JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 7 (1996) 531–541

Crosslinking of tissue-derived biomaterials in 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)

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In contrast to bifunctional reagents such as glutaraldehyde or polyfunctional reagents such as polyepoxides, carbodiimides belong to the class of zero-length crosslinkers which modify amino acid side-groups to permit crosslink formation, but do not remain as part of that linkage. The authors have compared the effects of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and glutaraldehyde (the de facto industrial standard crosslinker) on the hydrothermal, biochemical, and uniaxial mechanical properties of bovine pericardium. EDC crosslinking was optimized for maximum increase in collagen denaturation temperature using variables of pH, concentration, and ratio of EDC to N-hydroxysuccinimide (NHS): a reagent for formation of activated esters. EDC and glutaraldehyde crosslinked materials were subjected to hydrothermal denaturation tests, biochemical degradation by enzymes (collagenase, trypsin) and CNBr, amino acid analysis for unreacted lysine, and to high strain rate mechanical tests including: large deformation stress–strain studies (0.1 to 10 Hz), stress relaxation experiments (loading time 0.1 s) and small deformation forced vibration (1 and 10 Hz). A protocol for EDC crosslinking was developed which used 1.15% EDC (2:1 EDC:NHS) at pH 5.5 for 24 h. The increase in denaturation temperature for EDC (from 69.7 ± 1.2 °C to 86.0 ± 0.3 °C) was equivalent to that produced by glutaraldehyde (85.3 ± 0.4 °C). Both treatments equivalently increased resistance to collagenase and CNBr degradation; however, after denaturation, the EDC-treated tissue was slightly more resistant to collagenase, and markedly more resistant to trypsin. EDC-treated materials were more extensible and more elastic than glutaraldehyde-treated materials. Despite the differences in crosslinking mechanism, EDC and glutaraldehyde-treated materials are very similar. Subtle but intriguing differences in biochemical structure remain to be investigated.

1. Introduction

With the increased dissatisfaction over the performance of glutaraldehyde (GLUT) as a pre-implantation treatment for tissue-derived biomaterials, several alternative collagen crosslinking treatments have been explored in the literature. These include: (1) other homobifunctional reagents such as diisocyanates [1–4] and diisothiocyanates [5], (2) homopolyfunctional epoxides [6–9], and (3) zero-length crosslinking methods using carbodiimides [1–3, 10–12], acyl azide [13–15], or dye-mediated photo-oxidation [16, 17]. The term "zero-length crosslinker" is reserved for reagents or processes which modify side-groups on proteins to make them reactive with other side-groups (so that a crosslink can be formed) but which do not themselves remain in the linkage [18]. If stable covalent linkages are produced, this method can claim the advantage of precluding depolymerization and release of residual (potentially toxic) reagent.

Carbodiimides are available in a variety of molecular structures; however, by far the most widely used is 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (see Fig. 1). This reagent has found widespread use in peptide synthesis [19], for protein conjugation and complex formation [20–22], cellular modification [23], heparin binding to collagen [24, 25], and hapten binding for immunohistochemistry [26, 27]. Recently, Olde Damink [1] has used EDC on extracted ovine dermal collagen and demonstrated both significant crosslinking and interesting *in vitro* differences when compared with glutaraldehyde treatment. Performance in cell culture and *in vivo* has also been promising [2, 3]. Cyanamide (the smallest of the carbodiimides) has been used by Weadock and colleagues [10] either in solution or vapour form as a treatment for pure collagen gels and extruded fibres. They found it to be a weak crosslinker best used in combination with a dehydrothermal treatment [11, 12, 28]. By contrast, when cyanamide solution was used by Pereira *et al.* [7] on intact pericardium, they found no increase in collagen denaturation temperature or changes in mechanical properties consistent with any action other than degradation in acid pH conditions. Taken together, these results suggest both a

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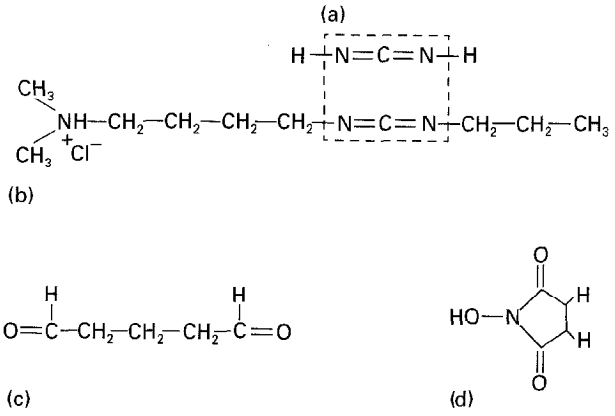

Figure 1 Molecular structures of crosslinking reagents (a) cyanamide, (b) EDC, and (c) glutaraldehyde. Note that the two carbodiimide reagents (a) and (b) differ in molecular size but share common carbodiimide structure (dashed box). Also shown is the structure of the activated ester forming reagent NHS (d).

dependence of action on carbodiimide structure and a potential difference in effectiveness when acting on purified collagen or on intact tissue.

In an effort to clarify these effects, we have used the EDC treatment of dermal collagen by Olde Damink [1] as a starting point to develop a treatment protocol for intact tissues—in this case, bovine pericardium. We have used collagen denaturation temperature rather than total reacted lysine residues as a basis for selection of processing parameters. The relationship between these two variables is quite different under EDC and glutaraldehyde crosslinking—at least in extracted collagen [1]. Failing to demonstrate any crosslinking of intact tissues by cyanamide, we have then compared the effects of glutaraldehyde and EDC treatment on the hydrothermal denaturation, biochemical degradation, and mechanical properties of the resulting materials.

2. Methods

The first part of this study dealt with development of a protocol for crosslinking of bovine pericardium in EDC by maximizing collagen denaturation temperature. Since we were interested in the effect of molecular structure on carbodiimide crosslinking, we also examined the crosslinking produced by the much smaller cyanamide molecule (Fig. 1.) The second part of the study investigated some mechanical and biochemical characteristics of this optimized EDC-crosslinked pericardium, and compared these characteristics with those of untreated pericardium and the industry-standard GLUT-crosslinked pericardium.

2.1. Tissue preparation

Bovine hearts with intact pericardia were obtained from an abattoir within a few minutes of slaughter of 6 9 month old calves. The hearts were transported at ambient conditions in Hanks' physiological solution to our laboratory. For each heart, all adherent fat was gently stripped from the ventral surface of the pericardium. Suture markers were sewn into the pericardium near the base of the aorta and near the apex of the heart to indicate the base-to-apex direction. A rectangular section of ventral pericardium — including the suture markers — was excised and washed in fresh Hanks' solution for 30 min (three washes at 10 min each).

2.2. Development of EDC crosslinking protocol

Rectangular strips measuring 4 mm by 25 mm were cut from the excised ventral pericardial section. Fresh tissue strips were immediately tested for their thermal stability and served as controls. The remaining strips were subjected to one of the crosslinking protocols described below. During crosslinking, all solutions were constantly agitated. Tissue strips was removed from the crosslinking solutions at 1 h, 2 h, 3 h and 24 h, and washed for 30 min in 0.1 M Na_2HPO_4 (three washes at 10 min each) prior to testing for their thermal stability.

Effect of controlling pH during crosslinking. The crosslinking solutions were unbuffered. Two identical 1.15% EDC (Sigma) solutions were made using a molar ratio of 1:1 between EDC and the blocking agent N-hydroxysuccinimide (NHS, Sigma). An equal number of tissue strips were immersed into each solution. During crosslinking, one of the solutions was held constant at pH 5.5 by the addition of drops of 0.1 M HCl or 0.1 M NaOH solution, while the pH of the other solution was simply recorded.

Effect of EDC concentration. Equal numbers of tissue strips were immersed into EDC solutions (1:1 EDC:NHS, pH constant at 5.5) with concentrations of 0.58%, 1.15% and 2.30%.

Effect of EDC concentration in the absence of NHS. Equal numbers of tissue strips were immersed into EDC solutions (pH constant at 5.5) with concentrations of 0.58%, 1.15% and 2.30%.

Effect of amount of NHS. Equal numbers of tissue strips were immersed into 1.15% EDC solutions with molar ratios of EDC:NHS of 1:2, 1:1, 2:1 and 4:1 (pH constant at 5.5).

Effect of molecular structure. Equal numbers of tissue strips were immersed into equimolar solutions of EDC or cyanamide: i.e. 1.15% EDC or 0.25% cyanamide solutions. Each solution had a molar ratio of EDC:NHS or cyanamide:NHS of 2:1 or 1:1 (pH constant at 5.5).

2.3. Characterization of EDC- and glutaraldehyde-crosslinked bovine pericardium

Based on the results above, the parameters for EDC crosslinking of bovine pericardium were chosen (1.15%, EDC:NHS = 2:1, pH constant at 5.5, room temperature). The mechanical and biochemical char-

acteristics of EDC-crosslinked bovine pericardium were compared with those of GLUT-crosslinked tissue. Untreated tissue was used as a control.

Six cleaned and suture-marked ventral pericardia were washed in Hanks' physiological saline. From each pericardium, six rectangular pieces measuring 2 cm wide by 3 cm long were cut with their long sides parallel to the base-to-apex direction. Each treatment group received two pieces—one for biochemical digestion tests and the other for mechanical and thermal stability tests. In order to reduce any effect of regional differences in the pericardium, each treatment group was assigned pieces from a different location with each subsequent pericardial sac. This method ensured that, with pericardia from six animals, each test group within each treatment (e.g. EDC tissue for biochemical digestion) had representation from each of the six locations on the ventral pericardial surface.

The treatment groups were prepared as follows:

(1) Untreated (fresh) tissue was tested for its thermal stability and mechanical properties no later than 6 h after slaughter. Fresh tissue for biochemical digestion was frozen in Hanks' solution and prepared as described below.

(2) GLUT crosslinking was carried out according to the protocol described by Ionescu *et al.* [29]. The pericardial pieces were immersed in 0.5% GLUT (EM grade, Sigma) buffered to pH 7.4 in 0.067 M phosphate buffer for 24 h. The pieces were then removed and washed in 0.1 M Na_2HPO_4 for 30 min (three washes at 10 min). GLUT tissue for biochemical digestion was frozen in Hanks' solution and prepared as described below.

(3) EDC crosslinking was carried out using the parameters developed in the first part of this study for optimized crosslinking. An unbuffered 1.15% EDC (2:1 EDC:NHS) solution was made by dissolving 1.15 g of EDC and 0.34 g of NHS in 100 ml of distilled water. The pH of the EDC solution was adjusted to 5.5 by the addition of 0.1 M HCl or 0.1 M NaOH solution. The pericardial pieces were immersed in the solution within one hour of solution make-up. The tissue remained in the EDC solution for 24 h, and throughout the fixation, the solution was maintained at pH 5.5. The pieces were then removed and washed in 0.1 M Na_2HPO_4 for 30 min (three washes at 10 min). EDC tissue for biochemical digestion was frozen in Hanks' solution and prepared as described below.

(4) Cyanamide treatment was carried out under the conditions described above for EDC crosslinking: i.e. equimolar concentration of cyanamide were substituted for EDC. These samples were subjected to thermal stability testing only due to the reagent's failure to introduce crosslinks (see results below).

2.3.1. Hydrothermal stability test

Tissue samples measuring 4 mm × 25 mm were mounted in a custom-built six-sample Denaturation Temperature Tester (Centre for Biomaterials, University of Toronto) interfaced to an Apple Macintosh computer with data-acquisition hardware (National Instruments' NB-MIO-16L A/D board) and software (National Instruments' LabVIEW®). The mounted specimens were immersed in a bath of distilled water at room temperature, loaded to 50 g and held at constant extension. The bath was then heated to 95 °C at a rate of approximately 2 °/min. The computer monitored the temperature of the bath and the loads on the six specimens, and recorded these measurements at 1 °C increments. When the collagen denatured, the specimen contracted and a sharp increase in load was observed at the denaturation temperature [30].

2.3.2. Biochemical tests

Pericardial sections from all treatment groups were thawed and washed with several changes of distilled water for 2 h (four washes at 30 min each) in order to remove any excess crosslinking agents. Tissue sections were subsequently defatted (using 1: 1 v/v chloroform: methanol, 100% methanol, 50% methanol, and distilled water) and freeze dried. The freeze dried tissues were then minced and stored in the freezer.

Enzymatic degradation with collagenase. Samples (20 – 30 mg) of finely minced tissue were weighed and placed in Beckman polycarbonate ultracentrifuge tubes. A 5 mg/ml stock collagenase solution was prepared by dissolving bacterial collagenase (*C. histolyticum*, Sigma Type I) in 0.05 M Tris-HCl/10 mM $CaCl_2$ buffer (pH 7.4). Appropriate amounts of the collagenase stock solution and Tris-HCl buffer were added to the tissue for a final 0.5% (w/w) enzyme to tissue ratio in a final volume of 2 – 3 ml. Incubations were carried out at 37 °C for 18 h. At the end of the incubation period, samples were centrifuged for 20 min under 62 000 g (30 000 rpm) at 4 °C. The supernatant was removed and the remaining pellets were washed with distilled water, re-centrifuged and freeze dried. The percentage of the initial mass remaining in the residue was used as a measure of resistance of the tissue to enzymatic degradation.

Effect of collagenase on denatured collagen. Tissue samples from different treatment groups were immersed in 0.05 M Tris-HCl/10 mM $CaCl_2$ buffer (pH 7.4) and heated in a 90 °C heating block for 30 min. Collagenase treatment followed as described above.

Enzymatic degradation with trypsin. To establish optimal conditions for the enzymatic action of trypsin on bovine pericardium, we assessed trypsin performance under several experimental conditions. Generally, 20–30 mg of finely minced tissue was weighed and placed in Beckman polycarbonate ultracentrifuge tubes. Incubation with trypsin (Sigma, Type XIII) was done in 0.1 M Tris-HCl/20 mM $CaCl_2$ buffer (pH 8.0) in a final volume of 1 ml per 10 mg tissue. During incubations, samples were constantly agitated. The percentage mass remaining after digestion was determined as above.

To establish conditions for digestion, fresh samples were incubated at a 1:10 (w/w) enzyme:tissue ratio at either 4 °C, room temperature or 37 °C for up to 48 h. Next, fresh samples were incubated at 37 °C with trypsin at concentrations of 1:20, 1:10, and 1:5 (w/w) enzyme:tissue ratio for up to 48 h.

Since trypsin digestion was ineffective on undenatured tissue, fresh samples were denatured by immersion in 0.1 M Tris-HCl/20 mM $CaCl_2$ buffer (pH 8.0) and heating in a 90 °C heating block for 30 min. Trypsin was then added at a 1:10 enzyme to tissue ratio and the samples were incubated at 37 °C for 48 h. To determine the weight loss due to denaturation (loss of solubilized protein or other constituents), freeze dried pellets were again prepared as above, and the weights compared to the weights before denaturation.

Once conditions for trypsin digestion of denatured fresh tissue were established, we applied the same protocol to EDC- and GLUT-crosslinked bovine pericardia. Trypsin was added to denatured samples at a 1:10 enzyme:tissue ratio, and the samples were then incubated in a 37 °C shaking water bath for 48 h.

Chemical degradation with CNBr. Minced pericardia from different treatment groups were accurately weighed (20–30 mg) and placed in Beckman polycarbonate ultracentrifuge tubes. CNBr was directly measured into a tube previously flushed with N_2. An appropriate amount of 70% v/v formic acid was added to make up a 25 mg/ml CNBr solution. The tubes containing tissue were then bubbled with N_2 for approximately 3 min. The CNBr/formic acid solution was then added to the tissue at a ratio of 0.1 ml per 10 mg tissue. The tubes were flushed with more N_2, capped and agitated for 24 h (at room temperature) under the fumehood. At the end of the incubation period, CNBr was diluted with 10 volumes of distilled water. The tubes were then left uncapped under the fumehood overnight. The next day, the percentage weight remaining was determined as above.

Amino acid analysis. The amino acid analyses were performed by HSC/Pharmacia Biotechnology Service Centre (Banting Institute, University of Toronto). Briefly, pericardial samples from all treatment groups were hydrolyzed by a vapour phase/liquid phase reaction, using 6N HCl with 1% phenol at 110 °C for 24 h. After hydrolysis, the excess HCl was vacuum-dried and samples were redried using a solution of methanol:water:triethylamine (2:2:1). The samples were then derivatized for 20 min at room temperature using methanol:water:triethylamine:phenylisothiocyanate (7:1:1:1). The derivatized samples were dissolved in sample diluent and placed on a Waters PICO-TAG system. The amino acids were detected at 254 nm at 0.01 AUFS range.

2.3.3. High strain rate mechanical tests

High strain rate mechanical tests were carried out using a slightly modified version of the protocol described more fully by Lee *et al.* [31]. Testing was conducted on an Instron 8500 servo-hydraulic test machine interfaced to an Apple Macintosh computer with high-speed data acquisition hardware (National Instruments' NB-MIO-16L A/D board) and software (National Instruments' LabVIEW® 2.0). The computer was also equipped with VideoSpigot™ image grabbing hardware and software. A tissue test sample measuring approximately 4 mm × 20 mm was cut from each of the rectangular sections with its long side parallel to the base-to-apex direction. In order to obtain a measure of sample thickness without disruption, the thickness of an adjacent piece of tissue was measured with a non-rotating thickness gauge (Mitutoyo) [32]. The test sample was then mounted into brass grips such that the sample's grip-to-grip gauge length was approximately 10 mm. The grip faces were lined with #240 grit water-proof sandpaper to prevent tissue slippage. The upper grip was attached to a fixed load-cell (Transducer Techniques GS-500; 500 grams maximum load). The lower grip was attached to the base of a plexiglass tank containing Hanks' solution held constant at 37°C (± 0.2°C). The tank, in turn, was secured to the movable hydraulic actuator of the Instron machine. The Instron's actuator contained a Linearly Variable Differential Transformer (LVDT) for measuring the actuator's —and hence the test sample's— displacement. In order to measure the test sample's actual gauge length, a small load of 0.5 g was applied to the sample to remove all kinks. An image of the 0.5 g loaded sample in the grips was then captured by the VideoSpigot™ image grabber via a video camera with 30 × zoom capabilities and stored for later analysis with our own SoftDigitizer© dimensional analysis software. The test sample was then subjected to the following mechanical testing protocol:

Preconditioning. The sample was mechanically preconditioned by loading and unloading the sample between 0 g and 80 g at 1 Hz for 25 cycles using a triangular deformation waveform.

Stress–strain. The sample was loaded/unloaded, from 0 g to 80 g to 0 g, using a single triangular deformation waveform at 0.1 Hz. Load–extension data was collected and stored for later conversion to stress–strain data. The sample was also tested in this manner at 1 Hz and 10 Hz with preconditioning before each test.

Stress relaxation. The sample was preconditioned and then loaded from 0 g to 40 g in 0.1 s using a single ramp deformation waveform and held at constant extension for 100 s. Load–time data was collected and stored for later conversion to stress–time data. The sample was then preconditioned and the stress relaxation test repeated using a maximum load of 80 g.

Forced vibration. The sample was preconditioned and then loaded to 40 g. A cyclic sinusoid waveform with a small amplitude of 0.05 mm was executed at 1 Hz and load–extension–time data was collected and stored for later conversion to stress–strain–time data. The sample was also cycled at 10 Hz about 40 g,

TABLE I Effect of processing parameters on collagen denaturation temperature

Effect of exposure time					
Time (h)	0	1	2	3	24
T_d (°C)	68.4 ± 0.3	73.4 ± 0.4	75.7 ± 0.5	76.7 ± 0.3	76.4 ± 0.3

Effect of EDC concentration		
EDC concentration	T_d at 24 h with NHS (°C)	T_d at 24 h without NHS (°C)
0.58 %	77.3 ± 0.5	73.2 ± 0.8
1.15 %	76.4 ± 0.3	75.0 ± 0.0
2.3 %	79.5 ± 0.3	76.2 ± 0.8

Effect of EDC:NHS ratio				
EDC:NHS	1:2	1:1	2:1	4:1
T_d (°C)	71.2 ± 0.8	76.4 ± 0.3	86.0 ± 0.6	86.9 ± 0.5

Concentration EDC = 1.15%, EDC:NHS = 1:1, pH = 5.5, T = 23°C (n = 6 for each group)
EDC:NHS = 1:1, pH = 5.5, T = 23 °C (n = 6 for each group)
EDC concentration = 1.15%, pH = 5.5, T = 23°C (n = 6 for each group) Mean ± SEM

cycled at 1 Hz about 80 g, and cycled at 10 Hz about 80 g. Preconditioning was performed before each of these tests.

2.3.4. Analysis of mechanical data

The image of each 0.5 g loaded test sample was analysed by our custom-written SoftDigitizer© software. Since the width of the grips was known, the magnification of the image was determined. The grip-to-grip gauge length and sample width were then calculated. Using the sample's thickness, width and gauge length, load–deformation–time data was converted to stress–strain–time data by the following methods. Strain was calculated as the change in length per unit gauge length. Stress was calculated as the force exerted per unit cross-sectional area assuming the test strip maintained a constant volume during the test. Stress relaxation was calculated as the percentage decay in stress with respect to the peak stress. In forced vibration, the dynamic modulus E^* was calculated as a complex number with magnitude $|E^*|$ and phase angle θ. The magnitude of the modulus $|E^*|$ was calculated as the ratio of the amplitude of the stress waveform to that of the strain waveform. The phase angle θ was calculated as the phase difference between the stress and strain sinusoids. Measures of the stress and strain waveforms' amplitudes and phase shifts were obtained by a non-linear least squares fit using the Levenberg–Marquardt method in LabVIEW®. The analysis of this data is more completely described in Lee *et al.* [31].

To examine the effects of EDC or GLUT crosslinking on the fresh bovine pericardium, analysis of variance (ANOVA) was used with Fishers' least significant difference test for multiple comparisons (StatVIEW®4, Abacus Concepts). Differences among the three test groups with $p \leqslant 0.05$ were accepted as statistically significant. For the amino acid analyses (where smaller sample sizes were used), both the ANOVA and Kruskal–Wallis non-parametric analyses were performed. All data are presented as the mean ± one standard error of the mean (SEM).

3. Results

3.1. Processing parameters for carbodiimide crosslinking

Thermal denaturation studies of bovine pericardium crosslinked with 1.15% EDC solution (EDC:NHS 1:1, pH 5.5 held constant) showed rapid crosslinking within the first 3 h, but no significant change in denaturation temperature (T_d) thereafter (Table I). Without the pH being held constant during fixation, the solution became gradually more basic, reaching pH 6 after 24 h. During this treatment, T_d values were slightly lower at 3 h (compared to the pH-controlled experiments), but were not significantly different after 24 h.

Crosslinking increased with EDC concentration, the 2.3% EDC solution (EDC:NHS = 1:1) showing greater final T_d than did 0.58% or 1.15% solutions (Table I). Removing the NHS reduced the maximum crosslinking achieved. Also, when the ratio of EDC:NHS was increased, greater crosslinking was achieved up to a maximum of 2:1; increasing the ratio to 4:1 had no significant effect.

3.2. Effect of molecular structure: ineffectiveness of cyanamide

When pericardium was treated in 1.15% cyanamide, with ratio of cyanamide:NHS at 2:1, pH 5.5, no increase in denaturation temperature was observed up to 24 h treatment. Denaturation temperature at 24 h was 68.5 ± 0.3°C, no different from the 69.7 ± 1.2 °C value for fresh tissue (n = 6). No further characterization of the cyanamide-treated material was undertaken.

3.3. Comparison of EDC- and glutaraldehyde-crosslinked materials with fresh bovine pericardium

3.3.1. Hydrothermal stability

The denaturation temperature for fresh bovine pericardial tissue was 69.7 ± 1.2 °C (n = 6). Both GLUT-crosslinked and EDC-crosslinked tissues were more thermally stable than fresh tissue ($p < 0.0001$); however, the slight difference between the two crosslinked tissues was not statistically significant ($p = 0.518$) (see Table II).

3.3.2. Biochemical tests

Collagenase resistance. Under reagent conditions where fresh pericardial tissue had little resistance to attack by collagenase, both GLUT-crosslinked and

TABLE II Denaturation temperatures, chemical and enzymatic degradation

Material	Denaturation Temperature	Percent Mass Remaining after CNBr	Precent Mass Remaining after Collagenase	Percent Mass Remaining after Trypsin
Undenatured				
Fresh	69.7 ± 1.2	11.0 ± 1.2	20.1 ± 1.5	- - -
Glut.	86.0 ± 0.3*	97.0 ± 0.5*	90.4 ± 1.5*	- - -
EDC	85.3 ± 0.4*	98.0 ± 0.3*	93.4 ± 0.6*	- - -
Denatured				
Fresh	- - -	- - -	9.7 ± 0.6	5.8 ± 0.6
Glutaral-dehyde	- - -	- - -	77.3 ± 0.7**	36.3 ± 0.8**
EDC	- - -	- - -	81.8 ± 0.9**	83.2 ± 0.4**

*EDC and glutaraldehyde crosslinked materials different from fresh tissue, but not from each other ($p < 0.05$)
**EDC and glutaraldehyde crosslinked materials different from fresh tissue and from each other ($p < 0.05$)
Mean ± SEM. ($n = 6$ for each group)

EDC-crosslinked tissues showed markedly higher resistance (Table II, $p < 0.0001$). Again, there was no significant difference in the resistance to collagenase between the two reagents ($p = 0.14$). Hydrothermally denatured collagen was more susceptible to enzymatic degradation by collagenase. After denaturation, the EDC-treated tissue was slightly—but significantly—more resistant to collagenase than was GLUT-treated tissue (Table II, $p < 0.05$).

Trypsin resistance. In the undenatured form, fresh pericardial tissue was highly resistant to attack by trypsin. After 48 h of incubation with trypsin at 4 °C, fresh tissue had 81.3 ± 0.7% of its mass remaining ($n = 4$), and 70.8 ± 0.2% ($n = 4$) when incubated at 37 °C (significant difference with $p < 0.05$). There was no significant difference between trypsin digestion at 1:10 and 1:5 enzyme to tissue ratios. At 1:10, 81.8 ± 1.5% of the fresh tissue mass remained intact ($n = 3$), compared to 74.7 ± 2.4% ($n = 6$) when the enzyme was available at twice the concentration.

Denaturation alone caused some weight loss in the pericardium due to solubility. Fresh samples remaining 97.4 ± 0.4% insoluble ($n = 4$), GLUT-crosslinked samples 99.9 ± 0.02% insoluble ($n = 4$), and EDC-crosslinked samples 98.9 ± 0.5% insoluble ($n = 4$). The difference in solubilities between fresh and crosslinked materials was significant with $p < 0.05$.

Trypsin digestion was extremely effective against denatured fresh tissue (see Table II). Crosslinking with EDC substantially inhibited digestion while GLUT was significantly less effective ($p < 0.05$).

CNBr resistance. EDC and GLUT crosslinking produced equivalent increases in resistance to solubilization by CNBr (see Table II). There was no statistically significant difference in the resistance to CNBr between the two crosslinked tissue groups ($p = 0.435$).

Amino acid analysis. When amino acid analysis is used to assess GLUT crosslinking, the decrease in available lysine (Lys) residues is an indication of the extent of reaction—but not necessarily the extent of crosslinking [1]. To normalize for the amount of collagen present, we have used the ratio of lysine to leucine (Leu) which is not expected to react [17].

The Lys/Leu ratio was 0.86 ± 0.01 in the fresh tissue. This ratio was much reduced in GLUT-crosslinked tissue (0.20 ± 0.02), while it remained unchanged in EDC-crosslinked (0.88 ± 0.03) tissue (overall $p < 0.0001$ by ANOVA, $p = 0.060$ by Kruskal–Wallis). As with the unchanged lysine values, there was no change in the numbers of the aspartic or glutamic acid residues in EDC crosslinked tissue. Although the crosslinks formed in the EDC reaction were hydrothermally stable (as shown in denaturation testing), they did not survive the acid hydrolysis used in the amino acid analysis.

Curiously, there was a marked increase in a peak which eluted in the position of histidine (His) in EDC-crosslinked samples (His/Leu = 1.16 ± 0.11), when compared with either fresh (0.20 ± 0.002) or GLUT-crosslinked (0.18 ± 0.004) samples (overall $p < 0.0001$ by ANOVA, $p < 0.02$ by Kruskal–Wallis). The identity of this component was not established.

3.4. Mechanical tests

The stress-strain curves for GLUT or EDC-treated materials were similar, both showed a ramping shape at low stress and a less defined transition from low to high slope than was seen in fresh tissue (Fig. 2). When strain under 200 kPa stress was examined, both GLUT-crosslinked and EDC-crosslinked tissues were more extensible than fresh tissue, and EDC-crosslinked tissue was more extensible than GLUT-crosslinked tissue (Table III).

In stress relaxation tests, EDC-crosslinked tissue displayed the least load decay (i.e. was most elastic) over a period of 100 s at fixed extension. The relaxation of the EDC material was less than for both fresh and GLUT-crosslinked materials—which were in turn different from each other (Fig. 3, Table III).

The lower stiffness of the EDC and GLUT-treated at a given stress level was confirmed in forced vibration tests. For both the 40 g and 80 g mean load levels,

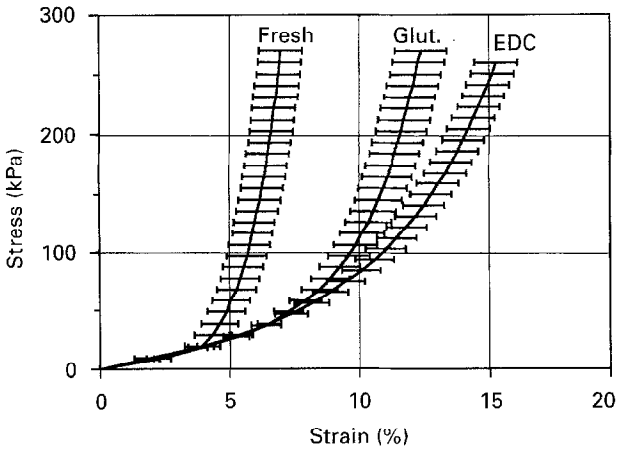

Figure 2 The stress–strain curves for glutaraldehyde (Glut.) and EDC-treated pericardium were significantly more extensible than fresh tissue in 1 Hz ramp deformation ($p < 0.01$). The EDC-treated material was the most extensible at higher stress ($p < 0.02$). Mean strain ± SEM ($n = 6$ for each group).

TABLE III Extensibility and stress relaxation data

Extensibility expressed as strain (%) at 200 kPa stress			
Frequency	Fresh	Glutaraldehyde	EDC
0.1 Hz	3.1 ± 0.5	9.0 ± 0.8*	12.3 ± 1.0**
1 Hz	3.0 ± 0.5	8.7 ± 0.9*	12.2 ± 0.8**
10 Hz	2.2 ± 0.4	5.6 ± 0.8*	8.5 ± 0.8**

* Difference between glutaraldehyde/ EDC treatments and fresh tissue significant across frequencies with $p < 0.02$.
** Difference between glutaraldehyde and EDC treatments significant across frequencies with $p < 0.01$. ($n = 6$ for all groups)
Mean ± SEM

Stress relaxation expressed as percentage stress remaining at 100 s			
Initial load	Fresh	Glutaraldehyde	EDC
40 g	52.2 ± 3.0	63.8 ± 1.0*	76.5 ± 1.7**
80 g	57.2 ± 2.6	68.4 ± 1.9*	75.3 ± 1.2**

* Difference between glutaraldehyde and fresh groups significant at both initial loads with $p < 0.02$.
** Difference between EDC and other two groups significant at both initial loads with $p < 0.002$. ($n = 6$ for all groups)
Mean ± SEM

and at both 1 Hz and 10 Hz cycling, EDC-crosslinked tissue had the lowest magnitude of dynamic modulus, and fresh tissue the highest (Table IV). The phase angles for all three tissue groups were each below 5° (i.e. the materials were quite elastic in small vibrations), and there were no statistical differences between the groups.

4. Discussion

Since the late 1960s and Carpentier's identification of glutaraldehyde as a useful fixation agent to prolong the *in vivo* lifetime of bioprosthetic heart valves, this reagent has become the *de facto* industrial standard for crosslinking of tissue-derived devices [33]. While glutaraldehyde is a very effective biocide and improves the resistance of collagen to biodegradation, it is now

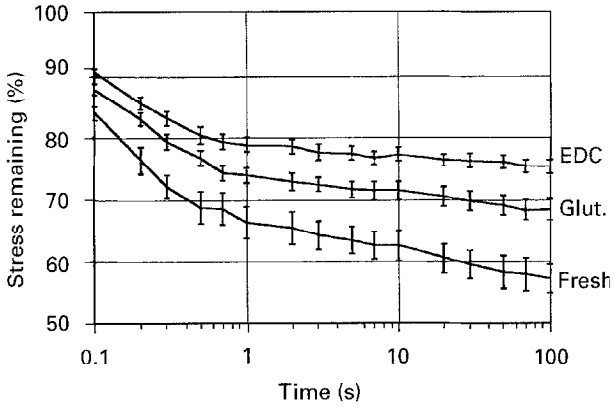

Figure 3 While both glutaraldehyde (Glut.) and EDC treatments reduced the stress relaxation which occurred over 100 s ($p < 0.0002$), EDC produced the greatest effect ($p < 0.02$). Data shown are for loading time of 0.1 s and are valid for $t \geq 0.4$ s. Mean ± SEM ($n = 6$ for each group except Fresh where $n = 5$).

TABLE IV Forced vibration data

Magnitude of dynamic modulus (MPa)				
Material	40 g; 1 Hz	40 g; 10 Hz	80 g; 1 Hz	80 g; 10 Hz
Fresh	27.8 ± 4.0	30.8 ± 4.2	40.2 ± 5.6	44.8 ± 6.4
Glut.	15.4 ± 2.2**	14.7 ± 1.2*	22.4 ± 1.9*	24.0 ± 1.9*
EDC	6.7 ± 0.8**	7.2 ± 0.9*	12.3 ± 1.4*	13.9 ± 1.5*
Phase angle (degrees)				
Fresh	1.6 ± 0.6	1.3 ± 0.5	0.7 ± 0.3	2.5 ± 1.4
Glut.	1.1 ± 0.5	2.0 ± 1.1	1.3 ± 0.5	1.7 ± 0.8
EDC	2.1 ± 0.4	0.2 ± 0.2	1.6 ± 0.5	0.6 ± 0.5

* EDC and glutaraldehyde crosslinked materials different from fresh tissue, but not from each other ($p < 0.05$).
** EDC and glutaradehyde crosslinked materials different from fresh tissue and from each other ($p < 0.05$)
Mean ± SEM. ($n = 6$ for each group)

clear that it cannot be considered an ideal reagent for tissue treatment.

First, while glutaraldehyde reduces the severity of immunological reactions against xenograft tissues, antigenic responses against crosslinked collagen can be identified in implanted animals [34, 35]. It may be that the principal strength of any crosslinking reagent in this respect is simply to limit release of solubilized antigenic fragments after enzymatic degradation by host or bacterial cells. Second, glutaraldehyde has been identified as potentiating calcification of bioprosthetic materials [36–38]. Third, desorption or possibly depolymerization of glutaraldehyde may be linked to local cytotoxicity and slowed or eliminated repopulation by host cells (e.g. endothelium) [2, 39, 40]. Fourth, glutaraldehyde produces significantly altered mechanical properties in treated tissues [41–46]. While these may be modulated by control of strain or stress during crosslinking [41, 47–49], a clear understanding of the relationship between crosslinking and resulting mechanical properties has not yet emerged.

The results of the present study demonstrate that EDC carbodiimide crosslinking of pericardium produces results which are strikingly similar to those of glutaraldehyde—this despite the very different mode of action at the biochemical level. Collagen denaturation

temperature, resistance to collagenase (without denaturation), stress–strain response, and forced vibrational properties were all similar after glutaraldehyde or EDC treatment. Some differences were nonetheless observed. After hydrothermal denaturation of its collagen, EDC-treated tissue showed slightly greater resistance to collagenase and markedly greater resistance to trypsin. As well, extensibility and elastic behaviour (seen as reduced stress relaxation) were both greater after EDC treatment.

Use of a battery of biochemical and enzymatic degradation tests is more informative regarding the extent and possible location of crosslinks than would be a single test. CNBr, for instance, cleaves denatured collagen at peptide bonds after methionine (Met), an amino acid which occurs only 4–10 times per chain in mammalian collagen molecules [50, 51]. Bacterial collagenase catalyses hydrolytic cleavage of undenatured collagen in *non-polar* regions (interbands in electron microscopy), either in a single α-chain or simultaneously across three chains of the triple helix in lateral fashion [52, 53]. By contrast, trypsin, chymotrypsin, pepsin, and pronase (and other common proteases) can normally only digest the non-helical ends of undenatured collagen, without disrupting the main helical structure [52, 54]. As expected, in our experiments trypsin had negligible effect on undenatured collagen, but was quite effective after hydrothermal denaturation of the triple helix. In denatured collagen, trypsin readily cleaves peptide bonds after the basic amino acids lysine (Lys), hydroxylysine (HO-Lys) and arginine (Arg) in the *polar* regions of the collagen molecule (bands in electron microscopy) [55]. Between the three agents, there was therefore a variety of cleavage sites and preferences for substrate conformation.

Examination of the primary structure of bovine type I collagen [56, 57] sheds light on the results obtained with chemical and enzymatic digestion. Because Lys or HO-Lys residues occur in triplets 22 times across the two α1 (I) and the α2 (I) chains, glutaraldehyde is capable of forming crosslinks either; (i) between adjacent α chains (*probably easy*) or (ii) between adjacent triple helix molecules or between fibrils (*possibly more difficult*).

While the former crosslinks may be achievable by glutaraldehyde monomer alone, the number of the latter crosslinks will depend on the distance to be spanned and the degree of polymerization of the glutaraldehyde. The situation for EDC is quite different. Katz and David [58] have pointed out that, in the amino acid triplet (Gly-X-Y) typical of collagen, a charged residue (Asp, Glu, Lys, Arg) is matched more often than not by a residue of the opposite charge either in the same triplet or an adjacent triplet. Indeed, within a given α chain, such charge or "salt" linkages only require that the acid and base group be separated by no more than two other residues. Further, the spatial packing of the fibril generates the spatial conditions for such charge linkages between adjacent triple helices. Since this interaction is fundamental to the stability of the triple helical structure, there are a large number of appropriate Asp-Lys or Glu-Lys interactions within and between α chains which are suitable for crosslink formation by carbodiimide action. Thus, EDC-derived crosslinks may occur within an α chain, between α chains, or as intermolecular or interfibrillar linkages.

It is not surprising, therefore, that EDC crosslinks were sufficient to increase the denaturation temperature above 85°C and to convey significant resistance to enzymatic digestion. The observed greater decrease in stress relaxation and shrinkage-related increase in extensibility under EDC treatment suggests that both of these two properties are significantly determined by intermolecular/interfibrillar crosslinking [9]. This notion is further borne out by the gross flexural stiffening of EDC-treated tissues [59]. If pliancy in pericardium is achieved by shearing between fibrils (and fibre layers), then increased intermolecular (and interfibrillar) crosslinking should stiffen the material, as observed.

Given that EDC-treated materials were: (i) *equivalently* resistant to solubilization by CNBr and collagenase (before denaturation), but (ii) *more* susceptible to solubilization by collagenase (after denaturation) and trypsin (after denaturation), we must consider four possible hypotheses which are not mutually exclusive. First, it is simply possible that more crosslinks formed under EDC treatment than under glutaraldehyde treatment, and that the extra crosslinks were sufficiently broadly distributed on the collagen to better prevent solubilization of fragments cleaved off by trypsin or collagenase. This hypothesis has been put forward by Weadock *et al.* [10] on the basis of the large numbers of available carboxyl groups on Asp and Glu residues in collagen (available to participate in carbodiimide crosslinking) versus smaller numbers of ε-amino groups on Lys or HO-Lys residues (the only sites available for both ends of a glutaraldehyde crosslink). The data of Olde Damink [1] using purified ovine dermal collagen has shown that fewer amino groups react with EDC/NHS (approximately 20/1000 residues) than with glutaraldehyde (approximately 30/1000). However, each glutaraldehyde crosslink requires two amino groups for a maximum of 15 crosslinks/1000 residues. By contrast, each carbodiimide-produced crosslink requires only one amino group—for a maximum of 20 crosslinks/1000 residues.

A second hypothesis is that EDC treatment was better capable of masking sites where trypsin cleaves collagen. Trypsin cleaves at Lys or Arg residues and Lys participates in the crosslinking reactions for both EDC and glutaraldehyde. EDC crosslinking may have been more effective in blocking trypsin action through its unique ability to form intra-α-chain linkages. These linkages would not be expected to affect denaturation temperature, mechanical properties, or the action of CNBr or collagenase, but could influence trypsin sensitivity: the property most differential between the two treatments. We note also that EDC treatment was carried out at pH 5.5 and some conformational alterations in the collagen may have been preserved by crosslinking, perhaps masking trypsin cleavage sites.

The third hypothesis derives from the hypothesis of Cheung *et al.* [60] that during treatment with

glutaraldehyde, collagen fibres become covered with a coating of glutaraldehyde polymer which precludes further penetration of the reagent to crosslink the core of the fibril. They found that their treated tissues were much more susceptible to pronase digestion after denaturation when the inner, ostensibly less-crosslinked molecules of the fibre were exposed to enzyme. It is possible that this effect occurred with glutaraldehyde treatment, but not with EDC treatment: the reagent is not incorporated in the crosslink and polymerization is not possible. The absence of an outer blockage to reagent penetration might result in greater crosslinking deep in the fibre bundles and greater resistance to both collagenase and trypsin in denatured collagen—as observed.

The final hypothesis is that access to trypsin and/or collagenase cleavage sites was blocked by greater binding of glycosaminoglycans to collagen with EDC. Since EDC can attack carboxyl groups on sugar residues as well as on proteins, it is possible that EDC increases the amount of bound glycosaminoglycans both on the surface of fibrils and fibre bundles and within them. This should be verifiable by further biochemical analysis.

The use of NHS to improve the performance of EDC crosslinking is well documented in the literature [18, 20, 61, 62]. Carbodiimides react with carboxyl groups of Asp or Glu residues (in collagen or other proteins) to form an activated O-urea. This group may either then react with an available amino group on a Lys residue or hydrolyse, reforming the carboxyl group and releasing a soluble product. Carbodiimides hydrolyse rapidly in aqueous solution, an effect exacerbated by common buffers such as phosphate, acetate, Tris, etc. [63]. Also, the lifetime of the activated O-urea is extremely short and therefore the crosslinking yield with the reagent alone can be quite low. Both NHS and the charged N-hydroxysulfosuccinimide (NHSS) [64] can react with the carboxyl group or activated O-urea to form an activated ester which is much more stable in solution. The ester can then form a crosslink with release of a soluble urea or can (after a longer period) also hydrolyse, restoring the carboxyl group. With appropriate concentrations of NHS, very high crosslink yields have been reported in conjugation of proteins [20, 27, 64]. The crosslink which is formed is a peptide bond between or within collagen chains. As the present results confirm, this bond is hydrothermally stable (high collagen denaturation temperature) but hydrolyses in acid, restoring the Asp or Glu and Lys residues (unchanged amino acid analysis results). The apparent increased histidine values observed remain unexplained, but may reflect degradation of residual reagent or the urea by-product and subsequent co-elution with histidine in the reverse phase chromatography system. The importance of reagent residuals in the treated tissue for biocompatibility must be further assessed.

The results of the present cyanamide treatments confirm the observations of Pereira *et al.* [7] who found no effect after 48 h in 1% cyanamide at pH 5.4 on intact tissue. Denaturation temperature was unchanged and mechanical properties were suggestive of, if anything, a slight degradation of the tissue during storage in buffer. The results of that study may have been compromised by the presence of the acetate buffer which, like phosphate and other common buffers, has been shown to increase the rate of hydrolysis of carbodiimides in aqueous solution [63]. In the present study, however, we have used an unbuffered system plus the addition of NHS to block hydrolysis of the activated O-urea—with the same result. Silver and co-workers have extensively employed cyanamide/dehydrothermal treatment of purified collagen products—most recently by exposure to cyanamide vapour after severe drying [65]. Although they have observed an increase of some *in vitro* mechanical behaviour, *in vivo* resorption of the materials remained rapid and there is no convincing evidence of significant crosslinking by the reagent. By way of explanation, Brown *et al.* [66] have noted a paucity of short-range binding sites for crosslinking between lysine and acid residues in their static collagen model. They felt that activated esters of short carbodiimide reagents were therefore unlikely to be effective. We conclude, therefore, that cyanamide is at best a very poor carbodiimide crosslinker for intact tissue collagen even under nearly optimal conditions. We caution, however, that performance in intact tissues is much more of a challenge than in pure collagens given the wide variety of reactive groups present in other molecular moieties and variations in pH and buffer conditions: a problem noted in previous use of EDC for immunohistochemistry [27].

The future of EDC crosslinking for pre-implantation treatment of intact tissues will depend on several factors. First, its biocidal effect needs to be assessed and, if inadequate, a suitable ancillary sterilizing treatment developed. EDC's crosslinking efficiency across a broad range of proteins and its documented effect on cell membranes suggests that it may be a good biocide [23]. Second, the long-term resistance of EDC-treated materials to *in vivo* degradation needs to be assessed. If, as Cheung *et al.* [59] have suggested, *in vitro* resistance to enzymatic degradation is indicative of *in vivo* stability, the present results suggest that performance matching or exceeding that of glutaraldehyde is available. Certainly, *in vivo* and *in vitro* results with EDC-crosslinked purified collagen suggests that cellular invasion is enhanced in the absence of the type of depolymerization and reagent release typical of glutaraldehyde-treated materials [2, 39]. Third, the resistance of EDC-crosslinked materials to *in vivo* calcification must be investigated. Fourth, the effectiveness of EDC treatment in limiting immunospecific responses needs validation. Previous studies suggest that EDC treatment of proteins and even cells may serve to reduce immunogenicity [21, 67]. To what extent this is linked to conformational changes, blocking of recognition sites, or reduced dissolution and release of antigens remains unclear. Fifth, the identity of the compound co-eluted with histidine after acid hydrolysis needs to be identified. This compound has not previously been described since Olde Damink used the TNBS method to analyse for reacted lysyl residues in his previous study, rather than the reverse phase chromatography

system used herein for amino acid analysis [1]. Finally, in situations where mechanical properties are important (e.g. heart valves, vascular grafting, patching), it must be a concern that mechanical properties are altered in EDC by as much or more than in glutaraldehyde. While all crosslinking techniques necessarily produce alterations in mechanical properties in tissue-derived materials, means to modulate those changes may be available by controlling fibrous protein conformation or fibril/fibre separation during treatment [68].

Acknowledgements

The authors would like to thank Grober Farms, Cambridge, Ontario for providing intact pericardial tissue for this work. We also wish to express our gratitude to Dr Leon Olde Damink, University of Twente, Netherlands for guidance in developing the crosslinking protocol, and Dr John Ramshaw, CSIRO Division of Biomolecular Engineering, Melbourne, Australia for valuable discussion of results. This work was supported by contracts from the Ontario Centre For Materials Research (OCMR) and a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC).

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Received 2 May
and accepted 16 November 1995