ELSEVIER

Biomaterials 20 (1999) 921–931

Biomaterials

Successive epoxy and carbodiimide cross-linking of dermal sheep collagen

Raymond Zeeman[a,*], Pieter J. Dijkstra[a], Pauline B. van Wachem[b], Marja J.A. van Luyn[b], Marc Hendriks[c], Patrick T. Cahalan[c], Jan Feijen[a]

[a] *Department of Chemical Technology, and Institute of Biomedical Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, Netherlands*
[b] *Faculty for Medical Sciences, Cell Biology and Biomaterials, University of Groningen, Bloemsingel 10/B2, 9712 KZ, Groningen, Netherlands*
[c] *Medtronic Bakken Research Center B.V., Endepolsdomein 5, 6229 GW Maastricht, Netherlands*

Received 13 November 1998; accepted 27 November 1998

Abstract

Cross-linking of dermal sheep collagen (N-DSC, $T_s = 46°C$, number of amine groups = 31 (n/1000)) with 1,4-butanediol diglycidyl ether (BDDGE) at pH 9.0 resulted in a material (BD90) with a high T_s (69°C), a decreased number of amine groups of 15 (n/1000) and a high resistance towards collagenase and pronase degradation. Reaction of DSC with BDDGE at pH 4.5 yielded a material (BD45) with a T_s of 64°C, hardly any reduction in amine groups and a lower stability towards enzymatic degradation as compared to BD90. The tensile strength of BD45 (9.2 MPa) was substantially improved as compared to N-DSC (2.4 MPa), whereas the elongation at break was reduced from 210 to 140%. BD90 had a tensile strength of 2.6 MPa and an elongation at break of only 93%. To improve the resistance to enzymes and to retain the favorable tensile properties, BD45 was post-treated with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) in the presence of *N*-hydroxysuccinimide (NHS) to give BD45EN. Additional cross-linking via the formation of amide bonds took place as indicated by the T_s of 81°C and the residual number of amine groups of 19 (n/1000). BD45EN was stable during exposure to both collagenase and pronase solutions. The tensile properties (tensile strength 7.2 MPa, elongation at break 100%) were comparable to those of BD45 and glutaraldehyde treated controls (G-DSC). Acylation of the residual amine groups of BD45 with acetic acid *N*-hydroxysuccinimide ester (HAc-NHS) yielded BD45HAc with a large reduction in amine groups to 10 (n/1000) and a small reduction in T_s to 62°C. The stability towards enzymatic degradation was reduced, but the tensile properties were comparable to BD45.

Keywords: Collagen; Cross-linking; Epoxy compounds; Carbodiimide; In-vitro degradation; Mechanical properties

1. Introduction

Cross-linking of collagen-based materials is an effective method to modify the stability towards enzymatic degradation and to optimize the mechanical properties. Furthermore, the antigenicity will be decreased affording more biocompatible materials [1–4]. Traditionally, glutaraldehyde has been used as a cross-linking agent, but with the increased dissatisfaction over the performance of glutaraldehyde cross-linking as a pre-implantation treatment of tissue-derived biomaterials [4–11], several alternative cross-linking methods have been investigated. These methods can be divided into two groups. The first group comprises bifunctional or multifunctional reagents such as diisocyanates [12] and epoxy compounds [13], which bridge amine groups between two adjacent polypeptide chains. The second group covers reagents which activate carboxylic acid groups of glutamic or aspartic acid residues to react with amine groups of another chain providing cross-links with the formation of amide bonds. Carbodiimides have been generally used to generate the active esters [14, 15].

Reagents based on glycidyl ethers have gained increased attention to cross-link collagen-based tissue. Cross-linking with such reagents results in collagen-based tissues with a good stability towards enzymatic degradation in combination with excellent mechanical properties [16–20].

* Corresponding author. Fax: +0031-53-4893823.

0142-9612/99/$ – see front matter
PII: S0142-9612(98)00242-7

In a previous study [21], the use of 1,4-butanediol diglycidyl ether (BDDGE) for cross-linking of dermal sheep collagen has been evaluated. Collagen cross-linking was performed at pH values of 8–10. Under these conditions, cross-linking will mainly involve amine groups. Acidic conditions (pH 4–6) during the fixation process will evoke a different reaction mechanism in which the epoxide groups react with carboxylic acid groups [21–23]. These different cross-linking conditions resulted in large differences in material properties. The resistance towards enzymatic degradation of dermal sheep collagen (N-DSC) cross-linked with BDDGE at pH 8–10 was much higher than materials cross-linked at pH 4–6. However, DSC cross-linked at pH 4.5 had a higher tensile strength and elongation at break as compared to DSC cross-linked at pH 8–10. Moreover, N-DSC cross-linked at pH 4.5 was flexible and pliable, whereas DSC cross-linked at pH 8–10 was rigid and stiff.

In order to retain the excellent tensile properties of collagen cross-linked at pH 4.5, and to increase the resistance towards enzymatic breakdown, a subsequent cross-linking step using a water-soluble carbodiimide was introduced. In this paper, the two-step cross-linking procedure of N-DSC will be described. The resulting materials will be evaluated with respect to their stability in contact with enzyme solutions and their mechanical properties.

2. Methods

2.1. Preparation of non-cross-linked dermal sheep collagen (N-DSC)

Dermal sheep collagen (DSC) was obtained from the Zuid-Nederlandse Zeemlederfabriek (Oosterhout, Netherlands). In brief, the skin was depilated and immersed in a lime-sodium sulfide solution to remove the epidermis. Non-collagenous substances were removed using proteolyic enzymes whereafter the skin was split to obtain the dermal layer. The remaining fibrous collagen network was washed with water (4 times), with acetone (2 times) and with deionized-water (2 times) before lyophilization (N-DSC) [24].

2.2. Cross-linking

About 1 g of N-DSC was immersed in 100 ml of a buffered solution containing 4 wt% 1,4-butanediol diglycidyl ether (BDDGE, Fluka, Buchs, Switzerland). The solution was buffered either with 0.05 M 2-[*N*-morpholino]ethanesulfonic acid (MES, Merck, Darmstadt, Germany) at pH 4.5 or with 0.025 M di-sodiumtetraborate-decahydrate ($Na_2B_4O_7 \cdot 10H_2O$ z.A., Merck, Darmstadt, Germany) at pH 9.0. Cross-linking was performed at room temperature for 7 d. After cross-linking, the samples were extensively washed with deionized-water before lyophilization.

In addition, samples cross-linked at pH 4.5 were either treated with HAc-NHS (Procedure A) or with EDC and NHS (Procedure B).

Procedure A: Amine groups were acylated by reaction with acetic acid *N*-hydroxy succinimide ester (HAc-NHS, Sigma Chemicals, St. Louis). The cross-linked sample was immersed in a buffered solution (0.05 M MES; pH = 6.8) containing 2.6 g of HAc-NHS (25 times molar excess with respect to the collagen amine groups). After 16 h of reaction at room temperature, the sheet was washed with deionized-water before lyophilization.

Procedure B: A second cross-linking step was carried out by immersing a collagen sample in a buffered solution (0.05 M MES; pH = 5.5) containing 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide. HCl (EDS z.S., Merck-Suchardt, Hohenbrunn, Germany) and *N*-hydroxy-succinimide (NHS z.S., Merck-Suchardt, Hohenbrunn, Germany) in a molar ratio to the initial collagen carboxylic acid groups of 5:2:1. After cross-linking for 2 h at room temperature, the sample was washed with 0.1 M NaH_2PO_4 for 2 h and with deionized water before lyophilization.

Glutaraldehyde (GA, purified [25] by distillation (b.p. 80°C, 16 mmHg) from 25% aqueous solution z.S., Merck, Darmstadt, Germany) cross-linking was performed by immersing 1 g of N-DSC in 100 ml of a 0.5 wt% GA solution in a phosphate buffer (0.054 M Na_2HPO_4, 0.013 M NaH_2PO_4, pH 7.4) for 1 h at room temperature. After cross-linking the sample was rinsed with tap-water (15 min), washed with 4 M NaCl (2 times 30 min) and deionized-water (4 times 30 min) before lyophilization.

2.3. Shrinkage temperature

The degree of cross-linking of the samples was related to the increase of the shrinkage (denaturation) temperature (T_s). T_s values were determined using an apparatus similar to that described in IUP/16 [26]. Test specimens were cut, mounted and hydrated. A heating rate of 2°C/min was applied and the onset of shrinkage was recorded as T_s. Thermal analysis of (non)-cross-linked DSC were performed on a Perkin-Elmer DSC-7 Differential Scanning Calorimetry which was calibrated with Indium and Gallium. A collagen sample (3–6 mg) was put in a volatile sample pan (Perkin-Elmer, stainless steel) and 50 l of a phosphate buffered saline solution (PBS, 0.14 M NaCl, 0.01 M Na_2HPO_4, 0.002 M NaH_2PO_4, pH 7.4, NPBI, Emmercompascuum, Netherlands) was added. The reference contained 50 l PBS. A heating rate of 2°C/min was used and a temperature interval between 30 and 95°C was chosen. The peak temperature, the transition enthalpy (ΔH_s) and the transition interval (ΔT) were determined from the thermograms.

2.4. Amine group content

The primary amine group content (lysine residues) of cross-linked samples was determined using 2,4,6-trinitrobenzenesulfonic acid (TNBS). To a collagen sample of 2–4 mg of subsequently 1.0 ml of a 4 wt% $NaHCO_3$ solution (pH 9.0) and 1.0 ml of a freshly prepared 0.5 wt% TNBS (Fluka, Buchs, Switzerland) solution in distilled water was added. After reaction for 2 h at 40°C, 3.0 ml of 6 M HCl was added and the temperature was raised to 60°C. Solubilization of collagen was achieved within 90 min. The resulting solution was diluted with 5.0 ml demi-water and the absorbance was measured at 345 nm. A control was prepared applying the same procedure except that HCl was added before the addition of TNBS. The amine group content was expressed either as the number of amine groups per 1000 amino acids (n/1000). An ε of 14.600 l/mol cm was used for the calculations.

2.5. Determination of the amount of pendant groups

Cross-linked materials weighing 0.10 g were immersed in 10 ml of a buffered solution (0.064 M $NaHCO_3$, 0.036 M Na_2CO_3, pH 10.0) containing 0.5 M lysine methyl ester dihydrochloride (Sigma Chemicals, St. Louis, USA) for 72 h at 20°C. After reaction with aldehyde or epoxide groups, the materials were thoroughly washed with 1.0 M NaCl (3 times 30 min) and 5 times 30 min with deionized water before lyophilization. The amount of pendant epoxide or aldehyde groups was calculated from the difference in amine group content, which is determined via a TNBS-assay as described above, before and after reaction with lysine methyl ester.

2.6. Enzymatic degradation

2.6.1. Collagenase

The degradation of (non)-cross-linked collagen samples was performed using bacterial collagenase from *Clostridium histolyticum* (EC 3.4.24.3, Sigma-Chemical, St. Louis, USA) with a collagenase activity of 283 U/mg (one unit will release peptides from native collagen, equivalent in ninhydrin color to 1.0 μmol of L-leucine in 5 h at pH 7.4 at 37°C in the presence of calcium ions). In a typical experiment, 15 mg samples of (non)-cross-linked DSC were immersed in 1.0 ml of a 0.1 M Tris-HCl buffer (pH = 7.4) containing 0.005 M $CaCl_2$ and 0.05 mg/ml NaN_3. After 1 h, 1.0 ml collagenase solution (200 U/ml) in Tris-HCl buffer (37°C) was added to give the desired concentration (100 U/ml).

The degradation was discontinued at the desired time interval by addition of 0.2 ml 0.25 M EDTA (Titriplex III, Merck, Darmstadt, Germany) solution. The mixtures were cooled on ice. A gravimetrical method was used to determine the weight-loss of the samples. The collagenase solution was decanted whereafter the remaining pellet was washed with Tris-HCl buffer (3 times for 15 min) and 3 times with deionized water before lyophilization. The changes in weight of partially degraded samples were expressed as the percentage of the initial weight.

2.6.2. Pronase

A pronase stock solution was prepared by dissolving a calculated amount of pronase (from *Streptomyces griseus*) lyophilizate (Boehringer Mannheim, 7000 U/g lyophilizate) into a 0.1 M Tris-HCl buffer solution (pH = 7.4) containing 0.005 M $CaCl_2$ and 0.05 mg/ml NaN_3. The final pronase concentration was 20 U/ml. This stock solution was incubated at 37°C for 1 h.

In a typical experiment, 15 mg of (non)-cross-linked DSC was immersed in 5.0 ml pronase solution (37°C). The degradation was discontinued at the desired time interval by addition of 0.5 ml 0.25 M EDTA, after which the mixtures were cooled on ice. The pronase solution was decanted and the remaining disk was washed with Tris-HCl buffer (3 times 15 min) and with demi-water (3 times 15 min) before lyophilization. The remaining weight of the disk was determined gravimetrically.

2.7. Mechanical properties

Stress–strain curves of DSC samples were determined by uniaxial measurements using a Zwick (Z020) mechanical tester. Because of variations in the mechanical properties of different parts of the sheep skin, only samples were taken from the IUP/2 [27] sampling area parallel to the backbone.

Tensile bars (40.0 mm × 4.0 mm × 1.4 mm) were cut using a dumb-bell shaped knife and hydrated for at least 1 h in PBS at room temperature. The thickness of the samples were measured in triplicate using a spring-loaded type micrometer (Mitutoyo, Tokyo, Japan). An initial gauge length of 10 mm was used and a crosshead speed of 5 mm/min was applied until rupture of the test specimen occurred. A pre-load of 0.05 N was applied to pre-stretch the specimen before the real measurement. The tensile strength, the elongation at alignment, the elongation at break, the low strain modulus and the high strain modulus of the sample were calculated from five independent measurements.

3. Results

3.1. Cross-linking

Cross-linking of N-DSC (**I**) using a diglycidyl ether (Fig. 1) involves reaction of the epoxy groups with amine groups of (hydroxy)lysine residues (**II**) when alkaline conditions are applied or with two carboxylic acid residues (aspartic or glutamic acid) if cross-linking is performed

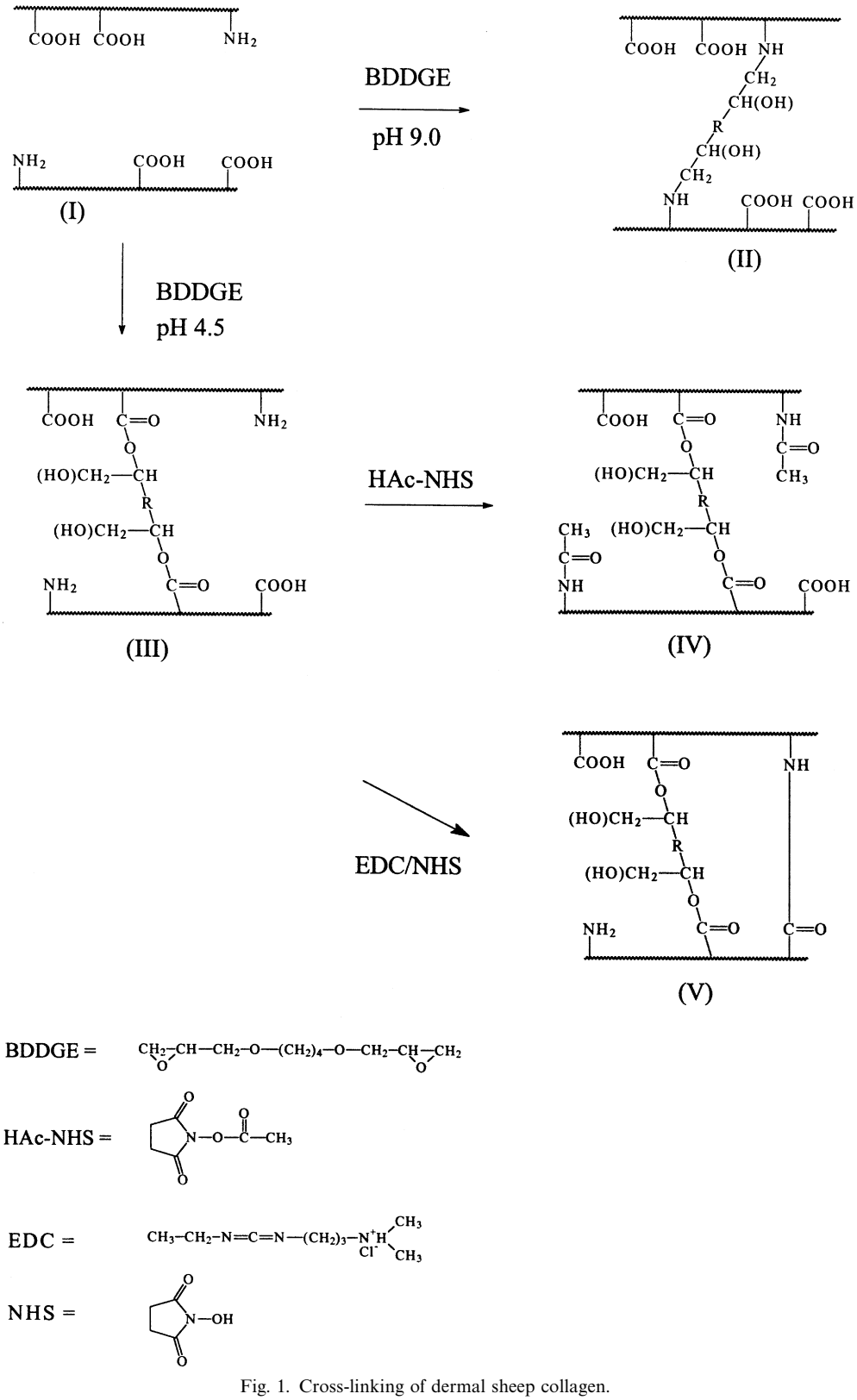

Fig. 1. Cross-linking of dermal sheep collagen.

under acidic conditions (**III**). Acylation of the remaining amine groups of (**III**) leads to structure (**IV**). No additional cross-links are formed during this reaction. Cross-linking of (**III**) using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide.HCl (EDC) and *N*-hydroxysuccimide (NHS) will result in the formation of additional cross-links. EDC/NHS cross-linking involves the activation of the residual carboxylic acid groups with EDC to give an

Table 1
Shrinkage temperature, amine and pendant aldehyde or epoxide group contents of (non) cross-linked dermal sheep collagen

Material	Shrinkage temperature (°C)	Amine group content [n/1000]	Pendant aldehyde or epoxide groups [n/1000]
N-DSC	46 ± 1	31.0 ± 1.0	—
G-DSC	70 ± 1	9.1 ± 0.7	5.2 ± 1.5
BD90	69 ± 4	14.6 ± 1.0	0.5 ± 0.3
BD45	64 ± 4	27.0 ± 0.3	0.5 ± 0.1
BD45HAc	62 ± 4	10.5 ± 3.0	1.5 ± 0.4
BD45EN	81 ± 3	17.7 ± 2.6	1.8 ± 1.0

Note: N-DSC = non-cross-linked collagen.
G-DSC = Glutaraldehyde cross-linked collagen (0.5 wt%, 1 h, phosphate buffer (pH 7.4)).
BD90 = Butanediol diglycidylether cross-linked collagen at pH 9.0 (4 wt% solution, 7 d).
BD45 = Butanediol diglycidylether cross-linked collagen at pH 4.5 (4 wt% solution, 7 d).
BD45HAc = Butanediol diglycidylether cross-linked collagen at pH 4.5, followed by treatment with the NHS ester of acetic acid (0.05 M MES buffer, molar ratio HAc-NHS:NH_2 (collagen) = 25:1, 16 h).
BD45EN = Butaneidiol diglycidylether cross-linked collagen at pH 4.5, followed by EDC/NHS cross-linking (0.05 M MES buffer, molar ratio EDC:NHS:COOH (collagen) = 5:2:1, 2 h).
The amine, the pendant epoxide and aldehyde group contents are expressed as the number of amine groups per 1000 amino acids (n = 3).

O-acylisourea group. In the presence of NHS, this group is converted to a NHS activated carboxylic acid group, which is highly reactive towards amine groups and a cross-link is formed through formation of an amide bond ('zero'-length [28] cross-link).

The shrinkage (denaturation) temperature (T_s) and the decrease in amine groups of the cross-linked materials were determined and used as a measure of the degree of cross-linking (Table 1). Cross-linking of the dermal sheep collagen under the different conditions applied results in an increase of the shrinkage (denaturation) temperature. Glutaraldehyde cross-linking (G-DSC) which involves reaction with the amine groups present in the collagen results in an increase of the T_s to 70°C and in a large decrease of the content of amine groups to 9.1 per 1000 amino acid residues. A similar T_s value was obtained upon cross-linking with the bis-epoxy compound (BDDGE) at pH 9.0. The content of amine groups was reduced to 14.6 per 1000 amino acid residues. Cross-linking with BDDGE at pH 4.5 appeared to give collagen materials with an increase in T_s to 64°C but only a small decrease of amine groups to 27.0 per 1000 amino acid residues. When the collagen cross-linked at pH 4.5 was treated with the acylating agent HAc-NHS, the content of amine groups decreased to a value of 10.5 per 1000 amino acid residues. This modification step of the collagen resulted in a slightly lower shrinkage temperature. Applying a second cross-linking step to the epoxy cross-linked material (BD45) using EDC and NHS resulted in a material with a high T_s (81°C) and a decrease of amine groups from 27 to 17.7 per 1000 amino acid residues. The content of free epoxide groups after cross-linking is very low (Table 1), whereas G-DSC contained about 5 n/1000) free aldehyde groups.

Table 2
Characteristics of the shrinkage endotherm of (non) cross-linked DSC

Material	T_{peak} (°C)	ΔT (°C)	ΔH_s (J/g tissue)	Peak shape
N-DSC	39.4	12.3	15.1	Non symmetric
G-DSC	68.8	10.0	10.7	Non symmetric
BD90	72.8	8.8	7.6	Non symmetric
BD45	69.6	4.0	16.9	Symmetric
BD45HAc	66.7	5.6	16.6	Symmetric
BD45EN	81.2	8.5	19.3	Symmetric

The abreviations of the materials are explained in Table 1.
ΔT: width of the transition peak (Fig. 2).

The endothermic shrinkage transition of the collagen samples was also analyzed by differential scanning calorimetry (Table 2). Cross-linking not only increases the T_s, but also results in a more narrow transition. BDDGE cross-linking of N-DSC under acidic conditions (BD45) resulted in a material having a T_s with a narrow and symmetrically shaped transition peak. On the contrary, collagen cross-linked via the amine groups (G-DSC and BD90) afforded materials with a lower transition enthalpy (ΔH_s) and a non-symmetric transition peak (Fig. 2).

3.2. Macroscopic appearance

There were some differences in the macroscopic appearance of the materials obtained. N-DSC was a white, soft and pliable tissue, while G-DSC was yellow, stiff and rigid. BD90 was a white colored material, which was rigid. On the contrary, the BD45 materials retained the macroscopic characteristics of N-DSC and were slightly

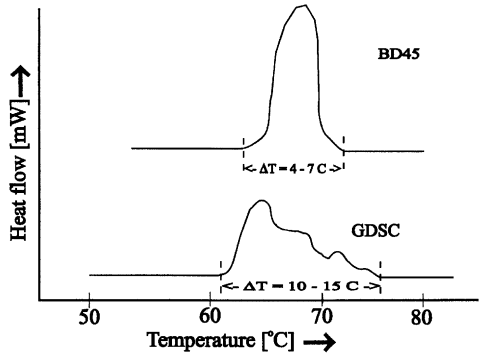

Fig. 2. Differential scanning calorimetry curves of 1,4-butanediol diglycidyl ether (BD45) and glutaraldehyde (GDSC) cross-linked dermal sheep collagen.

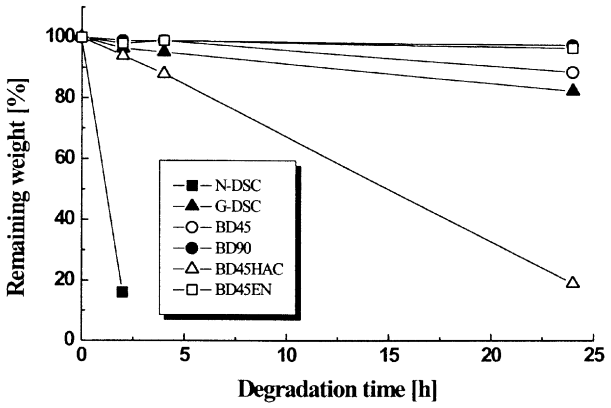

Fig. 3. Remaining weight of collagen samples as a function of the degradation time during exposure to a bacterial collagenase solution (pH 7.4, 37°C, $n = 3$, abbreviations are explained in Table 1).

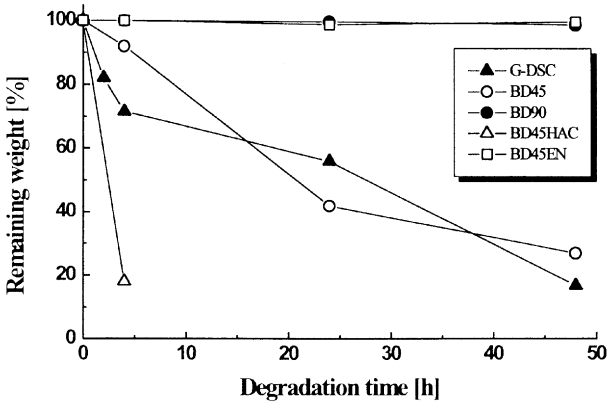

Fig. 4. Remaining weight of collagen samples as a function of the degradation time during exposure to a pronase solution (pH 7.4, 37°C, $n = 3$, abbreviations are explained in Table 1).

yellow colored. Treatment of BD45 with either HAc-NHS or EDC and NHS did not alter the macroscopic properties.

3.3. Enzymatic degradation

The degradation of the N-DSC samples were studied by exposing materials to either a collagenase or a pronase solution. The change in weight of the different collagen samples was determined as a function of the degradation time.

N-DSC was degraded within 5 h in the collagenase solution and within 30 min in the pronase solution. Figures 3 and 4 show the excellent resistance against enzymatic attack of collagen cross-linked with BDDGE at pH 9.0 (BD90) and the collagen first cross-linked by BDDGE at pH 4.5 and subsequently by the EDC/NHS method (BD45EN). Dermal sheep collagen cross-linked with BDDGE at pH 4.5 (BD45) afforded a less stable material, that was slightly degraded after 24 h in a collagenase solution and much more degraded after 48 h in a pronase solution. The resistance towards enzymatic degradation was dramatically decreased when the remaining amine groups were acylated (BD45HAc). Glutaraldehyde cross-linked collagen (G-DSC) has a similar stability as BD45.

3.4. Mechanical properties

The influence of the cross-linking process on the mechanical properties of the collagen material was determined by uniaxial tensile measurements (Table 3). Generally, cross-linking increase the tensile strength and both the low and high strain modulus and decreases the elongation at break.

There is a significant difference in mechanical properties between DSC cross-linked with BDDGE at 4.5 (BD45) or at pH 9.0 (BD90). The latter had both a low tensile strength and elongation at break, whereas BD45 had a very high tensile strength and a moderate elongation at break. The tensile properties of BD45 were comparable to those of G-DSC.

Successive cross-linking of BD45 with EDC/NHS to give BD45EN did not alter the mechanical properties to a large extent. Compared to BD45, BD45EN had a somewhat lower elongation at break. If the remaining amine groups of BD45 were acylated with HAc-NHS to give BD45HAc, only the tensile strength and the high strain modulus were slightly decreased.

4. Discussion

Polyglycidyl ethers have gained much attention as cross-linking reagents for collagen based biomaterials during recent years [13, 16–20, 29]. It has been shown that these reagents are effective cross-linkers for pericardium, vascular grafts and porcine aortic heart valves. Fixation of collagen using polyglycidyl ethers gives materials with good mechanical properties [18, 19, 30], a decreased calcification and a lower cytotoxicity [31] compared to glutaraldehyde cross-linked tissue. However, use of these reagents results in an undefined cross-linked structure because of the poly-functionality. Furthermore, it is likely that a considerable amount of pendant epoxide groups are introduced, which are still able to react with proteins.

Recently, cross-linking of dermal sheep collagen (N-DSC) using 1,4-butanediol diglycidyl ether (BDDGE) at pH between 8.5 and 10.5 has been described [21]. Cross-linking involves the reaction of amine groups of (hydroxy) lysine residues with epoxide groups of the BDDGE molecules, resulting in formation of secondary amines. The enzymatic resistance of the materials was excellent as shown by the absence of degradation after 48 h of incubation in a bacterial collagenase or a pronase

Table 3
Mechanical characteristics of (non) cross-linked dermal sheep collagen

Sample	Tensile strength (MPa)	Elongation at break (%)	Elongation at alignment (%)	Low strain modulus (MPa)	High strain modulus (MPa)
N-DSC	2.6 ± 0.4	210 ± 40	44 ± 8	0.8 ± 0.1	2.4 ± 0.4
G-DSC	7.9 ± 0.2	160 ± 50	34 ± 8	2.0 ± 0.4	5.7 ± 1.0
BD90	2.6 ± 0.1	90 ± 10	15 ± 2	1.8 ± 0.5	4.3 ± 0.6
BD45	9.4 ± 0.2	130 ± 20	27 ± 5	2.9 ± 0.8	9.4 ± 1.0
BD45HAc	7.1 ± 0.6	140 ± 20	23 ± 6	2.3 ± 0.2	7.0 ± 0.6
BD45EN	7.2 ± 0.5	100 ± 5	23 ± 2	2.4 ± 0.4	9.5 ± 1.5

solution. Furthermore, the tensile strength was slightly increased while elongation at break was largely reduced as compared to N-DSC [32].

Reaction of epoxide groups with carboxylic acid groups of glutamic or aspartic acid residues of N-DSC can be achieved at pH 4.5 (BD45) resulting in formation of ester containing cross-links [21]. However, the stability of this material towards collagenase and pronase is lower than of DSC cross-linked at pH 8.5–10.5. On the other hand, the mechanical properties of BD45 appeared to be superior as reflected in a high tensile strength and a high elongation at break. Moreover, a flexible material was found if cross-linking was carried out under acidic conditions, while cross-linking under alkaline conditions resulted in a material with a high stiffness in flexure [32]. In order to obtain a material with the same mechanical and macroscopic properties of BD45 but with a higher resistance towards enzymatic degradation, an additional cross-linking step was applied with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) in combination with *N*-hydroxysuccinimide (NHS). In this procedure, carboxylic acid groups are activated by EDC and NHS which subsequently react with adjacent amine groups forming amide bonds [14].

The denaturation temperature (T_s) and the percentage of amine groups after cross-linking using the above described reagents and conditions are summarized in Table 1. Glutaraldehyde cross-linking of N-DSC resulted in an increase of T_s from 46 to 70°C and in a decrease in amine group content from 31.0 to 9.1 (n/1000). N-DSC cross-linked with BDDGE at pH 9.0 (BD90) had a comparable T_s of 67°C as G-DSC. However, the content of amine groups after cross-linking was considerably higher, namely 14.6 (n/1000). This suggests that the efficacy of BDDGE cross-linking is higher than the GA cross-linking. Both materials were treated with an excess of lysine methylester to block the residual epoxide or aldehyde groups. An increase in amine groups of 5.2 (n/1000) was obtained for G-DSC. For BD90, the same treatment resulted in an increase in amine groups of only 0.5 (n/1000), which is in agreement with previous results [33] and suggests that hardly any pendant epoxide groups were left in the matrix. These results prove the higher cross-linking efficacy of BD90 as compared to G-DSC.

BDDGE cross-linking of N-DSC under acidic conditions at pH 4.5 [21] involves carboxylic acid groups. The high content of residual amine groups ($27n$/1000) after cross-linking confirms this mechanism. Nevertheless, it appears that a few amine groups were involved in the cross-linking reactions. A slightly lower T_s was observed compared to BD90. The additional EDC/NHS cross-linking of BD45 increased the T_s from 64 to 81°C. Furthermore, the amine content decreased from 27.0 to 17.7 (n/1000), which means that 9 (n/1000) amine cross-links [14] were introduced. Obviously, BDDGE cross-linking does not inhibit further cross-linking and two different types of bridges will be present in the collagen matrix. Both BD45 and BD45EN contain a low amount of pendant epoxide groups after cross-linking, probably due to the high rate of hydrolysis of epoxide groups at pH 4.5 [21]. Blocking of the residual amine groups of BD45 with acetic acid *N*-hydroxysuccinimide ester (HAc-NHS), resulted in a slight decrease in T_s which is ascribed to the destabilization effect of the acylated amines [34, 35].

The influence of the cross-linking procedure on the T_s of the cross-linked material can also be determined using differential scanning calorimetry. In general, cross-linking results in a narrower transition peak [36, 37], and in a higher transition enthalpy as a result of a better organization and stabilization of the helices. Cross-linking with BDDGE at pH 4.5 gave a material showing a narrow, symmetric peak, combined with a high transition enthalpy while cross-linking at pH 9.0 resulted in a material exhibiting a non-symmetric and broad transition peak combined with a low transition enthalpy. This may indicate that cross-linking via the amines resulted in a less homogeneous distribution of the cross-links and in a lower degree of three-dimensional organization and packing of the collagen helices. The broad transition peak obtained in G-DSC, and hence the inhomogeneous distribution of cross-links in agreement with the hypotheses of Cheung et al. [38]. When the amine groups of BD45 were acylated by HAc-NHS (BD45HAc) or when a second cross-linking step was applied (BD45EN),

materials were obtained which showed shrinkage endotherms with similar characteristics as for BD45. The highest transition enthalphy (19.3 J/g) was measured for BD45EN, which implies that successive cross-linking resulted in a well-organized and stabilized material.

The degradation behavior of (non)-cross-linked collagen was determined with the enzymes, collagenase and pronase. Bacterial collagenase from *Clostridium histolyticum* is capable of cleaving peptide bonds within the triple helical stucture and has a specificity for the Pro-X–Gly–Pro-Y region, splitting betwen X and Gly. This region is found about 40 times in an α-chain. Pronase, which is a mixture of unspecific endo- and exo-proteases, from *Streptomyces grisseus* cleaves bonds in the non-helical regions of collagen molecules. Pronase might be able to cleave bonds between helices and (micro)fibrils, thus interrupting the aggregates of collagen helices and microfibrils. This cleaving mechanism will lead to triple-helices and fragments of microfibrils from the collagen structure, which either will dissolve or become suspended as insoluble fragments in the enzyme solution.

Enzymatic degradation of collagen materials is dependent on and determined by its helical integrity, the degree of cross-linking and the availability of the cleavage sites. Whereas N-DSC was fully degraded within 5 h, cross-linking enhances the stability of collagen-based materials against enzymatic degradation. BD90 and BD45EN appeared to be the most stable materials and hardly any degradation was observed. In contrast, glutaraldehyde cross-linked collagen (G-DSC) revealed some degradation in collagenase solution and the material was almost fully fragmented in a pronase solution. Previous studies using G-DSC showed that almost no degradation took place after 24 h of exposure to a collagenase solution [7, 39]. On the other hand, studies with G-DSC using pronase also showed that a significant degradation took place.

BD45 appeared to be more susceptible to enzymatic degradation than BD90. Ten percent of the initial weight was lost upon exposure to collagenase for 24 h and 70% of the material was lost after 48 h of exposure to pronase. In contrast, BD90 was not degraded in both enzyme solutions. This difference in stability is most probably due to the lower degree of cross-linking of BD45 and the nature (esters versus secondary amines) of the cross-links. Ester bonds can be more easily cleaved by enzymes than the secondary amine bonds [40]. It was emphasized before [34] that blocking or shielding of amine groups results in a decreased rate of degradation because the cleavage sites were less accessible, but on the other hand, masking can lead to a certain loss of the helical integrity. Treatment of BD45 with acetic acid *N*-hydroxysuccinimide ester resulted in a reduction of the percentage of amines from 27.0 to 10.7 (n/1000). This means that about 16–17 amine groups were acylated. The T_s of the material was slightly decreased, which is a result of local destabilization or distortion of the triple-helix conformation of the collagen molecules or a more random molecular packing of the collagen molecules, owing to the side branches created by acylation of the (hydroxy) lysine residues [17, 35]. BD45HAc had a much lower resistance against collagenase than BD45. Therefore, it appears that partial distortion of the triple-helix caused by acylation is more important than shielding or blocking of specific sites for enzymes in determining the degradation rate of this material.

It can be concluded that cross-linking of N-DSC can lead to reactions which cause blocking or shielding of specific cleavage sites for enzymes and which partially distort or denature the helical structure enhancing degradation by specific enzymes. Furthermore it is generally observed that by increasing the degree of cross-linking, the resistance against enzymatic attack is increased. This is illustrated nicely by the excellent resistance against enzymatic breakdown of BD45EN, which was cross-linked in two-steps versus the low resistance of BD45, only cross-linked with BDDGE.

Linear relationships are observed for the remaining weight of collagen samples as function of degradation time using collagenase (Fig. 3). There is a trend that the rate of degradation decreases with increasing T_s of the material studied. No linear correlation between the remaining weight and the digestion time was obtained for the degradation of the materials with pronase, indicating that the level at which degradation takes place is different from collagenase or that different degradation mechanisms occur at the same time. Because pronase cleaves bonds in the nonhelical telo-peptides, cross-links formed in these regions would diminish the degradation rate. However, the telo-peptides of collagen type I do not contain amine residues and only a few carboxylic acid residues [41]. This indicates that hardly any cross-links will be formed in the telo-peptides. Therefore it is most likely that pronase mainly breaks or interrupts aggregates of collagen (micro) fibrils by cleaving native bonds. Furthermore, ester-containing cross-links can be slowly degraded by pronase [32].

Lee et al. [18] distinguished between two types of cross-links. The first type are intrahelical cross-links, which are formed between two polypeptide chains in the same helix, and will mainly influence properties like T_s and stress relaxation. The second type are interhelical cross-links which are formed between polypeptide chains of two adjacent helices, and which influence the swelling and the apparent extensibility. However, they did not mention the effect of interhelical cross-links on the T_s. Cross-links can also be formed between two adjacent microfibrils, if the distance between two microfibrils (1.3–1.7 nm) is smaller than the length of the reagent. For instance, BDDGE can bridge between two chains which have a distance up to 2.1–2.6 nm, while EDC/NHS can couple groups which are located within 1.0 nm from each

other. Therefore it can be assumed that BDDGE can and EDC/NHS cannot form intermicrofibrillar cross-links. Distances between fibrils and fibers are at least one order of magnitude higher [42], which means that no cross-links will be formed at these levels.

The enzymatic degradation experiments showed that the stability of a collagen material is mainly determined by the intrahelical and interhelical cross-links. BD90, which has a high T_s, was very stable towards enzymatic breakdown. BD45, which contained a lower degree of intrahelical and interhelical cross-links as indicated by the T_s, showed a lower enzymatic resistance. An additional EDC/NHS step improved the stability to a large extent. Because, EDC/NHS activation can only lead to intrahelical and interhelical cross-links, these cross-links determine the increased stability. The sharp increase in T_s confirms the introduction of intrahelical and interhelical cross-links. BD90 was not degraded by pronase, implying that also intermicrofibrillar cross-links were present. The susceptibility of BD45 for pronase indicates that few intermicrofibrillar cross-links were present or that the ester-groups of the cross-links are attacked by pronase.

Differences between the concentration of intrahelical, interhelical or intermicrofibrillar cross-links present in BD45 and BD90 might also explain the remarkable differences in macroscopic properties. Cross-linking via the amine groups resulted in a stiff and rigid material probably caused by intermicrofibrillar cross-links. These cross-links also lead to some planar shrinkage of the material, which might reduce the pliability. The same is observed during glutaraldehyde cross-linking [18, 43]. The flexibility and pliability of BD45 implies that hardly any intermicrofibrillar bridges were formed. This is further substantiated by the fact that after acylation (no additional cross-linking) or EDC/NHS cross-linking (only intrahelical and interhelical bonds) the flexibility of the material was not changed.

Cross-linking will affect the mechanical properties [19, 41, 43, 44]. The stress–strain curve of (non) cross-linked dermal sheep collagen can be divided into four distinctive parts. Initially, the fiber bundles are randomly oriented and only low stresses are needed to straighten the bundles (low strain modulus). The initial orientation of the fiber bundles can be expressed as the elongation at alignment. As more and more bundles become taut, an increase in the modulus is observed. The linear part of the curve at high strains is called the high strain modulus. At a sufficient stress, the material starts to yield and finally breaks.

Stress–strain curves of the cross-linked DSC reveal an increase in tensile strength, and modulus but a reduction of both elongation at alignment and break as compared to N-DSC (Table 3 and Fig. 5). Contradictory results of the effect of cross-linking on the mechanical properties of collagen-based materials were obtained in literature.

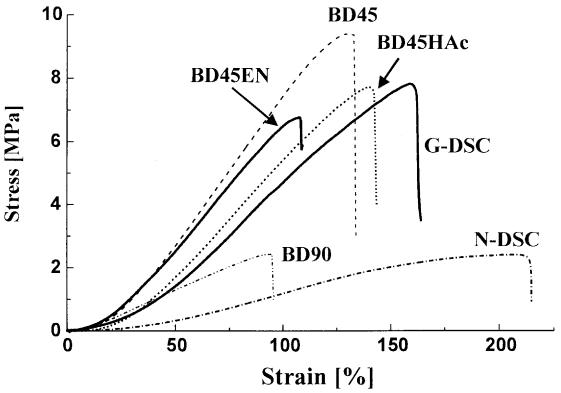

Fig. 5. Uniaxial stress–strain curves of (non)-cross-linked dermal sheep collagen (20°C, distance between clamps: 1 cm, pull speed: 10 mm/min, $n = 5$).

Porcine aortic leaflets and bovine pericardium which were cross-linked with epoxy compounds or glutaraldehyde had a slight increase in elongation at break and a similar or slightly reduced tensile strength as compared to non-fixed materials [19, 44]. The higher extensibility can be ascribed to the crimping of the fiber network during cross-linking which increases the angle of weave of the fiber bundles, resulting in a higher elongation at alignment and a higher elongation at break. Fixation of pericardium with glutaraldehyde results in shrinkage of the tissue, which also results in higher extensibilities [43]. Cross-linking of pericardium with EDC and NHS exhibited a higher elongation at break and a low stress relaxation [15], while DSC cross-linked with EDC/NHS had a similar elongation at break but a lower tensile strength compared to non-cross-linked DSC [14]. Furthermore, the low strain modulus was increased which was explained by an increased modulus or stiffness of the fibers. The effect of cross-linking on the mechanical properties of reconstitued collagen, which does not contain this highly ordered hierarchy as found in DSC, is different. Cross-linking of these collagen fibers with different physical and chemical methods always increased the tensile strength to a large extent [45–47].

Nevertheless it is still not completely clear which level of the collagen structure determines the mechanical properties of highly structured fibrous collagen. The interactions between the fiber/fiber or fibril/fibril surfaces are important parameters. During the initial part of the stress–strain experiment, the collagen fibers and bundles of DSC will align. The presence of chemical cross-links should not affect this behavior much, but the cross-linked samples have a lower elongation at alignment and a higher low strain modulus (Table 3 and Fig. 5). It is hypothesized that cross-links or branches (one-side reactions) which occur at the outer surface of fibrils and fibers alter the interactions between the fibril/fibril and fiber/fiber surfaces. Furthermore, the modulus or the stiffness

of the fibrils and fibers is increased by cross-linking. These changes in interactions and stiffness may also account for the higher strain modulus (4.3–9.5 MPa) of cross-linked DSC in relation to N-DSC (2.4 MPa).

The significant difference in tensile properties between BD90 and BD45 may be partly explained by the content or the nature (ester or secondary amine bonds) of intermicrofibrillar cross-links. These cross-links may hamper alignment and will hinder slippage of the fibrils and fibers, consequently creating internal stresses in the material. If the intermicrofibrillar cross-link density is high, a reduction in elongation at break is expected as observed in BD90. BD45 demonstrates a higher extensibility and a much higher tensile strength possibly due to the lower interfibrillar cross-link density as compared to BD90. The additional treatments performed on BD45 with HAc-NHS or EDC/NHS did not affect the mechanical properties to a large extent because no additional intermicrofibrillar cross-links were formed. BD45EN shows a lower elongation at break probably because of the increased stiffness of the fibrils caused by the zero-length cross-links. No additional effect of the modified fibril and fiber surfaces due to acylation of amine groups of BD45 with HAc-NHS was observed.

Finally, it seems that there is a correlation between the mechanical properties and macroscopical pliability of the cross-linked materials. BD90 which is stiff and rigid displays a low tensile strength and elongation at break. BD45, BD45HAc, and BD45EN which were pliable and flexible had a higher tensile strength and elongation at break. The properties of G-DSC are somewhat in between.

5. Conclusions

The results in this study reveal that cross-linking of dermal sheep collagen (DSC) with 1,4-butanediol diglycidyl ether can either occur via the carboxylic acid groups (reaction at pH 4.5) or via the amine groups (reaction at pH 9.0). Cross-linking of DSC at pH 4.5 resulted in good macroscopic and mechanical properties, but in relatively poor resistance towards collagenase and pronase. An additional EDC/NHS cross-linking step improved the enzymatic resistance without altering the other properties. Cross-linking of DSC at pH 9.0 resulted in materials with a high degree of cross linking and excellent enzymatic resistance, but with less desirable mechanical properties.

The material properties, such as T_s, swelling, in-vitro degradation and tensile properties can be correlated with the presence of interhelical, intrahelical and intermicrofibrillar cross-links. Cross-links between and in the helices will affect the T_s and the resistance against collagenase, while the mechanical properties and degradation by pronase are apparently dependent on the intermicrofibrillar cross-links (degree and nature) and the type of groups available at the outer surface of (micro) fibrils and fibers.

References

[1] Rault I, Frei V, Herbage D, Abdul-Marak N, Huc A. Evaluation of different chemical methods for cross-linking collagen gels, films and sponges. J Mater Sci: Mater Med 1996;7(4):215–22.
[2] Simmons DM, Kearney JN. Evaluation of collagen cross-linking techniques for the stabilization of tissue matrices. Biotech Appl Biochem 1993;17:23–9.
[3] Weadock K, Olsen RM, Silver FH. Evaluation of collagen cross-linking techniques. Biomater Med Dev Art Org 1983–84;11(4): 293–318.
[4] Sabelman EE. Biology, biotechnology, and biocompatibility of collagen. In: Williams DF, editor. Biocompatibility of tissue analogs. Boca Raton: CRC Press Inc, 1985.
[5] Cooke A, Oilver RF, Edward M. An in vitro cytotoxicity study of aldehyde treated pig dermal collagen. Br J Exp Pathol 1983; 64:172–6.
[6] Hey KB, Lachs CM, Raxworthy MJ, Wood EJ. Crosslinked fibrous collagen for use as a dermal implant: control of the cytotoxic effects of glutaraldehyde and dimethylsuberimidate. Biotechnol Appl Biochem 1990;12:85–93.
[7] Wachem PB van, Luyn MJA van, Olde Damink LHH, Dijkstra PJ, Feijen J, Nieuwenhuis P. Biocompatibility and tissue regenerating capacity of crosslinked dermal sheep collagen. J Biomed Mater Res 1994;28:353–63.
[8] Grimm M, Eybl E, Grabenwöger M et al. Glutaraldehyde affects biocompatibility of bioprosthetic heart valves. Surgery 1992; 111:74–8.
[9] Nimni ME, Cheung D, Strates B, Kodana M, Sheikh K. Chemically modified collagen: a natural biomaterial for tissue replacement. J Biomed Mater Res 1987;21:741–71.
[10] Wachem PB van, Luyn MJA van, Olde Damink LHH, Feijen J, Nieuwenhuis P. Tissue interactions with dermal sheep collagen implants: a transmission electron microscopical evaluation. Cells Mater 1991;1(3):251–63.
[11] Gong G, Ling Z, Seifter E, Factor SM, Frater RWM. Aldehyde tanning: the villain in bioprosthetic calcification. Eur J Cardio-Thorac Surg 1991;5:288–93.
[12] Olde Damink LHH, Dijkstra PJ, Luyn MJA van, Wachem PB van, Nieuwenhuis P, Feijen J. Crosslinking of dermal sheep collagen using hexamethylene diisocyanate. J Mater Sci: Mater Med 1995;6(7):429–34.
[13] Tu R, Quijano RC, Lu CL et al. A preliminary study of the fixation mechanism of collagen reaction with a polyepoxy fixative. Int J Art Org 1993;16(7):537–44.
[14] Olde Damink LHH, Dijkstra PJ, Luyn MJA van, Wachem PB van, Nieuwenhuis P, Feijen J. Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. Biomaterials 1996; 17(8):765–74.
[15] Lee JM, Edwards HL, Pereira CA, Samii SI. Cross-linking of tissue-derived biomaterials in 1-ethyl-3-(dimethylaminopropyl)-carbodiimide. J Mater Sci: Mater Med 1996;7(9):531–42.
[16] Noishiki Y, Koyanagi H, Miyata T, Furuse M. Bioprosthetic valve. Patent EP 0 306 256 A2, 1988.
[17] Tu R, Shen SH, Lin D et al. Fixation of bioprosthetic tissues with monofunctional and multifunctional poly epoxy compounds. J Biomed Mater Res 1994;28:677–84.
[18] Lee JM, Pereira CA, Kan LWK. Effect of molecular structure of poly(glycidyl ether) reagents on crosslinking and mechanical properties of bovine pericardial xenograft materials. J Biomed Mater Res 1994;28:981–92.

[19] Xi T, Liu F. Effect of pretreatment with epoxy compounds on the mechanical properties of bovine pericardial bioprosthetic materials. J Biomater Appl 1992;7:61–75.
[20] Tang Z, Yue Y. Crosslinkage of collagen by polyglycidyl ethers. ASAIO J 1995;41:72–8.
[21] Zeeman R, Dijkstra PJ, Wachem PB van et al. Cross-linking and modification of dermal sheep collagen using 1,4-butanediol diglycidyl ether. J Biomed Mater Res 1998, in press.
[22] Shechter L, Wynstra J. Glycidyl ether reactions with alcohols, phenols, carboxylic acids, and acid anhydrides. Ind Eng Chem 1956;48(1):86–93.
[23] Shechter L, Wynstra J, Kurkjy RP. Glycidyl ether reactions with amines. Ind Eng Chem 1956;48(1):94–7.
[24] Gulik TM van, Klopper PJ. The processing of sheepskin for use as a dermal collagen graft—an experimental study, Neth J Surg 1987;39(3):90–4.
[25] OldeDamink LHH, Dijkstra PJ, Luyn MJAv, Wachem PBv, Nieuwenhuis P, Feijen J. Glutaraldehyde as crosslinking agent for collagen based biomaterials. J Mater Sci: Mater Med 1995; 6:460–72.
[26] IUP/16. Measurement of shrinkage temperature. J Soc Leather Technol Chem 1963:122–6.
[27] IUP/2. J Soc Leather Trades' Chem 1960:44.
[28] Graberek Z, Gergely J. Zero-length cross-link procedure with the use of active esters. Anal Biochem 1990;185:131–5.
[29] Sung HW, Chen WH, Chiu IS, Hsu HL, Lin SA. Studies on epoxy compound fixation. J Biomed Mater Res Appl Biomater 1996;33:177–86.
[30] Shen SH, Sung HW, Tu R et al. Characterization of polyepoxy compound fixed porcine heart valve bioprostheses. J Appl Biomater 1994;5:159–62.
[31] Nishi C, Nakajima N, Ikada Y. In vitro evaluation of cytotoxicity of diepoxy compounds used for biomaterials. J Biomed Mater Res 1995;29:829–35.
[32] Zeeman R, Dijkstra PJ, Wachem PB van et al. In vitro degradation of dermal sheep collagen cross-linked with 1,4-butanediol diglycidyl ether. J Biomed Mater Res 1998, Submitted.
[33] Zeeman R, Dijkstra PJ, Wachem PBv et al. The kinetics of 1,4-butanediol diglycidyl ether cross-linking of dermal sheep collagen. J Biomed Mat Res 1998, Submitted.
[34] Diamond AM, Gorham SD, Etherington DJ, Robertson JG, Light ND. The effect of modification on the susceptibility of collagen to proteolysis. I. Chemical modification of amino acid side chains. Matrix 1991;11:321–9.
[35] Sung HW, Hsu CS, Wang SP, Hsu HL. Degradation potential of biological tissues fixed with various fixatives: An in vitro study. J Biomed Mater Res 1997;35:147–55.
[36] Loke WK, Khor E. Validation of the shrinkage temperature of animal tissue for bioprosthetic heart valve application by differential scanning calorimetry. Biomaterials 1995;16(3):251–8.
[37] Lee JM, Pereira CA, Abdulla D, Naimark WA, Crawford I. A multi-sample denaturation temperature tester for collagenous biomaterials. Med Eng Phys 1995;17:115–21.
[38] Cheung DT, Perelma N, Ko EC, Nimni ME. Mechanism of cross-linking of proteins by glutaraldehyde. III. Reaction with collagen in tissues. Connect Tissue Res 1985;13:109–15.
[39] Olde Damink LHH, Dijkstra PJ, Luyn MJA van, Wachem PB van, Nieuwenhuis P, Feijen J. Influence of ethylene oxide gas treatment on the in vitro degradation behaviour of dermal sheep collagen. J Biomed Mater Res 1995;29(2):149–55.
[40] Stryer L. Biochemistry, 4th ed. San Francisco, CA: W.H. Freeman and Company, 1995.
[41] Heidemann E. Fundamentals of leather manufacturing, 1st ed. Munich: Hanser Publishers, 1993.
[42] Ramachandran GN, Structure of collagen at a molecular level. In: Ramachandran GN, editor. Treatise on collagen. vol. I. Chemistry of collagen. London: Academic Press, 1967:103–84.
[43] Gratzer PF, Lee JM. Altered mechanical properties in aortic elastic tissue using glutaraldehyde/solvent solutions of various dielectric constant. J Biomed Mater Res 1997;37:497–507.
[44] Lee JM, Haberer SA, Boughner DR. The bovine pericardial xenograft: I. Effect of fixation in aldehydes without constraint on the tensile viscoelastic properties of bovine pericardium. J Biomed Mater Res 1989;23:457–75.
[45] Bellincampi LD, Dunn MG. Effect of cross-linking method on collagen fiber-fibroblast interactions. J Appl Polym Sci 1997;63: 1493–8.
[46] Pins GD, Huang EK, Christiansen DL, Silver FH. Effects of static axial strain on the tensile properties and failure mechanisms of self-assembled collagen fibers. J Appl Polym Sci 1997;63:1429–40.
[47] Kato YP, Silver FH. Formation of continuous fibers: evaluation of biocompatibility and mechanical properties. Biomaterials 1990;11:169–75.