall tissue)

[2] Swelling in PBS at 20°C: leaflet tissue 650% and aortic wall 330%

consists of about 1000 of these microfibrils. A collagen fiber contains about 500 of these fibrils and a number of these fibers together form bundles or tendons. Figure 3 given an impression of the composition of such a collagen bundle [104-113].

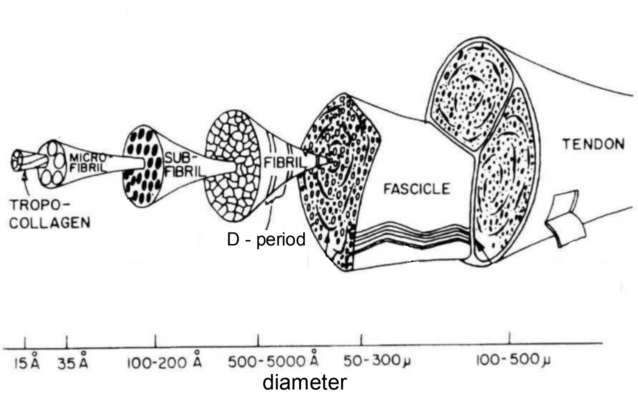


Figure 3, *Structural organization of collagen (reproduced with with permission) [114]*

Elastin is an insoluble, elastic protein of high tensile strength found in intercellular spaces of the connective tissues of large arteries, trachea, bronchi and ligaments. The precursors of elastin are called tropo-elastin and natural occurring cross-links between the lysine residues of this tropoelastin form the elastin molecules [106]. Elastin molecules contain relatively large hydrophobic side-chains that do not interact with eachother by forming hydrogen bonds. This gives elastin the opportunity to strech. It is worthwile to mention that elastin has an affinity for calcium ions and literature suggests a relationship between chronic arteriosclerosis and increased calcium concentrations found in elastin in aortic wall [107]. Although development of most cross-linking technologies focused on cross-linking of collagen, it may not be excluded that cross-linking affects the stability of elastin and its associated calcification [108].

Leaflets consist primarily of very small elastic and collagenous tendons relatively loosely arranged. These collagenous tendons, which are the major protein component of the leaflets, are unusually small: 300 - 500 A (98). Collagen types I and III are predominant collagen constituents (99 %) of heart valve tissue. A low content of methionine is found, whereas a high content of hydroxylysine is present, which contributes in the formation of stable native cross-links in the tissue [101]. The collagen tendons are not completely straight but follow wavy courses. This arrangement, usually referred to as crimping, allows changes in geometry of collagen-containing structures without substantial increase in tension (102). Collagen comprises 60 % of the total dry weight of human aortic leaflets. Due to aging this content drops after 80 years to 40 % [101].

In cross-section, the leaflet has three distinct layers [98, 103], the fibrosa, spongiosa and ventricularis (see figure 4). The fibrosa is a very dense layer, arranged as a series of parallel tedious cords in a rigid sheet of tissue. The collagenous fibers are mainly oriented in a circumferential direction. This layer provides the essential strength of the leaflets. The spongiosa is a very loose, watery connective tissue of varying thickness, consisting of fiber components, glycosaminoglycans (GAGS) and cells. Its sparse collagenous fibers and cells are oriented radially. It has a negligible structural strength but appears to perform an important role in minimizing mechanical interaction between the two fibrous layers and in dissipating energy during closure [105].

Finally the ventricularis consists of a superficial elastic layer, two or several fibers thick. This layer is less organized than the fibrosa. It enables the leaflet to have minimal surface area when it is open but stretch to form a large coaptation area when back-pressure is applied.

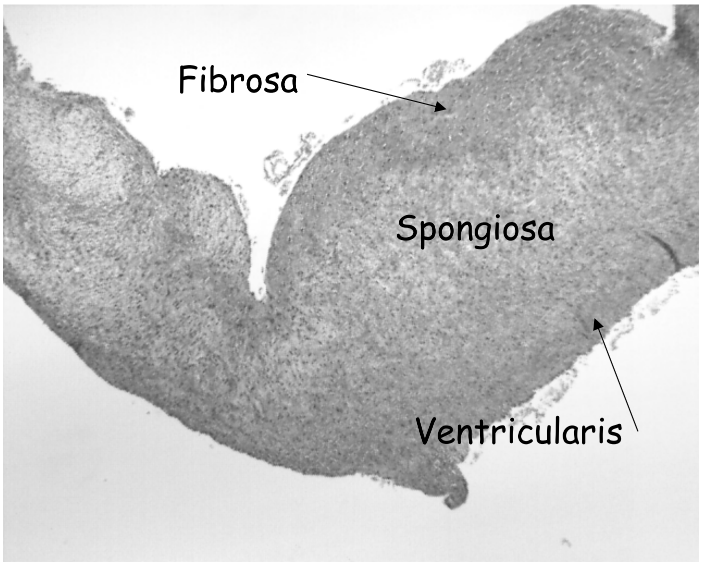


Figure 4; *H&E-staining of curved tissue leaflet that shows the fibrosa, spongiosa and ventricularis. The fibrosa faces the aorta and the ventricularis faces the left ventricle of the heart.*

As mentioned in table 1; in the aortic wall the structural proteins elastin and collagen constitute about 55% of the dry weight of the aortic wall. The elastin confers the elasticity of the aortic wall while collagen acts to stiffen the wall and to limit its extensibility [108]. The most abundant amino acids in the aortic wall are glycine, alanine, valine, proline and leucine, contributed to the high extent of elastine in the tissue. Furthermore, relatively high contents of hydrophilic amino acids, such as (hydroxy)lysine, glutamic acid, aspartic acid serine and thereonine residues are found [107].

Due to the dense structure of the wall matrix it is expected that diffusion of chemicals through wall material will take significantly longer time compared to leaflets and any cross-linking methods developed for wall tissue needs to take this into account [107].

In cross-section of the wall, three distinct layers are distinguished as well, called the intima, media and adventitia [109].

The intima has a continuous inner elastic lamina around its whole circumference. The media is mainly composed of bundles of smooth muscle fibers, which are present in large amounts; bundles of elastin and collagen fibers are seen in similar proportions. Finally the adventitia is composed of elastin and collagen fibers.

As indicated in table 1, aortic wall and leaflets contain besides elastin and collagen various additional components. As in most connective tissues, collagen is found in close association with proteoglycans. It is thought that they are involved in the in-vivo collagen fibril formation. Proteoglycans are composed of glycosaminoglycans (GAG) and core proteins such as aggregan, decorin, lu mican, perlecan and many more [103]. The GAGS are complex mucopolysaccharides and are covalently linked to the core protein by serine and threonine ester bonds. Due to the anionic nature of the GAGS which contain many carboxylate and sulfate groups, ionic interactions with the (hydroxy)lysine and arginine groups are present.

Furthermore, several categories of cellular components present in heart valves can be classified. Lining cells (such as endothelial cells) and connective tissue cells (such as fibroblasts, myofibroblasts and smooth muscle cells) are present [99]. Furthermore in both wall and leaflets many valvar interstitital cells (VIC's) have been identified [109] each with a specific feature (such as cytoskeleton, contractility, communication, matrix secretion and more).

Biomechanical studies on valves showed that both the aortic wall and leaflet have an important role in the optimal performance of a natural valve [114,115]. During performance the leaflets open and close in a well defined organized order (see figure 6). This in combination with dilatation of the aortic root is required assures optimal flow conditions for blood.

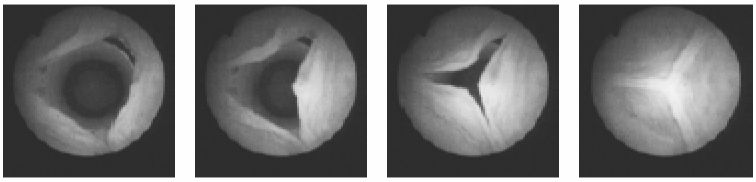


Figure 6, *Closing of a natural porcine valve and various stages mounted in a pulse duplicator*

Discussion

Tissue valves have been used for over 45 years now. In these years their performance has been much improved, partially through better stent designs and the implementation of post-fixation treatments like AOA™. Further performance improvement of tissue valves would require an answer to the ongoing problems of calcification and immuno-modulated inflammation. This need is further emphasized with the emerging utilization of stentless bioprosthetic heart valves, as their success is based on superior hemodynamic performance over stented valves. However, this advantage depends on maintaining flexibility of the valve components, and as such calcification can be expected to have an adverse effect. Results of a recent prospective randomized trial underscore this, as a correlation was found between calcification and left ventricular mass [97].

Many research groups are working on finding better techniques to process bioprostheses. Based on the information that was published and discussed in this chapter the reader may conclude that there are so many well promising cross-linking technologies developed that no issues with respect to in-vivo calcification and inflammation of bioprostheses remain at all and the reader may question why current commercial products still rely on old technology.

There are several underlying reasons that may explain this. First of all the valve manufacturers and physicans are conservative with respect to introducing new technologies. The development process of a totally new cross-linking platform for bioprostheses requires lot of effort with respect to money and time. Therefore new products, especially ‘high risk’ implantable medical devices that are introduced in the market typically are based on small modification of excisting technologies. In addition the issues with the Photofix™ process and termination of early clinical trials have slowed down development processes.

Furthermore, based on the information in literature it is very difficult, if not impossible to compare data to judge what cross-linking technology published gives best in-vivo performance. Each research group has its own animal models with associated procedures, processing techniques of native materials and standards. All of these parameters play a role in the in-vivo performance of the valve material.

In addition it can be expected that not all information is shared with the community for interlectural properties (IP) reasons.

Nevertheless successful development of a new tissue crosslinking process is very much dependent on the level of 'engineering' that is put into it. Each step in the process needs to be thoroughly characterized, in order to eventually have a fully controlled process with an established correlation to relevant performance measures, such as propensity to calcification or biomechanical properties.

This brings us to the title of this chapter; to our understanding, calcification alone is not the primary issue any more in the performance of bioprosthetic material. The major issue is to modify valvular tissue in such a way that decreased calcification combined with improved tissue mechanics and a reduced tissue response after implantation is obtained.
The biological response of the tissue, revascularization, cell diffusion or even replacing the complete bioprosthesis by the patient's own material is therefore investigated by many research groups.
Our strategy followed in this thesis is based on one, in our view well promising particular non-GA fixation technology, and on earlier work performed by Olde Damink [108] and Zeeman [109]. In our approach we focused on the development of a chemical process for bioprostheses providing predictable cross-links with respect to type and density. Furthermore the product should have a low in-vivo calcification and inflammation.

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Chapter 3

Reduced calcification of bioprostheses, cross-linked via an improved carbodiimide based method*

Abstract

Glutaraldehyde fixation of bioprosthetic tissue has been used successfully for almost 40 years. However, it is generally recognized that glutaraldehyde fixation is associated with the occurrence of bioprosthetic calcification. Accordingly, many efforts have been undertaken to develop an alternative non-glutaraldehyde fixation technology. In this chapter we describe a new enhanced carbodiimide based cross-linking method. Rather than cross-linking the tissue through its free primary amine groups, these groups were first blocked with butanal and the tissue was then cross-linked by by means of carbodiimide activation of tissue carboxylic acid groups followed by a reaction with a poly(propylene glycol)bis 2-(aminopropyl) ether, (Jeffamine™). It was demonstrated that cross-linked porcine leaflets had a calcification of less than 1 mg/g tissue after 8 wks sub-dermal implantion in rats. Furthermore, aortic wall calcification was reduced to 50 mg/g, compared to standard glutaraldehyde fixed tissue, which showed 120 mg/g tissue calcification in the 8 wks calcification model used.

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* Everaerts F.J.L, Torrianni, M, van Luyn, M, Hendriks, M, Feijen, J Reduced calcification of bioprostheses, cross-linked via an improved carbodiimide based method, Biomaterials. 2004 Nov;25(24):5523-30

Introduction

Improvements in healthcare and living standards of industrialized countries have resulted in a steady increase of the number of elderly people. Concurrently the number of patients with cardiovascular disorders such as artherosclerosis, aortic and mitral stenosis and calcification have increased as well. In 2004 nearly 250000 patients worldwide underwent valve replacement [1], of which about 70% were mechanical and 30% tissue based valves [1].
The most important parameters in bioprosthetic heart valve design are optimum haemodynamic function, good durability, minimal risk of thrombo-embolic complications and prevention of mineralization (calcification). Modern mechanical valves are durable and have excellent flow characteristics, but have the propensity for thrombo-embolic complications, necessitating life long anticoagulation therapy [2,3]. Stented porcine tissue valves, on the other hand, can be considered as hybrids of biological and mechanical structures. While central unobstructed flow characteristics are retained like in the native valve, the stent may contribute to partial reduction of the effective orifice area (4). The haemodynamic characteristics of the stented bioprosthesis lie therefore between homografts and the first generation of mechanical valves [5]. While tissue valves in general offer increased safety and efficacy in the short term they are prone to increased incidents of degeneration, calcification and cusp rupture compared to homografts, especially in young adults [3]. It has been shown that the differences in durability between mechanical and tissue valves are related to the mounting of the tissue onto the stent. Improperly mounted valve tissue leads to sub-optimal valve geometry and the development of improper stress loading in the leaflets. This finding has triggered the design of stentless valve products, in which the original wall of the porcine valve is used as a natural stent. Although haemodynamics were reported to be superior compared to stented valves [3], long-term performance is particularly diminished by calcification of the wall [2]. Although wall calcification is of less importance compared to calcification of the leaflet, a good functional stentless bioprosthesis can only be preserved by means of a crosslinking method, which will prevent both calcification of the wall and the leaflet. Results of a recent prospective randomized trial underscore the importance of this requirement, as a correlation was found between calcification of the wall and left ventricular mass [6]; the higher the calcification in the wall, the larger the left ventricle of the heart.
Vesely reported that for the development of alternative cross-linking techniques it is very

important that all aspects of a future valve design are considered [7]. Failures of some alternative cross-linking techniques are often due to a combination of factors not necessarily associated with the applied chemistry. The design of the stent and the technique used by the surgeon plays an important role as well [8].

According to Vesely's views, successful development of tissue cross-linking methodology relies upon establishing a correlation between the employed chemistry and the desired biological and/or mechanical performance. This understanding of the chemistry can only be achieved if every step in the process is thoroughly characterized. This, for example, can be achieved by quantitatively determining the number and type of functional groups involved, as to attain understanding of the impact of the employed chemistry on the mechanical behavior and final shape of both wall and leaflet. The other way around, the final mechanical performance of both wall and leaflet may dictate which chemistry has to be employed.

Following this "engineering approach", introduced in chapter 2 of this thesis [9], a new carbodiimide based cross-linking method was developed. In short, this new method involves blocking of free amine groups with butanal, followed by carbodiimide activated cross-linking of carboxyl groups using amine-terminated polypropylene glycols. The characterization of the cross-linked tissue is described, as well as the results achieved upon sub-dermal implantation of such cross-linked tissue materials in rats. It is demonstrated that the new carbodiimide based cross-linking method leads to a significant reduction in calcification when compared to standard glutaraldehyde fixed tissue.

Materials and Methods

Materials

Fresh porcine aortic root tissue was obtained from a local slaughterhouse (Premium Fleish, Emsland, Germany or Bleijlevens, Kerkrade, the Netherlands), rinsed free of blood with a buffered N-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid), HEPES (Sigma Aldrich, Zwijndrecht, Netherlands) physiological saline solution (Sigma Aldrich, Zwijndrecht, Netherlands) and trimmed to remove excess myocardium and adventitial tissue. Valves were rinsed for at least 24 h (maximum 48 h) in HEPES buffered saline (10 mM , pH 7.4, 4^{0}C) prior to starting the fixation chemistry.

Methods

Tissue was fixed according to the following procedures. A survey is given in table 1:

Table 1: *experimental setup*

Procedure	Blocking step	Cross-Linking step
0	None	None
1	None	GA (0.2 wt% for 24 h)
2	None	EDC/NHS
3	Butanal	Jeffamine™ 230
4	Butanal	Jeffamine™ 2001
5	Butanal	Jeffamine™ 400
6	Butanal	Jeffamine™ 600

1 After overnight storage (at least 12 h) in HEPES buffered saline (10 mM) at 4^{0}C, valves were each transferred to a fresh HEPES buffered saline solution (100 ml, 10 mM at pH 7.4) containing glutaraldehyde, GA (0.2 wt% ,Sigma Aldrich, The Netherlands). Reaction was continued for 24 h after which valves were rinsed in saline solution (0.15 M, 5 times 30 min). Finally valves were individually stored in HEPES (100 ml, 10 mM, pH7.4) with GA (0.2 wt%) at room temperature.

2 After overnight storage in HEPES buffered saline (10 mM) at 4^{0}C valves were each transferred to 2-(morpholino) ethane sulfonic acid, MES (100 ml, 0.05M, pH 5.5 Acros, Geel, Belgium) buffered solution containing N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide, EDC (0.03 M Aldrich, Zwijndrecht, The Netherlands) and N-hydroxysuccinimide, NHS(0.06 M Sigma, Zwijndrecht, The Netherlands). The cross-linking reaction was performed for 24 h at room temperature after which valves were rinsed in saline (0.15 M, 5 times 30 min) and individually stored in HEPES (100 ml, 10 mM, pH=7.4) containing isopropylalcohol, IPA (20wt%, Aldrich, Zwijndrecht, The Netherlands).

3 After overnight storage in HEPES buffered saline (10 mM) at 4^{0}C valves were each

	transferred to a MES buffer (100 ml, 0.25 M, pH 6.5) with butanal (8 ml, Aldrich, The Netherlands) and $NaCNBH_3$.(150 mM, Aldrich, The Netherlands).The blocking reaction was allowed to continue for 6 h whereby after 2 h and 4 h an additional amount of $NaCNBH_3$ (150 mM) was added. Thereafter valves were rinsed in saline (5 times for 15 min and valves were imbibed with the Jeffamine™ (Aldrich, Zwijndrecht, The Netherlands) spacer by having them each immersed in MES buffer (100 ml, 0.25M, pH=5.0) containing Jeffamine™ 230 (0.06 M) and after 30 min EDC (0.30 M) and NHS (0.06 M) was added. The cross-linking reaction was allowed to proceed for 14 h. Thereafter valves were rinsed in saline (3 times for 30 min) after which valves were individually stored in HEPES (100 ml, 10 mM, pH=7.4) containing IPA (20 wt%) at room temperature.
4	Same procedure as described in (3) but instead of Jeffamine™ 230, Jeffamine™ 2001 was used.
5	Same procedure as described in (3) but instead of Jeffamine™ 230, Jeffamine™ 400 was used.
6	Same procedure as described in (3) but instead of Jeffamine™ 230, Jeffamine™ 600 was used.

Tissue analysis

TNBS assay

The primary amine group concentration of tissue samples was determined using a colorimetric assay [10]. Tissue samples (cut-out valve leaflet, or 6mm punched disc from valve wall) were exposed to a solution of $NaHCO_3$ (2 wt%, pH 9.0, Aldrich, The Netherlands) and 2,4,6 trinitrobenzene sulfonic acid, TNBS (0.5 wt% Fluka, Switzerland). The reaction was continued for 4 h at 40^0C, after which the samples were rinsed in saline solution using a vortex mixer to remove unreacted TNBS.

Samples were freeze-dried overnight (HETO, Germany), after which the dry mass was determined. Dry samples were immersed in aqueous hydrochloric acid (2 ml, 6 M, 80^0C ,Aldrich, Zwijndrecht, The Netherlands) until fully dissolved. The obtained solution was then diluted with deionized water (8ml) and the absorbance was measured at 340 nm (Perkin Elmer, Fullerton, USA) The concentration of free amine groups was calculated using the follow equation:

$$[NH_2] = \frac{A \cdot V}{\varepsilon \cdot \ell \cdot m_{tissue}}$$

wherein:
$[NH_2]$ = free amine group content [mol / g tissue]
A = absorbance [-]
V = solution volume [ml]
ε = 14600 [ml/mmol.cm]
ℓ = path length [cm]
m_{tissue} = dry weight sample [mg]

The obtained value was used to determine the %-blocking which was calculated by the following formula:

$$\%\text{blocking} = \frac{[\text{NH2}]\text{native} - [\text{NH2}]\text{blocked}}{[\text{NH2}]\text{native}}$$

wherein:

%blocking=	% of blocked amine groups	[%]
[NH2]native=	free amine content of the native material	[mol/gram tissue]
[NH2]blocked=	free amine content of the blocked material	[mol/gram tissue]

Resistance to enzymatic degradation

Stability of the treated tissue was measured by the resistance to enzymatic degradation using a 1:1 mixture of protease and collagenase (20 Units/mg, Aldrich, Zwijndrecht, The Netherlands) (11). In this procedure samples were initially soaked in a HEPES buffered saline solution (10mM, pH 7.4) containing NaCl (9 g /liter) and glycine (7.5 g/liter, Aldrich, Zwijndrecht, The Netherlands) for 4 h at 37°C. Samples were then transferred to a solution of the same buffer containing the enzyme (3ml with 12mg enzyme per sample). To assure optimal enzymatic activity of the solution, the enzyme was added along with $CaCl_2$ (96 mg to 180 ml solution) Samples were incubated for 4 h, washed and freeze-dried. After renewed determination of the dry weight the actual weight loss due to enzymatic degradation was calculated.

Differential Scanning Calorimetry

Differental Scanning Calorimetry (Pyrus 1, Perkin Elmer Fullerton USA) was used to determine the shrinkage temperature of the various tissue samples.

Samples of leaflet or punched 6mm wall discs were immersed in NaH_2PO_4 (0.05 M, Aldrich, Zwijndrecht, The Netherlands) solution for 3 h. Samples were then removed and excess of buffer was blotted with a lint-free tissue. An individual sample was placed in the sample pan and the cover was crimped. For each sample three discs were analysed (n=3)

The shrinkage temperature of each sample was determined using a temperature scan between 30 and 95^{0}C and a scan rate of 2^{0}C/min. The shrinkage temperature was defined as the temperature at the maximum of the endothermic transition.

Subdermal implantation

In addition to the physico-chemical analyses, valves were also subdermally implanted in a Sprague-Dawley rat model for evaluation of the degree of calcification.

For subdermal implantation, 8 mm discs, punched from the valve wall and leaflets regions, were used. Prior to implantation, discs were washed with sterile saline solution (3 times for 2 min).

National Institute of Health guidelines for the care and use of laboratory animals (NIH 85-23 Rev. 1985) were followed.

Male, 25 days old rats (Sprague-Dawley, CD strain) were used. Backs were shaved and disinfected using ethanol, a mid-line incision was made in the skin and in two subcutaneous pockets created at each side of the spine a disc was inserted. The skin was closed with a single suture after which samples were explanted after 8 wks. Three disks of each sample were implanted (n=3).

Microscopy

After 8 wks, samples were explanted and cut into two halves. From one half of the explants, the surrounding capsule was removed and these were stored in HEPES/IPA (0.01 M/20 wt%) for further quantitative calcium analysis. The other half was immersion-fixed in GA (2%) in PBS (0.1 M, pH 7.4, Aldrich The Netherlands). After 24 h at 4^0C and subsequent dehydration in graded alcohols, the explant was embedded in glycol methacrylate (GMA) for light microscopic evaluation. Toluidine Blue (TB) staining was used for histological evaluation of the tissue. Calcification was evaluated by means of von Kossa staining.

Calcium and phosphor analysis

The calcium concentration in each tissue sample was quantified as a percentage of the total cross sectional area by image analysis (Leica Qwin, Leica Microsystems, Rijswijk, the Netherlands), by analysing the Von Kossa silver staining.

The calcium concentration was also determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Perkin Elmer Optima 3000, Fullerton, USA). Explanted samples, stored in HEPES/IPA (0.01M/20 wt%), were frozen in liquid nitrogen, after which they were freeze-dried for 12 h. The dry weight of each tissue sample was then recorded and freeze-dried samples were hydrolyzed in aqueous hydrochloric acid (15 ml, 6M) solution for 24 h followed by the addition of deionized water (10ml). Subsequently, the signal intensity of calcium and/or phosphor was determined with the atomic emission spectrometer (n=5 per sample). The concentration of calcium or phosphor per dry weight of tissue was calculated using a calibration curve obtained with standard solutions.

Results

It was shown that the shrinkage temperature of leaflets increased after cross-linking (see table 2). In this case only leaflet material was investigated since due to the small amount of collagen in wall no reproducible data for cross-linked wall tissue were obtained. It appears that the shrinkage temperatures of the Jeffamine™ cross-linked materials were the lowest. GA fixed samples had the highest shrinkage temperature, followed by the EDC/NHS group where the amine groups of the collagen were cross-linked directly with the carboxyl groups in the same matrix.

__Table 2__; Shrinkage temperatures of fresh leaflet material and leaflets after cross-linking

Procedure*	Treatment	Peak (°C)	N
0	Fresh	61.1±0.1	2
1	GA	83.3±0.3	3
2	EDC/NHS	81.1±0.4	3
3	B-J230	76.1±1	3
4	B-J2001	67±0.2	3

* See materials and methods section

The results of sample groups cross-linked with other Jeffamines™ with molecular weights varying from 230 to 2001 are presented in table 3. Here shrink temperatures of both wall and leaflet are presented. It can be observed that the shrinkage temperature for the cross-linked groups remains fairly constant but is lower for the Jeffamine™ 2001 series ($p<0.001$ with a student T-test). The free amine group content of the samples of the walls showed the same trend: almost constant for all samples, except for the Jeffamine™ 2001 cross-linked material where the content is lower ($p<0.001$) . For the leaflets however the free amine group content is almost constant but somewhat lower for the Jeffamine™ 600 cross-linked samples ($p<0.001$). Furthermore the free amine group content decreases during the blocking reaction and increases slightly during the cross-linking reaction.

The %-blocking of the amine groups of tissue with butanal was determined with the TNBS reaction. Typically about 70% of the amine groups in the wall had reacted while initial experiments with pure dermal sheep collagen showed that 95% or higher blocking of amine groups could be achieved. For leaflets it was found that a blocking efficacy of more than 95% could be achieved.

All groups cross-linked with Jeffamine™ showed a similar resistance to enzymatic degradation except for the Jeffamine™ 2001 series, which seems to be somewhat more resistant.

Table 3; *Influence of Jeffamine™ chain-length on shrinkage temperature, free amine group content and degradation with protease, and. 'W' stands for wall while 'L' stands for leaflet samples.*

		Shrinkagetemperature in °C (n=5)	Free amine groups in % (n=5)	Protease digestion in % remaining weight (n=5)
Fresh	W	58.5±0.3	100	40±5
	L	61.1±0.2	100	Digested
Blocked	W	58±1	28±2	41±3
	L	59±0	5±1	Digested
J230	W	72±1	35±1	76±4
	L	76±1	51±2	60±6
J400	W	74±1	36±1	73±6
	L	73±0	48±2	54±6
J600	W	73±0	31±2	75±1
	L	76±0	38±3	64±5
J2001	W	68±1	23±1	81±1
	L	67±0	45±9	79±3

Tissue discs were implanted according to the protocol and after 8 wks samples were explanted, further processed and analyzed.

Since both the calcium and phosphor concentration play a key role in the mineralization of bioprosthetic materials (9), both concentrations were determined with AAS and the analysis was performed prior and after implantation. Calcium concentrations in the wall tissue prior to implantation showed that groups that were cross-linked with the non-glutaraldehyde technology contained less calcium, while cross-linking of the samples did not affect the amount of phosphor present in the tissue (table 4). For leaflet material this evaluation was not performed.

***Table 4**, Calcium and phosphor content of wall tissue prior to implantation (n=3)*

Procedure	Wall	Ca (mg/g)	P (mg/g)
0	Fresh	0.53±0.08	0.96±0.02
1	GA	0.54±0.00	1.27±0.10
2	EDC/NHS	0.38±0.02	1.10±0.08
3	B-J230	0.30±0.06	0.95±0.02
4	B-J2001	0.26±0.04	1.07±0.07

***Table 5**, Calcium and phosphor content of wall and leaflet material in the explanted material (after 8 wks implantation)*

Procedure	Leaflets	Ca (mg/g)	P (mg/g)
0	Fresh	Not retrieved*	Not retrieved*
1	GA	194±16	94± 8
2	EDC/NHS	171±30	84±13
3	B-J230	1.3±0.6	1.7±0.7
4	B-J2001	2.2±0.5	1.3±0.4
	Wall	Ca (mg/g)	P (mg/g)
0	Fresh	155±22	75±10
1	GA	113±5	54±2
2	EDC/NHS	96±9	47±4
3	B-J230	49±19	24±10
4	B-J2001	49±26	24±12

**the fresh leaflet group was not retrieved because it was digested in the animal*

It was observed that both the calcium and phosphor concentrations varied in the samples after implantation. Both calcium and phosphor levels in cross-linked wall tissue were lower than in the non-treated material. Jeffamine™ cross-linked wall material contained about 50% lower calcium and phosphor compared to GA fixed samples, while in leaflets the concentration of calcium and phosphor was close to zero.

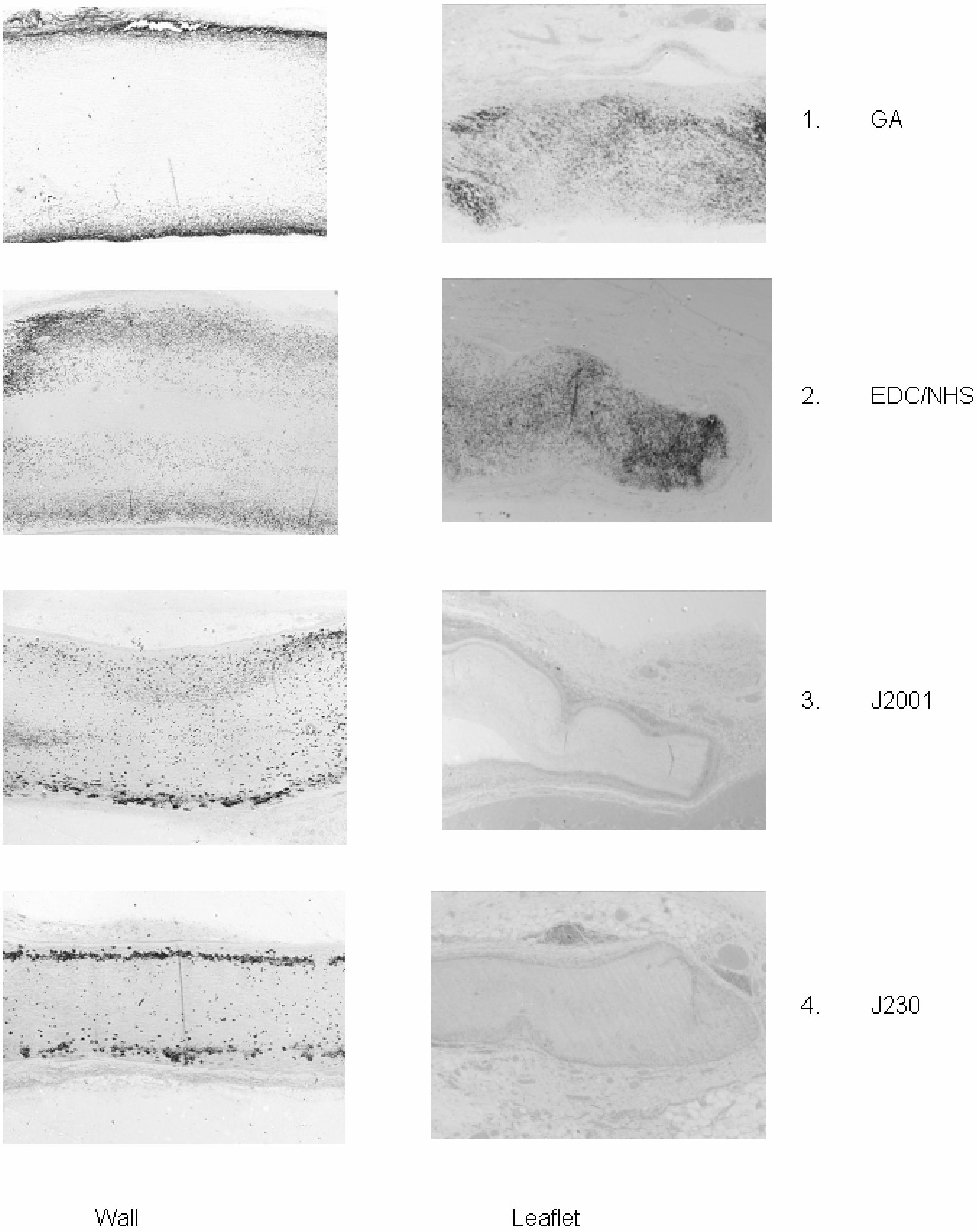


***Figure 1**, Von Kossa profiles of wall (left, 2.5x magnification) and corresponding leaflet (right, 5x magnification) explanted samples*

Figure 1 gives the Von Kossa staining results of the explanted samples. The difference between the GA and non-GA cross-linked wall samples is noticeable; while in GA heavily calcified area are primarily focused on the rims of the sample, in the non-GA cross-linked wall samples the calcification is concentrated in nodes and spread throughout the samples. In wall samples were Jeffamine™ is used the nodes are decreased in number, however increased in size. Leaflet samples show that calcification is almost absent in samples with blocked amine groups while in both GA and EDC/NHS samples the calcification is spread throughout the sample.

The implantation studies were repeated twice with Jeffamine™ 230 cross-linked tissue samples. The calcification results are depicted below. Repeatedly, all Jeffamine™ 230 wall samples demonstrated a 50% reduction in calcification in comparison to GA-fixed tissue, whereas in Jeffamine™ 230 treated leaflets calcification was almost absent.

Table 6, *Results of calcification of implants (3 studies) of butanal blocked and Jeffamine™ 230 cross-linked tissue compared with Glutaraldehyde cross-linked tissue*

Ca content (mg/gram), n=5	J230		GA	
	Wall	Leaflet	Wall	Leaflet
Study1	49.2±19.4	1.3±0.6	112±5	194±7
Study2	48.4±8.6	0.8±0.4	120±13	220±1
Study3	50.3±12.2	0.5±0.3	N.A.	N.A.

Discussion

The purpose of this study was to evaluate an alternative carbodiimide based cross-linking technology for its effectiveness to prevent in vivo calcification.Optimization of the cross-linking and blocking process was based on earlier work performed at the laboratories of Medtronic Bakken Research Center and University of Twente [11,11,13]. Most of these optimization studies were performed on Dermal Sheep Collagen (DSC) to get better insight in and understanding of the stoichiometry of the chemical reactions involved.

For the blocking reaction with butanal, the concentration was selected such that the molar ratio of butanal and free amino groups was higher than 10. The mole's of Jeffamine™ added to the reaction mixture in the second step was based on half the amount of free carboxylic acid groups available in the sample. This concentration was selected to minimize one-sided coupling of the Jeffamine™, thus maximizing effective incorporation of Jeffamine™ cross-links through reaction with two carboxyl groups. The TNBS results showed however that the free amine group concentration increases during the cross-linking reaction, an indication that some of the Jeffamine™ reacts with only one carboxyl group and dangling groups were formed. The concentration of EDC was selected such that the molar ratio of chemicals and available groups is higher than 10. The ratio of EDC:NHS was set on 5:2 as found to be optimal in earlier studies [11].

The blocking efficacy was assayed using a TNBS reaction [14] and typically 70% of amine groups in the tissue had reacted in wall. For leaflets it was found that more than 90% of all available groups reacted. Despite reaction times of 48 h, it is thought that a small number of free amine groups that are not blocked are not accessible due to steric hindrance and as such these groups are not able to participate at all in further reactions. In the calculation performed it is also assumed that no material is lost in the blocking reaction. A more detailed study has to be done to trace the location of these free groups in the tissue matrix.

In the second step the tissue is reacted with a diamine terminated spacer. By allowing the spacer molecule to penetrate into the tissue before the cross-linking reaction is initiated, it is thought that part of the diffusion limitation that occurs primarily in the tissue wall is overcome. A spacer molecule that was selected for this reaction is an amine terminated polypropylene

glycol, commercially available under the trade name Jeffamine™ .
Jeffamine™, in this case poly(propylene glycol)bis 2-(aminopropyl) ether contains primary amino groups attached to the terminus of a polyether backbone.

The spacer was selected because of its high intrinsic hydrophilicity and flexibility. A hydrophilic spacer is preferred because it has to penetrate deep in the tissue and it is hypothesized that the more hydrophilic the tissue becomes, the better tissue fluid infiltrates and diffuses through the tissue, whereby also the in-growth of capillary blood vessels is promoted. Moreover, cross-linking with Jeffamines™ will lead to longer chains in between cross-links, which is beneficial since it reduces the stiffness of the tissue.
To activate the carboxyl groups of aspartic acid and glutamic acid residues of the collagen, water-soluble carbodiimide (EDC) with the addition of N-hydroxysuccinimide (NHS) was used. NHS increases the efficacy of the activation reaction even more because it not only suppresses the formation of the by-product N-acylurea that is formed after intramolecular rearrangement, it also forms a much more stable activated carboxyl ester that has a half life of about 30 min instead of a few seconds under the reaction conditions that are employed (pH 5.0). See also chapter 5 of this thesis for more information regarding cross-linking using EDC and NHS.

Since the ε- amine groups of lysine and hydroxylysine have been blocked, primarily the free amines of the Jeffamine™ will react. This means that the formation of so-called zero-length cross-links is prevented, i.e., direct reaction between the amine and the carboxyl groups of the collagen matrix. These zero-length cross-links have been related to stiffening of the matrix [11].
As mentioned before, the more Jeffamine™ groups react at both sides, the more effective the cross-linking reaction. If a Jeffamine™ reacts only with one activated carboxyl group, pendant amine groups are introduced and stabilization of the tissue is less effective.
We hypothesize that the length of the Jeffamine™ spacer dictates the location where cross-linking takes place in the collagen. The diameter of a triple helix in collagen is approximately 15 Å and the total diameter of a microfibril, typically comprising 5 collagen triple helices next to each other, is approximately 40Å. Fibrils that are arranged to form a number of microfibrils have a diameter of about 500 Å [11]. The calculated chain lengths of Jeffamine™ are depicted in table 7.

Table 7, Calculated chain lengths of Jeffamine™ (end to end).

Jeffamine™ Molecular weight (gram/mol)	Chain length (in Å)
230	13
400	24
600	37
900	55
2000	123
4000	248

Taking these dimensions into account this means that Jeffamine™ with a molecular weight of 230 (J230) in theory only can form intramolecular cross-links inside a microfibril, while longer molecules also can form cross-links between different microfibrils.

Data depicted in Table 3 show that for leaflets, the number of free amine group's decreases when using higher molecular weight Jeffamines™. Since only the introduced amine groups during cross-linking in the tissue are Jeffamine™ based, this means that either less pendant groups are formed during the reaction, or that less Jeffamine™ is build into the matrix due to decreased diffusion or steric hindrance of the Jeffamine™. To prove this, a free-carboxyl group determination will have to be performed. Although a small decrease in shrinkage temperature occurs if longer Jeffamine™s are used, the resistance to enzymatic degradation indicates that the tissue is equally well stabilized.

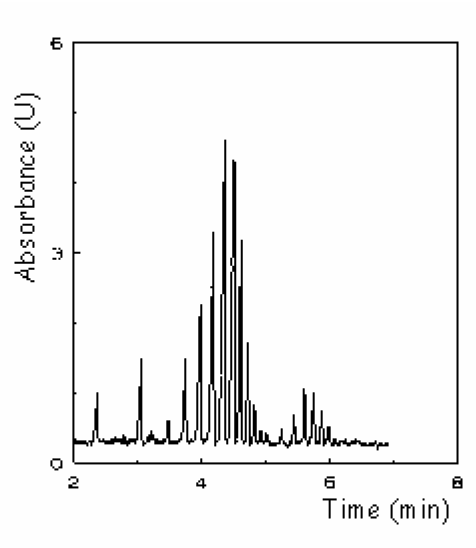
The shrinkage temperature is determined by the thermal stability of tissue where the collagen triple helix unfolds. The authors hypothesize that when two collagen triple helices are connected via a short spacer molecule more resistance to unfolding will be encountered than when a long spacer molecule is used.

One has to keep in mind that Jeffamine™ has a molecular weight distribution and that molecules can be incorporated in different ways according to their molecular weight.

Triepels et al [21] have characterized different Jeffamines™ with capillary electrophoresis. An example is depicted in Figure 2, Jeffamine ™ with a molecular weight of 400 (J400) has a molecular weight distribution between 190 and 596 g/mol. Furthermore the presence of several additional peaks indicates that this Jeffamine™ contains several additional components (not further investigated).

Accordingly, we regard the shrinkage temperature basically only as a process control measure. The shrinkage temperature itself has no physiological meaning. The combination of shrinkage temperature, TNBS assay, and enzymatic degradation and possibly other evaluation tests is needed to give a complementary, more relevant, picture of the physico-chemical condition of the tissue.

With respect to thermal characteristics, wall tissue showed a similar trend as leaflets, but with smaller differences. The resistance of wall tissue against enzymatic degradation is higher compared to leaflets and the number of free amine groups in wall tissue is lower as compared to leaflets. This may be explained by the higher concentration of elastin in the wall tissue compared to leaflets. This difference in tissue structure is thought to hinder fast diffusion and distribution of reactive compounds throughout the wall tissue.



Peak		J400
	(g/Mol)	Fraction
1	190	
2	248	0.07
3	306	0.11
4	364	0.18
5	422	0.27
6	480	0.20
7	538	0.11
8	596	0.05
	Mw	419.4

***Figure 2** (left); Fingerprint of Jeffamine™ 400, determined with capillary zone electrophoresis in Histidine/MES buffer (0.01M, pH adjusted to 6.0 with MES) at 20kV and detected at 240nm. Calculated molecular weight distribution of Jeffamine™ is depicted in Figure 2 (right). Reproduced with permission [21]*

The Von Kossa staining profiles of the wall samples show the difference between samples cross-linked with the GA and non-GA technology. While for wall the calcification in the GA cross-linked samples is primarily focussed on the outer rims of the samples; the EDC cross-linked samples show calcification throughout the sample. A possible explanation of this phenomenon could be that GA leaches into the surrounding tissue causing inflammation and calcification.

It was found that if amine groups are blocked the number of calcification spots decrease but increase in size. For an explanation the samples need to be investigated in more detail if the calcification has for example a specific initiation spot.

For leaflets both GA and non-GA show calcification but if amine groups are blocked and cross-links are formed trough Jeffamine™ the observed calcification was found to be almost absent. Due to the dense structure of wall matrix and the fact that chemicals need to diffuse into the matrix prior to reaction, the difference in diffusion rate between Jeffamines™ with increasing chain lenght, as mentioned before, could play a not clarified role in the different calcification patterns observed between the groups.

Different groups studied the effect of extraction of tissue or adding specific compounds to tissue [14,15,17,18] since the presence of cell remnants, phospholipids and non-collagenous proteins might be the key determinants in inducing calcification [14] The studies published showed that both approaches could lead to mitigation of calcification. Based on this, another explanation of the reduced calcification found in the amine-blocked groups with butanal, presented in this paper, could be the rinsing and cleaning up of the tissue matrix that occurs during the blocking process, possibly removing so far unidentified initiators for calcification.

It is important to mention that since in the subcutaneous pocket turn over of body fluids may be low, one has to keep in mind that leakage of a certain compound into the surrounding tissue from the implant could yield short-term low calcification results, whereas these results could be completely different, when conducting a long-term study or using a blood-contacting implant site [19,20]. To verify our findings in the rat model, we also have implanted of the tissue samples in a descending aorta sheep model. Similar results were achieved with a 90% reduction in calcification of J230 cross-linked leaflets compared to standard GA fixation (results not shown).

In conclusion it was repeatedly shown that with the EDC-based two-step process leaflet calcification of less then 1mg/gram could be achieved. Wall calcification was reduced as well, compared to a GA control, but was still present. It is concluded that the above-described technique forms a good starting point for the development of a new generation of non-glutaraldehyde fixed bioprostheses. Further optimization of the presented cross-linking process is believed to further reduce wall calcification in particular. Also the effect of the chemical modification to the inflammatory response of the tissue needs to be further investigated. Subsequent studies will be aimed at generating more insight in and understanding of the mechanisms that lead to the observed reduction in calcification.

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Chapter 4

Reduction of calcification of carbodiimide processed heart valve tissue by prior blocking of amine groups with monoaldehydes*

Abstract

Failure of implanted bioprostheses due to calcification is a commonly occurring phenomenon. In order to prevent calcification many alternative cross-linking methods to glutaraldehyde (GA) have been developed and evaluated. In a novel approach an improved carbodiimide cross-linking method that comprises a two-step process was developed. First the available amine groups in (tissue) collagen are blocked with a mono-aldehyde, followed by an N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) activated cross-linking reaction of the carboxyl groups in the tissue with a poly(propylene glycol) bis 2-(aminopropyl) ether (Jeffamine^{TM}). Samples processed via this method have shown a significantly reduced calcification in a sub-dermal juvenile rat model compared to samples with a standard glutaraldehyde treatment. In the study described in this paper heart valve tissue was blocked with various mono-aldehydes, followed by a reaction with Jeffamine using carbodiimide cross-linking chemistry. Leaflet calcification was almost eliminated using different aldehydes; whereas wall calcification was maximally 95% reduced when propionaldehyde was used as blocking agent, as compared to a carbodiimide cross-linked control without Jeffamine and blocked amine groups.

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*Everaerts F, Gillissen M, Torrianni M, Zilla P, Human P, Hendriks M, Feijen J, Reduction of caificiation in carbodiimide processed heart valves tissue by prior blocking of tissue amine groups with monoaldehydes, J Heart Valve disease, 2006 Mar; 15(2): 269-277

Introduction

Calcification of bio-prostheses after implantation is a commonly occurring phenomenon and may be caused by different factors. In order to mitigate the local inflammatory responses and associated enzymatic degradation that starts after implantation of the bioprosthesis, the tissue matrix is usually preserved, primarily by the introduction of cross-links between the collagen molecules in the matrix. Glutaraldehyde (GA) [1] based cross-linking of valvular bioprostheses is the current standard and these prostheses have a low incidence of thrombo-embolism and a satisfactory haemodynamic performance. Nevertheless problems in durability have been reported [2,3,4,5]. Clinical failure due to cuspal calcification has been registered in many cases [4]. This calcium deposition causes valve stiffness, tearing, rupture and results in stenosis and/or insufficiency [6]. The exact mechanism of calcification is still not well understood and numerous hypotheses have been described in literature. Most theories are based on the loss of components from the matrix, such as proteoglycans and glycosaminoglycans during the reaction with GA [7]. These components act as natural inhibitors of calcification. Other factors mentioned in the literature are changes in the morphology and the charge distribution in the valve after fixation, and the occurrence of local stress in the material [2,3,4,8].

In various studies, post GA fixation reactions were developed with the aim to reduce calcification. Some of these were successfully introduced in clinical practice [9]. In the last years, research has also focused on the use of alternative cross-linking agents [1]. Cross-linking based on water-soluble carbodiimide (EDC) in the presence of N-hydroxysuccinimide (NHS) has been reported to be a particularly promising alternative for GA [10,11,12]. Calcification after explantation has been reported lower compared to GA, however it has also been mentioned that direct cross-linking between existing free amine and carboxyl groups, so-called "zero-length cross-links", in the collagen chain causes stiffening of collagen based materials. In order to prevent the formation of these zero-length cross-links, a two-step reaction was developed. The first step comprises blocking of the amine groups of the collagen matrix, followed by an EDC and NHS activated cross-linking reaction with a poly(propylene glycol)bis 2-(aminopropyl) ether, (Jeffamine^{TM}) [13,14] The stabilized matrix with improved mechanical behavior showed a significantly decreased calcification of wall material in a sub-dermal juvenile rat model compared to the standard GA fixed control [15]

The present study follows through on these successful initial results. Various mono-aldehyde blocking agents were evaluated for their blocking efficacy. After optimization of the blocking reaction, porcine heart valves were cross-linked with Jeffamine and implanted subdermally in juvenile rats to evaluate calcification after 8 weeks implantation.

Materials and Methods

Materials

Fresh porcine aortic valve tissue was obtained from a local slaughterhouse (Bleijlevens, Kerkrade, the Netherlands), rinsed free of blood with a buffered N-(2-hydroxyethyl)piperazine-N'-(2-ethane sulfonic acid), HEPES (10 mM, pH 7.4, Sigma Aldrich, Zwijndrecht, The Netherlands) physiological saline solution (Sigma Aldrich, Zwijndrecht, The Netherlands) and trimmed to remove excess myocardium and adventitial tissue. Valves were rinsed for at least 24 h (maximum 48 h) in HEPES buffered saline (10mM, pH 7.4, 4^0C) prior to starting the fixation chemistry.

Fixation methods

Four different aldehydes were evaluated as blocking agents. These are butanal, 2-methyl butanal, propional and glyceral (P.A., all from Aldrich, Zwijndrecht, the Netherlands). For the blocking reactions 2-(morpholino)ethane sulfonic acid, MES (100 ml, 0.2 M, pH 6.4, Acros, Geel, Belgium) buffer was prepared and the selected aldehyde was added (0.5 M), followed by the addition of a valve. Three equal amounts of $NaCNBH_3$.(0.1 g, Aldrich, the Netherlands) over an 8 h interval were added. After 24 h reaction time, valves were rinsed in physiological saline solution (3 times 30 min). For each type of aldehyde 5 valves were treated. The blocking efficacy was determined using the TNBS method described later. Hereto 5 samples of each aldehyde group were taken randomly from the wall and 5 samples of each aldehyde group were taken randomly from the leaflets of 1 valve per aldehyde group. Remaining valves (n=4 for each aldehyde group) were subsequently imbibed in MES buffer (0.2M, pH 5.5) and Jeffamine 230 (0.06 M, Sigma, Zwijndrecht, the Netherlands) for 30 min and subsequently cross-linked using N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide EDC (0.3 M, Aldrich, Zwijndrecht, the Netherlands) and N-hydroxysuccinimide, NHS(0.12 M, Sigma, Zwijndrecht, the Netherlands). The cross-linking reaction was conducted for 24 h at room temperature after which the valves were rinsed 5 times for 30 min in physiological saline solution and thereafter individually stored in HEPES (100ml, 10mM, pH=7.4) containing isopropyl alcohol, IPA (20% Aldrich, Zwijndrecht, the Netherlands).

Because the blocking efficacy of glyceral processed wall tissue was relatively low, the reactions for this aldehyde were varied with respect to its concentration (0.2 and 0.5M), temperature (room temperature and 40^oC) and time (24 and 48 h). Other conditions were maintained as described before. For each combination of variables three valves were treated. The TNBS analysis taken of these valves was carried out on 5 randomly obtained wall samples.

Further optimization of process conditions

For further optimization of the process it is advantageous to use the lowest buffer and $NaCNBH_3$ concentrations, which still permit efficient blocking of amine groups and subsequent cross-linking.

First the MES buffer concentration was varied between 0.01M and 0.2M using propional (0.5M) and $NaCNBH_3$ added three times (0.1 g, total 0.3g over a period of 8h). Three valves per buffer concentration were treated in 100ml buffer solution each. The reaction was performed for 24h at room temperature. After the reaction valves were rinsed in physiological saline solution (3 times 30 min). The blocking of amine groups of 5 wall samples randomly taken from these three valves for each buffer concentration was determined with the TNBS method.

Secondly, keeping the MES buffer concentration at 0.05M, tissue wall samples (diameter of 1cm, randomly punched out of 10 fresh tissue valves) were treated with propional (0.5M) using different concentrations of $NaCNBH_3$. $NaCNBH_3$ was added at once or in three equal amounts over a period of 8 h. The blocking efficacy was determined again by analyzing 5 samples taken randomly from each concentration group.

Preparation of valves for the implant study

Samples for the implant study were processed (5 valves per sample group were prepared) with butanal (0.5M), 2-methyl-butanal (0.5M), propional (0.5M) or glyceral (0.2M). The reaction was carried out for 48 h at room temperature, using MES buffer (100ml per valve, 0.05 M, pH 6.5) and $NaCNBH_3$ (0.3g) was added at the beginning of the blocking experiment. The blocking efficacy was determined using a TNBS assay of 5 wall pieces per aldehyde group, punched randomly out of the valve material. Remaining valvular material was rinsed in MES buffer (0.05M, pH 6.5), subsequently imbibed in MES buffer (100 ml per valve, 0.2M, pH 5.5) containing Jeffamine 230 (0.06M) for 30 min and cross-linked by adding EDC (0.3M) and NHS (0.12 M) with a reaction time of 48 hours.

Valves were rinsed 5 times for 30 min in physiological saline solution and thereafter individually stored in HEPES (100ml, 10mM, pH=7.4) containing isopropyl alcohol, IPA (20% Aldrich, Zwijndrecht, the Netherlands) until further use.

The obtained sample groups were investigated in more detail by the determination of the tissue shrinkage temperature, enzymatic digestion, water uptake and static contact angle measurements. Before experiments were carried out, the valvular material was extensively washed in HEPES buffer saline solution (10mM, pH 7.4).

In this calcification study, two control groups were added being an EDC/NHS cross-linked sample group and group that was cross-linked in 0.2% GA for 1 week at 4°C . For the EDC/NHS group, fresh tissue was cross-linked in a MES buffer solution (0.2 M, pH 5.5) with the addition of 0.3 M EDC and 0.12 M NHS. Cross-linking was performed for 48 hours after which the samples were rinsed and stored as described above.

Tissue analysis

TNBS assay

The primary amine group concentration of tissue samples was determined using a colorimetric assay [14] Tissue samples (cut-out valve leaflet, or 6mm punched disc from valve wall) were exposed to a solution of $NaHCO_3$ (2 wt%, pH 9.0, Aldrich, The Netherlands) and 2,4,6 trinitrobenzene sulfonic acid, TNBS (0.5 wt% Fluka, Switzerland). The reaction was continued for 4 h at 40°C, after which the samples were rinsed in saline solution using a vortex mixer to remove unreacted TNBS.

Samples were freeze-dried overnight (HETO, Germany), after which the dry mass was determined. Dry samples were immersed in aqueous hydrochloric acid (2 ml, 6 M, 80°C ,Aldrich, Zwijndrecht, The Netherlands) until fully dissolved. The obtained solution was then diluted with deionised water (8ml) and the absorbance was measured at 340 nm (Perkin Elmer, Fullerton, USA) The concentration of free amine groups was calculated using the following equation:

$$[NH_2] = \frac{A \cdot V}{\varepsilon \cdot \ell \cdot m_{tissue}}$$

wherein:				
	$[NH_2]$	=	free amine group content	[mol / g tissue]
	A	=	absorbance	[-]
	V	=	solution volume	[ml]
	ε	=	14600	[ml/mmol.cm]
	ℓ	=	path length	[cm]
	m_{tissue}	=	dry weight sample	[mg]

The obtained value was used to determine the %-blocking, which was calculated by the following formula:

$$\%\text{blocking} = \frac{[\text{NH2}]\text{native} - [\text{NH2}]\text{blocked}}{[\text{NH2}]\text{native}}$$

wherein:

%blocking=	% of blocked amine groups	[%]
[NH2]native=	free amine content of the native material	[mol/gram tissue]
[NH2]blocked=	free amine content of the blocked material	[mol/gram tissue]

Resistance to enzymatic degradation

The resistance to enzymatic degradation was determined using protease (20 Units/mg, Aldrich, Zwijndrecht, The Netherlands) [14]. In this procedure wall and leaflet samples which were previously weighted after being washed in demineralised (Di) water in order to remove buffer-salt and freeze dried, were initially soaked in a HEPES buffered saline solution (10mM, pH 7.4) containing NaCl (9 g /liter) and glycine (7.5 g/liter, Aldrich, Zwijndrecht, The Netherlands) for 4 h at 37^{o}C. Samples were then transferred to a solution of the same buffer containing the enzyme (3ml with 12 mg enzyme per sample). To assure optimal enzymatic activity of the solution, the enzyme was added along with $CaCl_2$ (96 mg to 180 ml solution) Samples were incubated for 4 h, washed in Di-water and freeze-dried. After renewed determination of the dry weight the actual weight loss due to enzymatic degradation was calculated. The results are presented as remaining weight (in %).

Shrinkage temperature determination

Differential Scanning Calorimetry (Pyrus 1, Perkin Elmer Fullerton USA) was used to determine the shrinkage temperature (Ts) of the various tissue samples.

Samples of leaflets were obtained by cutting and samples of walls were punched (discs with a diameter of 6mm) and immersed in NaH_2PO_4 (0.05 M, Aldrich, Zwijndrecht, The Netherlands) solution for 3 h. Samples were then removed and excess buffer was blotted with a lint-free tissue. An individual sample was placed in the pan and the cover was crimped. For each leaflet or wall, three samples were analysed (n=5)

The shrinkage temperature of each sample was determined using a temperature scan between 30 and 95^0C and a scan rate of 2^0C/min. The shrinkage temperature was defined as the temperature at the maximum of the endothermic transition.

Water Content

To determine the water content of the tissue, 6 mm round discs of fresh, blocked or blocked-and-cross-linked wall samples were punched, blotted on lint free paper and freeze dried after weight determination. The weight was determined again and the weight loss recorded. Ten samples for each aldehyde group were used.

Contact Angle

Contact angle measurements of wall tissue samples were performed at room temperature (using the sessile drop technique) with image analysis. From each sample a 10μm thin slice was made with a cryotome. The material was sliced starting at the adventitia and after approximately 10 slices, 5 samples were retrieved (located in the middle of the tissue sample). With a static contact angle device (Dataphysics Contact Angle system OCA, Germany) the contact angle of a droplet of water on the surface of each sample was determined. Rinsed samples were placed on a microscope slide. The excess of solution was removed with lint-free tissue. The contact angle was determined 5 seconds after the water droplet was positioned. The sample was placed back in the saline solution and a fresh sample was taken for the next measurement allowing the other sample to re-hydrate completely. The experiment was repeated for each sample 10 times (giving a n=10).

Subdermal implantation

Finally a sub-dermal rat implant of the processed tissue was performed to determine the effect of the chemical modification on calcification.

After explantation of the tissue the degree of calcification was determined using Von Kossa staining and inductively coupled plasma atomic emission spectroscopy (ICP-AES). For subdermal implantation, 8 mm discs, punched from the valve wall and leaflets were used. Prior to implantation, these were washed 3 times for 2 min in sterile saline solution.

National Institute of Health guidelines for the care and use of laboratory animals (NIH 85-23 Rev. 1985) were observed and 5 wks old male Long Evans rats were used. After anesthetization with a mixture of halothane, N_2O and O_2, backs were shaved and disinfected using ethanol; a mid-line incision was made in the skin and in six created subcutaneous pockets at each side of the spine three discs were inserted. The skin was closed with a single suture and samples were explanted after 8 wks. From each sample group six disks from wall and six pieces of leaflet material were implanted in this study.

Analysis of calcium concentration

After 8 wks, samples were explanted and processed for further analysis. For quantitative calcium analysis, the surrounding capsule was removed after which the samples were stored in 0.01 M HEPES/20% IPA . Calcification was evaluated by means of von Kossa staining.
The calcium concentration in each tissue sample (mg / g tissue) was also determined by ICP-AES (Perkin Elmer radia Optima 3000). Explanted samples, stored in 0.01M HEPES/IPA, were frozen in liquid nitrogen, after which they were freeze-dried for 12 h. The dry weight of each tissue sample was determined. Then freeze-dried samples were hydrolysed in 15 ml 6M aqueous hydrochloric acid solution for 24 h followed by the addition of 10 ml deionised water. Subsequently, the signal intensity of calcium was determined with an atomic emission spectrometer using emission lines of 317.933 nm and 422.673 nm. The concentration of calcium per dry weight of tissue was calculated using a Ca^{2+} calibration curve obtained with Ca^{2+} standard solutions.

Results

Alternative blocking agents

The outcome of the study in which the different blocking agents were evaluated is depicted in table 1, showing the blocking efficacy for wall and leaflets. Leaflets have typical higher blocking efficacies compared to wall tissue ($p<0.01$, student T-test was used statistical analysis of data). The use of different aldehydes leads to comparable results with the exception of glyceral giving a significantly lower blocking efficacy for the wall ($p<0.01$).

***Table 1**, Blocking of amine groups of heart valve tissue with various mono aldehydes (5 valves were treated per aldehyde group)*

Aldehyde N=3	%-blocking wall	%-blocking leaflet
Butanal	67 ± 6	87 ± 3
Methyl-Butanal	73 ± 2	81 ± 2
Propional	75 ± 3	87 ± 3
Glyceral	58 ± 3	79 ± 4

Valves were processed in MES buffer (0.2M, initial pH 6.4), aldehyde (0.5M) and $NaCNBH_3$ (three additions of 0.1 g over a 8 h interval). Total reaction time 24 h. The TNBS analysis was carried out with 5 samples of wall and 5 samples of leaflet tissue obtained from one valve per aldehyde group randomly

Based on the less favorable results for glyceral, the blocking process for this compound was further investigated to further increase the blocking efficacy. The results of this study are depicted in table 2. In order to achieve a high blocking efficacy at least one of the parameters reaction temperature, the time or the concentration of glyceral needs to be increased ($p<0.01$). The most optimal reaction conditions are obtained if a long reaction time is used in the process. For convenience, a reaction at room temperature is preferred and since preparing a solution of glyceral at a concentration of 0.5 M is cumbersome, in further studies a concentration of 0.2 M was used.

For further optimization of the process two studies were performed in order to determine the lowest buffer and $NaCNBH_3$ concentration at which a degree of blocking of ±75% can be achieved. Table 3 depicts the results of the study with respect to the minimal buffer concentration that can be used for efficient blocking of the material. The results show that although the initial pH of 6.4 is not maintained under the conditions evaluated, the blocking efficacy achieved is not significantly influenced by the concentration of the buffer used in the range studied ($p<0.001$).

***Table 2**, Blocking of amine groups of heart valve wall tissue with glyceral as a function of the glyceral concentration, reaction time and temperature (three valves were processed for each experimental group*)

Concentration (M)	Reaction Temp (C)	Reaction time (h)	Blocking efficacy (%)
0.2	20	24	42±3
0.5	20	24	64±2
0.2	40	24	64±4
0.5	40	24	64±3
0.5	40	48	69±3
0.2	20	48	74±2
0.5	20	48	74±1
0.2	40	48	75±2

Wall samples were processed in MES buffer (0.2M, initial pH 6.4) with glyceral and $NaCNBH_3$ (three additions of 0.1 g over an 8 h interval). The TBNS analysis was carried out with 5 samples of wall tissue obtained randomly from each treated group.

***Table 3**, Blocking of amine groups of wall tissue samples of valves treated with propional in MES buffer with different concentrations.*

Concentrations (M)	Final pH	Blocking Efficacy (%)
0.01	9.2	76±2
0.02	8.6	74±2
0.05	8.0	76±2
0.1	7.6	73±4
0.2	6.9	75±3

Samples were processed in MES buffer (initial pH 6.4) at 5 different concentrations with propional (0.5M) and $NaCNBH_3$ (three additions of 0.1 g over an 8 h interval). Three valves per group were processed and the TBNS analysis was carried out with 5 samples of wall tissue obtained randomly from each treated group.

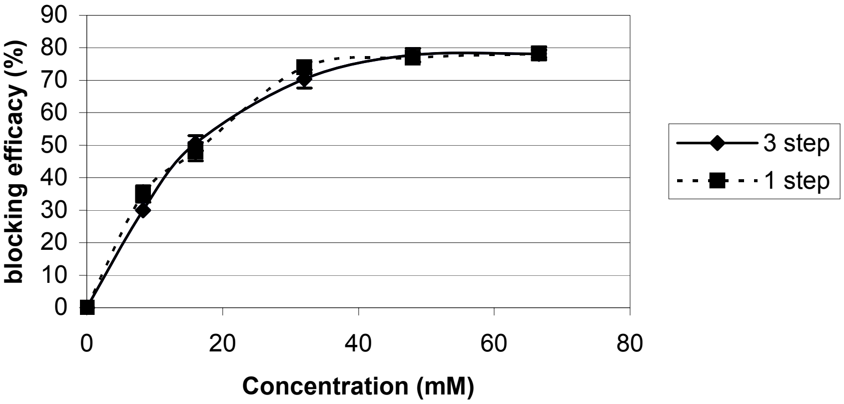


Figure 1, The blocking efficacy against the total concentration of $NaCNBH_3$ added to the reaction mixture in a single addition or added in three steps over an 8 h interval.
Samples were processed in MES buffer (0.2M, initial pH 6.4) with propional (0.5M) and different concentrations of $NaCNBH_3$ (three additions over an 8 h interval or added at the start of the experiment). The TBNS analysis was carried out with 5 samples of wall tissue obtained randomly from each treated group.

Figure 1 depicts the results of the blocking efficacy of wall tissue samples achieved with different concentrations of added $NaCNBH_3$ added in a single step of divided in three portions over an 8 h interval.
This study shows that in order to achieve the maximal blocking efficacy in wall tissue, a $NaCNBH_3$ concentration of minimal 50 mM needs to be employed and that in this case no significant difference was obtained by adding the $NaCNBH_3$ at the start of the experiment or divided in three equal additions ($p<0.001$). In further studies a 50 mM (0.3 g per 100 ml) $NaCNBH_3$ concentration was used, added at the start of the blocking reaction.

Characterization of samples for the implant study

Table 4 summarizes the sample groups prepared for further analysis and the results obtained in these studies. Here 5 valves per group were studied
The results show that for most aldehydes both wall and leaflet can be blocked for approximately 80%. For glyceral however these values were significantly lower ($p<0.01$). The shrinkage temperature (Ts) was determined after cross-linking and has increased for all groups compared to the fresh sample-group ($p<0.001$). Here the highest values were obtained with the glyceral blocked and EDC/NHS sample groups ($p<0.01$). For both the propional and glyceral groups, the shrinkage temperatures for

***Table 4**; Characterization of valves used for the implant study. Valves (5 per group) were treated using optimized conditions.*

Aldehyde		Concentration (M)	Blocking (%)	Ts (C)	Digest rem wt (%)
Fresh	Wall	-	0	61±2.1	5±2
EDC/NHS	Wall	N.A*	N.A.	80.1±2.1	87±2
Butanal	Wall	0.5	79±2	68.8±2.3	83±4
2 Methyl-butanal	Wall	0.5	79±3	70.3±1.8	75±5
Propional	Wall	0.5	81±3	72,7±2.8	64±2
Glyceral	Wall	0.2	72±4	79.9±2.4	63±3
Fresh	Leaflet	-	0	58.2±3.1	0
EDC/NHS	Leaflet	N.A.	N.A.	82.0±0.3	60±3
Butanal	Leaflet	0.5	84±2	75.8±1.2	50±5
2 Methyl-butanal	Leaflet	0.5	83±3	78.7±0.5	30±4
Propional	Leaflet	0.5	84±3	74.5±0.5	50±6
Glyceral	Leaflet	0.2	75±2	81.9±0.1	22±2

* Not applicable

Samples were processed in MES buffer (0.05M, initial pH 6.4) with aldehydes added (0.5 M except 0.2M for glyceraleral) and $NaCNBH_3$ (one single addition of 0.3 g at the start of the experiment). After determination of the blocking efficacy using the TNBS method, the valves were subsequently imbibed in Jeffamine 230 for 30 min and cross-linked for 48 h in 0.3M EDC and 0.12 M NHS. For the EDC/NHS tissue group, fresh valves were cross-linked directly for 48 h in 0.3M EDC and 0.12M NHS. Analyses were carried out on 5 samples of wall tissue obtained randomly from each treated group.

wall and leaflet were almost equal, while for the 2-methyl butanal and butanal groups the leaflets had a higher Ts compared to the wall ($p<0.01$).

Finally, the enzymatic digestion data indicates that all groups are more resistant to enzymatic degradation compared to the fresh sample group. The wall was found to be more resistant compared to the leaflets of all groups ($p<0.001$). The glyceral treated group showed the highest degradation for the leaflets ($p<0.01$). The EDC/NHS control group had the highest shrinkage temperature and the degradation of both wall and leaflets was the lowest ($p<0.01$).

Water content and contact angle

Figure 2 depicts the contact angles of blocked material (left) and blocked-and-cross linked wall material (right)

The contact angles of materials from the different groups vary depending on the treatment Samples become more hydrophilic after being blocked with aldehydes compared to a native fresh control (p<0.01, exception is butanal where the difference is not significant). After the cross-linking reaction is completed the contact angle measurements indicate that the materials become more hydrophobic in the range glyceral-butanal-propional.

Furthermore all samples have water contents between 70 and 75% and no significant difference between the groups was observed (p<0.01).

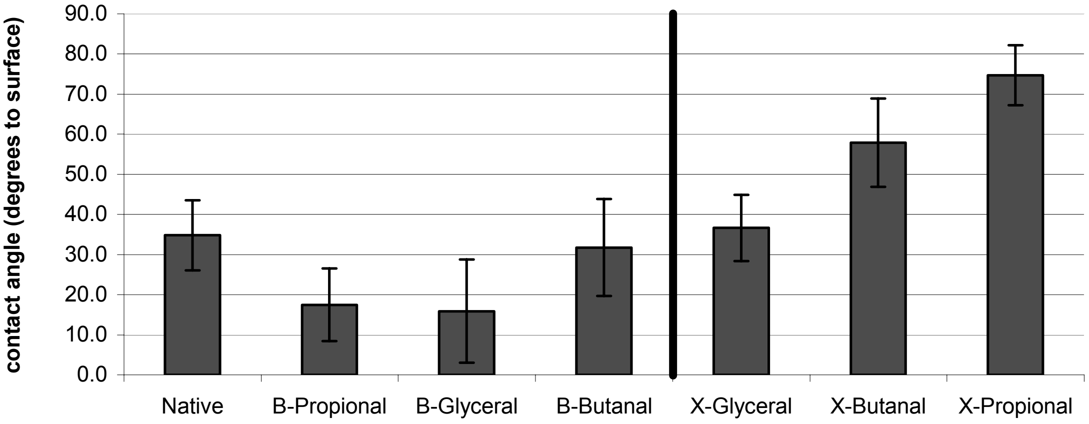


***Figure 2**, The contact angle of native, blocked (B) and blocked-and-crosslinked (X) wall tissue slices. Valves were processed as mentioned in table 6. The analysis was performed after the blocking step was performed and after the completion of the cross-linking. For the analysis 10 samples were obtained, punched randomly from the valves processed.*

Subdermal implantation

Samples were implanted subdermally in Long Evans rats (5 wks old) for 60 d and explanted and further processed for either histological or quantitative determination of Ca levels. The results of the calcium AAS determination are depicted below:

The amount of calcium in the wall samples was reduced in the groups having blocked amine groups. A decreasing Ca content was found in the series glyceral, butanal, 2 methyl-butanal and propional. Of the carbodiimide processed sample groups, the EDC/NHS control group showed the highest level of calcification. In leaflet tissue the calcium levels were found to be very low for all groups, whereby only the propional and methyl-butanal groups gave a significant reduction in calcification compared to the EDC/NHS group (depicted in the figure). The calcification of the GA fixed leaflet samples was found to be > 100µg/mg tissue and is not depicted in the figure.

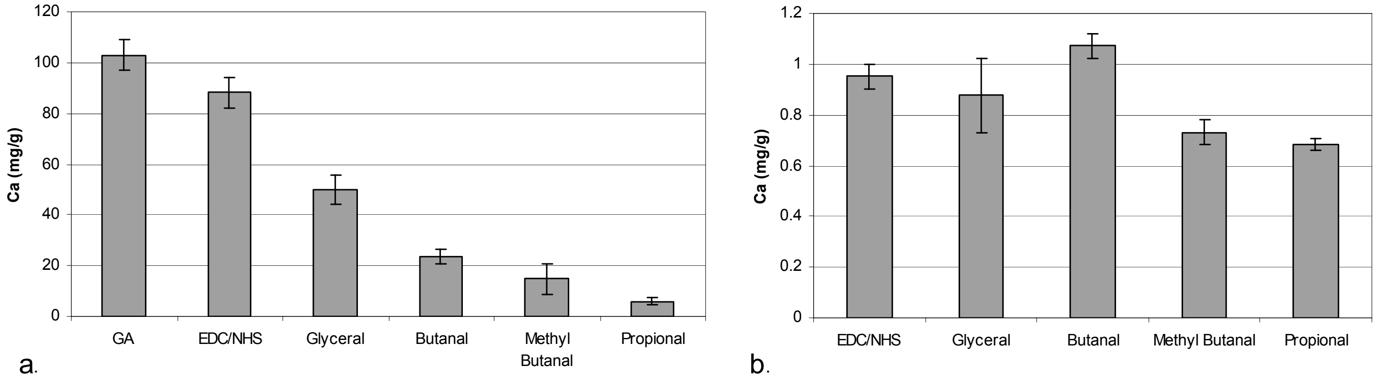
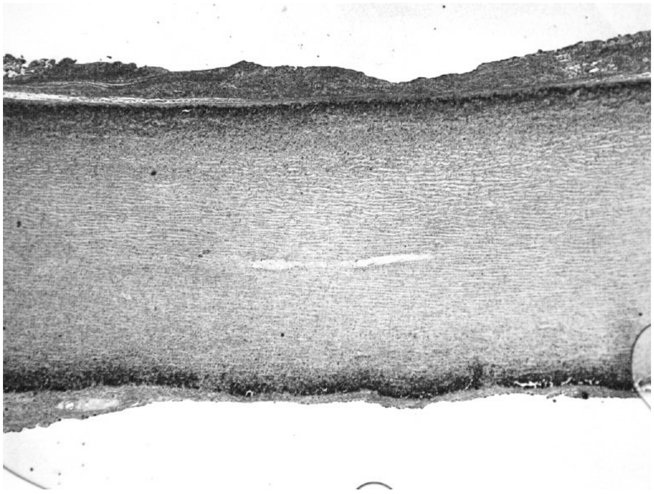
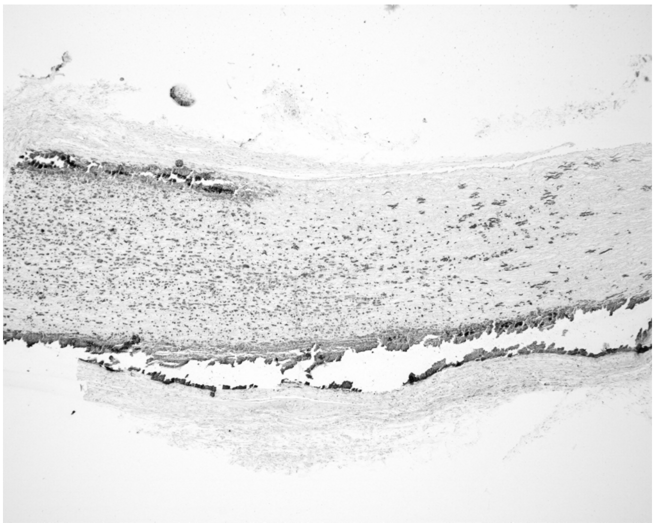


***Figure 3**, AES calcification results of the implanted wall samples (a) and leaflet samples (b). The type of aldehyde that was used is mentioned in the figure. Six wall and 6 leaflet samples per aldehyde group were implanted. The EDC/NHS control group was cross-linked without prior blocking of the amine groups. A second GA control group was added; wall calcification data is depicted; leaflet calcification data is not since this value was >100*mg*/g tissue*

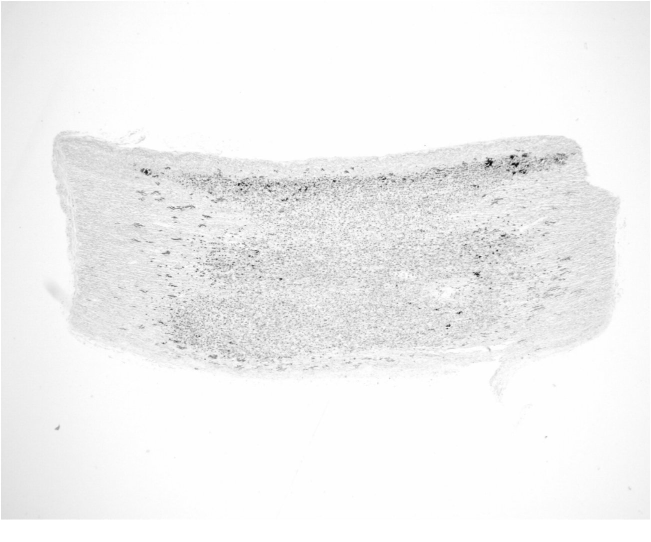
Figure 4 shows the Von Kossa results of wall tissue of the sample groups depicted in figure 3. In the control EDC/NHS tissue group calcification occurs throughout the material with two bands of complete calcification at the outer edges of the wall. Furthermore, the glyceral sample group shows the highest incidence of calcification within the aldehyde treated sample groups, with small calcium spots throughout the sample. For both butanal and methyl-butanal these condensed calcium spots are observed but these occur less frequently than the small calcium spots in the glyceral group (based on image analysis, $p<0.01$). Finally for the sample group processed with propional, calcification was almost absent. Results of leaflet material are not depicted since the calcification as measured with AAS could not be visualized with the Von Kossa staining.



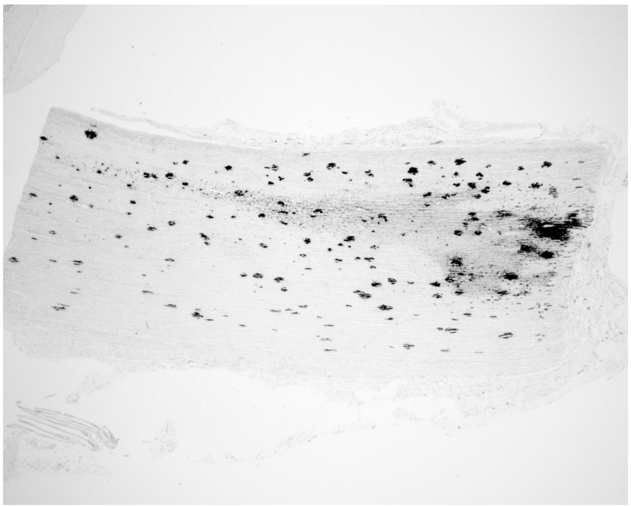
A.



B.



C.



D.

E.

F.

Figure 4, *Von Kossa histology performed on the wall samples as depicted in figure 3. A: GA (magnification 2x), B: EDC/NHS (magnification 4x), C: Glyceral (magnification 2x); D: Butanal (magnification 2x), E: Methyl Butanal (magnification 4x) and F: Propional (magnification 4x)*

Discussion

Before starting the experiments described in this paper a broad range of aldehydes were screened and evaluated as blocking agents for amine groups of dermal sheep collagen. These initial experiments showed that with many aldehydes nearly 100% of the primary amine groups could be blocked. Based on these successful results a selection of mono-aldehydes was further investigated for blocking of amine groups of tissue valves. Since all primary amine groups can be detected by the TNBS method and tissue material contains more compounds with free amine groups, which may be less accessible for the blocking agent then in dermal sheep collagen, the theoretical maximal blocking efficacy is expected to be less than 100 %. For the experiments described in this paper, glyceral, 2 methyl-butanal, propional and butanal were evaluated in detail. These aldehydes were selected based on their different hydrophobicity and the fact that they have a relatively low molecular weight. In this way diffusion limitations are minimized and the original valve matrix morphology can be maintained as much as possible.

After a feasibility study that showed that all 4 aldehydes could be successfully employed, glyceral was evaluated in more detail because the degree of blocking was lower compared to the other aldehydes. The study on glyceral showed that extending the reaction time, increasing the temperature or increasing its concentration during the reaction, all lead to a higher degree of blocking. Due to the fact that the dissolution of glyceral in the medium at high concentrations is cumbersome, a concentration of 0.2 M was used to assure that no solid glyceral particles were present during the blocking reaction. The reaction time for the process was increased from 24 to 48 h to assure that all chemicals reach and react also in the middle section of the tissue (wall) material [1]. This was confirmed by the presence of a single sharp peak in the calorimetry thermogram, from which the shrinkage temperature of treated samples is determined.

In order to further optimize the reaction conditions two studies were performed to determine the lowest $NaCNBH_3$ concentration still giving the highest blocking efficacy and to reduce the concentration of buffer still allowing efficient blocking of amine groups. The outcome of the studies showed that the minimal concentration of $NaCNBH_3$ is 50 mM (0.3 g per 100 ml) added at the start of the experiment. Since the pH of the buffer did not significantly affect the degree of blocking, a separate study was performed that showed that the MES buffer concentration during the blocking reaction could be reduced to 0.05 M as experimental work showed that with this concentration the pH did increase, however stayed within an acceptable range. MES is the preferred buffer since it is frequently used in biological systems. After optimization studies were completed, a wall blocking-efficacy close to 80% could be achieved. The analysis of the sample groups prepared for the implantation study show that glyceral blocked samples still have the lowest degree of blocking despite the fact that the process was optimized.

Because the shrinkage temperature of the glyceral-blocked and cross-linked samples was found to be the highest this may indicate that still free, unreacted amine groups were present and so-called zero-length cross-links between amine and carboxyl groups may have been formed. The enzymatic digestion of glyceral leaflet material was higher than the digestion of leaflets blocked with other aldehydes. This may be explained by a better accessibility of enzymes to the collagen structure, which has become more hydrophilic after glyceral treatment. However more diligent research on these findings needs to follow.

After explantation, the von Kossa analysis of wall samples showed differences in calcification patterns for the different groups. While in the EDC/NHS groups, a high calcification was observed in the edges and furthermore throughout the wall samples; in the aldehyde sample groups, the calcification concentrates in nodes throughout the material. It will have to be proven if a relationship can be found between these nucleation sites and reactions that have occurred in the tissue during blocking.

For leaflets the calcification of all sample groups were found to be very low. The von Kossa analysis of the leaflet samples did not show any calcification while AAS showed calcium levels of less then 1 mg/gram leaflet tissue. A GA fixed control sample group was added to the implantation study in order to be able to compare the outcome of the study with the carbodiimide processed sample groups to a 'standard' fixed sample group. The results show that indeed a significant decreased calcification compared to this GA fixed group could be obtained.

As discussed before [14], the experimental model selected to evaluate the calcification in-vivo does not predict the real outcome in terms of calcification. Therefore a propional-processed group was evaluated using a juvenile sheep descending thoracic aorta model and similar significant decreased calcification compared to a GA processed control group was achieved (data not published)

Contact angle experiments showed that the hydrophobicity of the different cross-linked samples after being blocked varies and together with the calcification data a decreasing calcification with increasing hydrophobicity was found ($p<0.01$). More studies on differently processed samples need to follow in order to verify if this observation also can be made for alternative non-carbodiimide cross-linked valve samples.

Available literature suggests 2 hypotheses to explain the observations made. The first is the decrease of transport of calcification promoting agents (like Ca^{2+}-ions) through the tissue with increased hydrophobicity. Since there is a relationship between hydrophobicity of a surface and surface charge, a second hypothesis is that there may be a link that each blocking agent has a different influence on the final surface charge of the matrix. Golomb and Era [17] proved that an impaired balance between positively and negatively charged amino acid residues resulted in affinity sides for Ca^{2+}. Zilla et al. [18] observed that the content of free aldehyde groups and polymeric GA cross-links has to be reduced to obtain low calcification, which could point in the same direction. Use of compounds like bisphophonate [24], aluminum-chloride [25], sodium dodecyl sulfate [26] and other metallic salts [27] bound to the tissue or present in the matrix, all change the surface charge of the tissue and each of these treatments were successful in mitigating the calcification. Since the observation of the relationship between calcification and hydrophobicity was based on 4 aldehyde samples only, a more detailed investigation needs to follow to reach final conclusions.

The third hypothesis is the removal of antigenic substances such as cellular elements and soluble proteins or potential nucleation sides for hydroxyapatite crystals that has been suggested in literature as being effective to reduce calcification. Various extraction methods are used like sodium dodecyl sulfate (SDS), trypsin and dimetylsulfoxide (DMSO) [21,22,23]. DMSO, a cryoprotective agent for tissue appears to extract compounds and it was suggested that it nullifies the nucleation sides that can attract Ca^{2+} [23]. In case of the chemistry described in this paper this leads to the second hypothesis that in the blocking reaction components are extracted from the matrix, or that the aldehyde reacts with amine groups of compounds, which are subsequently removed from the matrix. Initial extraction studies on processed tissue indicate indeed that compounds like proteins and lipids are removed during the blocking reaction and that the amount varies with the type of aldehyde used for the blocking reaction.

Although the achieved low calcification of the heart valve material is promising, the biomechanical properties were found to be in between the first generation of GA cross-linked valves and valves processed with Freestyle^{TM}. Therefore decreased tissue stiffness is desired as the biomechanical properties have preferably to be better or equal to the last mentioned commercial product.

It is expected that the mechanical properties will improve if valves are processed using root pressure or zero pressure fixation techniques [28]. Two alternative approaches investigated are increasing the chain-length of the Jeffamine used [14,15] and/or reducing the number of cross-links formed. For example by blocking a pre-defined number of carboxyl groups prior to cross-linking with the Jeffamine. Reducing the amount of EDC in order to decrease the number of cross-links formed leads to incomplete cross-linking and cross-links not well distributed in the matrix; an approach not desired.

Conclusion

In conclusion the blocking and cross-linking technology looks promising for the design of a next generation of tissue valves. Calcification was significantly reduced compared to GA cross-linking. The mechanistic insight about the decreased wall calcification is still unknown and based on the two hypothesis made, research is currently on going. The mechanical properties of the material need to be improved however and results of these studies will be available shortly.

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Chapter 5

Quantification of Carboxyl groups in Carbodiimide Cross-linked Collagen Sponges*

Abstract

One method for in-vitro characterization of cross-linked tissue and collagen matrices is the determination of the degree of cross-linking, usually performed by measuring the involvement of amine groups during the process.

One specific cross-linking method for collagen containing materials is based on water soluble carbodiimides (CDI). It is generally accepted that this cross-linking method leads only to the formation of amide linkages between tissue carboxyl and amine groups. Therefore, in absence of a reliable method to quantify tissue carboxyl groups until recently the degree of cross-linking of CDI cross-linked tissue and collagen was determined by quantifying amine groups as well. However it cannot be excluded that by using CDI, tissue carboxyl groups can react with other nucleophiles (such as hydroxyl groups) in the matrix.

In this chapter a new assay to determine residual carboxyl groups in CDI cross-linked collagen matrices is presented.

The concentration of carboxyl groups in unprocessed RDBC as determined with this new technique was equal to the concentration of carboxyl groups measured by amino acid analysis. Based on the concentration of residual carboxyl groups determined for CDI/NHS cross-linked RDBC and RDBC, in which the amine groups were blocked with propionaldehyde before CDI/NHS cross-linking, it was concluded that activated carboxyl groups can also react with other groups (such as hydroxyl groups) present in the matrix. This implies that the crosslink density of RDBC matrices after treatment with CDI/NHS is higher than expected on the basis of amide bond formation only, as determined by quantification of amines.

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Introduction

Cross-linking of bioprosthetic tissue is a common procedure to prevent degradation of the tissue during in-vivo application. Glutaraldehyde (GA) based cross-linking is the current standard procedure for the production of tissue heart valves, providing the prostheses with a low incidence of thrombo-embolism and satisfactory haemodynamic performance [1]. In spite of the positive effects of GA fixation, failure of these cross-linked materials in-vivo may occur mainly due to inflammation and calcification [2,3,4]. Therefore much research has been directed to the development of alternative cross-linking procedures to overcome these deficiencies [1].

Cross-linking of collagen based materials with a water-soluble carbodiimide (such as N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide , EDC) may be a promising alternative to GA cross-linking [5,6]. Carbodiimides are classified as zero-length cross-linking agents used to mediate the formation of amide linkages between carboxyl and amine groups [7]. The advantage of the use of these so called zero length cross-linking agents is that during cross-linking no additional chemical entities are introduced between the conjugating molecules [7,8,9]. This is in contradiction to the use of GA which leads to the formation of a variety of chemical structures after reaction with primary amines of lysine and hydroxylysine residues of tissue [10]. It has been demonstrated that diffusion of GA or GA based reaction products from GA cross-linked tissue matrices causes cytotoxic effects in vivo [3].

The use of zero length cross-linking agents has become more and more important for developing new biomaterials, such as collagen, for long term implantation and recently several articles have been published on tissue engineered constructs based on matrices cross-linked with EDC and N-hydroxysuccinimide (NHS) [5,6,11-20].

Insight in the extent of cross-linking of matrices that can be obtained using these reagents is essential for the design of matrices with optimal properties [1].

The degree of cross-linking of matrices in which only amine groups are involved in the cross-linking reaction, for instance with the use of GA, is usually determined by measuring the concentration of residual amine groups by a 2,4,6 trinitrobenzene sulphonic acid (TNBS) assay [18,21]. Furthermore determination of the shrinkage temperature provides indirect information about the degree of cross-linking of the material [18]. The degree of cross-linking of a matrix also has an effect on its resistance to enzymatic degradation [18]. In addition, in vitro cell based assays provide information about the biocompatibility of the cross-linked structures [15]. For the determination of the mechanical properties of cross-linked matrices a number of tensile tests have been developed [13]. The combination of these assays provides a complementary picture of the physical/chemical properties of the tissue after processing [18].

It is generally accepted that during EDC based cross-linking of collagen the carboxyl groups are activated and form amide linkages with tissue amine groups. However it was demonstrated in literature that activated carboxyl groups of hyaluronic acid are capable of forming ester linkages after reaction with hydroxyl groups [22]. In order to study if this reaction also occurs during EDC cross-linking of collagen a reliable test for quantification of residual carboxyl groups is required.

It has been found that titration techniques could not be used to assess the concentration of carboxyl groups of collagen based materials [23]. Labeling of residual carboxyl groups with dyes developed for histology proved to be a method for obtaining qualitative information [24]. Several techniques have been described in literature for the determination of carboxyl groups of water soluble materials [24-33]. It has been demonstrated that 5-bromomethyl fluorescein (5-BMF) in particular is a very effective labeling agent for carboxyl groups [28]. In the study described in this paper we have adapted existing schemes using 5-BMF to label carboxyl groups of reconstituted dermal bovine collagen matrices before and after cross-linking. After release of the label by mild hydrolysis, it was quantified by using capillary zone electrophoresis (CZE) with laser induced fluorescence (LIF) detection. Using a calibration curve relating carboxyl group concentrations of Sephadex™ standards with the fluorescence signal, it was possible for the first time to accurately determine the concentration of carboxyl groups in the collagen based samples before and after cross-linking.

Materials and Methods

Materials

All chemicals used were obtained from Sigma Aldrich (the Netherlands) and were of analytical grade. Acetonitrile, 18-crown-6 and 5 bromomethyl fluorescein (5-BMF) stock solutions (3mM) in acetonitrile were stored over molecular sieves, 5A (Supelco, the Netherlands) and kept at 4°C until use.

Reconstituted dermal bovine collagen (RDBC) was obtained in lyophilized form from Coletica (France). RDBC samples with a thickness of ca 3mm were cut into 2cm x 1cm samples and stored under anhydrous conditions over Drierite™ until use.

Methods

Amino acid analysis of RDBC

RDBC samples processed according to the method described in table 1 below were characterized by amino acid analysis (Ansynth Roosendaal, the Netherlands).

General procedure for 5-bromomethyl fluorescein (5-BMF) labeling of carboxyl groups of RDBC samples

Five samples containing approximately 100µg each of RDBC sponge material were swollen in DMSO (500 µl) in individual amber colored HPLC vials. After swelling, 96 µl of 18-crown-6 solution (3mM in acetonitrile) was added to the DMSO swollen samples. Thereafter anhydrous potassium carbonate (20mg) was added to each vial. The contents were agitated on a rotary shaker followed by the addition of 500 µl of 5-bromomethyl fluorescein (5-BMF) stock solution (3 mM in acetonitrile). The final mixture was incubated at 40°C, while the duration of incubation was varied over a period of 25h to perform a time course study for determination of the optimal reaction times for the labeling process.

At specific times after the start of the reaction, the 5-BMF labeled RDBC samples were rinsed with 2ml of acetonitrile for 1h. At the end of the first hour the rinse was analyzed for the presence of 5-BMF using fluorescence spectroscopy (Perkin Elmer, LS50B, Fullerton, USA with an excitation at 488 nm and emission at 520nm). The rinse and analysis processes were repeated at consecutive hour intervals until no fluorescence was detected in the rinse solutions.

To assess the amount of fluorescent label bound to available carboxyl moieties the label must be liberated from the substrate. To accomplish this, the washed labeled RDBC samples were blotted free of solution and transferred to amber colored HPLC vials containing NaOH (1ml, 0.1M). Hydrolysis times were varied up to 30 h in order to establish the time required for maximal release of 5-BMF. The resultant hydrolyzates were used for the determination of the concentration of free carboxyl groups in the original RDBC samples utilizing capillary zone electrophoresis (CZE), which is described below.

Capillary Zone Electrophorsis (CZE)

CZE analysis was performed on a MDQ (Beckman, Palo Alto, USA) equipped with a 488nm Laser Induced Fluorescence Detector (LIF), a 520 nm band pass filter and a fused silica capillary (50 μm I.D., 50cm from injection to detection). Pressurized injections for 20s at 10psi were used and the voltage was set at 15kV. Analysis took place in micellar electrokinetic chromatography (MEKC) mode using a borate buffer (pH 9.2, 10mM, adjusted with NaOH) containing sodium dodecyl sulphate (SDS, 10mM). A total analysis time of 20 min per sample was used. All analyses took place within 12 h after completion of the hydrolysis process. This was done to minimize possible deterioration of the fluorescent signal. Analytes were stored in the dark at 4°C in the CZE system during the experiment. All effluent was analyzed by the LIF detector. Peak areas were calculated using internal software (Beckman, Palo Alto, USA).

Calibration of the CZE method for quantifying carboxyl groups

A calibration curve was made by labeling carboxy-methyl modified Sephadex™ with know concentrations of carboxyl groups (obtained via GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Hereto the same labeling method as described for the RDBC samples was used. It was found that the Sephadex™ beads were compatible with the organic labeling system used in the 5-BMF reaction. The detection limit was determined at a signal to noise ratio of 3. The noise was determined using the mean peak to peak noise (n=3) of the electropherogram between 7 and 9 min after injection of water. The reproducibility of the method was demonstrated by quantifying the concentration of carboxyl groups of unprocessed RDBC samples on 5 different days. For each experiment a new calibration curve was made after labeling of fresh Sephadex™ standards.

RDBC samples used for determining concentrations of carboxyl groups.

Table 1 summarizes RDBC samples processed for the evaluation of the labeling method.

***Table 1**, Modifications of RDBC samples*

	Label	Chemical process
A	Unprocessed	None
B	Blocked-only	Blocking of amine groups with propionaldehyde
C	Cross-linked only	Formation of cross-links after activation of carboxyl groups by EDC/NHS
D	Blocked and cross-linked	Blocking of amine groups with propionaldehyde and subsequent formation of cross-links after activation of carboxyl groups by EDC/NHS.

Blocking of amine groups of RDBC [20]

RDBC samples were each submerged in 2-(morpholino) ethane sulphonic acid buffer (MES, 10ml, 0.05M adjusted to pH 6.4 with NaOH) for 1h. Then the MES buffer was removed and 10 ml of a reaction mixture containing the same MES hydration buffer supplemented with propionaldehyde (0.5M) and $NaCNBH_3$ (0.05M) was added to each individual reaction vessel. After 4 h, the RDBC samples were rinsed repeatedly in Di-water for a minimum of 16 h. The blocking efficacy was determined by the use of a colorimetric TNBS assay [20]. The concentration of residual carboxyl groups was determined with the 5-BMF labeling procedure as previously described.

Cross-linking of RDBC samples [20]

RDBC samples (blocked or unprocessed) were each immersed in MES buffer (10ml, 0.2M adjusted to pH 5.0 with NaOH for 1h). Then the MES buffer was removed and replaced with a cross-linking solution containing the same MES buffer (10ml) supplemented with N-hydroxysuccinimide, NHS (0.12M) and N’-(3-dimethylaminopropyl)-N-ethylcarbodiimide, EDC (0.3M). After 4 h the RDBC samples were removed and rinsed repeatedly in Di-water for a minimum of 16 h. After rinsing, samples were lyophilized and stored over Drierite™. The concentration of residual carboxyl groups was determined using the 5-BMF labeling procedure as described above.

Results

Amino acid analysis of RDBC

The concentration of amino acids in RDBC relevant for blocking and cross-linking is presented in Table 2.

Table 2, *Amino acids expressing free amine, carboxyl and hydroxyl groups present in RDBC samples.*

Functional group	Amino Acid	Number /1000 amino acids	Number of functional groups /1000 amino acids
NH_2	Lysine	20	
	Hydroxylysine	11	31
COOH	Glutamic acid	78	
	Aspartic acid	44	122
OH	Serine	33	
	Hydroxyproline	100	
	Hydroxylysine	11	144

Calibration of the CZE method for quantifying carboxyl groups

Figure 1 shows a typical electropherogram obtained during quantification of carboxyl groups of modified Sephadex™ or RDBC.

Two peaks were obtained: a small peak (a) which migrates prior to the main peak (b). The small peak appears in all samples in the same relative ratio to the main peak. The main peak is assigned to the fluorescent label that is released from carboxyl groups of the matrices after mild hydrolysis.

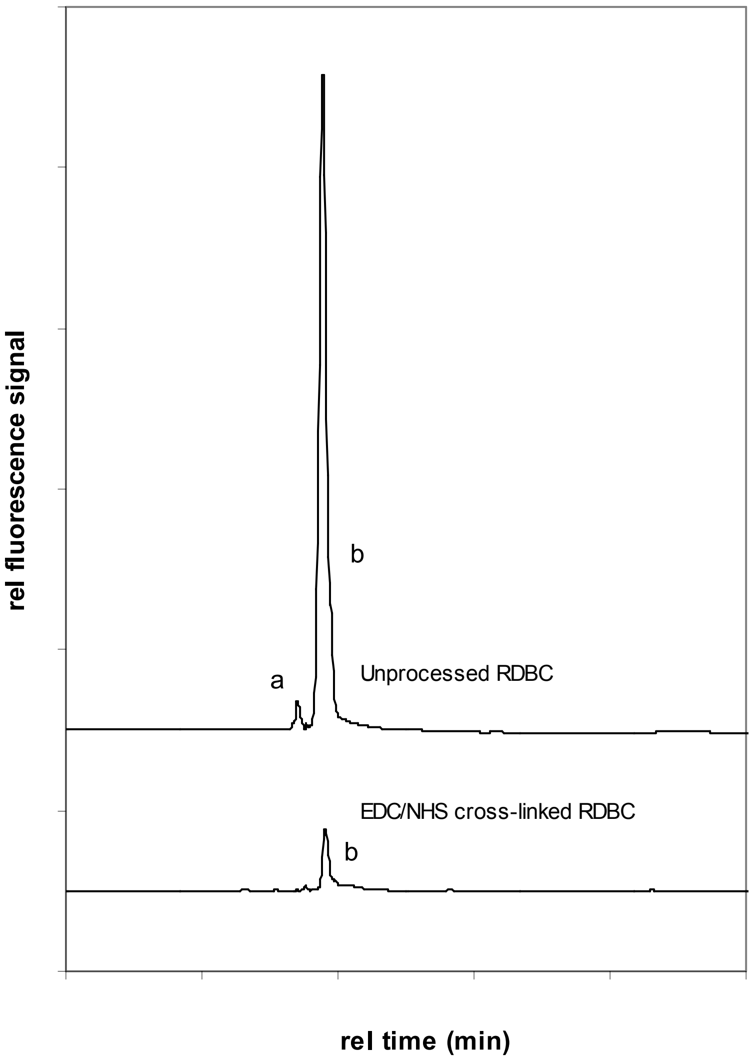

Figure 1, *Electropherograms showing peaks related to the released fluorescent label after hydrolysis unprocessed RDBC and EDC/NHS cross-linked RDBC. Peak (a) is always present in the same ratio to (b) and peak (b) is used for further analysis. Separation conditions: MEKC mode in borate buffer (10 mM, pH adjusted to 9.2) containing SDS (10mM). LIF detector (488nm) with detection at 520 nm was used.*

Sephadex™ beads represent an ideal model system for determining the concentration of carboxyl groups of insoluble substrates because they can be used for chemical reactions in an organic milieu without destroying the matrix. A linear calibration curve was obtained using Sephadex™ with a detection limit of 500 pmoles/ml. Reproducible results could be obtained (5 independent calibration curves were made with $p<0.01$, determined with a student T analysis).

General procedure for the labeling of carboxyl groups of RDBC samples with 5-bromomethyl fluorescein (5-BMF)

Figure 2a represents the results of the time course study for the reaction of 5-BMF with RDBC. It can be seen that the reaction of 5-BMF has reached a maximum conversion at approximately 12 h. In order to assure adequate label incorporation we further adopted 16 h as a standard time for the labeling reaction. For these studies a hydrolysis (fluorescent label liberation) time of 24 h was chosen.

Figure 2b represents the results of the time course study for liberating the ester linked fluorescent label residue from RDBC, utilizing a 16 h labeling reaction time. It can be seen that maximum release of the label was obtained at hydrolysis times > 15h. To assure optimum yields a hydrolysis time of 20h was used for further experiments.

Quantification of the concentration of carboxyl groups in RDBC samples

The amino acid analysis of the different RDBC groups (A-D in Table 3) showed that the concentrations of aspartic and glutamic acid residues were the same for each group as can be expected, because for the analysis the samples are completely hydrolyzed.

No significant difference was found between the total concentration of glutamic and aspartic acid present in unprocessed RDBC as determined with amino acid analysis compared to the results obtained with the 5-BMF labeling method.

Comparing group A and B indicates that as expected blocking of amine groups does not affect the concentration of carboxyl groups. It further illustrates that the results of the 5-BMF technique are not influenced by the presence of blocked amine groups. Blocking of amine groups with propionaldehyde resulted in a significant decrease of residual amine groups as determined with the TNBS method. Although it cannot be excluded that the presence of cross-links hinders the labeling reaction of residual carboxyl groups, it is assumed that the decrease in carboxyl group concentrations of samples which had been cross-linked is only due to involvement of these groups in forming cross-links.

After cross-linking of unprocessed RDBC approximately 57 carboxyl groups and 22 amine groups (per 1000 amino acid residues) are participating in cross-links. Interestingly, during cross-linking of RDBC with blocked amine groups a significant amount of carboxyl groups formed cross-links while no amine groups reacted. By comparing groups B and D it can be concluded that in both cases approximately 36 carboxyl groups per 1000 amino acids have formed cross-links with other reactive groups present in collagen.

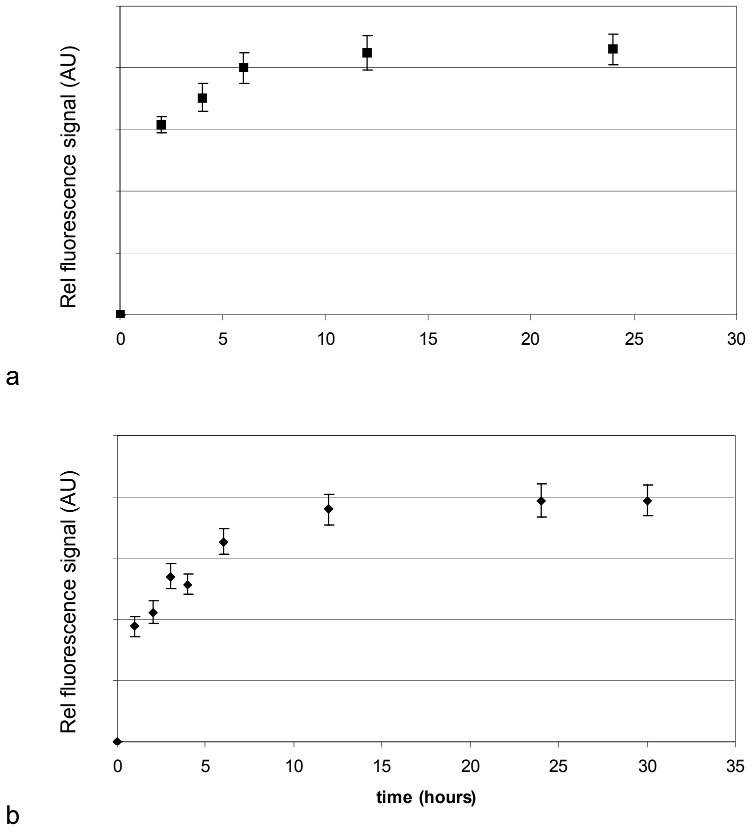

Figure 2*: Single factor optimization study for the time course of fluorescent label incorporation and release from carboxyl groups in RDBC matrices. 2a shows the incorporation of label as a function of time, using a hydrolysis time of 24 h, while 2b represents the release of the label from the matrix as a function of time, using a labeling reaction time of 16 h..*

Table 3*; Comparison of results from amino acid determination and CZE data*

N= number /1000 Amino Acids		COOH (AA)[1]	COOH (5-BMF)[2]	Δ_{COOH}[3]	NH_2 (TNBS)[4]	Δ_{NH2}[5]	$\Delta_{COOH} - \Delta_{NH2}$[6]
A	*Unprocessed*	*123±3*	*122±4*	*N.A.*	*31±3*	*N.A.*	*N.A.*
B	Blocked	121±2	122±2	0	4±2	~27	N.A.
C	Cross-linked	122±4	65±3	~57	9±2	~22	~35
D	Blocked and Cross-linked	121±3	86±4	~36	4±2	0	~36

[1] Carboxyl groups of aspartic and glutamic acid residues as determined by amino acid analysis, [2]Residual carboxyl groups as determined with 5-BMF labeling, [3] Carboxyl groups involved during cross-linking, [4] Amine groups as determined with the TNBS colorimetric assay, [5] Amine groups involved during blocking or cross-linking, [6] Difference in carboxyl group compared to amine group involvement in the reactions. All values are depicted in numbers per 1000 amino acid residues. 5 samples per group per method were analyzed. N.A. = not applicable

Discussion

Bioprosthetic devices have to be biocompatible and should not elicit cytotoxic or inflammatory responses. Although glutaraldehyde (GA) based cross-linking techniques are still used for most commercial products a number of different cross-linking agents for collagen based materials have been developed over the last years.

Many of these reagents react with tissue amine groups (examples are a variety of aldehydes, imidoesters, and hexamethylene diisocyanate) [1].

The degree of cross-linking of such materials is usually determined by a chemical analysis method using 2,4,6 trinitrobenzene sulphonic acid (TNBS) labeling of residual amine groups [21].

More recently, alternative cross-linking methods for collagen based materials have been developed, in which also other groups than amine groups are involved in the cross-linking reaction. An example of such a technique is based on the involvement of carboxyl groups. Olde Damink et al successfully demonstrated the use of N’-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC, a water soluble carbodiimide) in combination with N-hydroxysuccinimide (NHS) in the cross-linking of dermal sheep collagen [5,6,10]. Based on promising in-vivo results, published in his thesis in 1993, others have also applied water soluble EDC and NHS for the cross-linking of a wide variety of tissue matrices [11-20]. One of the reasons for the interest in using EDC and NHS is that cross-linking takes place without the incorporation of new chemical entities, As a result non-toxic matrices can be obtained, which offer ideal scaffolds for cell seeding and other tissue engineering purposes [12,14,15]. Other investigators have further optimized the carbodiimide based cross-linking technology by controlling the cross-linking reactions within biological matrices. By blocking of tissue amine groups followed by incorporation of a diamine terminated spacer (Jeffamine™) using EDC and NHS, flexible spacers between collagen carboxyl groups could be introduced. Porcine aortic wall samples, processed in this manner showed a significantly reduced in-vivo calcification as compared to porcine aortic wall samples cross-linked with EDC and NHS only [18,21].

Until now it is generally accepted that during EDC based cross-linking of collagen matrices activated carboxyl groups form amide bonds with amine groups present in the matrix [7,9,10]. Therefore quantification of residual amine groups with TNBS has also been used to estimate the degree of cross-linking of such matrices.

However, it cannot be excluded that activated carboxyl groups also react with other functional groups present in the matrix. This hypothesis is also based on the work of Tomihata et al [22], who demonstrated using FTIR that treatment of hyaluronic acid films with EDC yields cross-linked structures, rich in ester bonds.

In order to get a better insight in the degree of cross-linking of collagen based structures after treatment with EDC (and NHS) not only the concentration of residual amine groups has to be determined but also the concentration of remaining carboxyl groups. Up to now there is no reliable method available to quantify carboxyl groups of collagen matrices. As mentioned before, existing techniques based on titration of carboxyl groups or reaction of tissue samples with dyes developed for histology are cumbersome and do not have the sensitivity desired. Because of the lack of a direct method to quantify carboxyl groups, investigators were not able to relate the actual degrees of cross-linking of collagen matrices with physical-chemical properties like the shrinkage temperature (DSC), the resistance to enzymatic degradation and the biomechanical properties [18,21].

The work presented in this chapter is directed to the development of a reliable technique for determining the carboxyl group concentration of collagen based materials. Coletica's reconstituted dermal bovine collagen (RDBC) was chosen as a model system because its overall structure and amino acid composition are similar to other reconstituted materials on the market. RDBC was also selected because this material has a low sample to sample variation.

In this novel approach selective labeling of carboxyl groups with 5-bromomethyl fluorescein (5-BMF) was applied. The labeling technique is a modification of the method published by Mukherjee et al to determine the concentration of carboxyl groups of water soluble materials [28]. Figure 3 depicts the reaction of 5-BMF with functional groups present in the collagen matrix.

During the reaction of 5-BMF with functional groups of collagen, a fluorescent label becomes linked to the matrix. If 5-BMF reacts with carboxyl groups, the label is attached via an ester linkage (reaction A in figure 3). If 5-BMF reacts with amine groups, the label is linked through an amide bond to the matrix. After labeling, residual 5-BMF is removed by washing and part of the chemically bound fluorescent label (Fig.3 ,E) is retrieved after brief hydrolysis using NaOH(0.1M) without damaging the remaining collagen structure. In this procedure ester linkages between the label and the matrix are cleaved, whereas the label coupled through an amine bond remains attached to the matrix (Fig. 3, reaction C, product D). This is confirmed by comparing the concentrations of carboxyl groups of unprocessed RDBC with that of amine blocked RDBC (Table 3) both determined by the 5-BMF labeling procedure. The fact that the concentrations of carboxyl groups for both matrices are similar indicates that under mild hydrolytic conditions all the label bound via the ester groups will be released. By weighing the lyophilized samples before and after hydrolysis it was verified that no collagen material is lost during hydrolysis.

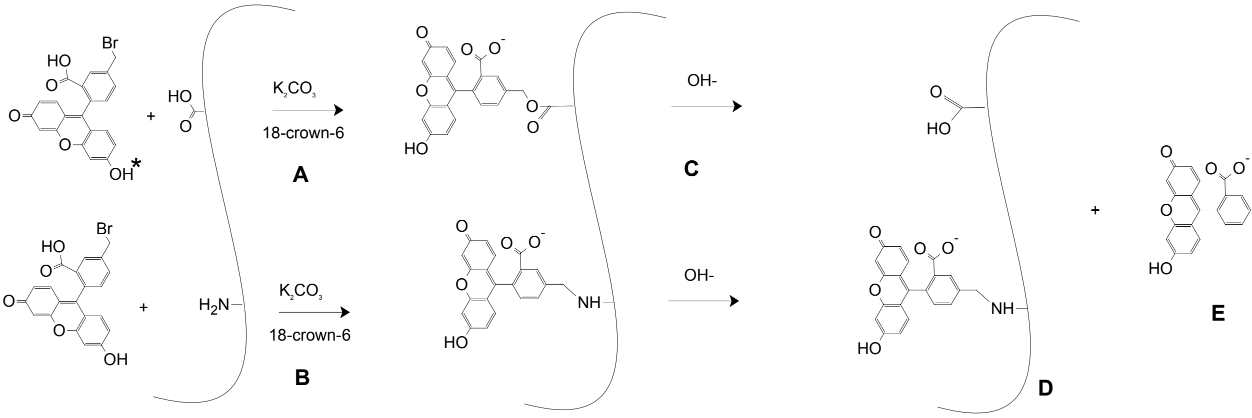

Figure 3: *Reaction scheme of 5-BMF with collagen related free carboxyl and free amine groups.*

Mukherjee et al [28] also postulated that under the labeling conditions employed, carboxyl groups of water soluble compounds may also react with the hydroxyl group of 5-BMF (indicated with a * in figure 3). There is no hard evidence that this reaction takes place. If the reaction should occur also ester bonds are formed between the label and free carboxyl groups of the collagen matrix, which will also be hydrolyzed after treatment with NaOH (0.1M).

Electropherograms obtained during the analyses of both processed and unprocessed RDBC show two peaks (similar to what was observed by Mukherjee et al [28]. A smaller leading peak was followed by a predominant peak and the ratio of the first peak to the second was always the same no matter which samples were analyzed. Spiking experiments with 5-BMF treated with 0.1M NaOH for times equivalent to those used to treat labeled collagen matrices resulted in similar electropherograms with the same ratio between the first and second peak. On the basis of these results the peak area of the main peak was used for determining the concentration of carboxyl groups in the original matrix.

In the course of developing this labeling process, some modifications on the method developed by Mukherjee had to be made [28]. First an appropriate concentration of 5-BMF with respect to reactive groups of RDBC had to be found. In order to fully convert available carboxyl groups of the matrix, a molar ratio of label to all available reactive groups in excess of 2000 was utilized. This is a common practice used for many colorimetric type reactions [21].

Another modification of the original 5-BMF labeling procedure concerned the reaction temperature. The optimal temperature for labeling as indicated by Mukherjee was approximately 75°C. It was reported that lowering the temperature to 40 °C, only decreased the labeling efficacy with a few percent [28]. In order to avoid structural changes of the RDBC matrix, which has a shrinkage temperature of 45° C, the labeling reaction was carried out at 40°C.

After optimization of the reaction conditions it was found that the concentration of carboxyl groups in unprocessed RDBC as determined with this newly developed method, using Sephadex™ to prepare a calibration curve, was equal to the concentration of carboxyl groups based on aspartic and glutamic acid residues as determined with amino acid analysis (see Table 3). Furthermore, as expected, prior blocking of amine groups of the RDCB matrix did not lead to changes of the concentration of carboxyl groups. Although it is generally assumed that during EDC cross-linking of collagen based materials activated carboxyl groups react only with amine groups, our results indicate that a significant part of carboxyl groups reacts with other groups of the matrix. This is also shown when RDBC samples having blocked amine groups were reacted with EDC and NHS. The data in table 3 show that carboxyl groups are consumed during cross-linking. It was estimated that both for the unprocessed RDBC and RDBC having blocked amine groups approximately 36 carboxyl groups / 1000 amino acid residues had reacted with other functional groups than amine groups. As hypothesized before, these carboxyl groups could have formed ester linkages with hydroxyl groups of the matrix. A detailed investigation on the formation of ester cross-links formed in RDBC matrices after treatment with EDC and NHS will be the subject of the next chapter.

Conclusion

This study describes a new assay based on the method of Mukherjee et all to determine the concentration of carboxyl groups of collagen containing (biological) matrices. Reconstituted collagen sponge (RDBC) was used as a model system. The concentration of carboxyl groups could be successfully determined after reacting carboxyl and amine groups of the matrix with 5-bromomethyl fluorescein (5-BMF) followed by selective cleavage of ester bonds to release the fluorescent label from carboxyl groups. After cross-linking of RDBC with N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS), it was found that based on the concentration of free carboxyl groups, that these groups had not only reacted with amine groups but also with other functional groups of the matrix. Most probably a significant percentage of the carboxyl groups had reacted with hydroxyl groups of the matrix to form ester cross-links.

This method provides a better determination of the cross-link density of cross-linked matrices and will help to understand the relationship between cross-link density and biophysical properties of cross-linked tissue such as shrinkage temperatures, resistance to enzymatic hydrolysis and mechanical properties. The method will also be very valuable for a better characterization and design of new matrices for bioprosthetic implants.

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Chapter 6

Biomechanical Properties of Carbodiimide Cross-linked Collagen: influence of the formation of ester cross-links*

Abstract

There is a growing interest in the use of collagen matrices for tissue engineering. In order to prevent rapid degradation and to improve their mechanical properties collagen matrices have been modified using different cross-linking agents. Among the different agents used, water soluble carbodiimides (such as N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide, EDC) in combination with N-hydroxysuccinimide (NHS) are attractive systems, because no additional chemical entities are incorporated in the matrix. EDC/NHS cross-linking leads to amide bond formation between activated carboxyl groups and amine groups. In chapter 5 it was proposed that in addition to amide bond formation also ester links between activated carboxyl groups and hydroxyl groups are formed. This was based on observations we made after development of a new method to quantify concentrations of carboxyl groups of collagen materials before and after cross-linking. The current study is directed to the influence of ester bond cross-links formed after cross-linking of collagen with EDC/NHS on its physical-chemical and bio-mechanical properties. Reconstituted dermal bovine collagen patches (RDBC) were used as model material and were cross-linked with EDC/NHS. In one RDBC group collagen amine groups were blocked with propionaldehyde prior to cross-linking while in the other group unprocessed RDBC was cross-linked without additional matrix modifications. It was shown that after activation of collagen carboxyl groups with EDC and NHS, amide cross-links as well as ester cross-links with collagen hydroxyl groups were formed. It was furthermore demonstrated that the ester cross-links of EDC/NHS cross-linked RDBC could be removed by mild hydrolysis affording collagen matrices with improved mechanical properties.

*Everaerts F, Torrianni, M, Hendriks M, Feijen J, Biomechanical properties of carbodiimide cross-linked collagen: influence of the formation of ester cross-links, J Biomed Mater Res A. 2007 Aug 29, (E-pub ahead of print)

Introduction

Water soluble forms of carbodiimides (CDI), such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), have been frequently applied as cross-linking agents for biological materials like collagen [1-5]. Carboxyl groups of aspartic and glutamic acid residues of the matrix are usually activated with EDC in the presence of N-hydroxysuccinimide (NHS). The activated carboxyl groups then react with nucleophiles, such as primary amine groups, to form stable zero-length amide bonds [6-12]. By-products of the reaction are easily rinsed away leaving a stabilized non-cytotoxic matrix. Accordingly, CDI have become popular in a wide range of tissue engineering based applications [13-21].

In spite of the positive results reported using CDI, there are also possible drawbacks. Introduction of zero length cross-links results in matrices with increased stability. On the other hand, this also leads to undesired stiffening of the tissue matrix [20]. The increased stiffness of the tissue may result in a deterioration of function and even in long term failure of the implant [22]. In order to improve the mechanical properties of the matrix, a process was developed where additional di-amine containing spacer molecules, such as 1,6 diamino hexane were introduced in the matrix [21].

Another approach, the so called “enhanced” carbodimide process we developed, is based on prior blocking of amine groups of the matrix with monofunctional aldehydes and subsequent cross-linking with EDC/NHS and Jeffamine™ spacers. This process results in stabilized tissue matrices with improved mechanical properties as compared to matrices with zero length cross-links. Furthermore, aortic wall and leaflet tissue processed according to this method showed significant less calcification than zero-length cross-linked controls after subdermal implantation in juvenile rats and even in a thoracic aorta position in juvenile sheep [17-20]. However, disappointingly, the “enhanced” process still resulted in relatively stiff materials [20].

Recently, a new method to quantify concentrations of carboxyl groups in collagen materials was developed and with this method the chemical reactions occurring in the process were further evaluated [23]. We demonstrated that during EDC/NHS cross-linking of matrices with blocked amino groups unexpectedly zero-length cross-links are formed [23]. Based on the work of Tomihata et al [24], who reported that EDC processing of hyaluronic acid yields cross-linked films rich in ester bonds, it was hypothesized that in EDC/NHS cross-linked collagen similar groups are present. If present these will give increased tissue stability, but at the same time also impart unwanted rigidity to the matrices.

The aim of the present study is to investigate whether during EDC/NHS cross-linking of RDBC, activated carboxyl groups form ester cross-links with hydroxyl groups of the matrix. Furthermore, if such ester groups are formed, a method to remove these groups has to be developed. The impact of ester groups present in the cross-linked matrix on the biomechanical properties will be investigated.

Materials and Methods

Materials

All chemicals used were obtained from Sigma Aldrich (the Netherlands) and were of analytical grade. Acetonitrile, 18-crown-6 and 5 bromomethyl fluorescein (5-BMF) stock solution in acetonitrile were stored over molecular sieves, 5A (Supelco, the Netherlands) and kept at 4°C until use.

Reconstituted dermal bovine collagen (RDBC) was obtained in lyophilized form from Coletica (France). RDBC samples with a thickness of ca 3mm were cut into 3cm x 5cm patches or 8 mm discs and stored under anhydrous conditions over Drierite™ until use.

Methods

Table 1 summarizes the different RDBC groups obtained after various chemical treatments, which were used in this study.

***Table 1**, Chemical treatment of RDBC matrices.*

	Treatment group	Chemical process
1	A	Unprocessed RDBC. Samples were stored at pH 7.2
2	A-hyd	Unprocessed RDBC. Samples were stored at pH 10
3	B	Formation of cross-links after activation of carboxyl groups with EDC/NHS. Samples were stored at pH 7.2
4	B-hyd	Formation of cross-links after activation of carboxyl groups with EDC/NHS. Samples were stored at pH 10
5	C	Blocking of amine groups with propionaldehyde. Samples were stored at pH 7.2
6	C-hyd	Blocking of amine groups with propionaldehyde. Samples were stored at pH 10
7	D	Blocking of amine groups with priopionaldehyde and subsequent formation of cross-links after activation of carboxyl groups with EDC/NHS. Samples were stored at pH 7.2
8	D-hyd	Blocking of amine groups with propionaldehyde and subsequent formation of cross-links after activation of carboxyl groups with EDC/NHS. Samples were stored at pH 10

Blocking of amine groups of RDBC [20]

Separate RDBC patches or lots of 20 RDBC discs were submerged for 1h in 2-(morpholino) ethane sulphonic acid buffer (MES, 50ml, 0.05M adjusted to pH 6.4 with NaOH). Then the

MES buffer was removed and 50 ml of a reaction mixture containing the same MES hydration buffer supplemented with propionaldehyde (0.5M) and $NaCNBH_3$ (0.05M) was added to each individual reaction vessel. After 4 h, the RDBC samples were rinsed repeatedly in MES buffer for a minimum of 16 h.

Cross-linking of RDBC samples [20]

Separate RDBC patches (blocked or unprocessed) or lots of 20 RDBC discs were immersed in MES buffer (50ml, 0.2M adjusted to pH 5.0 with NaOH for 1h). Then the MES buffer was removed and replaced with a cross-linking solution containing the same MES buffer (50ml) supplemented with N-hydroxysuccinimide, NHS (0.12M) and N’-(3-dimethylaminopropyl)-N-ethylcarbodiimide, EDC (0.3M). After 4 h, the RDBC samples were removed and rinsed repeatedly in MES buffer for a minimum of 16 h.

Storage of processed RDBC in solutions with different pH.

Samples were transferred from their final rinse solutions into two different storage solutions (6 patches or 100 discs per solution). Either HEPES buffered saline (500 ml, 10mM, pH 7.4) or borate buffered saline (500ml, 10mM, pH 10) was used. Both buffers were supplemented with 0.05% NaN_3 to inhibit bacterial contamination.

Experimental

Effect of storage of modified RDBC matrices in solutions with different pH on their physical/chemical properties.

RDBC discs, processed according to A, B, B-hyd, C,C-hyd, D and D-hyd as depicted in Table 1 were stored for 7 days either in solutions of pH 7.4 or pH 10 .

After rinsing in Di-water (3 times for 2 min) samples were lyophilized overnight.

Subsequently, the shrinkage temperature and the concentrations of free amine and free carboxyl groups were determined according to the analysis methods described below. In addition FTIR analyses were performed on sample sections.

Effect of storage of modified RDBC samples in solutions with different pH as a function of time on residual amine and carboxyl groups.

RDBC discs, processed according to B, B-hyd, D and D-hyd were either placed in solutions at pH 7.4 or pH10 at room temperature for various time periods. Five samples per time period (at day 1,2,3,4,5,6,7,14,20,30 and 40) were obtained, rinsed in MES buffer (0.2M, pH 5.0) for 1 h, followed by a quick rinse in Di-water (3 times 2 min). Samples were subsequently lyophilized. After all samples were collected, the concentrations of residual amine and carboxyl groups were determined as described below.

Effect of storage of RDBC groups in solutions of different pH on their tensile properties.
Unaxial tensile testing was performed on six processed RDBC samples per group as depicted in Table 1. These samples were stored for 30 days in solutions at pH 7.2 or pH 10 at room temperature. Prior to tensile testing the samples were retrieved from their solution and quickly rinsed with Di-water (3 times 2 min). The experimental conditions for tensile testing are described below.

Characterization of RDBC samples.
Processed RDBC samples were characterized as follows. The concentration of residual amine groups was determined with a colorimetric TNBS assay [20]. The resistance to enzymatic degradation of matrices was evaluated using a combination of collagenase and pronase enzymes [20]. The tissue shrinkage temperature was determined with differential scanning calorimetry (DSC) [20] and the concentration of residual carboxyl groups was determined after 5-BMF labeling according to the method given below [24]. FTIR analysis was performed on freeze dried samples (Excaliber series, Biorad, USA equipped with a Golden Gate ™, Specac USA).

General procedure for 5-bromomethyl fluorescein (5-BMF) labeling of carboxyl groups of RDBC samples
Five samples containing approximately 100μg each of RDBC lyophilized sponge material were swollen in DMSO (500 μl) for 1h in individual amber colored HPLC vials. After swelling, 96 μl of 18-crown-6 solution (3mM in acetonitrile) was added to the DMSO swollen samples. Thereafter anhydrous potassium carbonate (20 mg) was added to each vial. The contents were agitated on a rotary shaker followed by the addition of 500 μl of 5-bromomethyl fluorescein (5-BMF) stock solution (3 mM in acetonitrile). The final mixture was incubated at 40°C for

16h. Thereafter the samples were retrieved from its reaction solution and rinsed with 2 ml portions of acetonitrile for 1h (with changes approximately every 10 min). After 1h the rinse was analyzed for the presence of 5-BMF using fluorescence spectroscopy (Perkin Elmer, LS50B, Fullerton, USA with an excitation at 488 nm and emission at 520nm). The rinse and analysis processes were repeated at consecutive hour intervals until no fluorescence was detected in the rinse solutions.

To assess the amount of fluorescent label bound to available carboxyl moieties the label must be liberated from the substrate. To accomplish this, the washed labeled RDBC samples were blotted free of solution and transferred to amber colored HPLC vials containing NaOH (1ml, 0.1M) for 24h. The resultant hydrolyzate was used for the determination of the concentration of free carboxyl groups in the original RDBC samples utilizing capillary zone electrophoresis (CZE) on a MDQ (Beckman, Palo Alto, USA) equipped with a 488nm Laser Induced Fluorescence Detector (LIF), a 520 nm band pass filter and a fused silica capillary (50 µm I.D., 50cm from injection to detection). Pressurized injections for 20s at 10psi were used and the voltage was set at 15kV. Analysis took place in micellar electrokinetic chromatography (MEKC) mode with a borate buffer (pH 9.2, 10mM, adjusted with NaOH) containing sodium dodecyl sulphate (SDS, 10mM). A total analysis time of 20 min per sample was used and all samples wre stored in the dark and were analyzed within 12h after completion of the hydrolysis process. The area of the main peak was used and peak areas were calculated using internal software (Beckman, Palo Alto, USA) [23].

Uniaxial tensile testing

Tensile tests were performed on a Zwick apparatus. (2.5H1S, Zwick, Ulm Germany) with a 50N load cell and pneumatic operated grips. A grip to grip distance of 30mm was applied. RDBC samples were measured within 30s after removal from solution. A pre-load of 0.05N was applied for 10s after which a cross-head speed of 5mm/min was used until rupture of the sample occurred [25-26]. The average stress strain curve per sample was reconstructed from raw data in Microsoft Excel.

Statistical analysis

A student-T test was performed in order to establish statistically significant differences between sample groups ($p < 0.05$).

Results

Figure 1 depicts the FTIR fingerprint analyses of the sample groups presented in Table 1. The spectra were scaled to equal absorption at 2925cm^{-1}, assigned to the $-CH_2$ group which remains unchanged during the cross-linking reaction [23]. Cross-linking of RDBC samples in N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) in combination with N-hydroxysuccinimide (NHS), which were subsequently stored at pH 7.4 (group B) results in an increased absorption at 1175cm^{-1} and 1050cm^{-1} as compared to the unprocessed control stored at pH 7.2 (group A). The intensity of the peaks at 1175 cm^{-1} and 1050 cm^{-1} were similar for the control stored at pH10 (group B-hyd) as well as for the RDBC group having amine groups blocked and stored either at pH 7.2 or at pH10 (groups C and C-hyd respectively (not shown in Figure 1).

Significantly higher absorptions were measured for EDC/NHS cross-linked RDBC with blocked amine groups (D), compared to zero-length cross-linked RDBC (B), while for both groups the hydrolysis reaction (B-hyd and D-hyd) leads to significant decreased absorptions at the two mentioned wavelengths. Furthermore only in sample group D a peak was observed around 2850cm^{-1}.

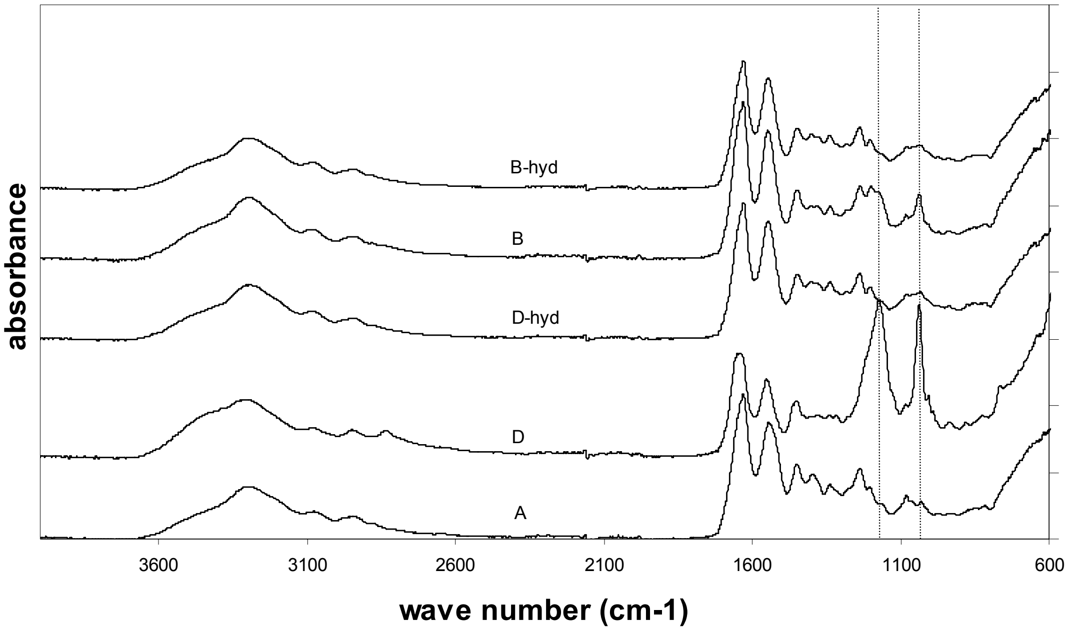

Figure 1, FTIR analysis of processed lyophilized collagen patches. Unprocessed RDBC (A), EDC/NHS cross-linked RDBC with blocked amine groups stored either at pH 7.2 (D) or at pH 10 (D-hyd), EDC/NHS cross-linked RDBC stored either at pH 7.2 (B) or at pH10 (B-hyd).

Shrinkage temperature and concentrations of amine and carboxyl groups of processed RDBC samples.

Table 2 lists the shrinkage temperature and concentrations of residual amine and carboxyl groups of RDBC samples processed as summarized in Table 1. Cross-linking with EDC/NHS leads to a significantly increased shrinkage temperature of RDBC samples as compared to the control groups. A slight but significant decrease in shrinkage temperature was observed for samples with blocked amine groups, which were not cross-linked but kept at pH 10 (C and C-hyd) as compared to unprocessed RDBC. Whereas storage of the cross-linked group (B) at pH 10 (B-hyd) did not lead to a decrease in shrinkage temperature, storage of cross-linked RDBC with blocked amine groups (D) at pH 10 (D-hyd) resulted in a significant decrease in shrinkage temperature. Furthermore all chemical modifications of RDBC samples lead to a strong decrease in the concentration of amine groups and storage at pH 7.2 or pH 10 had no effect on free amine content. Table 2 also lists that the concentration of residual carboxyl groups of RDBC samples decreases after reaction with EDC/NHS (compare groups A with groups B and D). Subsequent storage of EDC/NHS processed samples at pH 10 leads to an increase in the concentration of residual carboxyl groups (B and B-hyd, and D and D-hyd).

***Table 2**: Shrinkage temperature and concentrations of free amine and carboxyl groups determined for unprocessed RDBC (A), EDC/NHS cross-linked RDBC, stored at pH 7.2 (B) or pH 10 (B-hyd), RDBC having blocked amine groups, stored at pH 7.2 (C) or pH 10 (C-hyd) and EDC/NHS cross-linked RDBC after previous blockage of amine groups, stored at pH 7.2 (D) or pH 10 (D-hyd). 6 samples per group per analysis method were measured.*

Treatment group N=6	Shrinkage temperature (°C)	Amine groups (/1000 amino acids)		Carboxyl groups (/1000 amino acids)	
A	45±1	31±3	100%	122±2	100%
B	65±2	9±2	29%	65±3	64%
B-hyd	64±2	8±2	29%	85±3	70%
C	43±1	4±2	12%	122±2	100%
C-hyd	43±2	3±1	10%	122±2	100%
D	54±2	4±2	12%	86±4	70%
D-hyd	49±2	4±2	12%	108±2	89%

Carboxyl group concentration of EDC/NHS treated RDBC containing blocked amine groups as a function of time at pH 7.2 or 10.

Figure 2A shows the effects of storage time and pH on the concentration of free carboxyl groups of EDC/NHS processed RDBC having blocked amine groups (samples group D and D-hyd in Table 1). EDC/NHS treatment of RDBC with blocked amine groups leads to a reduction of the carboxyl group concentration. While prolonged storage of these samples at pH 7.2 has no significant effect on the residual carboxyl group concentration, prolonged storage under mild alkaline conditions results in free carboxyl groups. After 40 days, a plateau is reached approaching the concentration of free carboxyl groups found in unprocessed RDBC.
Figure 2B shows the theoretical ester concentration as function of time in EDC/NHS cross-linked RDBC samples with blocked amine groups at pH 10 (group D-hyd in table 1). The ester group concentration is calculated with the assumption that the increase in carboxyl group concentration is inversely related to the concentration of ester groups.
Furthermore, it was assumed that all esters present in the matrix originated from a reaction with these carboxyl groups. For the hydrolysis during the first 7 days a linear semi logarithmic plot of ester concentration against time (r=0.96) was obtained, indicating a first order reaction With the calculated k value a theoretical curve was constructed, which is depicted as a dotted line in figure 2.

Mechanical properties of (un) processed RDBC.
Figure 3 shows the results of the tensile testing of various processed sample groups as depicted in Table 1. Each curve reflects the average of 6 independent measurements. Reproducible results within a sample group were obtained. It is observed that cross-linking of all RDBC samples leads to increased stiffness as indicated by increased low and high strain moduli values. In addition, amine blocking of RDBC samples causes a significant increase in both high and low strain modulus. Furthermore EDC/NHS cross-linking of RDBC (group B) leads to a significantly stiffer matrix compared to EDC/NHS cross-linked RDBC having blocked amine groups (group D). For both groups B and D a decrease in both high and low strain modulus was observed after hydrolysis. The results indicate that in general processing of RDBC leads to increased strain values at break.

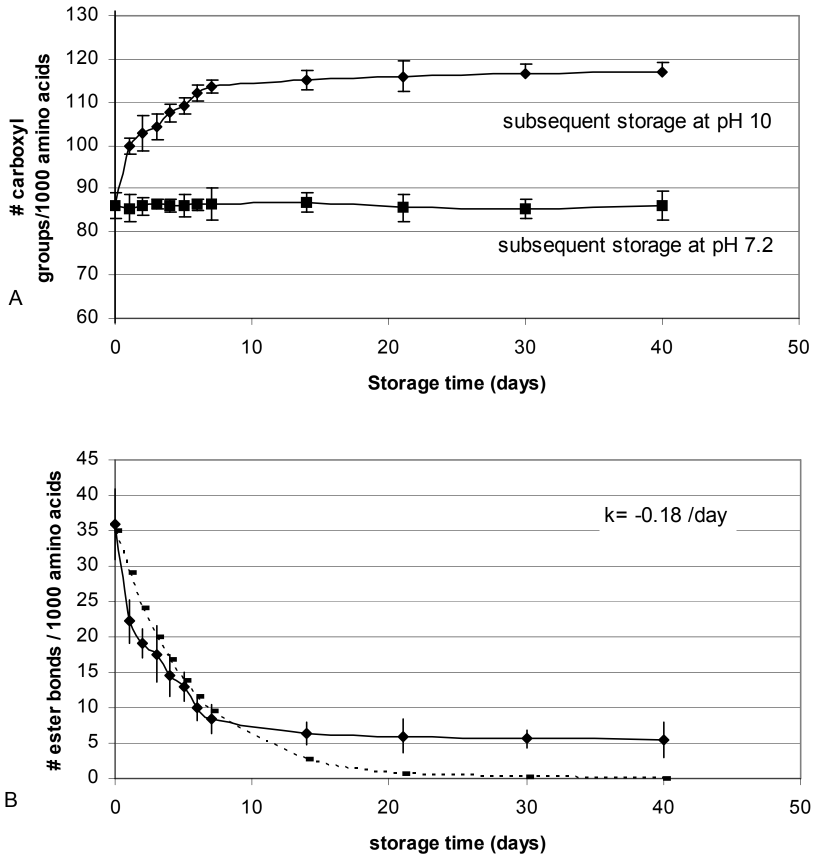

Figure 2. *The concentration of free carboxyl groups of EDC cross-linked RDBC samples having blocked amine groups as a function of time during storage at pH 7.2 or at pH 10 (Figure 2A), Concentration of ester bonds of the same RDBC samples as a function of time during storage at pH 10. (Figure 2B). The theoretical first order hydrolysis reaction with a k value that was calculated from this data (with a regression coefficient of 0.96) is depicted with a dotted line.*

Interestingly, processed RDBC having blocked amine groups has a reduced strain at a given stress as compared to unprocessed RDBC samples and has the highest stress at break of all samples.

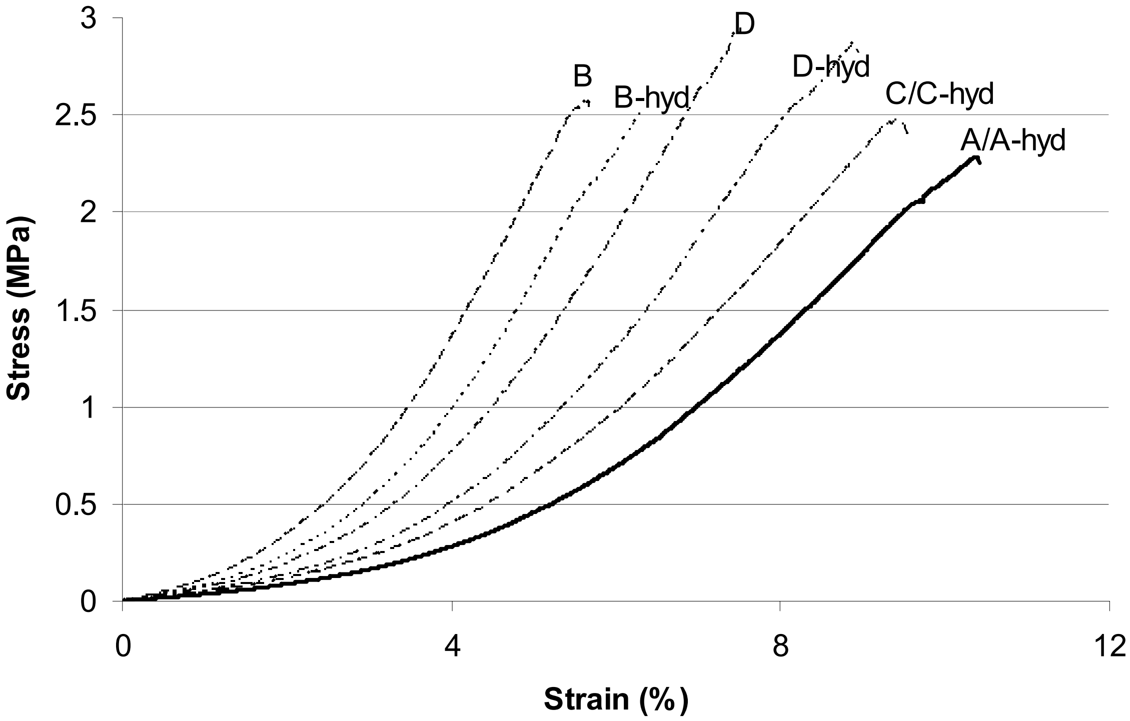

Figure 3; Uniaxial stress strain curves of wet unprocessed RDBC, storage solution pH 7.2 (A) and pH 10 (A-hyd), EDC/NHS cross-linked RDBC samples stored at pH 7.2 (B) and pH 10 (B-hyd), RDBC samples with blocked amine groups stored at pH 7.2 (C) or pH 10 (C-hyd) and EDC/NHS cross-linked RDBC samples having blocked amine groups stored at pH 7.2 (D) and pH 10 (D-hyd). Samples were kept for 7 days in the storage solution and 6 patches per group were tested. The curves reflect the average values. See experimental for sample processing methods

Discussion

While water soluble carbodiimides (such 3-dimethylaminopropyl)-N-ethylcarbodiimide, (EDC) in combination with N-hydroxysuccinimide, (NHS) have been used in protein conjugation chemistry for almost 4 decades their use in cross-linking of collagen based materials is more recent [3,4,5]. It is generally accepted that during carbodiimide cross-linking of collagen based materials residual carboxyl groups of glutamic and aspartic acid residues react with primary amines originating from lysine and hydroxylysine
residues. These cross-links are called ‘zero-length’ as no additional chemical entities between the conjugating molecules are introduced [1].
If so desired, di-amine terminated “spacer” molecules can be incorporated into the cross-linked structure to modify the physical/chemical character of the cross-linked matrix [21]. This is based on the idea that by incorporation of amine terminated molecules a matrix with an increased flexibility is obtained, as compared to a matrix with only zero-length cross-links.
In view thereof we previously developed a so called “enhanced” carbodiimide cross-linking process, based on blocking of matrix amine groups prior to the formation of cross-links with Jeffamine™ spacers using EDC/NHS [17-20]. This method provided stabilized tissue matrices with improved mechanical properties as compared to zero length cross-linked matrices. Furthermore, a significant decrease in calcification of aortic wall and leaflets processed according to this method as compared to zero-length cross-linked controls was obtained in selected animal implant models [20]. However, the stiffness of these materials was higher than expected suggesting that in addition to cross-links formed via the spacer molecules also other cross-links were formed.
In order to detect and quantify these cross-links an adequate characterization of the tissue matrices after cross-linking is required.

There are a number of methods routinely used to characterize cross-linked collagen based materials, such as tissue. The efficacy of blocking of tissue amine groups can be measured by determination of the concentration of residual amine groups using the 2,4,6 trinitrobenzene sulphonic acid (TNBS) assay [18,21]. The determination of the shrinkage temperature provides indirect information about the degree of cross-linking of the material [18]. Furthermore, the degree of cross-linking of a matrix also has an effect on its resistance to enzymatic degradation

[18]. The combination of these assays provides a complementary picture of the physical/chemical properties of the tissue after processing [18].

Until recently it was not possible to quantify tissue carboxyl groups of processed tissue. This hampered us in attaining complete understanding of processes occurring during cross-linking. Particularly this gap in our available characterization methods hindered us in finding an explanation of the observed tissue stiffening.

Recently however we successfully developed a 5-bromomethyl fluorescein (5-BMF) labeling method for quantifying carboxyl groups in a collagen model material [23].

The experiments performed to validate this labeling technique revealed that during cross-linking of collagen with EDC/NHS a possible reaction between carboxyl groups and other amino acid residues, notably hydroxyl groups, occurs. It was hypothesized that this (side) reaction yields zero-length ester cross-links in the collagen matrix.

This hypothesis is supported by the work of Tomihata et al. They created thin films of hyaluronic acid stabilized by EDC via the formation of ester bonds between endogenous hydroxyl groups of the sugars and activated carboxyl groups on neighboring molecules [23]. Gratzer et al reported that in tissue EDC/NHS activated carboxyl groups are capable of forming cross-links with hydroxyl groups from the carbohydrate components of glycosaminoglycans [16]. Zeeman et al postulated that the presence of decorating groups (such as cross-links and one sided reaction products) of the outer surface of collagen fibrils and fibers alters the interaction between fibril/fibril and fibril/fiber surfaces. This may result in an increase in stiffness of a matrix [26].

Accordingly our research first focused on demonstrating the formation of ester cross-links as a by product of EDC/NHS cross-linking of collagen based materials. Secondly, if formed indeed, a method to remove these ester cross-links without destroying essential tissue properties was investigated. Since porcine aortic roots are complex structures, a model collagen material was used for further studies. Coletica's reconstituted dermal bovine collagen (RDBC) was chosen because its overall structure and amino acid composition are similar to other reconstituted collagen materials. Moreover using a reconstituted material provides more consistent baseline data, with sample to sample variation significantly reduced.

RDBC samples were cross-linked with EDC/NHS directly or after blocking of amine groups. A sample group with blocked amine groups was included, since amide cross-links cannot be formed during EDC/NHS cross-linking of this material.

In general esters are susceptible to hydrolysis at alkaline pH [27]. Therefore, after cross-linking the processed RDBC samples were stored in a solution of pH 7.2 or pH 10 and it was investigated whether ester linkages in processed RDBC samples could be hydrolyzed. No higher pH value was selected in order to avoid harming the tissue integrity by hydrolysis. It was demonstrated earlier that exposure of collagen based materials to pH 10 hardly changes their chemical and physical properties. [28].

FTIR analyses of lyophilized processed RDBC samples were performed to study the presence of esters. It was found that after EDC/NHS cross-linking of all samples two sharp peaks were found at 1176 cm-1 and 1050 cm^{-1} respectively. For a matrix containing R-COOR' groups, in which R and R' are large molecules, very sharp peaks are expected in the region of 1277-1185 cm^{-1} and at 1160-1050 cm^{-1} representing the asymmetrical and symmetrical stretching frequencies of the C-O ester groups respectively [29]. Therefore the peaks at 1175 cm^{-1} and at 1050 cm^{-1} that appear in the FTIR spectrum of EDC/NHS cross-linked collagen samples are clearly due to ester groups.

The peak at 2850 cm^{-1} that only appeared in the EDC/NHS cross-linked RDBC group with blocked amine groups could not be identified. In this region many $-CH_x$ interactions are found. EDC/NHS cross-linking of RDBC samples leads to a decrease of carboxyl groups and an increase in the tissue shrinkage temperature (Table 2). Carboxyl groups become involved during the EDC/NHS reaction independent of the presence of amine groups. In the RDBC samples without blocked amine groups participation of amine groups during EDC/NHS cross-linking was observed. As indicated in figure 1, in RDBC samples with blocked amine groups more esters are formed during EDC/NHS cross-linking as compared to RDBC samples that were cross-linked directly.

FTIR analyses show that hydrolysis of processed samples results in loss of ester linkages. After hydrolysis of all EDC/NHS cross-linked RDBC samples a significantly decreased absorption at 1176 cm^{-1} and 1050 cm^{-1} was measured. Hydrolysis also resulted in a decrease in shrinkage temperature and more free carboxyl groups, while amine group concentrations remained the same.

Hydrolysis of EDC/NHS processed RDBC samples having blocked amine groups resulted in materials with free carboxyl group concentrations close to those of RDBC control samples having blocked amino groups, which were not reacted with EDC/NHS. Furthermore, hydrolysis of these samples led to materials with a decreased shrinkage temperature. However this value was still increased compared to the aforementioned control and indicates that there are still cross-links remaining.

These, results confirm that during EDC cross-linking in addition to amide type of cross-links also ester bonds are formed, which can be removed by mild hydrolysis.

The rate of hydrolysis of formed ester bonds was determined in a time course study. The effect of temperature was not investigated. In studies that utilize biological matrices careful attention needs to be paid to the reaction temperature in order to conserve the protein structures. For this feasibility study the hydrolysis reaction was performed at room temperature.

EDC/NHS cross-linked RDBC samples having blocked amine groups were hydrolyzed for various time periods at pH 10. As a control, the same materials were kept at pH 7.2. It was observed, as previously demonstrated, that at pH10 esters were hydrolyzed, whereas at pH 7.2 this hydrolysis did not occur. The hydrolysis reaction did not remove all ester bonds during the course of the study as approximately 108 carboxyl groups/1000 amino acids were measured after 40 days, while in unprocessed RDBC 122/1000 amino acids are present. It was assumed that no amide cross-links had been formed in this matrix during EDC/NHS cross-linking, as the few remaining residual amine groups after blocking did not participate during cross-linking. The theoretical ester concentration was determined with the assumption that during EDC/NHS cross-linking of a RDBC matrix having blocked amine groups all carboxyl groups that react form esters. As indicated in figure 2B, the hydrolysis process takes initially place via a first order process while after 7 days it starts to deviate and the hydrolysis rate changes.

It remains unclear whether residual carboxyl groups that could not be retrieved during hydrolysis are ester groups shielded by the complicated three dimensional structure of collagen matrix or that these groups cannot be accessed because of physical changes of the collagen. Finally the analysis method may not be accurate enough.

Prolonged storage at pH10 will reveal whether a few cross-links remain or that a statistically insignificant difference in carboxyl group concentration as compared to native collagen is found.

Now that we have shown that during EDC/NHS cross-linking of RDBC samples ester cross-links are formed and subsequently can be removed by hydrolysis it was evaluated if these ester cross-links are the underlying cause of the observed tissue stiffening as mentioned earlier. Therefore the biomechanical properties of processed RDBC samples (Table1) were determined with uni-axial tensile testing.

As expected, EDC/NHS cross-linking of RDBC resulted in an increase of the modulus of the collagen [25,26]. Hydrolysis of cross-linked matrices resulted in decreased moduli. Interestingly, it was demonstrated that RDBC in which only amine groups were blocked without further cross-linking (C) a significant increase in tissue modulus was measured as compared to an unprocessed control (A). In addition, a difference in tissue shrinkage temperature between the two groups was measured and as previously demonstrated; the blocking reaction resulted in a change in hydrophobicity of the material [20].

Similar as suggested by Zeeman et al that the presence of decorating side groups attached to collagen fibrils or fibers results in increased shear stress, possibly a similar mechanism is at play with materials of group C as well [25].

In this study it has been demonstrated that EDC/NHS cross-linking of collagen based materials results in both amide and ester linkages and that these esters significantly attribute to the biomechanics of processed tissue. It was furthermore demonstrated that ester bonds can be removed by an alkaline treatment after cross-linking.

In an additional study it was demonstrated that, ester formation during EDC/NHS cross-linking of RDBC and removal of these esters by hydrolysis are processes, which occur in more complicated matrices as well. Similar results as were discussed in this paper were found for EDC/NHS cross-linked bovine pericardium and porcine aortic roots [18]. These matrices contain many non structural proteins that may become imparted in the matrix via amide or ester linkages [25]. Removal of esters in EDC/NHS processed tissue could improve its biomechanical characteristics however it may also result in worsening of in-vivo calcification and inflammation.

A study on the implications of the removal of esters in cross-linked porcine aortic root material using the enhanced carbodiimide cross-linking technology on the in-vivo performance is currently on-going.

Conclusion

In an in-vitro study a detailed investigation on the type of cross-linking products formed during cross-linking of a reconstituted dermal bovine collagen (RDBC) material using 3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) in combination with N-hydroxysuccinimide (NHS) was made. Although it is generally accepted that during EDC/NHS cross-linking only amide cross-links are formed between the matrix carboxyl and amine groups it was demonstrated that additional ester cross-links with hydroxyl groups are formed.

Involvement of carboxyl groups during cross-linking was studied using a recently developed assay for quantification of carboxyl groups in EDC/NHS cross-linked RDBC samples. It was demonstrated that in a RDBC sample having blocked amine groups, during EDC/NHS treatment still cross-links were formed. With FTIR analyses of lyophilized processed RDBC samples it was demonstrated that these formed bonds are esters. Furthermore it was demonstrated that the additional ester bonds affect the mechanical properties of the material.

The observations made demonstrate that for an adequate estimation of the cross-linking density of EDC/NHS processed matrices residual carboxyl groups need to be quantified.

Since esters are in general susceptible for hydrolysis it was demonstrated that esters in a cross-linked RDBC matrix could be removed by storage of a cross-linked matrix in a buffer at pH10. As a result of removal of esters, EDC/NHS RDBC matrices with improved biomechanical properties were obtained.

The formation of ester bonds during cross-linking of collagen based materials may extent the application range of EDC/NHS for tissue engineering based applications. The effects of the presence of ester linkages on the in-vivo performance is currently under investigation.

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Chapter 7

Effects of ester bonds in carbodiimide cross-linked porcine aortic wall tissue: in vivo response

Abstract

The present study focuses on the effect that these ester cross-links have on the in-vivo performance of cross-linked porcine aortic wall tissue. Notably, calcification and inflammatory response of cross-linked aortic wall samples were evaluated after subcutaneous implantation in weanling Sprague Dawley rats for 8 wks. Hereto heart valve tissue was EDC/NHS cross-linked with Jeffamine™, molecular weight 230 or 400, after blocking free amine groups through a reductive amination reaction with propionaldehyde. From each processed group samples were stored at pH 7.2 (control group) and pH 10 (hydrolysis of ester bonds) for 2.5 months.

It was observed that while subtle but significant changes were noted in in-vitro tests typically used to characterize bioprosthetic tissue, generally these changes did not significantly affect the overall calcification and inflammatory response. Yet, interestingly a difference in origin of calcification initiation sites and calcification distribution pattern was observed between sample groups with either ester cross-links present or removed.

While eliminating the ester cross-links formed during the EDC and NHS process did not have dramatic consequences with respect to the in vivo calcification and inflammation responses of the bioprosthetic tissue, there are indications that removing extraneous ester cross-links improves its biomechanical properties. Such improvements in tissue suppleness could potentially increase the long term durability of bioprosthetic devices.

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*Frank Everaerts, Marja van Luyn, Mark Torrianni, Linda Brouwer, Marc Hendriks and Jan Feijen, Effects of ester bonds in carbodiimide cross-linked porcine aortic wall tissue: in vivo response, submitted to J Biomed Mater Res A

Introduction

Cross-linking of bioprosthetic tissue is a common procedure used to prevent autolytic degradation and to stabilize the tissue for in-vivo applications [1-4]. For tissue heart valves, glutaraldehyde (GA) based cross-linking is the current standard for providing the prostheses with a low incidence of thrombo-embolism and a satisfactory hemodynamic performance [1]. In spite of these positive attributes, the GA fixation process has several drawbacks. Early failure of GA cross-linked materials in-vivo is a common occurrence, primarily caused by calcification. Although the underlying mechanisms are still not fully understood, there are indications that GA itself plays a major role [5]. Various reaction products during cross-linking are formed and it has been described that some of these products in addition to traces of residual non-reacted GA are cytotoxic. [2,6,7,8,9]. Therefore many research programs have focused on developing alternative cross-linking agents to overcome these deficiencies [1]. Cross-linking of collagen based materials with a water-soluble carbodiimide (such as N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide, EDC in the presence of N-hydroxysuccinimide, NHS) is a particularly promising alternative [9,10,11]. By this method, non-toxic, so called direct 'zero-length' cross-links are formed between juxtaposed tissue carboxyl and amine groups [12]. Based on work by Olde Damink et al [9], others have explored EDC/NHS for various collagen based materials, including bioprostheses, aimed at increasing long term implant durability and diminishing calcification [13-24]. In some of these approaches EDC/NHS cross-linked matrices were used as scaffold for cell seeding [13,14,15] or for delivery of a biological active substance [24].

In spite of the positive results reported, there are possible drawbacks however. Introduction of zero-length cross-links results in increased stability as said, but this has also been related to undesired stiffening of the tissue matrix [19,23]. Increased stiffness of the tissue may result in a deterioration of function and even in long term failure of the implant [18,19,23].

Therefore we have developed a so-called “enhanced” EDC/NHS process, which involves prior blocking of amine groups of the matrix with mono-functional aldehydes and subsequent cross-linking with EDC/NHS and Jeffamine™ spacers. By using this process, stabilized tissue matrices are obtained with improved mechanical properties as compared to matrices with zero-length cross-links [16, 18, 19]. Furthermore, in porcine aortic wall and leaflet tissue, cross-linked with this method, significantly less calcification occurred than in zero-length cross-linked controls after subdermal implantation in juvenile rats and even in a thoracic aorta position in juvenile sheep [18,19]. However, disappointingly, this “enhanced” process still resulted in relatively stiff materials [19].

Recently however, we demonstrated that EDC/NHS activated carboxyl groups in collagen not only form cross-links with amines but also with available tissue hydroxyl groups. [17,25,26]. It was demonstrated that these ester bonds significantly contribute to the biomechanical behavior of processed tissue [26]. Fortunately these ester bonds can be hydrolyzed by a mild alkaline treatment [17,26]. With a model collagen matrix we demonstrated that the alkaline treatment gave a significant improvement in the tissue biomechanics, while we demonstrated that the hydrolysis reaction did not affect other physical/chemical properties of the material [26]. What is not understood as of yet is whether this removal of ester bonds has an effect on the otherwise favorable in-vivo performance of tissue cross-linked with the “enhanced” cross-linking process [19].

The goal of this study was to evaluate the effect of the (removal of) ester linkages on the tissue performance in-vivo. Hereto porcine aortic roots were EDC/NHS cross-linked after initial blocking of amine groups with propionaldehyde. Cross-links were established with two different diamine spacers: Jeffamine™230 and Jeffamine™400 [17,18]. These two Jeffamines™ were selected in the study as it was previously described that the Jeffamine™ molecular size will determine whether cross-links are formed inside collagen microfibrils alone or also in between microfibrils [17]. In conjunction with the foregoing aim, it thus is also of interest to study the effect of Jeffamine™ spacer length on further improvements in-vivo tissue response; particularly with regard to a possible relation between the length and position of the Jeffamine™ cross-links and number of ester linkages formed.

Hence, after cross-linking, a portion of the samples went through a hydrolysis treatment to remove ester bonds [26]. After an in-vitro assessment, 4 groups (cross-linked with either Jeffamine™230 or Jeffamine™400 with or without additional hydrolysis treatment) were implanted in weanling Sprague Dawley rats. The samples were explanted after 8 weeks and inflammatory response and calcification levels were determined.

Materials and methods

Materials

All chemicals used were obtained via Sigma Aldrich (the Netherlands) and were of ACS grade. N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and propionaldehyde were stored at 4^0C.

Jeffamines™ with molecular weights of 230 (referred as J230) and 400 (referred as J400) were obtained from Sigma Aldrich (the Netherlands). Fresh porcine aortic valves were obtained from slaughterhouses in the USA (obtained via Medtronic Santa Ana, CA, USA), rinsed free of blood and extraneous tissue debris with 0.9% sterile NaCl solution (saline). The valves were trimmed to remove excess myocardium and adventitial tissue. After cleaning the valves were again rinsed in saline solution. Subsequently, the valves were transferred to containers filled with 2-(morpholino)ethane sulphonic acid, MES buffer (0.05M, pH6.5) and stored overnight at 4^0C.

Methods

Table 1 shows how tissue valves were processed for the various experimental groups used in this study. Chemical processing details of the matrices are listed on the next page

Blocking of the aortic tissue amine groups

Prior to the blocking reaction, 20 randomly selected valves were transferred to 4 roller bottles (containing 5 valves each) containing MES buffer (1000 ml, 0.05 M, pH 6.5) at room temperature. After temperature equilibration, propionaldehyde (0.5 M) and $NaCNBH_3$ (50 mM) were added. A paddle and collar was inserted and the bottle was transferred to a roller bottle system. The blocking reaction was allowed to continue for 48 h. After the 48h blocking reaction, valves were extensively rinsed in saline solution with volume changes 3 times daily for 3 days.

***Table 1**, Chemical procedures used for treating tissue valves*

sample	Treatment group	Sample number	Treatment process
A	P-J230	N=5	Tissue amine groups blocked with propionaldehyde and EDC/NHS activated carboxyl groups were cross-linked with J230. Samples were stored at pH 7.4 in HEPES buffered saline with 0.05% NaN_3
B	P-J230-hyd	N=5	Tissue amine groups blocked with propionaldehyde and EDC/NHS activated carboxyl groups were cross-linked with J230. Samples were stored in borate buffered saline solution at pH10 with 0.05% NaN_3
C	P-J400	N=5	Tissue amine groups blocked with propionaldehyde and EDC/NHS activated carboxyl groups were cross-linked with J400. Samples were stored at pH 7.4 in HEPES buffered saline with 0.05% NaN_3
D	P-J400-hyd	N=5	Tissue amine groups blocked with propionaldehyde and EDC/NHS activated carboxyl groups were cross-linked with J400. Samples were stored in borate buffered saline solution at pH10 with 0.05% NaN_3
	Fresh Control	N=5	No further treatment. Samples were stored in saline containing 0.05% NaN_3

Cross-linking of aortic tissue

At the end of the rinse cycle the roller bottles were filled with MES buffer (350 ml, 0.25M, pH 5.0) containing either J230 (0.06M) or J400 (0.06M). After a 3 hours incubation time, a concentrated solution of N-hydroxysuccinimide (NHS, 350 ml, 0.45 M) and a concentrated solution of EDC (350ml, 0.9 M) both in MES buffer (0.25M, pH 5.0) containing either J230 (0.06M) or J400 (0.06M) were added. A paddle and collar was inserted and the roller bottle was closed with a hydrophobic vent cap. The cross-linking reaction was allowed to proceed for 48h on the roller bottle system. After completion of the cross-linking reaction the valves were extensively rinsed in saline solution with volume changes 3 times daily for 3 days.

Storage of processed aortic tissue

Fully processed valves were transferred from their final rinse solutions into two different holding solutions. 5 valves per group cross-linked with J230 or J400 were stored in HEPES buffered saline solution (500 ml, 10mM, pH=7.4) or borate buffered saline solution (500ml, 10mM, pH 10). Both buffers contained 0.05% NaN_3. All samples were stored for 2.5 month prior to further evaluation.

Tissue assessment

In vitro characterization (physical/chemical tests)

In vitro characterization was performed by a number of physical/chemical tests in order to evaluate the overall properties of the processed tissue groups. Discs (with a diameter of 6mm were) punched out of the post sinotubular aortic wall region of randomly selected valves. The residual tissue amine groups were characterized with a colorimetric TNBS assay [18], the resistance to enzymatic degradation was characterized with a combination of collagenase and pronase [18], the tissue shrinkage temperature was determined with differential scanning calorimetry (DSC) [18] and the residual carboxyl groups were determined after 5-bromomethyl fluorescein (5-BMF) labeling [24,25]. FTIR analyses (Biorad Excaliber series, USA equipped with a Golden Gate™, Specac USA) were performed on lyophilized porcine aortic wall samples [25]. For each of the analyses 6 discs per group were investigated.

In vivo characterization

Aortic wall samples of the valves were subdermally implanted in Sprague-Dawley rats for evaluation of the degree of calcification and inflammatory response for 8 weeks [17]. Three days before the experiment was performed; 3 valves were randomly selected from each valve group, washed with sterile saline (3 times 10 min) and transferred to a sterile saline holding solution (500 ml) containing 0.05% NaN_3. This solution was refreshed daily and it was verified that the pH of the solution did not change. One day prior to implantation, discs (8mm in diameter), of the post sinotubular aortic wall region, were punched free from the surrounding tissue. Thereafter the discs were stored in sterile saline solution (10ml per disc) free of NaN_3. Before implanting, the discs were washed with sterile saline solution (3 times for 2 min). National Institute of Health guidelines for the care and use of laboratory animals (NIH 85-23 Rev. 1985) were followed.

Male, 21 day old rats (Sprague-Dawley, CD strain) were used. After anesthetization with a mixture of halothane, N_2O and O_2, backs were shaved and disinfected using Betadine™. A mid-line incision was made in the skin and in two subcutaneous pockets created and at each side of the spine a disc was inserted with the intimal side facing the facial covering of the muscles of the back. From the J230, J400, J230-hyd and J400-hyd, 6 discs each were randomly implanted and the skin was closed with a single suture. After 8 wks, animals were anesthetized with a mixture of halothane, N_2O and O_2 followed by cervical disc relocation.

Following euthanasia the sample discs with the surrounding tissue were explanted and cut into two halves. From one half of the explants, the surrounding capsule was removed and these samples were stored in HEPES containing isopropanol (IPA, 20 wt%) for further quantitative calcium analysis. The other half was immersion-fixed in GA (2%) in phosphate buffered saline (PBS, 0.1 M, pH 7.4) for 24h at 4^0C. Samples were subsequently de-hydrated in a graded series of alcohols. Thereafter samples were processed trough increasing concentrations of glycol methacrylate (GMA) and eventually embedded in pure GMA. GMA blocks were then faced followed by thin sectioning to 5 µm in thickness.

Host response to processed porcine aortic wall samples.

Host response to implanted samples were quantitatively analysed after toluidine blue staining (TB). Three independent investigators counted macrophages (MØ), giant cells and lymphocytes in the cellular layer at the interface of the intimal side of the samples.

Total calcium

The calcium concentration was determined by atomic absorption spectroscopy (AAS; Perkin Elmer Optima 300, Fullerton, USA). Hereto samples were removed from the storage solution, blotted free of excess buffer and then frozen in liquid nitrogen followed by lyophilization. The dry weight of each tissue sample was recorded and samples were then hydrolyzed in aqueous hydrochloric acid (110°C, 15 ml, 6M) for 24 h. After hydrolysis Di-water (10ml) was added to each sample. The signal intensity of calcium was determined by atomic emission spectrometry (n=5 per sample). The concentration of calcium per dry weight of tissue was calculated using a calibration curve obtained with standard solutions [19].

Calcium distribution in explanted tissue samples

The distribution of calcium throughout the explanted samples was determined by using image analysis of Von Kossa stained histology sections. A TB counter stain was used to increase the visibility of the matrix background. Customized image processing software (Leica Q-Win, Rijswijk, the Netherlands) was used to distinguish calcification patterns and differentiate those from non calcified portions of the tissue matrix. The calcified area of the histology section was determined and presented as a percentage of the total tissue sample area.

Statistical analysis

A student-T test was performed on data in order to establish statistically significant differences between sample groups ($p< 0.05$).

Results

In vitro characterization (physical / chemical tests)

Table 2 summarizes the results of the in-vitro characterization of aortic wall samples from the various treatment groups of this study together with a fresh control group.

The percentage of free amine groups and free carboxyl groups are shown along with shrinkage temperatures and resistance to enzymatic degradation. Corrected FTIR values measured at 1176cm^{-1} and 1050cm^{-1} represent peak heights, relative to the absorbance measured at 2925cm^{-1}, of ester bonds present in the tissue matrix [26].

***Table 2**, Physical/chemical data for EDC processed porcine aortic root tissue. 6 samples per group and per analyses method were investigated*

	Fixation method* N=6	Shrinkage temperature (°C)	Carboxyl group concentration (% of fresh)	amine group concentration (% of fresh)*	Resistance to enzymatic digestion (%)	FTIR ratio $A_{1176}/A_{2925} - A_{1050}/A_{2925}$
A	P-J230	74.1±0.1	42±3	19±2	67.5±3.2	1.28-1.23
B	P-J230-hyd	71.8±0.5	51±2	17±2	63.4±2.2	1.14-1.15
C	P-J400	75.1±0.2	47±2	18±2	66.2±2.6	1.16-1.19
D	P-J400-hyd	72.0±1.1	56±2	20±1	64.7±3.1	0.95-0.98
	Fresh control	*62±2.1*	*100±4*	*100±2*	*40±5*	*0.27-0.31*

*determination of the amine group concentration of tissue samples after blocking but before cross-linking gave a value of 17±2%.

In general an increase in shrinkage temperature (Ts) was observed in all groups as compared to the fresh control caused by the formation of cross-links. Slight but significantly increased Ts's were found in the P-J400 compared to P-J230 cross-linked group. For both the P-J230 and P-J400 group, mild hydrolysis leads to significantly decreased Ts's.

Interestingly, the involvement of carboxyl groups during cross-linking was not found to be in line with measured Ts's. More carboxyl groups participated in the cross-linking reaction in the J230 group than in the J400 group. The hydrolysis reaction in turn resulted in liberation of carboxyl groups, whereby the relative difference in level of participation of carboxyl groups between the two groups was maintained.

It can be observed that after blocking of the amine groups still approximately 13% residual amine groups had not reacted with propionaldehyde. Furthermore no significant differences in residual free amine groups were determined between the 4 groups.

A significantly increased resistance to enzymatic degradation was observed in all sample groups as compared to the fresh control, which is the result of the cross-linking process. No difference in resistance to enzymatic degradation was observed between the groups processed with either J230 or J400. For both groups hydrolysis did not result in a statistically significantly decreased resistance to the enzymatic degradation. However by comparing the resistance to enzymatic degradation of the two hydrolyzed groups combined, to that of the non-hydrolyzed groups, a statistically significant decrease in resistance to enzymatic degradation was found. Finally, increased FTIR ratios at 1176 and 1050 cm^{-1}, relative to the absorbance measured at 2925cm^{-1}, indicative for the presence of ester bonds, were measured after cross-linking in all sample groups [26]. In the samples cross-linked with J230, more ester bonds were found than in the samples cross-linked with J400. For both groups the hydrolysis reaction resulted in loss of ester bonds.

In vivo characterization

Figure 1a represents the AAS values for explanted wall samples. The calcium found in the samples ranged from 15.8-20.4 mg/gram of dry weight tissue. Figure 1b is a graphical representation of the area occupied by calcium containing deposits in the treated tissues. These values range from 1.2-3.7 percent of the total matrix. The differences between sample groups observed with the latter methodology appear to give the same trend as seen with AAS.

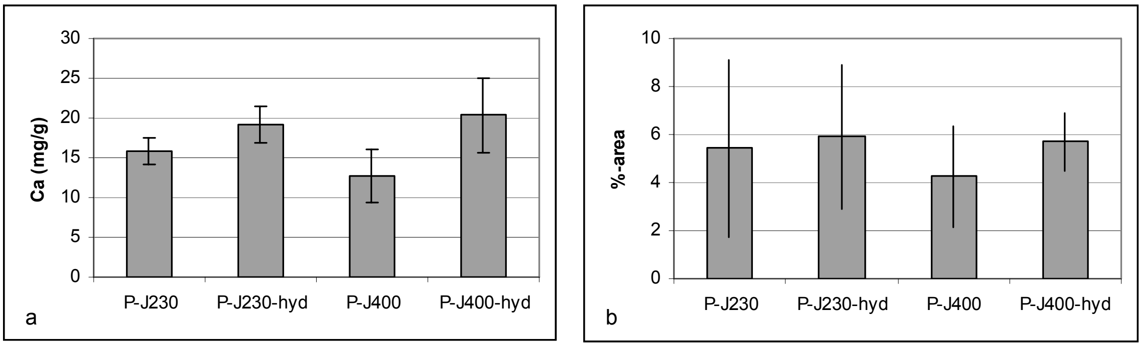

Figure 1, *Calcification of the samples processed. The amount of calcium in µg/mg tissue is determined with AAS, while the calcification in % total area is determined using image analysis of Von Kossa stained samples.*

Figure 2 is a panel of the Von Kossa histology slides of the various explanted aortic wall samples. The staining pattern represents the distribution of calcium salts within the extracellular matrix of the aortic wall. Inset pictures are high magnification micrographs. From the images it is concluded that in non-hydrolyzed samples (see Figure 2a,c) the calcification spots are associated with the extracellular matrix, while in hydrolyzed samples calcification is associated with remaining cells (Figure 2b,d). Furthermore there are changes in the pattern of calcification observed, in that without hydrolysis calcification is mainly located in the center of the wall tissue, while after hydrolysis calcification is concentrated on the adventitial side.

Figure 3 is a panel of TB stained histology sections of the various explanted aortic wall sample groups. In each of the panels the large micrographs represent the implant itself and the interface with the surrounding tissue capsule. Aortic wall sections are seen at the right-hand side in each panel with the capsule on the left, separated by an interfacial layer of inflammatory cells. The inserts are high magnification images of the cellular composition of the inflammatory cell layer at the interface between implant and host tissue.

Histological analysis after TB staining revealed no significant difference in the foreign body reaction between groups. Within all groups a small enveloping capsule had formed at the implant-tissue interface and some blood vessels were present in the surrounding tissue (see arrow in Figure 3). The absolute number of macrophages and giant cells was low, with a small layer containing these cells only observed at the implant-tissue interface. Also, only low numbers of lymphocytes were observed in the interfacial layers.

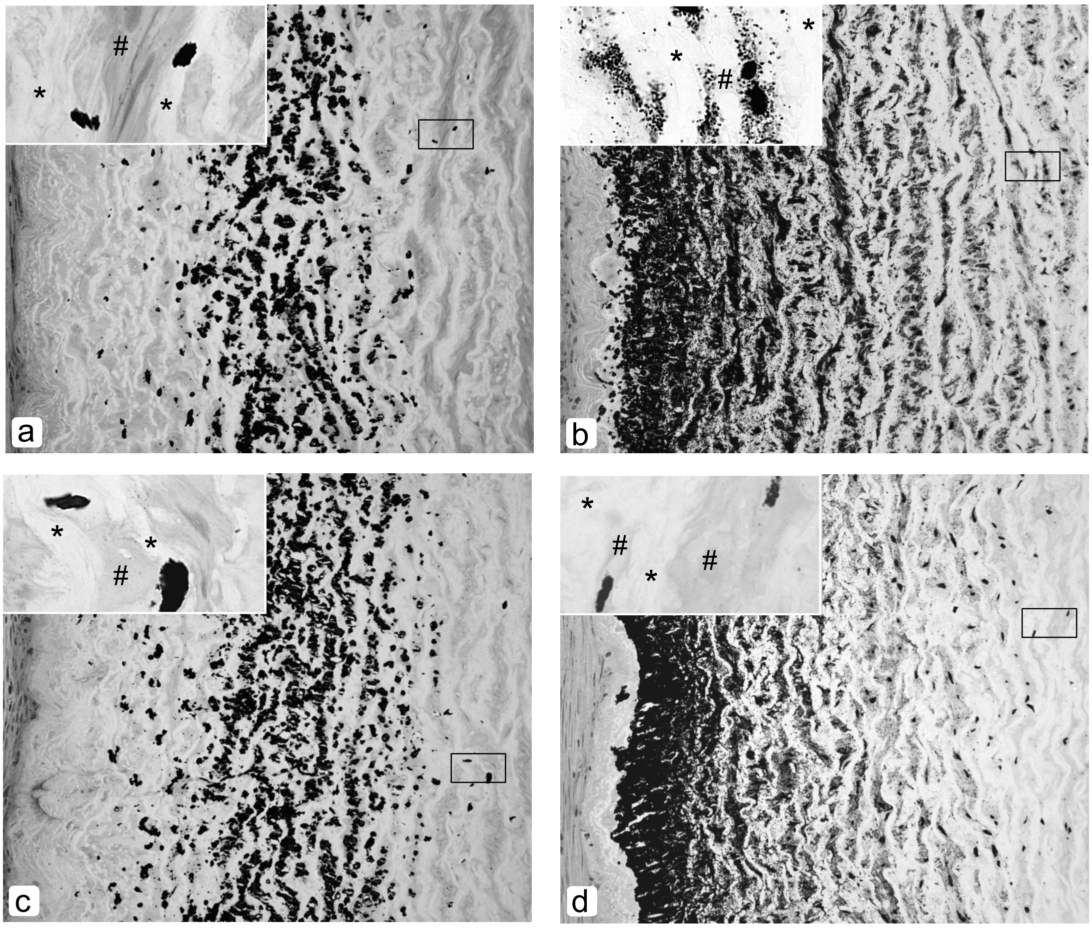

Figure 2: 200x magnifications of Von Kossa stained profiles of the J230 (A), J230-hyd (B), J400 (C) and J400-hyd (D) samples, after 8 weeks of implantation. A Toluidine Blue counter-stain was used to enhance the background contrast to show the distribution of the mineral deposition within the matrix. Inset pictures are 1000x (oil immersion) magnifications of selected areas within the large panel demonstrating the association of mineral deposition with either cells (#) (or extracellular matrix (*).

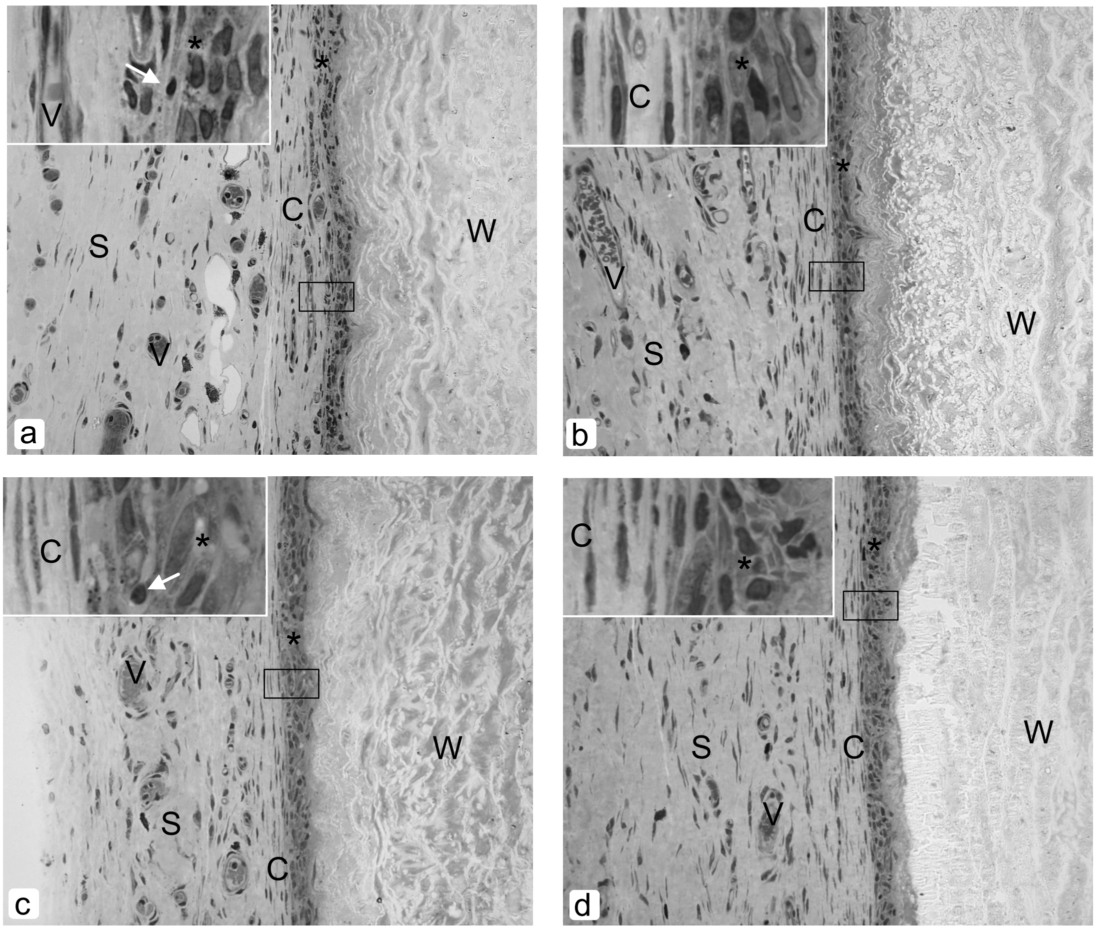

Figure 3*; 200x magnification of Toluidine Blue staining of the J230 (A), J230-hyd (B), J400 (C) and J400-hyd (D), after 8 weeks of implantation. Inset pictures are 1000x (oil immersion) micrographs of the cellular interface between the host capsule and the aortic wall implant.*

The capsule (C) and the surrounding tissue (S) are populated with small blood vessels (V). The Interface between the aortic wall (W), is populated with macrophages and lymphocytes.

Discussion

Water soluble carbodiimides (such as N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide, EDC) represent a class of cross-linking reagents that show promise for treatment of bioprosthetic devices. In literature, EDC cross-linking, in combination with N-hydroxysuccinimide (NHS), of collagen based materials is described for a wide variety of applications [9-26].

It is generally accepted that EDC/NHS cross-linking of collagen based materials results in the formation of amide bonds between activated carboxyl groups and amines [9,12, 23]. One of the side effects of EDC/NHS cross-linking is that such cross-linked collagen based materials have shown a tendency to increase in stiffness with a concomitant tissue contraction of the matrix [9,19,23].

In order to overcome this, we developed a so called "enhanced" EDC/NHS cross-linking process, based on blocking of matrix amine groups prior to the formation of cross-links with Jeffamine™ spacers using EDC/NHS [17-20]. This method provided stabilized tissue matrices with improved mechanical properties as compared to zero-length cross-linked matrices. Furthermore a significant decrease in calcification of aortic wall and leaflet tissues as compared to zero-length cross-linked controls was obtained in selected animal implant models [18,19]. However, the stiffness of these materials was higher than expected, suggesting that in addition to cross-links formed via the spacer molecules also other type of cross-links were formed [19]. More recently we demonstrated that during the EDC/NHS cross-linking process of a collagen model material additional ester cross-links between activated carboxyl groups and hydroxyl groups are formed [25,26].

As a result, we have developed a methodology to reduce or eliminate these ester bonds in an attempt to minimize stiffness [17,26]. By prolonged storage of EDC/NHS cross-linked collagen in an alkaline solution (borate buffered saline, pH 10) it was demonstrated that esters were hydrolyzed, yielding an improvement in biomechanics, while not influencing other physical/chemical properties [25]. A pH 10 storage solution was selected because it has been demonstrated that under these condition ester bonds are effectively removed, while no damage to the structure of the extracellular matrix occurs [26]

While physical/chemical properties are important parameters indeed, they however do not address biocompatibility requirements that exist for these cross-linked materials.

The goal of this study was therefore to investigate whether the hydrolysis process affects the otherwise favourable in-vivo inflammation and calcification results. Hereto porcine aortic wall samples were processed under conditions equal to what was described earlier [18,19]. Tissue groups were processed with two Jefamines™, with a molecular weight of 230 (J230) or 400 (J400), after blocking of amine groups with propionaldehyde [19]. Both J230 and J400 were included in the experimental design. We previously described that J230 forms cross-links only inside a collagen microfibril, while J400 can also form cross-links in between collagen microfibrils [18]. By using both J230 and J400 we investigated the effect of the location of the cross-link on the in-vivo tissue response and whether this location of the Jeffamine™ cross-link had an effect on the number of esters formed in the matrix.

After cross-linking the processed valves were stored for 2.5 months in a storage buffer at either pH 7.2 or pH 10 after which material characterization was done [26]. This storage time was chosen to assure that optimal hydrolysis of ester linkages occurred. Notably, it was verified that, by determination of free carboxyl groups in wall samples from both groups, no change in free carboxyl groups was observed after 50 days in pH 10 storage solution (data not published). This observation was in line with what previously found using a model collagen material [26].

The in-vitro assessment of tissue wall samples showed that no differences in residual free amine group concentration were measured between all cross-linked samples and the non cross-linked, amine-blocked control group. This suggests that the free remaining residual amine groups did not participate in cross-linking and the hydrolysis reaction had no effect on amide cross-links in the matrix [18,26].

Furthermore, in tissue processed with P-J400 a slightly, but significantly, higher shrinkage temperature (Ts) was measured as compared to the P-J230 sample group. The Ts of a matrix is determined by the temperature at which the collagen triple helix unfolds [18] and it provides information on the overall number of cross-links in the matrix (amide and esters linkages) [19,27].

Interestingly, by determining the free carboxyl groups directly it was found that overall less cross-links were formed in the P-J400 group compared to the P-J230 group. Furthermore with FTIR analyses it was demonstrated that in the P-J400 group also less ester linkages were formed than in the P-J230 group.

The observation that J400 has a higher Ts as compared to J230 was in line with previous results [18], and was explained by the fact that by using J400 in addition to intra-fibrillar cross-links, also cross-linking in between the collagen micro-fibrils occurs. The fact that in the J400 group apparently less cross-links result in a higher Ts (as compared to the J230 group) suggests that in addition to the number of cross-links also the location of the cross-links (intra-fibrillar or inter-fibrillar) will have an effect on the Ts and its related matrix stability.

For both P-J230 and P-J400 groups, it was demonstrated that the hydrolysis process removed cross-links as a decrease in Ts, increase in free carboxyl groups and decrease in resistance to enzymatic degradation was found. By comparing free carboxyl groups measured before and after hydrolysis for both P-J230 and P-J400 group it was concluded that independent of the Jeffamine™ spacer length equal amounts of esters were removed by hydrolysis. Furthermore, in line what previously was observed [26], FTIR data show that for both the P-J230 and P-J400 sample groups still esters remain present in the matrix after hydrolysis. In addition in the P-J230-hyd group significantly more esters remain than in the P-J400 group. It was described before that the remaining ester bonds are likely protected by the complicated three dimensional matrix structures [26]; but possibly also a well buffered micro-environment could prevent hydrolysis of ester bonds. More research will be needed to elucidate this. It is suggested that by adding well chosen surfactants to the hydrolysis buffer more esters can be hydrolyzed, however these surfactants may permanently change the matrix properties [28,29]. Overall the in-vitro data suggests that J400 is a better choice than J230 for cross-linking of aortic wall matrices, as similar in-vitro performance with less cross-links is achieved. Furthermore the data suggests that by using J400 fewer ester linkages are formed, and remain after hydrolysis, as compared to J230. However additional research on the location of cross-links formed in relationship to the molecular weight of the Jeffamine™ in the collagen matrix is needed in order to fully understand and control the reactions in the tissue matrix [1]. Adding further complexity is the fact that aortic wall material contains besides collagen and elastin many additional non-structural proteins having free amine, carboxyl and/or hydroxyl groups, which during cross-linking could become attached to the matrix [20,23]. This means that these components could participate during cross-linking and thus affect the physical/chemical characteristics of matrices.

Next, samples were evaluated in an in-vivo implantation study using a juvenile rat model. Analyses of explanted materials focused on the material's propensity to inflammation and calcification, both of which are critical factors in attaining long term success with bioprosthetic implants.

It was again confirmed that the tissue samples cross-linked via the enhanced EDC/NHS method showed a high reduction in levels of calcification when compared to glutaraldehyde treated or EDC/NHS zero-length cross-linked controls [18,19]. Although the AAS data suggest that P-J400 has a lower calcification than P-J230, this was not found to be statistically significant. The difference in calcification pattern between the 4 sample groups is remarkable. Of special note is the shift in tendency toward cell based calcification as seen in the hydrolyzed samples, and matrix based calcification as found in the non-hydrolyzed tissue samples. This finding could mean that ester bonds mask nucleation sites for calcification on residual cells, cell debris, or other extracellular matrix components for that matter. While both calcification and inflammation are low in all groups included in this study, no correlation can be drawn between inflammation and the onset of calcification. Furthermore in spite of the observed differences in calcification patterns, there nonetheless is calcification of both cells and extracellular matrix in all samples, which brings us no closer to solving the question of the origin of calcification. It would be interesting to conduct similar studies on decellularized matrices to identify matrix components that would initiate calcification [30,31,32]

All in all, it is fair to say that neither the selection of either J230 or J400, nor additional hydrolysis after cross-linking have had significant effects on general calcification and inflammation responses, although there appear to be subtle differences. The relevance and significance of these remain to be seen and is under continued investigation.

The low in-vivo calcification and the now additional improvements in bio-mechanical properties of heart valve material [19,33,34] make the technology highly promising in the design of the next generation tissue valves [18,19].

Conclusions

An in-vivo study was performed to evaluate the effect of removal of ester bonds, formed as a by-product, during cross-linking of porcine tissue aortic walls using N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) on calcification and inflammatory reactions. Previously it was demonstrated that these esters linkages had an impact on the physical/chemical properties of the matrices.
Hereto porcine aortic valves were cross-linked by EDC/NHS activation of tissue carboxyl groups with two types of Jeffamines™. The tissue amine groups were blocked with propionaldehyde prior to this cross-linking step.
It was demonstrated that the selection of the Jeffamine™ and removal of esters caused subtle but significant changes in tissue properties. It was furthermore demonstrated that these changes did only slightly affect in-vivo performance. While intriguing results were noted between hydrolyzed and non-hydrolyzed cross-linked wall samples with respect to calcification origin and distribution, there was no significant difference noted in the overall calcification and inflammatory host response.
Taking the obtained results into account, and with the added notion that elimination of ester cross-links was found to improve biomechanical properties of cross-linked tissue, it is safe to conclude that addition of the hydrolysis step after cross-linking as part of the overall enhanced EDC/NHS cross-linking process is very much warranted.

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Epilogue

The aim of the project described in this thesis was the development of a non-glutaraldehyde based cross-linking technology for bioprosthetic heart valves, with a low propensity for calcification, a high durability and a good biocompatibility.

The approach we took resulted in the development of a so-called ‘enhanced’ carbodiimide (e-EDC/NHS) based cross-linking process. In this process, tissue amine groups are first blocked by an aldehyde followed by incorporation of a diamine terminated spacer (Jeffamine™) in the matrix using N’-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS). The underlying idea was that by blocking of the amine groups no direct cross-links between juxtaposed amine and carboxyl groups (also referred to as zero-length cross-links) could be formed. It was expected that the biomechanical properties of the cross-linked structure could be controlled by varying the concentration and length of the Jeffamine™ spacer. Each reaction step was characterized and tissue samples were analyzed in order to get insight in the degree and type of cross-links formed. After the development of a method to quantify carboxyl groups in a model collagen it was demonstrated that during EDC/NHS cross-linking in addition to amide linkages between matrix activated carboxyl groups and Jeffamine™ amine groups, also ester bonds between matrix activated carboxyl groups and matrix hydroxyl groups were formed. This caused an undesired stiffening of the matrix. It was subsequently demonstrated that these additional linkages can be removed by hydrolysis resulting in improved biomechanical properties of the matrix.

Porcine aortic wall samples, processed with the optimized e-EDC/NHS method, have a significantly reduced in-vivo calcification as compared to porcine aortic wall samples cross-linked with glutaraldehyde (GA) or EDC/NHS only. After optimization of the reaction conditions, no leaflet calcification occurred as shown with a sub-dermal juvenile rat calcification study.

Furthermore, with in-vitro performance tests it was demonstrated that the valves processed with the e-EDC/NHS technology performed very well. With accelerated wear tests it was demonstrated that completely processed valves were still in good shape after 25 million cycles while the GA cross-linked control valves matrices started to deteriorate [1,2].

In our experiments we have used a GA cross-linked valve as a control. Since cross-linking of a matrix with EDC/NHS or GA occurs via a totally different mechanism resulting in cross-linked matrices with different properties one has to be very careful in comparing results of in-vitro and in-vivo tests. An example is the biomechanical properties of the matrix (more popular: the stiffness of the matrix) significantly different for an e-EDC/NHS as compared to a GA cross-linked valve matrix. Furthermore, the number of standardized performance tests for heart tissue valves is limited and these are mainly designed to evaluate GA cross-linked heart valves. In an accelerated wear test with stented e-EDC/NHS cross-linked leaflets mechanical failure of all specimens occurred initially. Analyses revealed that the cause of failure was due to mechanical interaction between the leaflets and the stent. As the shape of the stent used was optimized for GA cross-linked leaflets, and e-EDC/NHS processed leaflets have a different opening and closing behavior during testing these leaflets hit the stent and became damaged. Similar to what we observed, this mode of failure also caused termination of a clinical study of a Photofix™ cross-linked stented valve, one of the reasons why 'alternative cross-linking techniques' have a somewhat negative reputation [3].

This example demonstrates that, as mentioned in chapter 2, the 'level of engineering', needed to develop a new cross-linking technology is not restricted to the biochemical performance of the material alone. In addition new assays and performance tests need to be developed as well and by testing materials processed with different technologies one needs to be cautious not to draw wrong conclusions by comparing 'apples and oranges' and rely too much on historical data.

We have tried to explain the underlying mechanisms causing the low calcification of the e-EDC/NHS modified tissue. We demonstrated that especially for the aortic wall the selection of the aldehyde used for blocking the amine groups had a significant effect on the calcification. In chapter 4, it was hypothesized that during the blocking reaction components that may cause calcification are extracted from the matrix.

In an additional study, not published yet, with SDS-PAGE analysis of extracts from processed tissue wall tissue samples it was confirmed that during the blocking reaction proteins are extracted from the matrix. Initial results show a relationship between the type of blocking agent, number of extractable proteins remaining in the matrix and calcification in-vivo. Furthermore it was demonstrated that non cross-linked aortic wall samples in which the amine groups were blocked with propional a similar degree of calcification occurred as compared to an e-EDC/NHS processed control. The work suggests that unknown components (not necessary limited to proteins only) are removed from the matrix during the blocking step of the reaction causing the low calcification in-vivo.

This work, combined with in vivo evaluation of EDC/NHS and e-EDC/NHS cross-linked decellularized valve matrices is currently under investigation and will be the subject of forthcoming publications by Torrianni et all (University of Groningen, the Netherlands).

Furthermore, it was demonstrated that the reaction with the aldehyde changes the hydrophobicity of the surface and as can be found in chapter 4, we were able to show a relationship between the hydrophobicity of the surface and the calcification. Since there is also a relationship between the hydrophobicity of the surface and the surface charge and furthermore surface charge with biological response to a matrix [4,5], the change in matrix properties due to the e-EDC/NHS process could explain the low amount of calcification as well.

Despite the promising results of the e-EDC/NHS cross-linking technology as published in this thesis, more research may result in further improvements and understanding of the effect of chemical modifications on the performance in-vivo.

An example is that by bringing the 'biology' back in the matrix better adaptation of the bioprosthesis to the host may occur. Pieper et al [6] demonstrated that loading of an EDC/NHS cross-linked matrix with glycosaminoglycans (GAG's) results in improved in-vivo performance. In addition the presence of GAG's linked to the matrix has been associated with improved tissue mechanics as GAG's prevent leaflets from 'buckling' (see also Figure 4 in Chapter 2 demonstrating this phenomenon) [7].

Ex-vivo introduction of the patient's own material (cells, non structural proteins) to e-EDC/NHS processed valves may result in significant improvements with respect to adaptation of the bioprosthesis to the host and 'healing' of the matrix. If so the case, it will cause additional challenges (most logistical based) if implemented clinically, however it is worthwhile to investigate.

Furthermore, Zilla et al [8] demonstrated that the combination of GA and e-EDC/NHS cross-linking of valves results in reduced valve calcification as compared to GA alone. This is an example of a 'combination' technology that may promote adaptation of alternative cross-linking technologies in the bioprostheses arena. However, at the other hand, since the permutations of reaction products and type of linkages formed in the matrix are almost endless it is the question if this approach is desired.

Finally, we suggest that before an alternative cross-linking technique for GA in general clinically is evaluated on a complicated versatile matrix such as a heart valve, a simpler matrix needs to be selected first. Exploring, for example, the e-EDC/NHS technique on a collagen patch used as a tissue filler or drug containing matrix is easier accepted, has less risk associated and is therefore easier adapted clinically. With such an approach very viable clinical performance information of the material will be obtained. This data is needed for the development of optimized cross-linking procedures for complicated matrices and in addition development of the appropriate in-vitro performance tests to be used.

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Summary

Due to some form of heart valve disease, approximately 250000 patients worldwide undergo surgery to receive a new heart-valve each year. In about 70% of the cases a mechanical heart valve is used despite the fact that permanent anticoagulant therapy for the patient is required. The remaining 30% of the patients receive a bioprosthesis, made from porcine or bovine material. Since a porcine heart is similar to a human heart also complete porcine valves can be used. If bovine material is used, the valve leaflets are made from sheets of pericardium. Either bioprostheses which are constructed by mounting valve leaflets on a supporting rig (a stent) or complete stentless porcine valves including leaflets and root (wall) are used.

Although the bioprosthetic valve has initially in many ways a superior performance compared to the mechanical valve, the reason why it is currently only employed in such a relatively small patient population is because after several years it is prone to increased incidents of degeneration, calcification and cusp rupture. These phenomena occur especially in young adults. Modern mechanical valves, on the other hand, are durable and have excellent flow characteristics, but have the propensity for thrombo-embolic complications, necessitating life long anticoagulation therapy with the result of increased risk of bleeding. This has triggered the desire to develop a better, i.e. more durable bioprosthesis and many attempts have been described in literature.

In general, the process of producing porcine bioprostheses starts in an abattoir. Here the valves are collected and transported on ice to a production facility. Suitable valves are selected and trimmed down to remove all not necessary material (such as remaining muscle). In a next step, the degradation process that has started at the moment the valve was obtained from its donor is arrested and compounds causing antigenic reactions are removed or at least neutralized. Hereto the valve material undergoes some form of chemical treatment to cross-link the collagen and after several critical inspections and performance tests it is packed in a container with holding solution and shipped to the customer. Less than ten percent of the valves that are collected in an abattoir make it to a final product.

The aim of the research presented in this thesis was to develop a new cross-linking technology for preparing stentless bioprostheses with a significantly reduced wall and leaflet calcification compared to commercially available glutaraldehyde (GA) cross-linked valves.

Chapter 2 presents a literature review about the chemical modification technologies for heart

valves developed so far and general information on the compositions and structures is provided. Many processes have been published and it is striking that GA is the cross-linking agent that is still used for most commercial heart valves, despite the fact that GA has been implicated in the calcification process. An explanation may be that GA has been used for almost 45 years and lots of clinical data are available. Furthermore, the reputation of alternative cross-linking techniques that have been developed is somewhat negative, since a few clinical failures of alternative cross-linked valves have been reported over the last years. In chapter 2, the term ‘engineering of the tissue’ (in contradiction to tissue engineering) is introduced as we are convinced that successful development of a new tissue cross-linking process is very much dependent on the level of engineering (from many aspects) that is put into it. Each step in the process needs to be thoroughly evaluated in-vitro, in order to have a fully controlled process and to be able to create a tissue matrix that performs according to the desired specifications. It has been demonstrated that during GA cross-linking, for example, many uncontrolled reactions occur and numerous reaction products are formed in the matrix.
In chapter 3, as we named it, an “enhanced carbodiimide’’ based cross-linking method is described. Rather than cross-linking the tissue through its free primary amine groups, these groups were first blocked with butanal and subsequently cross-links were formed between the carboxyl groups of the tissue matrix using a poly(propylene glycol)bis 2-(aminopropyl) ether (Jeffamine™) spacer. N’-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) were used to initiate this cross-linking reaction.

It was demonstrated that porcine leaflets cross-linked in this way had a calcification of less than 1 mg/g tissue after subcutaneous implantation in juvenile rats for 8 weeks. Furthermore, aortic wall calcification was reduced to 50 mg/g, compared to standard glutaraldehyde fixed tissue, which showed 120 mg/g tissue calcification in the 8 wks calcification model used.

In chapter 4 a study is described in which processing conditions are further explored and optimized. Several aldehydes for blocking of the amine groups were selected and in-vitro optimization studies with repect to the reaction conditions were performed. After cross-linking, calcification of valve wall and leaflet samples was studied in-vivo for 8 weeks. It was demonstrated that no leaflet calcification had occurred, whereas wall calcification was maximally 95% reduced when propional was used as blocking agent, as compared to a valve cross-linked with GA.

Despite these promising results, the new technique caused unexpected stiffness of processed valves.

In chapter 5 a new assay to determine residual carboxyl groups in EDC/NHS cross-linked collagen matrices is presented. In the method, carboxyl groups were labeled with 5-bromomethyl fluorescein (5-BMF). Subsequently, the fluorescent label was released by mild hydrolysis and quantified with capillary zone electrophoresis (CZE). A calibration curve relating the concentration of carboxyl groups with peak intensities was obtained using modified Sephadex™.

The concentration of carboxyl groups in unprocessed collagen as determined with this new technique was equal to the concentration of carboxyl groups measured by amino acid analysis. With the 5-BMF method in hand the EDC/NHS cross-linking technique was investigated. The concentration of residual carboxyl groups of EDC/NHS cross-linked collagen and collagen, in which the amine groups were blocked with propional was determined and based on the results it was concluded that activated carboxyl groups do not only react with amine groups but also can react with other functional groups present in the matrix. Literature data suggest that these functional groups might be hydroxyl groups.

This implies that the cross-link density of collagen matrices after treatment with EDC/NHS is higher than expected on the basis of amide bond formation only as determined by a standard assay for quantification of amine groups. Furthermore, if hydroxyl groups are also involved in cross-links, the formed ester linkages between juxtaposed carboxyl groups and hydroxyl groups will render the matrix stiffer than when only amide cross-links were formed .

In chapter 6, a study toward the type of cross-links formed during EDC/NHS cross-linking of collagen materials is described in an effort to relate the actual degrees of cross-linking with their physical-chemical and bio-mechanical properties. With FTIR analysis it was demonstrated that ester bonds are formed during the cross-linking reaction. It was further shown that these ester cross-links significantly affect the mechanical properties of matrices. By applying a mild hydrolysis process on EDC/NHS cross-linked collagen matrices it was demonstrated that the ester groups could be removed without affecting the overall tissue integrity. Furthermore, this additional reaction step led to improved tissue mechanics.

As the removal of ester groups might have an impact on the performance of cross-linked matrices in-vivo this was studied as described in chapter 7. Porcine aortic valves were EDC/NHS cross-linked with Jeffamine™ (with a molecular weight of 230 or 400) after a

blocking reaction of matrix amine groups with propional. From each processed sample group valves were stored at pH 7.2 (control group) and pH 10 (in order to hydrolyze ester bonds) for 2.5 months. Followed by these procedures, the calcification of aortic wall samples devoid of extraneous ester bonds were evaluated compared to control aortic wall samples by subcutaneous implantation in weanling Sprague Dawley rats for 8 wks.

This study demonstrated that while subtle but significant changes in properties were noted using in-vitro tests typically used to characterize bioprosthetic tissue, these changes did not significantly affect the in-vivo performance of the materials. Interestingly, the calcification profile changed totally as a result of the hydrolysis reaction.

The cross-linking process developed for collagen-based materials presented in this thesis was optimized for stentless porcine tissue heart valves and successfully evaluated in-vitro and in-vivo. Through 'engineering the tissue', chemical processes were optimized resulting in a significantly decreased calcification of porcine aortic wall material compared to GA control samples in a juvenile subcutaneous rat model. The leaflet calcification was found absent in the samples evaluated. The implant study was repeated in juvenile sheep (descending thoracic aorta model) and here a significantly reduced calcification compared to GA controls was achieved as well. An improvement in tissue mechanics was obtained after removal of ester groups.

It is suggested that effective removal of components that cause calcification in-vivo during the blocking reaction, in combination with alterations in surface charge/matrix hydrophobicity are responsible for the promising performance of valve material cross-linked with the 'enhanced carbodiimide' technology.

Based on the observations made the author suggests that the use of this technology will result in longer lasting heart-valves and therefore is a serious candidate to replace the existing GA cross-linking technology in the future. Due to its versatility it also may lead to the development of other type of collagen based bioprostheses.

Samenvatting

Wereldwijd wordt bij ongeveer 250000 patiënten per jaar een hartklep vervangen. Dertig procent van de patiënten krijgt een bioprosthese, een weefselklep gemaakt van dierlijk materiaal (voornamelijk varken of rund). In de overige 70% van de patiënten wordt een mechanische hartklep toegepast. Aangezien een varkenshart nogal wat overeenkomsten heeft met een menselijk hart kunnen varkenskleppen in zijn geheel gebruikt worden. Een klep van runderoorsprong wordt gemaakt uit pericardium (de hartzak). De klepblaadjes van de hartklep worden gemonteerd op een kunststof drager (een stent) of er wordt een complete varkensklep met de bijbehorende aorta toegepast (zogenaamde stentloze configuratie).

Ondanks dat een bioprosthese aanvankelijk in verschillende opzichten superieur is ten opzicht van een mechanische hartklep wordt dit type klep slechts toegepast in een relatief kleine patiëntenpopulatie. De onderliggende reden is dat de bioprosthese een verhoogde kans heeft op degeneratie, verkalking (calcificatie) en scheuren van de klepblaadjes. Dit effect treedt vooral op bij jonge patiënten. Ondanks het feit dat moderne mechanische hartkleppen weliswaar goede mechanische eigenschappen bezitten (zoals een geoptimaliseerde stroming van het bloed langs de klepbladen), bestaat er voor de patiënt een verhoogde kans op trombose. Patiënten dienen daarom voor de rest van hun leven anti-stollingmiddelen te gebruiken. Dit is de reden dat er veel onderzoek is gedaan naar het verbeteren van de bioprosthesen.

De productie van een bioprosthese begint in een slachthuis waar varkenskleppen worden verzameld en verpakt op ijs naar een fabrikant gestuurd. Daar worden bruikbare kleppen geselecteerd en onbruikbare stukken (zoals resten hartspier) mechanisch verwijderd. Vervolgens wordt de degradatie van de klep gestopt en stoffen die het afweersysteem van de patiënt kunnen activeren na implantatie (zoals cellen en eiwitten in de matrix) verwijderd of inactief gemaakt. Tijdens deze behandelingen worden vooral de collageen vezels in de matrix vernet (ook wel gecrosslinkt of gefixeerd genoemd). Vervolgens wordt de klep opnieuw geïnspecteerd, verpakt en naar de klant verzonden. In de praktijk levert minder dan tien procent van de hartkleppen die in het slachthuis verzameld worden een bruikbaar product op. Het onderzoek dat is uitgevoerd en beschreven in dit proefschrift is gericht op de ontwikkeling van een stentloze varkenshartklep met een significant gereduceerde in-vivo verkalking vergeleken met commercieel verkrijgbare hartkleppen. Tijdens het onderzoek is gekeken naar de verkalking van zowel de klepwand als het klepblad.

In hoofdstuk 2 zijn de resultaten beschreven van een literatuurstudie naar de diverse crosslinktechnieken die zijn ontwikkeld voor hartkleppen. Verder wordt de samenstelling en structuur van hartkleppen nader beschouwd. Er zijn verschillende veelbelovende chemische modificaties bekend. Het is echter typerend dat glutaraldehyde (GA) nog steeds de verbinding is die toegepast wordt voor commercieel verkrijgbare weefselkleppen. Dit ondanks het feit dat er een relatie bestaat tussen het gebruik van GA en verkalking van de klep. Een mogelijke verklaring zou kunnen zijn dat in de ongeveer 45 jaar dat GA toegepast wordt, veel klinische data is verkregen en dat de reputatie van alternatieve technologieën voor GA geschaad is door een aantal mislukte klinische studies.

Verder wordt in dit hoofdstuk gesteld dat voor het ontwikkelen van een goed functionerende klep 'dedicated engineering' nodig is. Wij zijn ervan overtuigd dat het effect van iedere reactie stap op het weefsel nauwkeurig moet worden onderzocht, teneinde een matrix te verkrijgen waarvan de chemische samenstelling bekend is en die voldoet aan de specificaties die gesteld zijn.

In hoofdstuk 3 wordt een, zo door ons genoemde, "verbeterde carbodiimide" gebaseerde crosslinking techniek besproken. Hierbij wordt in tegenstelling tot het creëren van crosslinks gebruik makend van de vrije amine- en carboxyl groepen van het collageen, crosslinks gemaakt tussen carboxyl groepen van het collageen met behulp van een Jeffamine™. Crosslinks worden gemaakt door gebruik te maken van N'-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) en N-hydroxysuccinimide (NHS). De amine groepen van de collageen vezels worden eerst geblokkeerd met butanal. Na in-vitro karakterisatie van de op deze wijze gecreëerde kleppen, werden klepwand en klepblad monsters subdermaal geïmplanteerd in jonge ratjes. De uitkomsten van deze verkalkingstudie lieten zien dat er een verkalking van minder dan 1mg/g weefsel voor de klepblaadjes na 8 weken implantatie opgetreden was. Er werd een verkalking van 50mg/g weefsel voor de klepwand gevonden. De verkalking van deze monsters is significant lager in vergelijking met de 120 mg/g verkalking gevonden voor een klepwand van een monster behandeld met GA. In hoofdstuk 4 worden de resultaten besproken van een serie optimalisatie experimenten met de doelstelling het blokkeringproces te optimaliseren en het beste aldehyde te vinden. De resultaten van een in-vivo experiment laten zien dat de verkalking van de klepblaadjes bijna volledig geëlimineerd is na deze optimalisatie, terwijl een reductie van de verkalking van de klepwand van bijna 95% verkregen kan worden bij gebruik van het aldehyde propional in vergelijking met het GA gecrosslinkte materiaal. Ondanks dat de verkregen resultaten veelbelovend zijn, werd een niet verwachte toename van de stijfheid van de kleppen waargenomen na toepassing van de nieuwe techniek.

Omdat er geen methode voorhanden was om carboxyl groepen te kwantificeren werd tot dusver de crosslink-dichtheid geschat met een aantal methoden die slechts indirecte informatie verschaffen. Hoofdstuk 5 beschrijft de ontwikkeling van een methode om carboxyl groepen in collageen materialen te kunnen bepalen met de doelstelling de crosslink-dichtheid direct te kunnen kwantificeren. Hierbij werd gebruik gemaakt van het label 5-bromomethyl fluorescine (5-BMF). Na reactie van het label met carboxyl groepen van een collageen model matrix werd eerst niet-gereageerd label verwijderd. Door een hydrolyse reactie werd vervolgens het label gescheiden van de matrix om vervolgens gekwantificeerd te worden met behulp van een capillaire zone elektroforese techniek. Een ijklijn werd verkregen door gebruik te maken van een gemodificeerde Sephadex™ met een bekende hoeveelheid carboxyl groepen.

Er werd aangetoond dat de concentratie carboxyl groepen in collageen bepaald met de 5-BMF methode gelijk was aan de concentratie van carboxyl groepen in dezelfde matrix bepaald met behulp van aminozuur analyse.

Verder kon worden aangetoond dat gedurende het crosslinken van collageen met EDC/NHS niet alleen amide crosslinks maar ook additionele crosslinks gevormd worden. Op basis van literatuurgegevens kon worden geconcludeerd dat deze additionele crosslinks mogelijk gevormd werden door een reactie tussen carboxyl en hydroxyl groepen leidend tot ester groepen.

In het onderzoek dat beschreven staat in hoofdstuk 6 is het type crosslinks dat in collageen gevormd wordt onder invloed van EDC/NHS nauwkeuriger onderzocht. Met behulp van infrarood metingen (FTIR) kon worden aangetoond dat de additionele crosslinks die gevormd werden esters zijn.

Een biomechanische evaluatie van gecrosslinkte lapjes collageen liet zien dat deze esters een significante bijdrage leveren tot de stijfheid van het materiaal. Omdat deze ester groepen relatief makkelijk te hydrolysereen zijn wordt in hoofdstuk 6 een methode beschreven om deze ester crosslinks te verwijderen gebruik makend van een buffer met een pH van 10. Er werd aangetoond dat dit resulteert in materialen met een verbeterd biomechanisch gedrag.

Aangezien het niet bekend was of de aanwezigheid van ester crosslinks een effect heeft op de biocompatibiliteit van de matrix, werden vervolgens klepmaterialen met en zonder ester

crosslinks in-vivo geëvalueerd.
In hoofdstuk 7 worden de resultaten beschreven van deze in-vivo studie. Varkens hartkleppen werden EDC/NHS gecrosslinkt met Jeffamine™ (molekuulgewicht 230 of 400) na een blokkeringsreactie met propional. Van iedere groep werden kleppen gedurende 2.5 maand bewaard in een buffer met een pH van 7.2 (controle groep) of pH 10 (om ester groepen te hydrolysereen). Hierna werd een in-vivo subdermale verkalkingstudie uitgevoerd op stukjes klepwand materiaal. De resultaten van deze studie lieten zien dat ondanks het feit dat er in-vitro meetbare verschillen waren tussen de eigenschappen van de klepmaterialen van de verschillende groepen deze verschillen geen invloed hadden op de biocompatibiliteit en absolute hoeveelheid verkalking in-vivo. Het verkalkingspatroon van de groepen bewaard bij pH 7 en pH 10 was echter zeer verschillend.

De crosslinktechnologie die beschreven staat in dit proefschrift is geoptimaliseerd voor stentloze varkenshartkleppen en is zowel in-vitro als in-vivo geëvalueerd. De meest belangrijke chemische processen werden geoptimaliseerd met als resultaat een significant gereduceerde verkalking van de klepwand in vergelijking met klepwandmonsters gecrosslinkt met GA. De optredende calcificatie van de klepblaadjes was verwaarloosbaar. Een volledige klep werd ook geëvalueerd in-vivo in een functioneel model (de klep werd hiertoe geïmplanteerd in de aorta van een jong schaap) en ook hier werden significant lagere calcificatie waarden gevonden, vergeleken met GA behandelde kleppen. Met additioneel onderzoek naar het EDC/NHS crosslinking proces kon worden aangetoond dat gedurende crosslinking van een collageen matrix amide en ester bindingen gevormd worden. Deze ester groepen, gevormd als bijproduct zorgen voor een niet gewenste verstijving van de matrix. Het bleek echter mogelijk deze estergroepen te hydrolyseren zonder de matrix verder essentieel te veranderen.
De schrijver van dit proefschrift is van mening dat in de nabije toekomst de in dit proefschrift beschreven technologie een serieus alternatief voor de GA technologie kan worden. De nieuwe technologie zal niet alleen gebruikt kunnen worden voor de vervaardiging van hartkleppen met een langere levensduur maar zal ook toepasbaar zijn voor het behandelen van diverse andere collageen bevattende producten.

Curriculum Vitae

Frank Everaerts werd geboren op 25 augustus 1973 in Sittard en bracht zijn jeugd in Weert door. Hij volgende zijn middelbare schoolopleiding aan het Bisschoppelijk College in Weert en behaalde zijn VWO diploma in 1991. Hetzelfde jaar begon hij aan de opleiding Scheikundige Technologie aan de Technische Universiteit van Eindhoven en deed zijn stage en afstudeeropdracht onder leiding van Prof. Dr. Ir. F.M. Everaerts bij de voormalige vakgroep TIA. Dit onderzoek met de titel 'Determination of the composition of collagen based materials using Capillairy Electrophoresis' werd praktisch uitgevoerd bij het Medtronic Bakken Research Center (BRC) in Maastricht. Na het afstuderen in 1997 werden zijn werkzaamheden gecontinueerd en vanaf 1999 combineerde hij dit met een promotieonderzoek onder leiding van Prof. Dr. J. Feijen in Twente, Dr. Ir. M. Hendriks in Maastricht en M. Torrianni, MSc in Santa Ana Californië. Het meeste praktisch werk werd uitgevoerd in Maastricht en Santa Ana. De resultaten van dit onderzoek staan beschreven in dit proefschrift waarop de auteur hoopt te promoveren op 19 oktober 2007 Vanaf 1 april 2007 is hij werkzaam bij Philips Applied Technologies in Eindhoven.

Frank Everaerts was born on August 25, 1973 in Sittard (The Netherlands), grew up in Weert and studied Chemical Engineering at the University of Technology in Eindhoven. His practical work period and graduation project was performed at Medtronic Bakken Research Center in Maastricht (BRC, the Netherlands) under supervision of Prof. Dr. Ir. F.M. Everaerts on the subject of 'Determination of the composition of collagen based materials using capillary electrophoresis. After his graduation in 1997 his professional career started at the BRC. In 1999 he combined this assignment with a PhD project at the Twente University (The Netherlands), under supervision of Prof. Dr. J Feijen, Dr. Ir. M Hendriks and M. Torrianni MSc. Most practical work was performed at the BRC and Medtronic Valves in Santa Ana (USA). The study is published in this thesis and the author hopes to complete his PhD project on October 19, 2007. Since April 1 2007 he got employed at Philips Applied Technologies in Eindhoven, the Netherlands.

Jasmijn and Milan, August 2007