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Cross-linking of dermal sheep collagen using a water-soluble carbodiimide

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A cross-linking method for collagen-based biomaterials was developed using the water-soluble carbodiimide 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC). Cross-linking using EDC involves the activation of carboxylic acid groups to give O-acylisourea groups, which form cross-links after reaction with free amine groups. Treatment of dermal sheep collagen (DSC) with EDC (E-DSC) resulted in materials with an increased shrinkage temperature (T_s) and a decreased free amine group content, showing that cross-linking occurred. Addition of *N*-hydroxysuccinimide to the EDC-containing cross-linking solution (E/N-DSC) increased the rate of cross-linking. Cross-linking increased the T_s of non-cross-linked DSC samples from 56 to 73°C for E-DSC and to 86°C for E/N-DSC samples, respectively. For both cross-linking methods a linear relation between the decrease in free amine group content and the increase in T_s was observed. The tensile strength and the high strain modulus of E/N-DSC samples decreased upon cross-linking from 18 to 15 MPa and from 26 to 16 MPa, respectively. The elongation at break of E/N-DSC increased upon cross-linking from 142 to 180%.

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The high enzymatic turnover rate of collagen in the body makes stabilization of collagen-based biomaterials by chemical cross-linking methods necessary to give materials that maintain the desired mechanical properties and stability during the desired implantation period. Several cross-linking methods have been reported and in principle they can be divided into two groups. First, bifunctional reagents can be used to bridge amine groups of lysine or hydroxylysine residues of different polypeptide chains by monomeric or oligomeric cross-links. Second, amide-type cross-links can be formed by activation of the carboxylic acid groups of glutamic and aspartic acid residues followed by reaction of these activated carboxylic acid groups with amine groups of another polypeptide chain.

Based on the use of bifunctional reagents for cross-linking, glutaraldehyde (GA) has generally been applied for the stabilization of collagen-based materials[1]. The use of hexamethylene diisocyanate (HMDIC) as a cross-linking agent was introduced by Chvapil *et al.*[2]. A promising class of cross-linking agents for collagen more recently described is the polyepoxy compounds[3,4].

GA cross-linking involves the formation of short (branched) aliphatic chains[1] and pyridinium compounds[5,6], while in HMDIC cross-linking aliphatic chains containing urea bonds are introduced between two adjacent amine groups[7]. Both GA and HMDIC cross-linking may lead to the presence of unreacted functional groups (probably aldehyde or amine groups after hydrolysis of isocyanate groups) in the collagen matrix, which can result in a cytotoxic reaction upon degradation of the collagen[8,9]. Furthermore, it has been reported that GA cross-linked collagen-based biomaterials releases toxic GA (related) molecules from the material[10,11], which may result from unreacted GA present in the samples or from hydrolytic or enzymatic degradation products[12]. This may also contribute to the cytotoxic reactions elicited by these materials both *in vitro* and *in vivo*[13–15].

Recently, cross-linking methods based on the concept of cross-linking by activation of carboxylic acid groups have been developed. The use of cyanamide for the cross-linking of reconstituted collagen was first reported by Weadock *et al.*[16]. In a patent application these authors suggested the use of several other carbodiimides[17]. Petite *et al.*[18] used the acyl azide activation method for cross-linking of pericardium. Using these methods, direct cross-linking of the polypeptide chains occurs, resulting in the formation of amide-type cross-links. In principle, no unreacted groups will be left in the material during cross-linking provided that reagents used for the activation of the carboxylic acid groups are easily removed. Cross-linking of collagen-based biomaterials using these

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methods resulted in materials with a similar resistance against degradation by bacterial collagenase compared with GA cross-linked materials[16,18].

Because of the multistep reaction pathway used in the acyl azide method and the high toxicity of cyanamide, we investigated the use of another activation method of carboxylic acid groups. Here we describe the results obtained from a study on the cross-linking of dermal sheep collagen (DSC) using the water-soluble carbodiimide 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC). The influence of *N*-hydroxysuccinimide (NHS) on the activation of the carboxylic acid groups and subsequent cross-linking of the collagen material was studied.

MATERIALS AND METHODS

Purification of DSC

DSC was obtained from the Zuid-Nederlandse Zeemlederfabriek (Oosterhout, The Netherlands). In brief, the sheep skin was depilated and immersed in a lime–sodium sulphide solution to remove the epidermis. Non-collagenous substances were removed using proteolytic enzymes, whereafter the skin was split to obtain the dermal layer[19]. The remaining fibrous collagen network was washed four times with water, twice with acetone and twice with water before freezing and lyophilizing.

Cross-linking

Cross-linking with EDC

In a typical experiment, cross-linking of non-cross-linked DSC (N-DSC) with EDC to give E-DSC was performed by immersing N-DSC samples weighing 1 g (1.2 mmol carboxylic acid groups) in 100 ml of an aqueous solution containing 1.15 g (6.0 mmol) EDC (z.S., Merck-Schuchardt, Hohenbrun, Germany) at room temperature for 18 h. During the reaction, a pH of 5.5 was maintained by addition of 0.1 M HCl using a pH Stat apparatus (702 SM Titrino, Metrohm, Herisau, Switzerland). The added volume was monitored over time. The molar amount of carboxylic acid groups (COOH) of N-DSC samples was calculated assuming that 120 carboxylic acid group-containing residues are present per α-chain (1000 amino acids)[20] and that each α-chain has a molecular weight of 100 000. After cross-linking, E-DSC samples were washed for 2 h in a 0.1 M Na_2HPO_4 solution to hydrolyse any remaining O-acylisourea groups and subsequently washed four times with distilled water before lyophilization.

The influence of the molar ratio of EDC to COOH on the degree of cross-linking was determined using EDC to COOH ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. The influence of the pH of the cross-linking solution on the degree of cross-linking of N-DSC was determined by varying the pH between 4.0 and 5.5. The changes in degree of cross-linking as a function of cross-linking time were determined for time periods up to 18 h.

Cross-linking with EDC and NHS

In a typical experiment, cross-linking of N-DSC with EDC and NHS to give E/N-DSC was performed by immersing N-DSC samples weighing 1 g (1.2 mmol carboxylic acid groups) in 100 ml aqueous solution containing 1.15 g (6.0 mmol) EDC (z.S., Merck-Schuchardt) and 0.69 g (6.0 mmol) NHS (z.S., Merck-Schuchardt) at room temperature for 4 h. The pH of the solution was adjusted at 5.5 by the addition of HCl. During the reaction, a pH of 5.5 was maintained by addition of 0.1 M NaOH using a pH Stat apparatus. After cross-linking, the samples were washed as described above.

The influence of the molar ratio of EDC to COOH on the degree of cross-linking was determined using EDC to COOH ratios of 1:1, 1:2, 1:3, 1:4 and 1:5, respectively. During these experiments an NHS to EDC ratio of 1:1 was used. The influence of the pH of the cross-linking solution on the degree of cross-linking of N-DSC was studied by varying the pH from 4.0 to 5.5. The degree of cross-linking as a function of cross-linking time was determined by cross-linking N-DSC for time periods up to 6 h. The influence of the molar ratio between NHS and EDC on the degree of cross-linking was determined using a ratio of EDC to COOH of 5:1 and NHS to EDC ratios of 1:5, 2:5, 3:5, 4:5 and 5:5, respectively.

Activation of carboxylic acid groups

The number of NHS-activated carboxylic acid groups present after cross-linking of N-DSC with EDC and NHS was determined by measuring the amount of NHS released from the activated carboxylic acid groups as a function of cross-linking time. Either N-DSC samples or N-DSC samples with blocked amine groups were used in these experiments.

The free amine groups of N-DSC samples were blocked using the acylating agent acetic acid NHS ester (HAc–NHS). N-DSC samples weighing 1 g were immersed in 50 ml of an aqueous solution containing 1.35 g HAc–NHS (Sigma, St Louis, MO, USA) for 5 h at room temperature. Every 2 h, an additional amount of 0.2 g HAc–NHS was added. The pH of the solution was maintained between 6.5 and 7.5 by the addition of concentrated NaOH.

Samples of N-DSC and N-DSC with blocked amine groups (total of 15 samples weighing 20 mg each, 0.36 mmol carboxylic acid groups) were incubated in 15 ml of an aqueous solution containing 0.345 g (1.8 mmol) EDC and 0.08 g (0.72 mmol) NHS (molar ratio of EDC to NHS to COOH = 5:2:1) at pH 5.5 for time periods up to 120 min. After the desired time interval a sample was washed for 2 min in 100 ml of 0.2 M NaH_2PO_4 buffer (pH 4.5) to remove unreacted NHS. Hydrolysis of the NHS-activated carboxylic acid groups was achieved by immersing the sample in 5 ml of 0.1 M Na_2HPO_4 (pH 9.1) for 2 h. The amount of NHS released was measured spectrophotometrically in 260 nm against the buffer solution[21].

Reaction of EDC with poly(acrylic acid)

The N-acyl shift of EDC-activated carboxylic acid groups was studied using poly(acrylic acid) (PAA) as a

model compound. A solution of 0.14 g PAA in 50 ml of water was adjusted to the desired pH value of either 4.0 or 5.5. To the solution, 0.88 g of EDC was added (molar ratio of EDC to CooH = 5:1) and the reaction mixture was left for 18 h at room temperature. During all experiments the pH was maintained at the desired value using a pH stat apparatus. The reaction was discontinued and the formed O-acylisourea groups were hydrolysed by the addition of a concentrated Na OH solution to a final pH of 9.1. After 2 h the resulting mixture was concentrated by ultrafiltration at 4 bar (10YM10, Diaflo, Amicon, Ireland) and washed by flushing the resulting solution four times with 120 ml distilled water using an Amicon CDS 10 apparatus (Amicon, Ireland). 1-Ethyl-3-(3-dimethyl aminopropyl)urea resulting from hydrolysis of O-acylisourea groups was removed by ion exchange using an Amberlite IR 120 column (E. Merck, Darmstadt, Germany). Conductometrical titration of the carboxylic acid groups of PAA was performed using 0.1 M NaOH. The influence of NHS on the reaction of EDC with PAA was determined by adding 0.21 g NHS to the reaction mixture (molar ratio of EDC to NHS to COOH = 5:2:1). The reaction was carried out for 2 h. Further sample handling was performed as described above.

Characterization

The degree of cross-linking of the E-DSC and E/N-DSC samples was related to the increase in shrinkage temperature (T_s) after cross-linking[22]. T_s values of cross-linked or non-cross-linked DSC samples immersed in water were determined as described previously[12]. The free amine group content of the samples was determined spectrophotometrically after reaction of the primary amine groups with 2,4,6-trinitrobenzenesulphonic acid[12] and is expressed as the number of amine groups present per 1000 amino acid residues (*n* per 1000).

Mechanical properties

Stress–strain curves of non-cross-linked and cross-linked DSC samples were determined by uniaxial measurements using an Instron mechanical tester[12]. Tensile test samples (30.0 × 6.0 × 0.8 mm) were cut using a blade knife and were hydrated for at least 30 min in phosphate-buffered saline (0.14 M NaCl, 0.01 M Na_2HPO_4, 0.002 M NaH_2PO_4, pH 7.4, NPBI, Emmercompascuum, The Netherlands) at room temperature. 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. Because of variations in the mechanical properties of different parts of the sheep skin[23], the change in mechanical properties of cross-linked samples was compared only with the mechanical properties of matching non-cross-linked controls. Samples used to study the influence of cross-linking on the mechanical properties were always taken from the IUP/2[24] sampling area parallel to the backbone and were either cross-linked or kept as control.

The mechanical properties of E/N-DSC samples were studied as a function of the degree of cross-linking of the samples. Samples with different T_s values were obtained by cross-linking N-DSC samples for 2 h at pH 5.5 using molar ratios of EDC to NHS to COOH of 0.2:0.08:1, 0.5:0.2:1, 1:0.4:1 and 5:2:1, respectively.

RESULTS

Cross-linking

The increase in T_s and the decrease in free amine group content of cross-linked DSC samples were monitored to optimize the degree of cross-linking. The T_s of DSC samples cross-linked either with EDC (E-DSC) or with a mixture of EDC and NHS (E/N-DSC) as a function of the molar ratio of EDC to COOH is presented in *Figure 1*. Cross-linking of DSC with EDC for 18 h using a molar ratio of EDC to COOH of 1:1 increased the T_s of N-DSC from 56 to 72°C for E-DSC. An increase of the ratio of EDC to COOH to 5:1 resulted in only a small additional increase in T_s to 73°C. Addition of NHS to the EDC solution resulted in higher T_s values for the cross-linked samples. A T_s of 77°C for E/N-DSC was determined when N-DSC samples were cross-linked for 4 h using an EDC to NHS to COOH ratio of 1:1:1. An additional increase in T_s to 82°C was observed when a molar ratio of 5:5:1 was used during cross-linking.

The free amine group content of E-DSC and E/N-DSC samples as a function of the molar ratio of EDC to COOH used during cross-linking is presented in *Figure 2*. The initial free amine group content of 34 per 1000 amino acid residues for N-DSC samples is in good agreement with values reported in the literature[20]. Cross-linking of N-DSC samples using a molar ratio of EDC to COOH of 1:1 for 18 h decreased the free amine group content from 34 per 1000 to 28 per 1000 amino acid residues. The use of higher

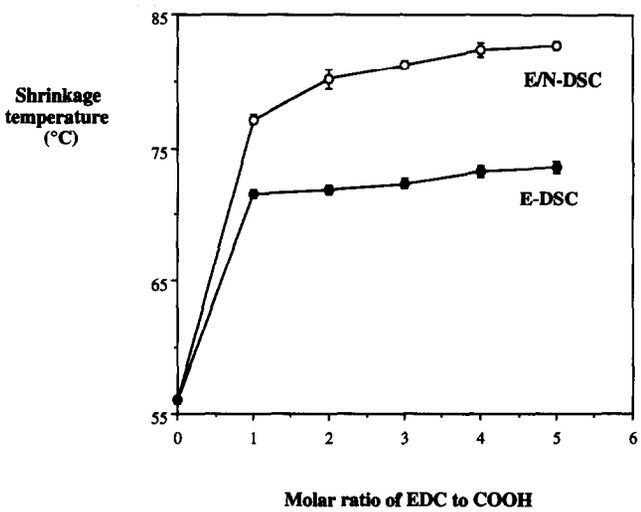


Figure 1 Shrinkage temperature as a function of the molar ratio of EDC to the carboxylic acid groups of DSC during cross-linking with EDC (●) or with EDC and NHS (○). Room temperature, pH 5.5, $n = 3$; E-DSC: 18 h; E/N-DSC: ratio of EDC to NHS = 1:1, 4 h.

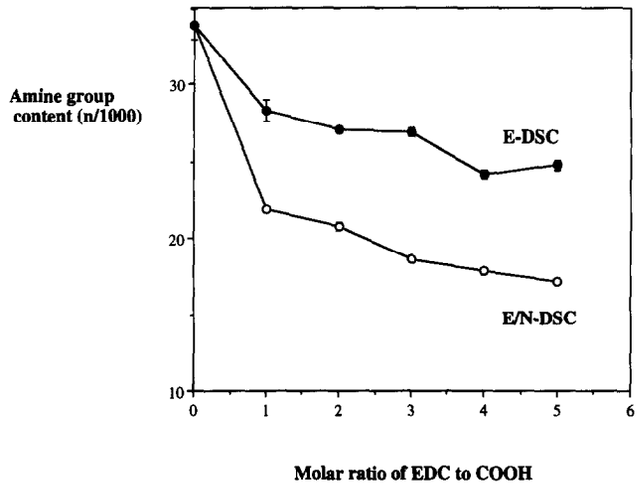


Figure 2 Free amine group content as a function of the molar ratio of EDC to the carboxylic acid groups of DSC during cross-linking with EDC (●) or with EDC and NHS (○). Room temperature, pH 5.5, $n = 3$; E-DSC: 18 h; E/N-DSC: ratio of EDC to NHS = 1:1, 4 h.

ratios of EDC to COOH during cross-linking resulted in slightly lower values of free amine group content of 25 per 1000 amino acid residues. Addition of NHS to the EDC solution during cross-linking resulted in a larger decrease in free amine group content for E/N-DSC samples compared to E-DSC samples. Using a molar ratio of EDC to NHS to COOH of 5:5:1 and cross-linking for 4 h resulted in E/N-DSC with a free amine group content of 17 per 1000 amino acid residues. From these results, a molar ratio of EDC to COOH of 5:1 and a molar ratio of EDC to NHS to COOH of 5:5:1, respectively, were selected for further experiments.

Cross-linking of N-DSC using either EDC or EDC and NHS was also studied at pH values between 4.0 and 5.5 (*Figure 3*). The T_s values of the E-DSC samples varied only between 72 and 73°C as a function of pH.

In contrast, the T_s of the E/N-DSC samples was much more dependent on the pH. While a T_s of 76°C was observed for E/N-DSC samples cross-linked at pH 4.0, a plateau value of 82°C was observed for samples cross-linked at pH values of 5.0 and 5.5. At all pH values, higher T_s values were obtained for E/N-DSC samples compared with E-DSC samples.

During cross-linking of DSC with EDC, the pH of the EDC solution drifted to higher values and HCl had to be added to keep the pH at the desired value. In contrast, addition of NHS to the EDC-containing cross-linking solution resulted in a drift to lower pH values. The amount of NaOH necessary to maintain the pH at a level of 4.0 was independent of the presence of N-DSC in the cross-linking solution.

From *Figure 4* it can be seen that the rate of cross-linking of N-DSC with EDC at pH 5.5 is increased by the addition of NHS to the cross-linking solution. Maximal T_s values of 82°C were determined for E/N-DSC samples after 2 h cross-linking. Cross-linking of N-DSC with EDC resulted not only in a lower rate of cross-linking, but also a lower maximal T_s value of 72°C, even after 18 h cross-linking. A cross-linking time of 2–4 h was selected for further experiments using EDC and NHS in the cross-linking of N-DSC samples.

The effect of the molar ratio between NHS and EDC on the increase in T_s and the decrease in free amine group content after cross-linking is presented in *Figure 5*. Highest values of T_s and lowest values of the free amine group content, indicating the highest degree of cross-linking, were found when a molar ratio of EDC to NHS to COOH of 5:2:1 was used.

Activation of carboxylic acid groups

To study which intermediate is involved in the cross-linking reaction with the free amine groups the following experiments were carried out.

First, the total number of NHS-activated carboxylic acid groups during cross-linking of N-DSC samples

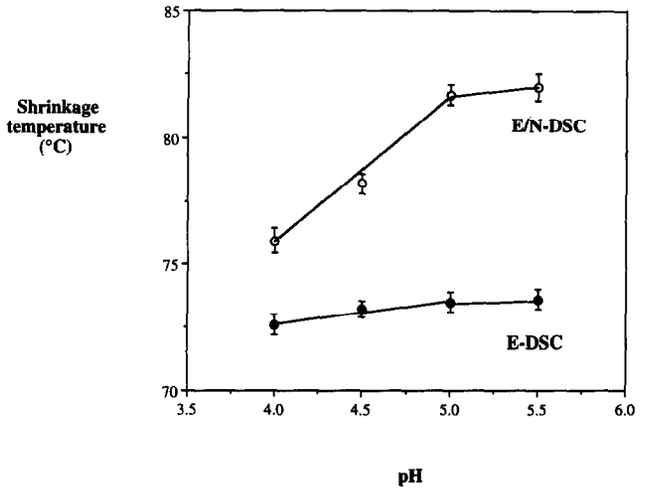


Figure 3 Shrinkage temperature as a function of pH during cross-linking of DSC with EDC (●) or with EDC and NHS (○). Room temperature, $n = 3$; E-DSC: ratio of EDC to COOH = 5:1, 18 h; E/N-DSC: ratio of EDC to NHS to COOH = 5:5:1, 4 h.

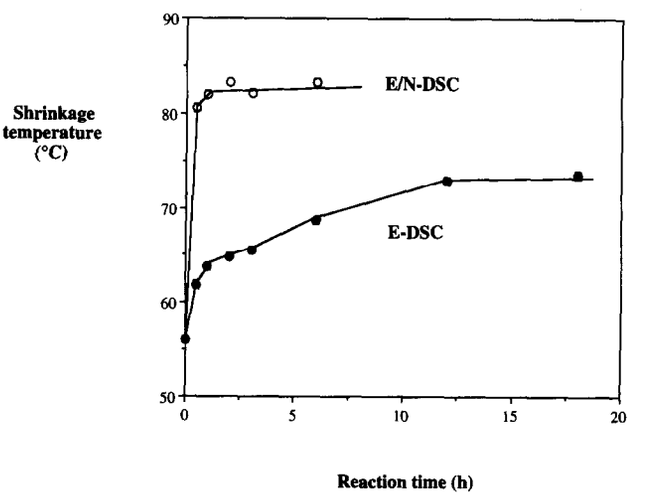


Figure 4 Shrinkage temperature as a function of the reaction time during cross-linking of DSC with EDC(●) or with EDC and NHS (○). Room temperature, pH 5.5, $n = 3$; E-DSC: ratio of EDC to COOH = 5:1; E/N-DSC: ratio of EDC to NHS to COOH = 5:5:1.

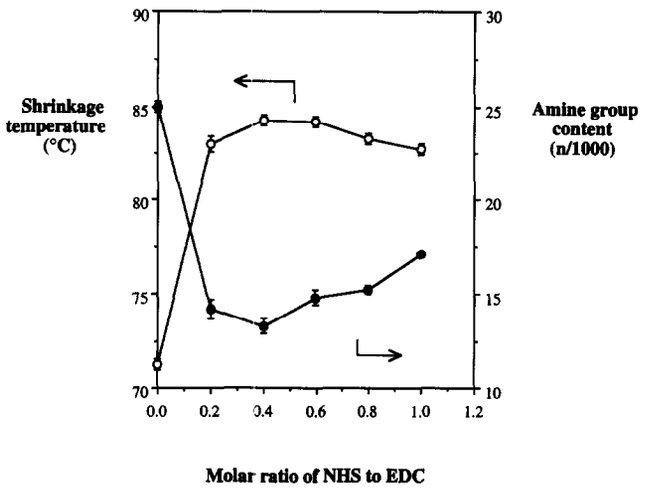


Figure 5 Shrinkage temperature (○) and free amine group content (●) as a function of the molar ratio of NHS to EDC during cross-linking of DSC. Room temperature, pH 5.5, 4 h, molar ratio of EDC to COOH = 5:1, $n = 3$.

with EDC and NHS was determined. This was performed by treating N-DSC samples with blocked amine groups with a mixture of EDC and NHS using a molar ratio of EDC to NHS to COOH of 5:2:1 and spectrophotometrically monitoring the NHS release from the activated N-DSC samples at 260 nm. Blocking of the amine groups was necessary to prevent NHS release due to cross-linking reactions and was performed using HAc–NHS as an acrylating agent. Treatment of N-DSC with HAc–NHS decreased the free amine group content from 33 per 1000 amino acids for N-DSC to 6 per 1000 amino acids for HAc–NHS-treated N-DSC samples. No increase in T_s was found when N-DSC samples with blocked amine groups were subsequently treated with EDC and NHS, indicating that blocking of sufficient amine groups was achieved to prevent cross-linking. From model experiments it was concluded that NHS-activated carboxylic acid groups did not react with either hydroxyl or carboxylic acid groups.

Second, the number of carboxylic acid groups present in cross-linked DSC samples that can be activated with NHS was determined spectrophotometrically by monitoring the NHS release upon basic hydrolysis. The difference between the number of carboxylic acid groups that can be activated by reaction with NHS in N-DSC and in E/N-DSC samples is equal to the number of NHS-activated carboxylic acid groups that has been involved in the formation of a cross-link.

The number of NHS-actiated carboxylic acid groups as a function of reaction time for either N-DSC or N-DSC with blocked amine groups is presented in *Figure 6*. For both materials an initially sharp increase in the number of NHS-activated carboxylic acid groups was found. The difference between the number of NHS-activated carboxylic acid groups reached a constant value of 20 per 1000 amino acid residues after 60 min reaction and did not change at longer reaction times. This number was comparable with the decrease in free amine group content of the cross-linked N-DSC samples in this experiment, which was found to be 19.0 ± 2.4 per 1000 amino acids.

Reaction of EDC with PAA

The O-acylisourea group formed upon reaction of a carboxylic acid group with EDC can rearrange via an N-acryl shift to a hydrolytically stable N-acylurea group[25]. The influence of NHS on the N-acyl shift was studied by performing model reactions using PAA. PAA was allowed to react with EDC or a mixture of EDC and NHS. The occurrence of the N-acyl shift was related to differences in the number of free carboxylic acid groups present before and after treatment of PAA with EDC or EDC and NHS. Carboxylic acid groups were determined by titration. The results of these experiments are presented in *Table 1*.

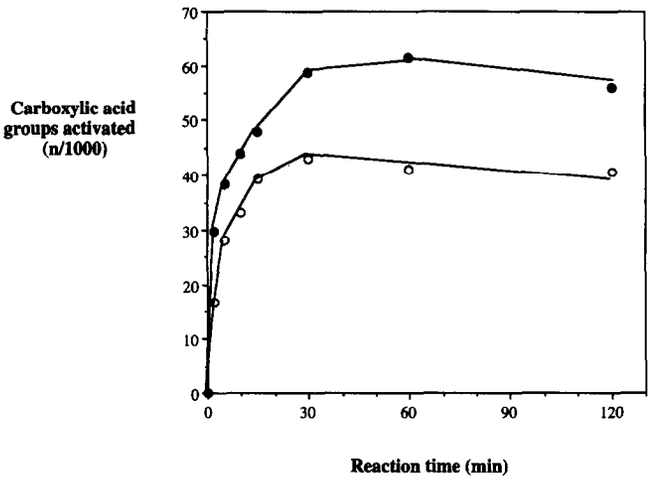


Figure 6 Number of activated carboxylic acid groups as a function of reaction time during treatment of N-DSC (○) or N-DSC with blocked amine groups (●) with EDC and NHS. Room temperature, pH 5.5, molar ratio of EDC to NHS to COOH = 5:2:1.

Table 1 Reaction of 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride with poly(acrylic acid)

Sample	pH	NHS added	Reaction time (h)	Carboxylic acid groups substituted (%)
1	4.0	no	18	8.8 ± 1.6
2	4.0	yes	2	11.1 ± 1.3
3	5.5	no	18	22.0 ± 1.1
4	5.5	yes	2	9.4 ± 1.4
5	5.5	yes	18	12.2 ± 2.0

During reaction of EDC with PAA a ratio of EDC to NHS to carboxylic acid groups of 5:2:1 was used ($n = 3$).

At a pH of 4.0, addition of NHS to the reaction solution did not result in a decrease in the amount of carboxylic acid groups substituted. With or without the addition of NHS during the reaction of PAA with EDC, 8.8 ± 1.6% and 11.1 ± 1.3%, respectively, of the carboxylic acid groups were converted into N-acylurea groups. Reaction of EDC with PAA at pH 5.5 resulted in 22.0 ± 1.1% of the carboxylic acid groups being converted. Addition of NHS during the reaction decreased this number to 9.4 ± 1.4%. These results reveal that NHS can be effective in suppressing the N-acyl shift.

A control experiment was performed to investigate the influence of the hydrolysis procedure at pH 9.1 on the number of carboxylic acid groups converted into N-acylurea groups. During the control experiment (sample 5), the NHS-activated carboxylic acid groups were hydrolysed for 18 h at pH 5.5 instead of for 2 h at pH 9.1. Compared with sample 4, no significant difference in the number of carboxylic acid groups substituted was found, indicating that the hydrolysis procedure at pH 9.1 did not increase the number of carboxylic acid groups substituted by the N-acyl shift.

Mechanical properties

The mechanical properties of E/N-DSC samples as a function of degree of cross-linking are presented in *Table 2*[12]. A small but significant decrease in tensile strength was found for E/N-DSC samples with increasing degree of cross-linking. The tensile strength decreased from 18.1 MPa for N-DSC with T_s of 56°C to 15.1 MPa for E/N-DSC with a T_s of 86°C. Independent of the degree of cross-linking, for all samples an average elongation at alignment of 60% was found. The elongation at break initially increased as a function of degree of cross-linking from 142% for N-DSC with a T_s of 56°C to 209% for E/N-DSC with a T_s of 66°C. At higher degrees of cross-linking, the elongation at break tended to decrease. The low strain modulus increased upon cross-linking, but at a higher degree of cross-linking this increase levelled off to 3.8 MPa. In contrast, the high strain modulus decreased upon cross-linking from 26 MPa for N-DSC with a T_s of 56°C to 14 MPa for E/N-DSC having a T_s of 66°C. Introduction of more cross-links did not result in an additional decrease of the high strain modulus.

DISCUSSION

Cross-linking of collagen-based biomaterials using carboxylic acid group-activating methods has gained increasing interest recently. These cross-linking procedures, like the acyl azide[18] or the cyanamide methods[16], involve the activation of carboxylic acid groups present in the polypeptide chains followed by reaction with free amine groups of other polypeptide chains. Contrary to the use of bifunctional reagents like GA, these methods do not result in the incorporation of cross-linking agents in the material. Based on the concept of cross-linking by activation of the carboxylic acid groups, the water-soluble carbodiimide EDC was selected for the cross-linking of DSC.

Treatment of N-DSC samples with EDC (E-DSC) resulted in materials with an increased T_s and a decreased free amine group content (*Figures 1* and *2*), indicating that cross-linking was achieved. Similar results have been reported for the treatment of reconstituted collagen fibres with EDC[26]. Cross-linking using EDC involves the activation of the carboxylic acid groups of glutamic or aspartic acid residues (**I**) by EDC (**II**) to give O-acylisourea groups (**III**), as shown in *Scheme 1*. Cross-links (**IV**) are formed after nucleophilic attack by free amine groups of lysine or hydroxylysine residues[27]. During the latter reaction, the EDC-related water-soluble compound 1-ethyl-3-(3-dimethyl aminopropyl)urea (**VIII**) is liberated.

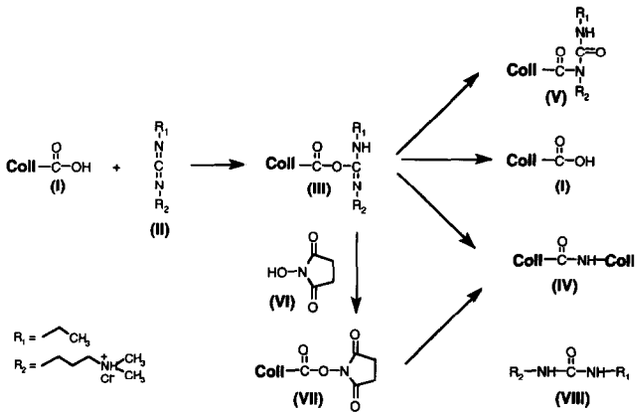
Addition of the nucleophile NHS to the EDC-containing solution was very effective in increasing the number of cross-links introduced into the N-DSC matrix. Cross-linking of N-DSC samples by a mixture of EDC and NHS (E/N-DSC) resulted in materials with a larger increase in T_s (*Figure 1*) and decrease in free amine group content (*Figure 2*) compared with E-DSC samples. Similar results were observed when the influence of the pH of the cross-linking solution on the degree of cross-linking of E-DSC and E/N-DSC samples was studied (*Figure 3*). From *Figure 4* it is clear that addition of NHS to the cross-linking solution not only results in materials with a higher degree of cross-linking (82°C for E/N-DSC versus 72°C for E-DSC), but also increases the rate of the cross-linking reactions.

The beneficial effect of NHS during cross-linking is based on the suppression of two side reactions (*Scheme 1*) that can occur when EDC is used for the activation of carboxylic acid groups[25]. First, water can

Table 2 Mechanical properties of non-cross-linked and cross-linked dermal sheep collagen

Sample	Sterilization procedure	Tensile strength (MPa)	Elongation at alignment (%)	Elongation at break (%)	Low strain modulus (MPa)	High strain modulus (MPa)
N-DSC*	56.5 ± 0.3	18 ± 2	59 ± 8	142 ± 12	1.6 ± 0.4	26 ± 1
E/N-DSC†	66.2 ± 0.7	19 ± 2	72 ± 8	209 ± 10	2.8 ± 0.8	14 ± 1
	76.9 ± 0.6	17 ± 2	76 ± 5	190 ± 12	4.2 ± 1.0	13 ± 1
	82.8 ± 0.4	16 ± 1	64 ± 7	184 ± 5	3.7 ± 0.2	16 ± 1
	85.9 ± 0.3	15 ± 1	59 ± 3	180 ± 11	3.8 ± 0.9	16 ± 1

*Non-cross-linked DSC.
†DSC cross-linked with EDC and NHS.
E/N-DSC samples with different T_s values were obtained using molar ratios of EDC:NHS:COOH of 0.2:0.08:1, 0.5:0.2:1, 1:0.4:1 and 5:2:1, respectively. All samples were cross-linked at pH 5.5 for 2 h. All mechanical properties are given as mean ± s.d. of five measurements.



Scheme 1 Cross-linking of DSC with EDC and NHS.

act as a nucleophile, resulting in the hydrolysis of the O-acylisourea group to give the substituted urea (**VIII**) and the starting carboxylic acid group (**I**). Second, the highly reactive O-acylisourea group can rearrange to a stable N-acylurea group (**V**). This N-acyl shift is independent of the presence of a nucleophile. In the presence of NHS, the O-acylisourea group is converted to the NHS-activated carboxylic acid group (**VII**), which is less susceptible to hydrolysis at acidic pH values compared with the O-acylisourea group.

The use of NHS appeared effective in preventing the N-acyl shift of the O-acylisourea group. This is supported by the results from model experiments using PAA as presented in *Table 1*. The relative number of substituted carboxylic acid groups of PAA after reaction with EDC at pH 5.5 was suppressed by addition of NHS to the reaction solution.

Using a mixture of EDC and NHS for the cross-linking of DSC, it may be expected that both the O-acylisourea group and the NHS-activated carboxylic acid group can simultaneously be involved in the reaction with the free amine group. However, the decrease in free amine group content of E/N-DSC samples after cross-linking (19 per 1000 amino acid residues) was equal to the number of NHS-activated carboxylic acid groups that reacted during cross-linking (*Figure 6*). This indicates that both the reaction of NHS with **III** and the reaction of **VII** with a free amine group of collagen to give **IV** are rapid reactions. This suggests that the reactive species towards the nucleophilic attack of the free amine group is the NHS-activated carboxylic acid group rather than the O-acylisourea group.

From *Figure 6* it can be seen that, during the first hour of reaction, the number of NHS-activated carboxylic acid groups that have reacted with a free amine group increased rapidly to the maximal number of 20 per 1000 amino acid residues. This indicates that cross-linking was essentially complete after 1 h which agrees with the results obtained from the kinetic study as presented in *Figure 4*. In this figure it is shown that, during this time period, the increase in T_s of the E/N-DSC samples was almost complete. However, after 1 h cross-linking 40 carboxylic acid groups per 1000 amino acid residues are still activated (*Figure 6*), showing that the formation of additional cross-links is prevented owing to spatial limitations rather than the lack of activated carboxylic acid groups.

During cross-linking of N-DSC samples with EDC, the pH of the cross-linking solution drifted to higher values, although no protons are consumed during cross-linking. A possible explanation is that, owing to the N-acyl shift during dross-linking, the amount of carboxylic acid groups in the DSC matrix is depleted, which results in the observed pH drift. In contrast, when a mixture of EDC and NHS is used for cross-linking, a pH drift to lower values was observed. This pH drift was independent of the presence of N-DSC samples in the cross-linking solution. This suggests that this drift is not related to cross-linking reactions involving DSC samples, but to reactions between EDC and NHS. It has been reported that reaction between carbodiimides and NHS leads to the formation of unstable compounds, which give CO_2 upon hydrolysis[28]. Partial dissolution of the CO_2 in the cross-linking solution will be responsible for the observed pH drift. The reaction between EDC and NHS results in the depletion of EDC from the cross-linking solution, especially when high ratios of NHS to EDC are used. This results in a lower T_s value and a higher free amine group content for the E/N-DSC samples cross-linked at high NHS to EDC ratios (*Figure 5*).

Previously, we studied the cross-linking of DSC with GA[12]. Cross-linking of N-DSC samples with GA using optimized conditions increased the T_s of N-DSC from 56 to 78°C. In comparison, cross-linking using a mixture of EDC and NHS produced materials with considerably higher T_s values (86°C). Although this suggests that E/N-DSC samples have a higher degree of cross-linking, care has to be taken when the T_s values of samples cross-linked using different methods is directly related to the degree of cross-linking of the samples. Since the T_s of collagen-based materials depends on the degree of swelling of the material[22], it will not only be influenced by the degree of cross-linking of the material, but also by the thermal stability and chemical structure of the cross-links introduced into the material. While cross-linking of DSC with GA involves the formation of short aliphatic chains or pyridinium compounds[12], during cross-linking of DSC with a mixture of EDC and NHS, two polypeptide chains are directly coupled together[29]. It is evident that these differences between the types of cross-links introduced may also have an effect on the degree of swelling of the materials.

The T_s values of samples cross-linked either with EDC or a mixture of EDC and NHS may be directly related to the degree of cross-linking of the samples, since both methods result in the formation of similar cross-links. This is supported by the linear relationship between the decrease in free amine group content and the increase in T_s of the E-DSC and E/N-DSC samples, as can be seen in *Figure 7*. The increase in T_s is proportional to the number of cross-links introduced, i.e. the number of free amine groups reacted.

The mechanical properties of fibrous collagens like DSC depend on the mechanical properties of the fibre bundles, which are highly structured aggregates of fibres and fibrils, respectively. Also, the spatial arrangement of the fibre bundles and the interweaving of fibres from one bundle to another influence the

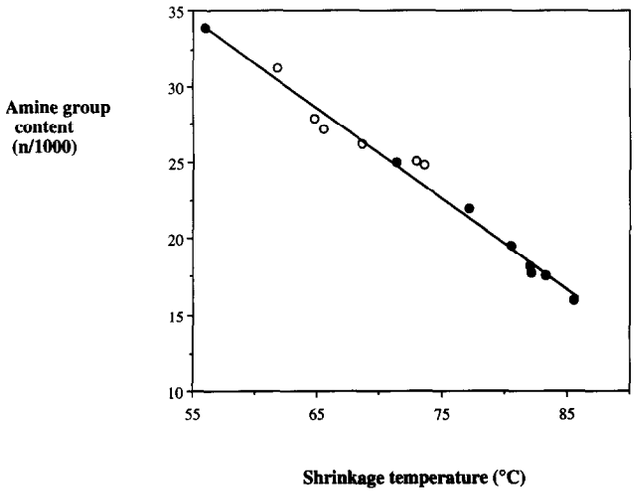


Figure 7 Free amine group content as a function of shrinkage temperature for the cross-linking of N-DSC with EDC (○) and with a mixture of EDC and NHS (●).

mechanical properties of the material. Previously, it was reported that the tensile strength was not changed and the high strain modulus decreased upon cross-linking of DSC samples using GA[12]. From these results it was concluded that cross-links are introduced only within the fibres. Amine groups located on two adjacent fibres or fibre bundles were apparently too far apart to be bridged by the cross-links. Aligning of the fibres by applying a pre-strain to the samples during cross-linking could overcome these spatial limitations[12].

The results obtained from the determination of the mechanical properties of E/N-DSC samples as presented in *Table 2* reflect these previously reported results. Cross-linking of N-DSC samples using a mixture of EDC and NHS resulted in materials with a decreased high strain modules. The tensile strength of N-DSC samples appeared to decrease upon cross-linking using a mixture of EDC and NHS. The decrease in tensile strength was observed for E/N-DSC samples having T_s values of 83° and higher and may be explained by the production of local stress concentrations due to the early failure of brittle collagen fibres formed after extensive cross-linking[30].

CONCLUSIONS

Treatment of DSC with EDC resulted in materials (E-DSC) with an increased T_s and a decreased free amine group content, indicating that cross-linking occurred. The use of NHS during the EDC cross-linking (E/N-DSC) not only increased the rate of cross-linking, but also resulted in materials with higher T_s values compared with E-DSC samples. Substituting the formed O-acylisourea groups by succinimate esters reduced the N-acyl shift of the O-acylisourea to the N-acylurea groups. Also, the lower susceptibility of the succinimate ester groups to hydrolysis accounts for the more effective cross-linking of the collagen. For both cross-linking methods the same linear relation between the decrease in free amine group content and the increase in T_s was observed. The influence of cross-linking on the mechanical properties of the E/N-DSC samples revealed that cross-links were introduced within fibres rather than between fibres.

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