Crosslinking of Porcine Aortic Leaflets with Butane-1,4-diol Diglycidyl Ether

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SUMMARY: Currently, efforts are being made to reduce xenograft calcification and to optimize biomechanical properties by applying different crosslinking methods and techniques. Porcine aortic leaflets could be stabilized with a bisepoxy compound, butane-1,4-diol diglycidyl ether (BDDGE), under acidic (pH 4.5) or alkaline conditions (pH > 8.5). Maximum values of the shrinkage temperature (83 °C), which are comparable to glutaraldehyde-crosslinked materials, are achieved at pH 9.0 and 10.0 within 48 h with a BDDGE concentration of 4 wt%. The crosslinking efficacy decreased at higher pH values or at prolonged reaction times due to a higher proportion of one-side or masking reactions. Crosslinking of aortic leaflets at pH 4.5, which consisted in a reaction between the epoxide groups of BDDGE and the carboxylic acid groups of the tissue, afforded crosslinked material with a shrinkage temperature of 76 °C after 7 days of reaction.

Introduction

Replacement of damaged or diseased heart valves can be done with either mechanical or tissue heart valves[1-3]. The major advantages of the use of tissue valves over mechanical valves are their good hemodynamic properties and that an anticoagulant therapy is not necessary[1,2].

Tissue heart valves are nowadays made of either porcine aortic heart valves or bovine pericardium[2]. Because non-treated tissue is prone to enzymatic degradation upon implantation, commercial implantable devices are crosslinked with glutaraldehyde (GA)[4], which forms crosslinks mostly within the collagen component of the tissue. Unfortunately, the durability of such crosslinked bioprostheses is limited, mainly due to cuspal calcification. Although the mechanism and the main determinants of tissue calcification are not understood, it is generally accepted that the interaction between the host and implant, mechanical factors[5,6] as well as the GA crosslinking are the main determinants in the occurrence of calcification[7].

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Taking these events into account, new crosslinking methods have been developed. Model studies on a type I collagen matrix, dermal sheep collagen, proved that butane-1,4-diol diglycidyl ether (BDDGE) is an effective crosslinker at pH > 8.5 leading to well-stabilized materials. At relatively high pH, crosslinks can be formed between the amino groups of hydroxylysine residues and at low pH between carboxylic acid groups of aspartic and glutamic acid. Materials which were crosslinked under alkaline conditions showed high stability to enzymatic degradation, a moderate tensile strength and a high modulus at low strains[8,9]. The reaction under acidic conditions yielded materials with good mechanical properties but they proved to be less stable to enzymatic attack. However, stable materials could be obtained after successive crosslinking with a carbodiimide[10]. Subcutaneous implantation of BDDGE-crosslinked materials in male Albino Oxford rats revealed that these materials were biocompatible and non-calcifying[11].

In this paper we describe the use of BDDGE to stabilize porcine aortic heart valves. The effect of reaction time, pH and BDDGE concentration on the crosslinking density, as measured by an increase in shrinkage temperature (T_s), and the change in the amino group content were evaluated and compared by the conventional GA method. Because the use of bifunctional reagents also yields one-side or masking reactions, the effect of monofunctional glycidyl ether on the T_s of aortic leaflets was examined as well.

Materials and methods

Tissue preparation

Fresh porcine aortic heart valves were selected and dissected at a slaughterhouse (Premium Fleisch Emsland, Lingen, Germany). Residual fat and myocardium were removed as much as possible. After harvesting, the valves were rinsed with and stored in ice-cold saline (0.9 wt% sodium chloride, NPBI-Braun, Emmercompascuum, Netherlands). After transport to the laboratory, the valves were washed with a 0.01 M 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Sigma-Chemical, St. Louis, USA) buffered solution (pH 7.4) before an overnight storage in 0.01 M HEPES at 4 °C.

Crosslinking

Glutaraldehyde (GA)

Fresh valves were immersed in 100 ml of a 0.01 M HEPES-buffered solution (pH 7.4) containing 0.2 wt% glutaraldehyde (GA, 25 % aqueous solution, z.S., Merck, Darmstadt, Germany) for 1, 2, 4 or 24 h at 20 °C. After the reaction, the valves were extensively washed with deionized water before lyophilization.

Butane-1,4-diol diglycidyl ether (BDDGE)

Fresh valves were immersed in 100 ml of buffered solution containing 4.0 wt% BDDGE (technical purity, > 95 % (GC), Fluka, Buchs, Switzerland) for 144 h at 20 °C. The following buffers were chosen: 0.1 M 2-morpholinoethane-1-sulfonic acid (MES) at pH 4.5, 0.025 M $Na_2B_4O_7.10H_2O$ (z.A., Merck) at pH 8.5 and 9.0 and a carbonate buffer (0.064 M $NaHCO_3$, 0.036 M Na_2CO_3) at pH 10.0 and 10.5. After reaction, the valves were washed with deionized water before lyophilization. Subsequently, to study the effect of the reaction time on the crosslinking density, aortic valves were immersed in buffered solutions (pH 4.5, 9.0 and 10.0) containing 4.0 wt% BDDGE for 2, 4, 24, 48 and 144 h at 20 °C. The effect of the BDDGE concentration on the crosslinking rate was studied by immersing the valves in a buffered solution (pH 4.5 or 9.0) containing 0.5, 1.0, 2.0, 4.0 or 5.0 g BDDGE per 100 ml solution for 72 h (pH 9.0) or 144 h (pH 4.5) at 20 °C. After crosslinking, the valves were washed before lyophilization.

Modification

Fresh valves were immersed in 100 ml of a 0.1 M MES-buffered solution (pH 4.5) containing 4.0 g glycidyl isopropyl ether (PGE, Fluka) or in 100 ml of a carbonate (0.064 M $NaHCO_3$/ 0.036 M Na_2CO_3) buffered solution (pH 10.0) containing 1 or 4 g of PGE. The reaction was allowed to proceed for 48 h at 20 °C. The tissue was washed with deionized water followed by lyophilization.

Extent of crosslinking

Shrinkage temperature

Thermal analysis of (non)crosslinked leaflets was carried out with a Perkin-Elmer DSC7 differential scanning calorimeter. A piece of leaflet tissue, 2-4 mg, was put in a volatile sample pan (Perkin-Elmer, stainless steel) and 50 µl of phosphate-buffered saline (PBS, pH 7.4, NPBI, Emmercompascuum, Netherlands) was added. The reference pan contained only 50 µl of PBS. A heating rate of 2 °C/min was applied and the temperature range between 30 and 95 °C was chosen. The peak temperature was recorded and set as the shrinkage temperature (T_s).

Amino groups

The content of primary amino groups was determined using a slightly modified procedure as described before[8]. A weighed amount of lyophilized leaflet (2 - 6 mg) was incubated in 1.0 ml of $NaHCO_3$ (4 wt%) solution. After 30 min, 1.0 ml of a 0.5 wt% 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS, 1 M in water, Fluka) solution was added and the reaction was carried

out at 40 °C for 3 h. After derivatization, the solutions were decanted and the tissue samples were washed thoroughly with deionized water to remove the residual TNBS. Then the sample was immersed in 1.0 ml 6 M HCl and hydrolysis was carried out at 110 °C for 20 h. The hydrolyzed samples were diluted with 9.0 ml of deionized water, followed by measurement of absorbance at 345 nm. The content of primary amino groups present after crosslinking was expressed as the percentage of the initial amine content (%).

Tissue composition

Collagen content

The total collagen content of the leaflets was assessed by determining the hydroxyproline content. Because hydroxyproline originates from both collagen and elastin, an independent determination of the elastin content with elastase is used as described below. The collagen content was related to hydrolyzed control samples of noncrosslinked dermal sheep collagen (N-DSC), which is composed of 100 % type I collagen.

Fresh and lyophilized aortic leaflet samples (2-4 mg) were hydrolyzed in 1.0 ml of 6 M hydrochloric acid at 110 °C for 20 h. After hydrolysis, 1.0 ml of 6 M sodium hydroxide was carefully added to neutralize the solution to pH ~ 7.0. The solution was diluted by addition of 7.5 ml of citric acid/acetate buffer (14.25 g sodium acetate trihydrate, 9.38 g trisodium citrate dihydrate and 1.38 g citric acid monohydrate, all Merck) in 100 ml isopropyl alcohol p.A. diluted to 250 ml with deionized water (pH 6.0). Exactly 1.0 ml of the buffered hydrolyzate was reacted with 1.0 ml of 0.5 wt% chloramine T solution in citric acid/acetate buffer for 15 min at 20 °C. Thereafter, 2.0 ml of Ehrlich's reagent (7.0 g of 4-(dimethylamino)benzaldehyde in 12.5 ml of 70-72 % perchloric acid, diluted to 100 ml with isopropyl alcohol p.A., all Merck) was added and reacted for 20 min at 65 °C. The samples were cooled to 20 °C and absorbances were measured with a Uvikon 930 spectrophotometer (Kontron Instruments, Switzerland) at 555 nm. The blank was prepared by treating 1.0 ml of citric acid/acetate buffer containing no tissue sample as described above.

Elastin content

The elastin content of porcine aortic leaflets was determined by selective degradation with elastase. Elastin contains a small amount of hydroxyproline (1-2 %)[12]. The remaining elastase solutions were analyzed for the presence of hydroxyproline taking pure elastin samples as controls. Elastase (type I, from porcine pancreas, Sigma, activity 100 U/mg; 1 U will solubilize 1 mg of elastin in 20 min at pH 8.8 at 37 °C) stock solution (activity 15 U/ml) was prepared by dissolving the desired amount of elastase in 0.1 M Tris-HCl (Sigma) buffer (pH

8.8) containing 0.005 M $CaCl_2$ and 0.05 mg/ml NaN_3. This stock solution was allowed to stand at 37 °C for 1 h before use. To a tissue sample weighing 4 - 6 mg, 1.0 ml of elastase solution in Tris-HCl buffer (37 °C) was added, and degradation was carried out at 37 °C for 24 h. The reaction was terminated by the addition of 0.1 ml of 0.25 M EDTA (Titriplex III, Merck). As control materials, similar weights of N-DSC and elastin (from bovine neck ligament, Sigma) were taken and the same hydroxyproline assay as described above was used. N-DSC was not degraded by elastase, which confirms that elastase can only degrade elastin and denatured collagen.

Degree of collagen denaturation

A leaflet sample weighing 4 - 6 mg was immersed in 1.0 ml elastase solution (15 U/ml) in Tris-HCl buffer (pH 8.8) as described above. Elastase can only degrade denatured collagen[13] and, consequently, a higher degree of denaturation will lead to a higher degree of degradation. Degradation with elastase was carried out for 24 h at 37 °C. The reaction was terminated by addition of 0.1 ml of 0.25 M EDTA. An aliquot of the supernatant (0.5 ml) was hydrolyzed with 0.5 ml of 6 M HCl at 110 °C for 20 h. After hydrolysis, 0.5 ml of 6 M NaOH was added to neutralize the solution, followed by addition of 3.5 ml of citric acid/acetate buffer (pH 6.0). The amount of degraded collagen was determined by performing a hydroxyproline assay as described above on 1.0 ml of this buffered solution. The amount of hydroxyproline which is derived from collagen was calculated by subtracting the amount of hydroxyproline which originates from elastin from its total concentration in the hydrolyzate. The degree of denaturation was defined as the amount of collagen degraded by elastase divided by the initial content of collagen present in the leaflet.

Results and discussion

Composition

Porcine aortic heart leaflets are complex structures consisting of several components such as collagen, elastin, proteoglycans, glycosaminoglycans, and cells. The leaflet is composed of 58 wt % collagen, 13 wt% elastin, and 14 wt% glycosaminoglycans. The remaining 15 wt% is composed of cellular components (fibroblasts), lipids, proteoglycans, glycoproteins, etc. Under physiological conditions (pH 7.4, 37 °C), the leaflets contain about 83 wt% of water.

Crosslinking

Glutaraldehyde

Glutaraldehyde-crosslinked porcine aortic leaflets were prepared to provide a reference material. The influence of the reaction time on both the shrinkage temperature (T_s) and the

primary amino group content was determined. Glutaraldehyde crosslinking of leaflets is almost complete in 1 h (Fig. 1). The maximum value of the shrinkage temperature is 85 °C. However, the content of residual amino groups still decreased from 44 % after 1 h to 25 % after 24 h. This implies that amino groups react with glutaraldehyde without the formation of crosslinks (masking reactions)[13].

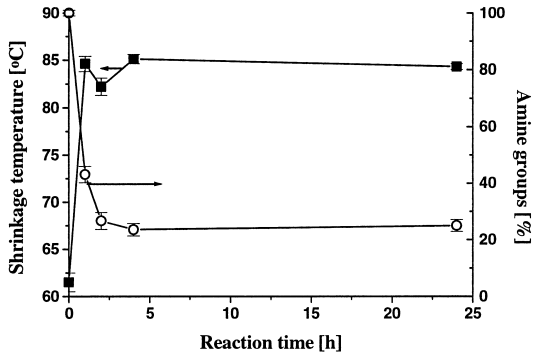
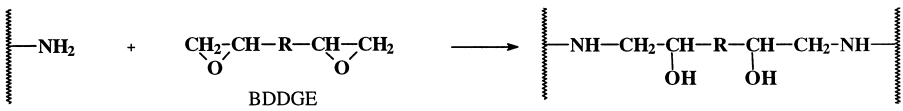


Figure 1. The shrinkage temperature (■) and the content of amino groups (O) after glutaraldehyde crosslinking of porcine aortic leaflets (0.2 wt% GA, 0.01 M HEPES, 20 °C, 100 ml, pH 7.4) as a function of the crosslinking time (n=2).

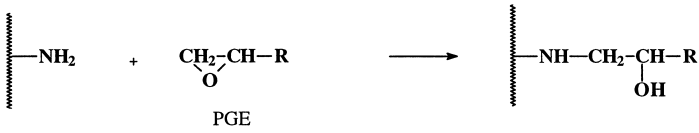
Butane-1,4-diol diglycidyl ether (BDDGE)

Butane-1,4-diol diglycidyl ether (BDDGE) has proved to be an effective crosslinker of collagen type I (Scheme 1); the reaction rate and mechanisms are dependent on the solution pH[8]. Contrary to GA fixation, the use of epoxy compounds affords more soft and pliable materials, which is advantageous in the intended application.

BDDGE crosslinking



PGE masking



Scheme 1. Reactions of free amino groups of collagen with BDDGE or PGE at alkaline pH.

The influence of pH on the T_s and the amino group content in porcine aortic leaflets after reaction with a 4 wt% BDDGE solution for 144 h were determined (Fig. 2).

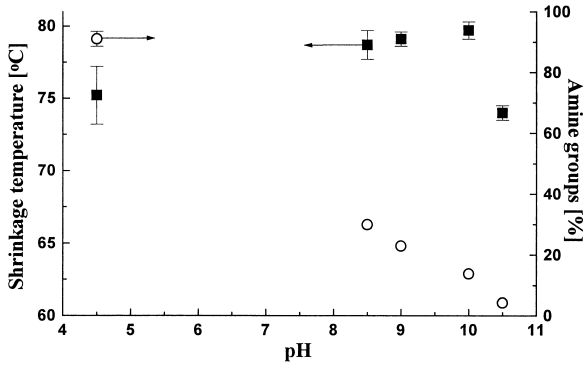


Figure 2. The shrinkage temperature (■) and the content of amino groups (○) as a function of the solution pH in the BDDGE crosslinking of porcine aortic leaflets (4 wt% BDDGE, 20 °C, 144 h, 100 ml).

Crosslinking of leaflets under alkaline conditions (pH > 8.0) caused a reaction with the amino groups of hydroxylysine residues. Changing pH from 8.5 to 10.5 resulted in a higher conversion of amino groups and in a less efficient crosslinking reaction (lower T_s). The highest T_s values were obtained at pH 9.0 or 10.0, while the lowest content of amino groups was found in materials which were crosslinked at pH 10.5. At high pH values, the reaction rate is high and most amino groups react with the BDDGE molecules at the early stage of the crosslinking process. The pendant epoxide groups may not find an adjacent amino group any longer to form a crosslink, which results in a considerable amount of one-side (masking) reactions causing a lower T_s due to the destabilization or denaturation of the triple helix. It also appeared that crosslinking with a 4 wt% BDDGE solution for 72 h was sufficient for optimum crosslinking of the leaflets. A lower concentration used under the same crosslink conditions resulted in materials with lower T_s. Moreover, application of a higher BDDGE concentration did not result in a higher crosslink density as judged from the T_s and the content of amino groups after reaction.

Crosslinking under acidic conditions (pH 4.5) demonstrated that predominantly carboxylic acid groups are involved in the crosslinking reactions. Crosslinking at pH 4.5 for 144 h led to a T_s of 75 °C without significantly altering the content of amino groups. In separate experiments, it was found that an increase in the BDDGE concentration accelerated the

crosslinking reaction and that the T_s of the leaflets could be further increased to 77 °C when a 5 wt% solution was used. The content of residual amino groups was approximately 90 % in all samples. The kinetics of the crosslinking reaction at pH 9.0 and 10.0 was also examined. The reaction at pH 10.0 was faster than that at pH 9.0 as manifested by a fast initial increase in T_s and reduction in the amino group content (Fig. 3).

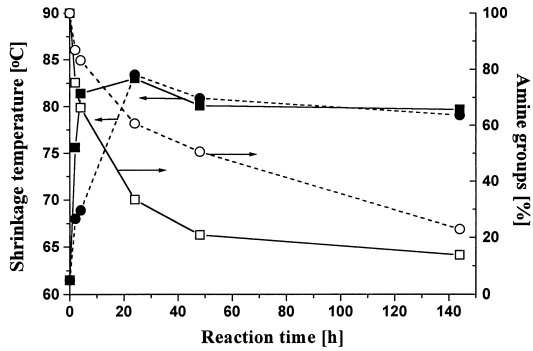


Figure 3. The shrinkage temperature (● pH 9.0 and ■ pH 10.0) and the amino group content (O pH 9.0, □ pH 10.0) as a function of the BDDGE (4 wt%) crosslinking time during fixation of aortic leaflets at pH 9.0 or 10.0 (0.025 M $Na_2B_4O_7 \cdot 10\ H_2O$ or 0.064 M $NaHCO_3$/ 0.036 M Na_2CO_3, 100 ml solution, 20 °C). (The error bars have been omitted for convenience of comparison.)

The maximum value of T_s at pH 9.0 or 10.0 was 83 °C, followed by a slight decrease to 78-79 °C. The content of amino groups still decreases after 48 h, while the T_s does not increase any longer.

Masking

In literature, minor attention has been paid to the influence of masking reactions (Scheme 1) on thermal stability of the collagen material. In a previous study[8], it was found that the reaction of dermal sheep collagen with glycidyl isopropyl ether at pH 10.0 caused a decrease in the T_s. Other groups found similar results after reaction of collagen with monofunctional reagents such as glycidyl methyl ether, or with modifying agents such as succinic or acetic anhydride; they concluded that masking or branching of the amino groups destabilized the collagen triple-helical conformation and packing[14-16]. The effect of masking amino groups with glycidyl isopropyl ether (PGE) on the T_s of porcine aortic leaflets was studied. Furthermore, the degree of collagen denaturation was determined by exposure of the modified leaflets to an elastase solution, which is able to degrade elastin and denatured collagen. The

degree of collagen denaturation was expressed as the amount of collagen degraded with elastase divided by the initial collagen content of the leaflets.

Using a higher concentration of PGE resulted in a larger decrease in the amino group content (Table 1). Furthermore, the T_s was dramatically reduced and a higher degree of collagen denaturation was observed when more amino groups reacted. Treatment of leaflets with PGE under acidic conditions resulted in a material with a slightly increased T_s and hardly any change in amino groups. No denaturation of collagen was found. The exact reason for this behavior is not known.

Table 1. The T_s (n=2), the content of amino groups (n=3) and the degree of collagen denaturation (n=3) for PGE-modified aortic leaflets.

Reagent	Reaction time (h)	PGE (wt%)	T_s (°C)	NH_2 (%)	Degree of denaturation (%)
None	--	--	61 ± 1	100 ± 3	0
PGE (pH 10)	48	1.0	59 ± 1	77 ± 2	6 ± 1
PGE (pH 10)	48	4.0	55 ± 1	36 ± 2	35 ± 2
PGE (pH 4.5)	48	4.0	64 ± 1	106 ± 6	0

It is concluded that the extent of masking reactions has to be minimized in order to prevent the introduction of reactive pendant groups, which are still able to react with proteins, and to avoid destabilization of the material. Additional studies have to be carried out with BDDGE-crosslinked heart valve tissue to evaluate the applicability of this method in fabrication of bioprosthetic heart valves.

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