

In vitro degradation of dermal sheep collagen cross-linked using a water-soluble carbodiimide

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Bacterial collagenase was used to study the susceptibility of dermal sheep collagen (DSC) cross-linked with a mixture of the water-soluble carbodiimide 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride and *N*-hydroxysuccinimide (E/N-DSC) towards enzymatic degradation. Contrary to non-cross-linked DSC (N-DSC), which had a rate of weight-loss of 18.1% per hour upon degradation, no weight loss was observed for E/N-DSC during a 24 h degradation period. The tensile strength of the E/N-DSC samples decreased during this time period, resulting in partially degraded samples having 80% of the initial tensile strength remaining. The susceptibility of E/N-DSC samples towards enzymatic degradation could be controlled by varying the degree of cross-linking of the samples. Ethylene oxide sterilization of E/N-DSC samples made the material more resistant against degradation compared with non-sterilized E/N-DSC samples. This may be explained by a decrease of the adsorption of bacterial collagenase onto the collagen owing to reaction of ethylene oxide with remaining free amine groups in the collagen matrix.

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Cross-linking of collagen-based biomaterials is often necessary to improve the resistance against enzymatic degradation. Currently, several methods are available for the cross-linking of collagen-based biomaterials. Glutaraldehyde is the most commonly used bifunctional reagent¹. Hexamethylene diisocyanate can also be used for the cross-linking of collagen². However, the use of these reagents in the cross-linking of collagen may result in the release of toxic products both *in vitro*³⁻⁵ and *in vivo*^{6,7}. A new class of cross-linking agents for collagen more recently described are the polyepoxy compounds^{8,9}.

The cross-linking of dermal sheep collagen (DSC) using a mixture of the water-soluble carbodiimide 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) has been described in the foregoing paper¹⁰. Cross-linking of DSC was performed by activation of the carboxylic acid groups of DSC through their succinimide esters followed by reaction with an amine group. The resulting materials (E/N-DSC) had a higher shrinkage temperature (T_s) compared with glutaraldehyde cross-linked DSC, which shows that cross-linking using a mixture of EDC and NHS is a good alternative for glutaraldehyde cross-linking.

Here we report on the *in vitro* degradation behaviour of E/N-DSC using bacterial collagenase as the degrading enzyme. Previously, ethylene oxide gas sterilization was selected as the preferred sterilization method for collagen-based biomaterials¹¹. Therefore, the influence of ethylene oxide sterilization on the *in vitro* degradation behaviour of E/N-DSC was also studied.

MATERIALS AND METHODS

Cross-linking of dermal sheep collagen

Dermal sheep collagen (DSC) was obtained from the Zuid-Nederlandse Zeemleiderfabriek (Oosterhout, The Netherlands) and prepared as reported previously¹². The fibrous collagen network was washed four times with water, twice with acetone and twice with water, and was subsequently frozen and lyophilized to give non-cross-linked dermal sheep collagen (N-DSC).

Cross-linking of DSC with EDC and NHS (E/N-DSC) was performed by immersing N-DSC samples weighing 1 g (1.2 mmol carboxylic acid groups) in 50 ml water containing 1.15 g (6.0 mmol) EDC (z.S., Merck-Schuchardt, Hohenbrunn, Germany) and 0.28 g (2.4 mmol) NHS (z.S., Merck-Schuchardt) for 2 h at room temperature to give E/N-DSC¹⁰. During cross-

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linking, the pH of the solution was maintained at 5.5 using a pH stat apparatus (702 SM Titrino, Metrohm, Herisau, Switzerland). After cross-linking, the E/N-DSC samples were washed with a 0.1 M Na₂HPO₄ solution for 2 h to hydrolyse any remaining NHS-activated carboxylic acid groups. Subsequently, the examples were washed four times with distilled water and lyophilized.

E/N-DSC samples with different degrees of cross-linking were obtained 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. All samples were cross-linked at pH 5.5 for 2 h.

Ethylene oxide sterilization

E/N-DSC samples were air dried before sterilization with ethylene oxide gas. Sterilization was performed by exposing the samples to a 100% ethylene oxide atmosphere at a relative humidity of 70% for 5 h at 55°C¹¹. After sterilization, the samples were aerated with a warm air flow at atmospheric pressure for at least 48 h to remove ethylene oxide from the DSC matrix before use in further experiments.

Characterization

The degree of cross-linking of DSC samples cross-linked with either a mixture of EDC and NHS was related to the increase in shrinkage temperature¹³. Shrinkage temperatures (T_s) of cross-linked or non-cross-linked DSC samples immersed in water were determined as described previously¹. The free amine group content of the samples was determined spectrophotometrically after reaction of the primary amine groups with 2,4,6-trinitrobenzenesulphonic acid¹ and is expressed as the number of amine groups present per 1000 amino acids (n per 1000).

Mechanical properties

Stress-strain curves of non-cross-linked and cross-linked DSC samples fully hydrated in phosphate-buffered saline (0.14 M NaCl, 0.01 M Na₂HPO₄, 0.002 M NaH₂PO₄, pH 7.4, NPBI, Emmercompascuum, The Netherlands) were determined by uniaxial measurements using an Instron mechanical tester¹. 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.

In vitro degradation

The degradation of non-sterilized and sterilized N-DSC and E/N-DSC was performed using bacterial

collagenase from *Clostridium histolyticum* (EC 3.4.24.3, Sigma Chemical Company, St Louis, MO, USA) with a collagenase activity of 255 U mg⁻¹ (one unit will release peptides from native collagen, equivalent in ninhydrin colour to 1.0 μmol of L-leucine in 5 h at pH 7.4 at 37°C in the presence of calcium ions). To a DSC sample weighing 10 mg, 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.4) containing 0.005 M CaCl₂ and 0.05 mg ml⁻¹ sodium azide was added. After incubation at 37°C for 1 h, 0.5 ml collagenase solution (200 U ml⁻¹) in Tris-HCl buffer (37°C) was added to give a final collagenase concentration of 100 U ml⁻¹. The absolute amount of collagenase present was 10 U mg⁻¹ DSC. During all degradation experiments the pH of the buffer remained constant. The degradation was discontinued at the desired time interval by the addition of 0.1 ml of 0.25 M ethylenediaminetetra-acetate (Titriplex III p.a., E. Merck, Darmstadt, Germany).

The weight loss of the DSC samples, expressed as the percentage of the initial weight remaining, was related to the hydroxyproline content of the supernatant, which was determined spectrophotometrically¹⁴. The tensile strength of the samples as a function of degradation time was determined by stress-strain measurements¹⁴. The change in mechanical properties of a degraded sample was only compared with the mechanical properties of a matching non-degraded control taken from an adjacent part of the skin. Samples used to study the influence of degradation on the mechanical properties were always taken from the IUP/2¹⁵ sampling area, parallel to the backbone, and were either degraded with collagenase or kept as control.

RESULTS

Initial properties

The results obtained from the T_s measurements and the free amine group content determinations of the N-DSC and E/N-DSC samples used during the degradation experiments are presented in Table 1. For N-DSC samples, a T_s of 56°C and a free amine group content of 34.4 per 1000 amino acid residues was measured. The latter value is in good agreement with values reported in the literature¹⁶. Cross-linking with EDC and NHS increased the T_s to 86°C and decreased the free amine group content to 16.3 per 1000 amino acid residues compared to N-DSC samples. Ethylene oxide sterilization of E/N-DSC samples resulted in lower values of T_s and free amine group content compared to non-sterilized E/N-DSC. After ethylene oxide

Table 1 Shrinkage temperature and amine group content of non-cross-linked and cross-linked dermal sheep collagen

Sample	Sterilization procedure	Shrinkage temperature (°C)	Amine group content (n per 1000)
N-DSC*	None	56.3 ± 0.3	34.4 ± 0.4
E/N-DSC [†]	None	85.8 ± 0.3	16.3 ± 0.6
	Ethylene oxide	82.4 ± 0.4	9.5 ± 0.5

*Non-cross-linked DSC.

[†]DSC cross-linked with EDC and NHS.

The free amine group content of DSC is expressed as the number of amine groups present per 1000 amino acids (triplicate measurements).

sterilization of E/N-DSC, a T_s of 82°C and a free amine group content of 9.5 per 1000 amino acid residues was found.

The initial mechanical properties of the N-DSC and E/N-DSC samples used during degradation are presented in Table 2. Ethylene oxide sterilization did not significantly change the tensile strength, elongation at alignment, high strain modulus and elongation at alignment of the E/N-DSC samples.

In vitro degradation

To study the *in vitro* degradation of N-DSC and E/N-DSC, samples were exposed to a bacterial collagenase solution. The weight and the tensile strength of the different DSC samples were determined as a function of degradation time. The tensile strength of DSC samples was monitored because it appeared to be more sensitive to degradation than the high strain modulus or the elongation at break¹⁴. The extent of degradation is expressed as percentage decrease in the original value of weight and tensile strength, respectively. Because partially degraded N-DSC behaved like denatured collagen, no cross-sectional area of the test specimens could be measured and consequently the tensile strength could not be determined. Therefore, the progress of degradation of N-DSC is represented by a dotted line from the start of degradation to the time at which the DSC samples desintegrated, when the degradation tube was vigorously shaken.

The percentage weight loss of N-DSC and E/N-DSC as a function of degradation time is presented in Figure 1a. The rate of weight loss is determined from the initial slope of the lines presented in Figure 1a. For N-DSC a rate of weight loss of 18% per hour was found. For E/N-DSC no weight loss was observed during a 24 h degradation period.

The degradation behaviour as a function of degree of cross-linking was determined using a series of E/N-DSC samples with increasing T_s values. Samples with T_s values of 66, 77, 83 and 86°C were obtained by cross-linking N-DSC samples 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. The change in weight of these samples as a function of degradation time is shown in Figure 1a. The rate of weight loss decreases with an increasing degree of cross-linking of the samples. For samples with a T_s of 56, 66, 77 and 83°C, a rate of weight loss of 18.1, 2.7, 0.7 and 0.05% per hour, respectively, was found.

The changes in tensile strength during degradation of N-DSC and E/N-DSC samples are presented in Figure

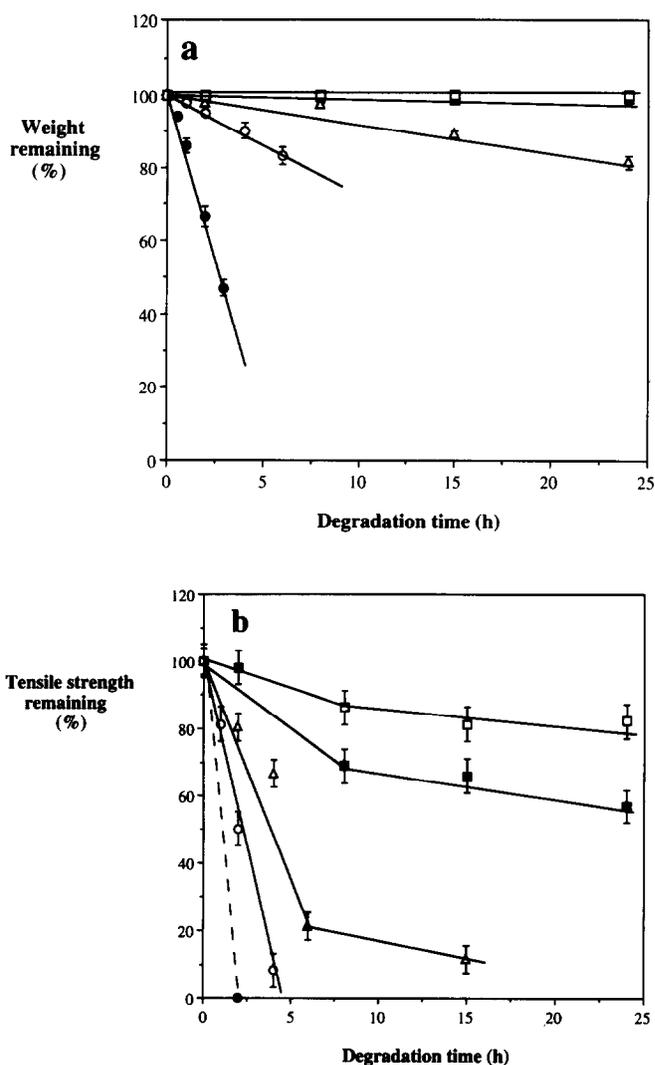


Figure 1 Changes in: a, weight and, b, in tensile strength as a function of degradation time during exposure of N-DSC with a T_s of 56°C (●), and E/N-DSC with a T_s of 66°C (○), 77°C (△), 83°C (■) and 86°C (□) to a bacterial collagenase solution (pH 7.4, 37°C, $n = 5$, \pm s.d.).

1b. N-DSC samples were most susceptible to degradation and had no tensile strength remaining after a 2 h degradation period. E/N-DSC samples with a high T_s showed a slow decrease in tensile strength. The tensile strength of partially degraded E/N-DSC samples with the highest degree of cross-linking (86°C) was 82% of the initial value after a 24 h cross-linking period.

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*	None	16 \pm 1	67 \pm 7	167 \pm 5	1.8 \pm 0.1	17 \pm 2
E/N-DSC†	None	11 \pm 1	76 \pm 5	186 \pm 11	3.4 \pm 0.3	8 \pm 1
	Ethylene oxide	10 \pm 1	69 \pm 6	171 \pm 9	3.8 \pm 0.2	8 \pm 1

*Non-cross-linked DSC.

†DSC cross-linked with EDC and NHS.

All mechanical properties are given as mean \pm s.d. of five measurements.

The influence of ethylene oxide sterilization on the change in weight and tensile strength during degradation of E/N-DSC samples is shown in Figure 2a and b, respectively. For both non-sterilized E/N-DSC and ethylene oxide-sterilized E/N-DSC, no weight loss was found during a 24 h degradation period (Figure 2a). During this period a slight decrease in tensile strength was observed for non-sterilized E/N-DSC (Figure 2b). Contrary to non-sterilized E/N-DSC, no loss in tensile strength was observed for the ethylene oxide-sterilized E/N-DSC samples during a 24 h degradation period.

DISCUSSION

Collagen-based biomaterials are generally stabilized by chemical cross-linking before *in vivo* application. In a previous paper we reported on the use of a mixture of the water-soluble carbodiimide EDC and NHS for the cross-linking of DSC¹⁰. Cross-linking using EDC and NHS involves the activation of carboxylic acid groups

of polypeptide chains to give NHS-activated carboxylic acid groups which form cross-links after reaction with free amine groups of other polypeptide chains. The results obtained revealed that treatment of N-DSC with E/N-DSC increased the T_s value from 56° for N-DSC to 86° for E/N-DSC samples¹⁰. In contrast, treatment of N-DSC with glutaraldehyde only increased the T_s from 56 to 78°C¹.

Recently, the *in vitro* degradation of glutaraldehyde cross-linked DSC samples (G-DSC) has been described¹⁴. Exposure of G-DSC samples to bacterial collagenase resulted in a weight loss of 3.5% after a 24 h degradation period (rate of weight loss 0.14% per hour). During this period, an initially fast decrease in tensile strength was observed. After an 8 h degradation period, the partially degraded G-DSC samples had only 35% of their initial tensile strength remaining. Longer degradation times resulted in partially degraded samples having only 25% of their initial tensile strength remaining after a 24 h degradation period. The degradation of E/N-DSC samples was performed applying identical conditions as used during the degradation of the G-DSC samples.

The influence of ethylene oxide gas treatment on the degradation behaviour of E/N-DSC samples was determined because this method has previously been selected as the preferred method for the sterilization of collagen-based biomaterials¹¹. Bacterial collagenase is capable of cleaving peptide bonds within the triple helical structure of collagen and has a specificity for the Pro-X-Gly-Pro-Y region, splitting between X and Gly^{17,18}. It has been shown that enzymatic degradation of polyesters occurs by a surface erosion process which is experimentally characterized by a zero order weight loss in the initial stage of the degradation^{19,20}. A zero order weight loss was always observed for the degradation of either non-cross-linked or cross-linked DSC. This suggests that the degradation of DSC can be depicted as a surface erosion process, as was described previously for reconstituted collagen²¹.

Cross-linking of DSC using a mixture of EDC and NHS gave materials with a T_s of 86°C (Table 1). The high degree of cross-linking of E/N-DSC samples is reflected in their resistance towards enzymatic degradation using bacterial collagenase. Both the decrease in weight (Figure 1a) and the decrease in tensile strength (Figure 1b) in time were significantly slower compared with N-DSC. It appeared that both the rate of weight loss and the decrease in tensile strength during degradation were controlled by the degree of cross-linking of the samples.

The decreased rate of weight loss of cross-linked versus non-cross-linked DSC samples during enzymatic degradation is most probably owing to interference of the penetration of the enzyme into the fibre¹. The reduced penetration will substantially decrease the surface area available for adsorption of the enzyme in the cross-linked collagen network and thus the surface degradation rate. Moreover, owing to the introduction of cross-links, on average more chains have to be cleaved before a degraded fragment can be solubilized.

These considerations can also be applied to describe the influence of cross-linking on the change in tensile

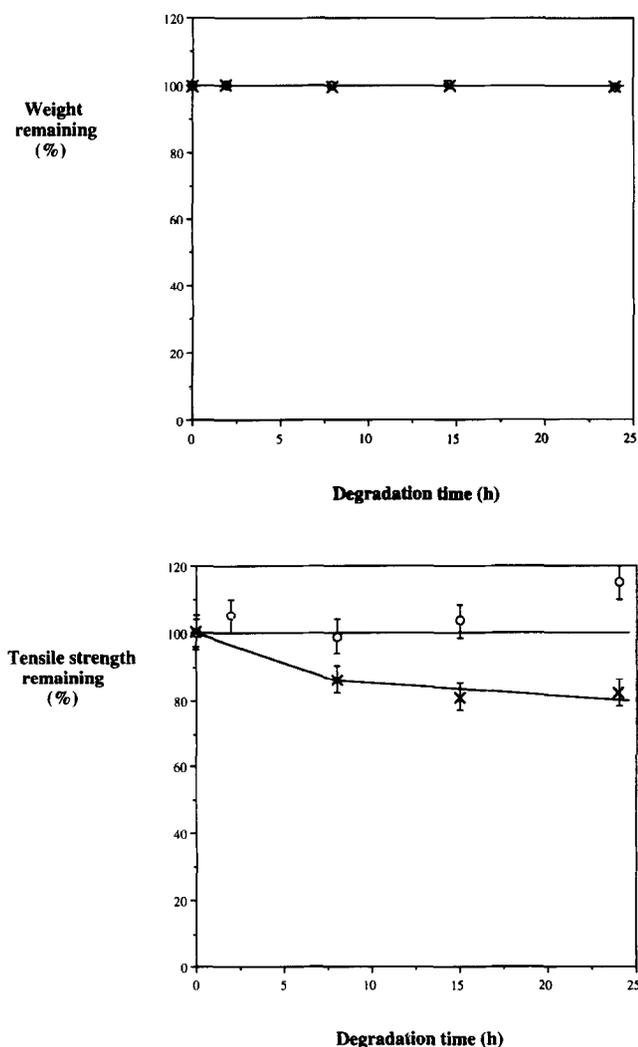


Figure 2 Changes in: a, weight and, b, in tensile strength as a function of degradation time during exposure of non-sterilized E/N-DSC (x) and ethylene oxide-sterilized E/N-DSC (o) to a bacterial collagenase solution (pH 7.4, 37°C, $n = 5$, \pm s.d.).

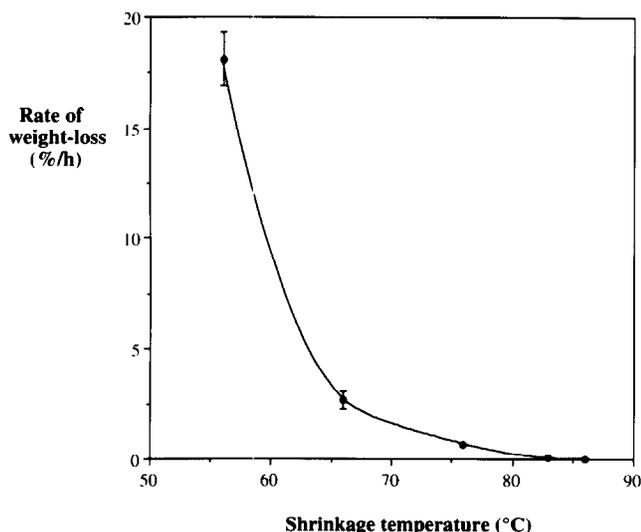


Figure 3 Rate of weight loss as a function of shrinkage temperature for E/N-DSC samples.

strength during degradation. If cross-linking prevents the penetration of the enzyme in the fibre structure and erosion occurs only at the fibre surface, the load-bearing area of the material will decrease more slowly for materials with a higher degree of cross-linking. Furthermore, cross-links present in the cross-linked materials will retain the strength of the samples for a longer period during degradation.

The rate of weight loss of E/N-DSC samples as a function of the T_s value is shown in Figure 3. This figure is constructed from the data presented in Figure 1a. It appears that two phases can be distinguished in this plot. At low degrees of cross-linking (T_s values up to 66°C), a small increase in T_s results in a large decrease in rate of weight loss. As a result of cross-linking between fibrils the penetration of the enzyme into the fibres is hindered. This will reduce the surface area available for adsorption of collagenase and will have a large influence on the degradation rate. At higher degrees of cross-linking (T_s values of 66°C and higher), a further increase in T_s only results in small changes in the rate of weight loss. The decreased rate of weight loss is probably based on the reduction of the rate of solubilization of degraded parts from the fibre surface.

When the degradation behaviour of E/N-DSC samples is compared with the previously reported degradation behaviour of G-DSC samples¹⁴, it is observed that E/N-DSC samples are more resistant towards *in vitro* degradation by bacterial collagenase. Contrary to G-DSC, no weight loss is observed during a 24 h degradation period for the E/N-DSC samples and only a gradual decrease in tensile strength during degradation is found, resulting in partially degraded samples having 82% of the initial tensile strength remaining after 24 h degradation.

Ethylene oxide gas sterilization improves the resistance of non-cross-linked and cross-linked DSC samples towards enzymatic degradation¹¹. During sterilization, ethylene oxide molecules react with the free amine groups of DSC. The N-2-hydroxyethyl

groups may interfere with the adsorption of the enzyme, thus decreasing the rate of degradation. Similar results were obtained in this study. In Table 1 a decrease in free amine group content is observed for ethylene oxide-sterilized E/N-DSC samples compared with non-sterilized samples, showing that reaction of ethylene oxide with amine groups occurred during ethylene oxide sterilization. The reaction of ethylene oxide with the free amine groups did not significantly change the mechanical properties of E/N-DSC (Table 2). No significant differences in weight loss were observed between non-sterilized and ethylene oxide-sterilized E/N-DSC samples during a 24 h degradation period (Figure 2a). However, a significant increase in resistance to degradation was observed when the tensile strength of the samples was monitored as a function of degradation time (Figure 2b). Contrary to non-sterilized E/N-DSC, the tensile strength of the ethylene oxide-sterilized E/N-DSC samples was not affected by exposure to bacterial collagenase for 24 h, indicating that ethylene oxide-sterilized E/N-DSC samples were more resistant to degradation.

CONCLUSIONS

The resistance against degradation by bacterial collagenase of E/N-DSC samples was increased compared to N-DSC. The susceptibility of E/N-DSC samples towards enzymatic degradation could be controlled by varying the degree of cross-linking of the samples. During ethylene oxide gas sterilization of the E/N-DSC samples, reaction of ethylene oxide with the free amine groups of E/N-DSC occurred. Ethylene oxide-sterilized E/N-DSC samples had an improved resistance to degradation resulting from an interference of the N-2-hydroxyethyl groups with the adsorption sites for bacterial collagenase.

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