HMDC crosslinking of bovine pericardial tissue: a potential role of the solvent environment in the design of bioprosthetic materials

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The need for alternative crosslinking techniques in the processing of bioprosthetic materials is widely recognized. While glutaraldehyde remains the most commonly used crosslinking agent in biomaterial applications there is increasing concern as to its biocompatibility—principally due to its association with enhanced calcification, cytotoxicity, and undesirable changes in the mechanical properties of bioprosthetic materials. Hexamethylene diisocyanate (HMDC), like glutaraldehyde, is a bifunctional molecule which covalently bonds with amino groups of lysine residues to form covalent crosslinks. Evidence within the literature indicates HMDC-treated materials are less cytotoxic than glutaraldehyde-treated materials; however, there is limited characterization of the material properties of HMDC-treated tissue. This study uses a multi-disciplined approach to characterize the mechanical, thermal, and biochemical properties of HMDC-treated bovine pericardial tissue. Further, to facilitate stabilization of the HMDC reagent, non-aqueous solvent environments were investigated. HMDC treatment produced changes in mechanical properties, denaturation temperature, and enzymatic resistance consistent with crosslinking similar to that seen in glutaraldehyde treated tissue. The significantly lower extensibility and stiffness observed under low stresses may be attributed to the effect of the 2-propanol solvent environment during crosslinking. While the overall acceptability of HMDC as a crosslinking agent for biomaterial applications remains unclear, it appears to be an interesting alternative to glutaraldehyde with many similar features.

1. Introduction

Engineering of bioprosthetic materials for cardiovascular surgery routinely includes a method of enhancing tissue stabilization. Crosslinking agents are commonly used for this purpose. Bovine pericardial xenografts are usually fixed in glutaraldehyde or glutaraldehyde/formaldehyde solutions with the aims of reducing antigenicity, sterilizing the device and preventing post-implantation collagen degradation [1–3]. Unfortunately, this treatment has also been associated with enhanced calcification, cytotoxicity and undesirable changes in mechanical properties [4–6]. In an attempt to overcome these problems potential new crosslinking agents are currently under investigation.

Hexamethylene diisocyanate (HMDC) is a bifunctional molecule capable of covalently bonding to amino acid residues. Like glutaraldehyde, it will bond with the epsilon amino groups in lysine residues and may form both intra and/or interhelical crosslinks through urea bonds (Fig. 1). These crosslinks are potentially important determinants of both the mechanical properties and the enzymatic resistance of the tissue [7, 8]. Theoretically, HMDC represents a promising alternative to glutaraldehyde; however there has been limited examination of its overall use in biomaterial applications. Previous studies have focused on the evaluation of *in vivo* degradation of HMDC-treated dermal collagen using microscopic methods [9–11]. HMDC-treated ovine dermal collagen has been shown to produce less severe cytotoxic effects than the same material crosslinked with glutaraldehyde [12, 13]. Recently, van Wachem *et al.* [14] have further modified the HMDC treatment which subsequently eliminated this cytotoxic effect. Material characterization of HMDC-treated ovine dermal collagen has also shown increased shrinkage temperatures and decreased free amine content, indicative of exogenous crosslink formation [8]. This HMDC-treated tissue showed no change in tensile strength and a decrease in high-strain modulus – consistent with glutaraldehyde-treated collagen.

HMDC is insoluble and highly reactive with aqueous solutions. To overcome this problem, HMDC stabilization and material penetration have previously been enhanced through the use of surfactants [8, 10–14]. While surfactant-mediated HMDC crosslinking of highly processed ovine dermal collagen has produced promising results, complications due to the use of a surfactant should not be ruled out. Surfactants

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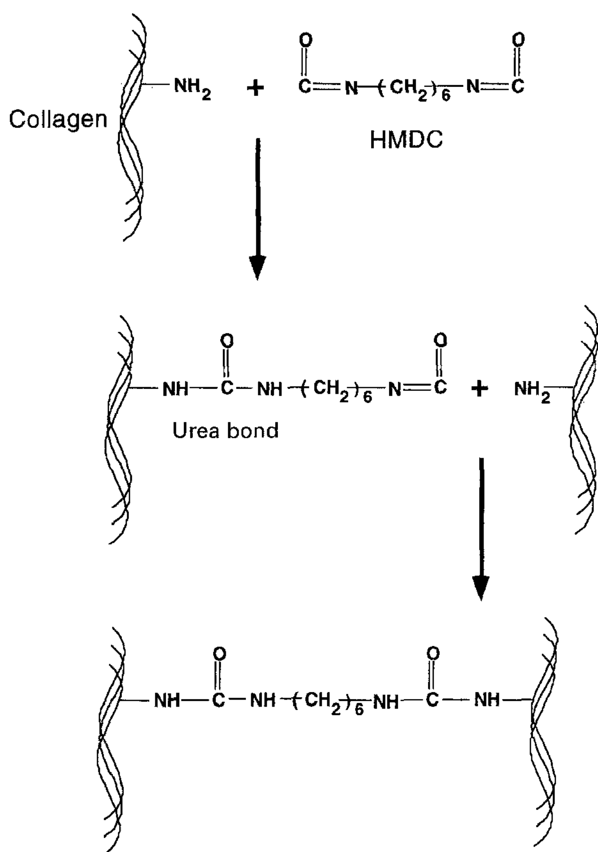

Figure 1 The diisocyanate reagent HMDC reacts with the ε-amino groups on lysine residues in collagen molecules to form stable, covalent crosslinks. The results are ideally two urea bonds; however, this presumes that after the first reaction step a second amino group is stereologically available.

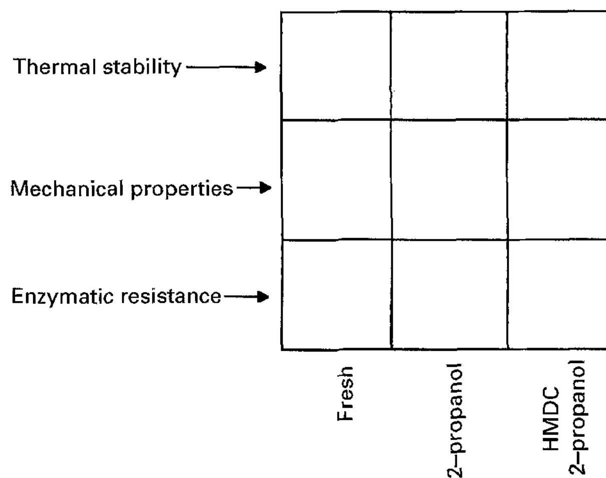

Figure 2 Each pericardial sample was subdivided into nine sections: three treatment groups × three characterization techniques. The location of each treatment/characterization rectangle was randomly assigned. Five pericardia were examined.

comprise a class of molecules with powerful interactive capabilities [15] and may play a role in the disruption of the material matrix through mechanisms such as extraction or increasing hydrophobicity. For example, sodium dodecyl sulfate (SDS) treatment has been shown to increase tissue susceptibility to enzymatic cleavage by elastase likely by opening up the non-polar elastin structure [16]. Primary cytotoxicity due to leakage of products from the material has been demonstrated to occur with HMDC-treated dermal sheep collagen [13]. Such potential concerns have warranted the investigation of other possible approaches for HMDC solubilization such as the change in solvent environment used here.

This study examines the effect of the diisocyanate crosslinking agent HMDC on the mechanical properties, denaturation temperature, and *in vitro* enzymatic resistance of bovine pericardial tissue. Comparison of these findings with previously studied glutaraldehyde-treated tissue provides insight into HMDC's potential as an alternative crosslinking agent. Further, use of an alternative solvent environment suggests new mechanisms for the engineering of specific material properties for biomaterial applications.

2. Materials and methods

2.1. Sample preparation

Bovine pericardial sacs (6–9 months) were obtained fresh from slaughter. The hearts and intact pericardia were transported from the abattoir in Hanks' solution (pH 7.4, 310 mOs) and prepared for testing promptly on return. Adherent fat was carefully stripped from the ventral pericardial surface and sutures were used to demarcate the base-to-apex direction. The ventral pericardial surface was sectioned into a rectangle, approximately 9 cm (circumferential) by 11 cm (base-to-apex), rinsed thoroughly in Hanks' solution and subdivided into nine sections (Fig. 2). Treatment groups consisting of: (i) fresh; (ii) hexamethylene diisocyanate/2-propanol; and (iii) a 2-propanol control, each consisted of three sections. One section of each treatment group was analysed for: (1) resistance to enzymatic degradation; (2) thermal stability (assessed by denaturation temperature); and (3) mechanical properties.

2.2. Optimization of crosslinking conditions

The initial portion of this study established conditions of maximal crosslinking. Denaturation temperature tests (see Section 2.5 for detailed description) were used for this purpose. An increase in denaturation temperature above that of fresh tissue indicated the presence of crosslinking [17]. This optimization process addressed pH, solvent medium, HMDC concentration and reaction time.

Comparison of crosslinking in 0.05% HMDC (1,6-Diisocyanatohexane, Sigma Chemical Co.) in aqueous buffer solutions at neutral pH (pH = 7.4, 0.07 M phosphate buffer) or basic pH (pH = 11.0, 0.1 M sodium hydroxide solution) revealed higher denaturation temperatures (T_d) at neutral pH (T_d = 79 °C compared to T_d = 69 °C at pH 11.0; for fresh, untreated tissue T_d = 68 °C at neutral pH). Unfortunately, during the crosslinking process, an undesirable white precipitate formed and was determined to be a product of HMDC reactivity in aqueous solutions. To eliminate this problem alternative solvent environments were explored.

Two 0.05% HMDC solutions in secondary alcohol solutions (50% 2-propanol/water and 100% 2-propanol (ACS Grade)) were compared for their effect on

tissue thermal stability. Tissue samples were also immersed in 50% and 100% 2-propanol solutions without HMDC to differentiate between solvent and HMDC/solvent effects. The 50% 2-propanol solution again produced a significant white precipitate while none was observed in the 100% 2-propanol medium. After 48 h in the HMDC/100% 2-propanol solution the thermal stability of the tissue was increased ($T_d = 77\,°C$) more than was seen in the HMDC/50% 2-propanol solution ($T_d = 72\,°C$).

The next step was to examine the effect of HMDC concentration (0.05%, 0.5%, and 5% HMDC) in 100% 2-propanol (v/v) on thermal stability. Denaturation temperature tests were performed at times 0 h (fresh pericardium), 15 min, 30 min, and 1, 2, 3, 24 and 48 h. The thermal stability produced by all three solutions reached a plateau after 2 h, with the 5% solution producing the highest denaturation temperature ($T_d = 83\,°C$ verses 73 °C in the 0.05% solution and 77 °C in the 0.5% solution).

From the above results, HMDC crosslinking of bovine pericardium was undertaken by (i) dehydration of the fresh pericardium in 100% 2-propanol prior to (ii) fixation in a 5% HMDC/100% 2-propanol solution for 2 h.

2.3. Tissue treatments

To differentiate between the effects of the crosslinking agent and the solvent environment, the following treatment protocol was used: (1) the HMDC/solvent group received a preliminary washing step (two changes of 100% 2-propanol for 30 min each) to remove all traces of water before immersing the tissue in a 5% HMDC/100% 2-propanol solution for 2 h; (2) the solvent control group was washed (as above) and immersed in 100% 2-propanol for 2 h; (3) the fresh samples were immersed in Hanks' physiological saline only and tested within 4 h.

2.4. Enzymatic resistance to tissue degradation

In its native, triple helical form collagen is susceptible to degradation by bacterial collagenases. The degree of degradation and subsequent solubilization in the presence of these enzymes is influenced by the degree of crosslinking within the tissue — the greater the degree of crosslinking the more resistant the tissue is to degradation. The addition of exogenous crosslinks further stabilizes the tissue limiting its solubilization. The degree of exogenous HMDC crosslink formation was assessed using these principles. Tissue from the three treatment groups was defatted (sequential extraction in 1 : 1 chloroform/methanol, 100% methanol, 50% methanol, and phosphate buffered saline, pH = 7.4) and freeze dried. Finely minced and accurately weighed samples were incubated with bacterial collagenase (C. histolyticum, Sigma Type 1) at a ratio of 100 : 1 tissue to enzyme (pH = 7.4, 0.05 M Tris-HCl buffer/10 mM Ca^{2+}) for 24 h at 37 °C. The residue remaining after incubation was separated from the supernatant (solubilized collagen) by centrifugation at 14000 rpm at 4 °C for 20 min. The residue was washed, re-centrifuged and freeze dried. The percentage of original mass remaining in the residue was used as a measure of the tissue's resistance to enzymatic degradation.

2.5. Denaturation temperature tests

The thermal stability of the three treatment groups was measured using a multi-sample denaturation temperature tester as previously described [17]. Briefly, six 4 × 20 mm tisue strips were mounted between sandpaper lined grips and loaded to 200 g. The tissue strips were held at a fixed extension over the course of the experiment and any resulting changes in load were monitored by the attached strain gauges. The apparatus was immersed in a water bath and the temperature was increased at a rate of 2 °C per minute.

Initially, there is a decrease in load due to stress relaxation. Eventually, the temperature of the bath provides enough energy to break hydrogen bonds which stabilize the highly ordered helical structure of this predominantly collagenous material [18]. This results in a conformational change into a random coil configuration which would produce observable shrinkage of the tissue were it unconstrained; however, since the tissue is held at a fixed extension (isometric constraint), the point at which the tissue denatures is registered as a sharp increase in the force exerted on the tissue strip. The inflection point derived from a plot of load versus temperature is termed the denaturation temperature (T_d).

2.6. Mechanical testing

Prior to testing, each sample was washed thoroughly in Hanks' solution and tissue strips (4 × 20 mm) were cut from each sample in the base-to-apex direction. Tissue thickness was measured on an adjacent section of tissue using a non-rotating Mitutoyo thickness gauge.

Each strip was tested in Hanks' solution at 37 °C (±0.5 °C) on an Instron servo-mechanical testing machine (model 4301) using the apparatus previously described [19]. The Instron machine consisted of a movable crosshead for applying deformations, a load cell for detecting forces, and a chart recorder which recorded these variables. The tissue strip was firmly held between two sandpaper-lined brass grips and the grips were secured such that the gauge length was approximately 10 mm. The upper grip was attached to the load cell which was mounted on the movable crosshead while the lower grip was secured to the bottom of a fixed plexiglass tank. The tank was maintained at constant temperature (37 °C) by a heating coil interfaced with a thermistor probe and an electronic temperature controller.

The cross-sectional area of the test strip was determined from digitized photographs of the tissue samples which were loaded to 0.5 g prior to the start of the experiment. To standardize gauge length, zero extension was thus taken at the point where a 0.5 g load was detected, and the extension gauge on the Instron was

then set to zero [20]. The extension rate for testing was 10 mm/min.

The following test protocol was used: (1) *preconditioning*: each strip was cyclically extended between 2 g and 95 g for 25 cycles to stabilize the load-elongation behaviour of the material; we note that this may alter tissue length, these changes are reported as plastic deformation; (2) *stress-strain relationship*: after preconditioning, the strip was brought back to zero load and a complete loading/unloading cycle, 0 g to 95 g to 0 g, was recorded on the Instron chart; (3) *stress relaxation*: the strip was extended from 0 g to 95 g at 10 mm/min and held at the corresponding constant extension for 1000 s while the decay of load was recorded as a function of time; (4) *plastic deformation*: after stress relaxation for 1000 s, the load was decreased to 0.5 g and the elongation which had occurred during the cyclic loading and relaxation tests was recorded from the Instron extension gauge; (5) *fracture*: after stress relaxation, the tissue was again preconditioned for 25 cycles, then extended at 10 mm/min from 0 g load until the tissue strip ruptured.

2.7. Analysis of mechanical data

Photographic prints (8″ by 10″) of the sample strips under 0.5 g load were digitized on a Kurta Series 2 digitizer (1000 point/inch resolution) interfaced with an Apple IIe computer. The standardized width of the grips was used to determine the magnification of the photograph and the gauge lengths and mean widths of the strips were obtained. Using tissue thickness data, strip cross-sectional areas were calculated. Stress versus strain data were obtained by digitizing the load–extension charts and converting load and extension to stress and strain, respectively. Strain was calculated as percentage change in length, and true stress was calculated assuming constant strip volume.

Stress relaxation was described as the percentage decay in stress with respect to time (t) at fixed extension:

$$\%\ \text{stress remaining} = \left(\frac{\sigma(t)}{\sigma(t=0)}\right) \times 100\%$$

Plastic strain was taken as the percentage deformation of the strip after preconditioning and relaxation for 1000 s. Fracture was taken to occur when the first decrease in load was detected during extension. Strain at fracture was taken as the percentage strain at the point of fracture, while the ultimate tensile strength (UTS) was taken as the force at fracture divided by the initial cross-sectional area. The tissue modulus (TM) was defined as the slope of the linear portion of the stress–strain curve prior to fracture: typically at stress > 1 MPa. A tangential modulus at 100 kPa was derived from the stress–strain data to also quantitatively assess material properties under lower loads.

All results are expressed as the mean ± SEM with $n = 5$ for each treatment group. An ANOVA with multiple comparisons (Fisher PLSD) was used to test for significant differences ($p < 0.05$) between treatments (StatView, Abacus Concepts).

3. Results

The 2-propanol (isopropanol) treatment resulted in a significant decrease in thickness: to 0.28 ± 0.01 mm from 0.49 ± 0.06 mm for fresh tissue. The HMDC treatment group (thickness = 0.42 ± 0.02 mm) did not, however, show this decreased thickness, suggesting that a thickness increase associated with planar shrinkage was superimposed on the decreased thickness due to the propanol treatment alone. The tissue became grossly rigid after immersion in the alcohol, and this effect was reversed after rehydration for the propanol only group. In the HMDC-treated group, however, the tissue remained quite rigid in flexure even after rehydration.

Formation of exogenous crosslinks in the HMDC treated tissue was confirmed by the denaturation temperature results. The HMDC group only showed a significant increase in denaturation temperature to 83.0 ± 0.3 °C from the 70.0 ± 0.4 °C for fresh tissue (Fig. 3). The biochemical assay for enzymatic resistance confirmed this observation. The undigested fraction of the HMDC-treated group was greatly elevated to 85.6 ± 0.9% from the 8.4 ± 0.3% for the fresh tissue (Fig. 4).

Crosslinking by HMDC in 2-propanol produced significant and interesting changes in the mechanical behaviour of the tissue. After fixation, the tissue was significantly more elastic (i.e. showed much less stress relaxation, Fig. 5). After 1000 s, 87.0 ± 0.6% stress was still present in the fixed tissue versus only 71.5 ± 1.6% in the fresh tissue. Plasticity was also reduced, with the plastic strain falling from 4.0 ± 0.8% for fresh tissue to only 1.0 ± 0.1% for fixed tissue. Along with the observed flexural stiffening of the material, the HMDC-treated tissue showed an unusual, nearly linear, stress–strain curve with a significantly lower tangential modulus at 100 kPa compared to both the fresh and 2-propanol groups (Fig. 6). The usual large initial extension under low loads was nearly absent in this material. HMDC/2-propanol treatment also produced significant effects in fracture

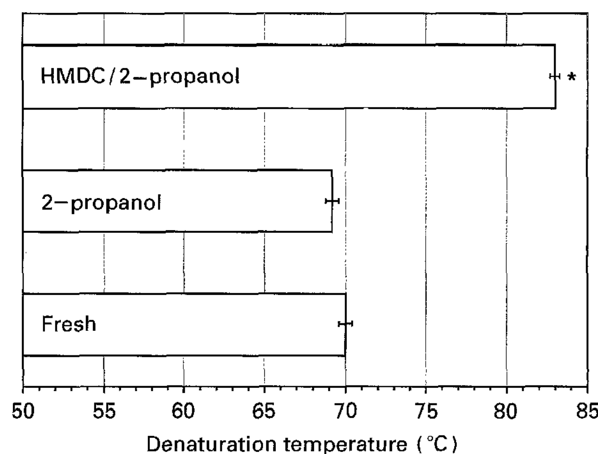

Figure 3 The denaturation temperature of the pericardial collagen was significantly increased by the HMDC/2-propanol treatment (effect of treatment significant with $p < 0.0001$); however, treatment in the 2-propanol alone had no significant effect. The increase in denaturation temperature is attributed to the formation of helix-stabilizing covalent crosslinks.

where the ultimate tensile strength (UTS) of the material fell by nearly 70%, and the strain at fracture rose by nearly 40% (Table I).

Treatment with 2-propanol alone had very little effect on the pericardium. The only significant changes observed were the decreased thickness, a slight increase in enzymatic resistance (Fig. 4) and a slight reduction in stress relaxation (Fig. 5).

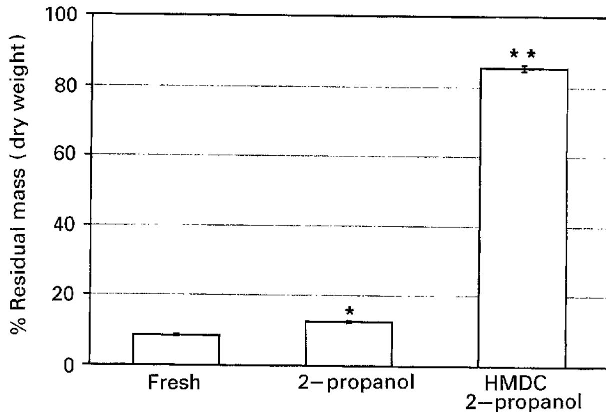

Figure 4 Exposure of the pericardial tissue to 2-propanol alone produced a slight increase in resistance to degradation by collagenase (effect of treatment significant with $p < 0.001$), perhaps due to either dehydration-induced crosslinking or modification of the collagen. By contrast, the HMDC/2-propanol treatment dramatically increased the resistance to collagenase, consistent with crosslink-induced reduction in solubility.

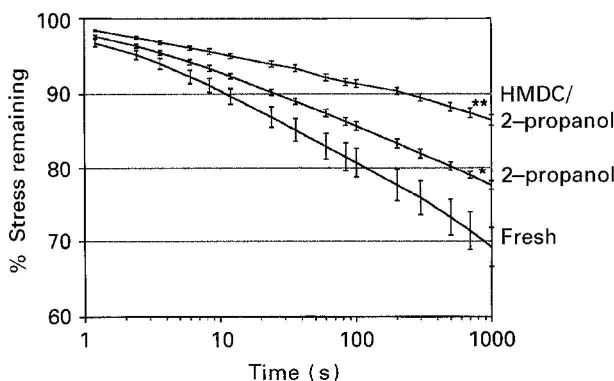

Figure 5 Both the 2-propanol treated pericardium and the HMDC treated pericardium were significantly more elastic than fresh pericardium (effect of treatment significant with $p < 0.0001$): i.e. they showed less stress relaxation. The nearly linear shape of the relaxation curves on a log time scale was preserved, however.

4. Discussion

Crosslinking of pericardium in HMDC is in many ways similar to that seen with glutaraldehyde. Both reagents are bifunctional, react with the ε-amino groups on lysine residues, and are capable of altering collagen denaturation temperatures, enzymatic resistance, and mechanical properties by formation of stable, covalent, crosslinks; however, they have substantially different intrinsic chemical reactivity. HMDC is highly reactive in aqueous solution; base catalysed hydrolysis results in the production of amine compounds (i.e. 1,6-hexadiamine) which are capable of polymerizing with remaining isocyanate groups. In our results, HMDC was only effective in aqueous solution at neutral pH, likely due to the relatively longer lifetime at neutral pH which permitted time for reaction with the amino groups. At pH 11, the HMDC was hydrolysed too quickly to produce significant crosslinking—even though the epsilon-amino groups of the lysine residues were deprotonated and reactive. The precipitate which formed in aqueous and 50/50 2-propanol/water solutions was evidence of a polymerization reaction which was not present in the 100% secondary alcohol solution (2-propanol). Once crosslinking was undertaken in alcohol only, maximal

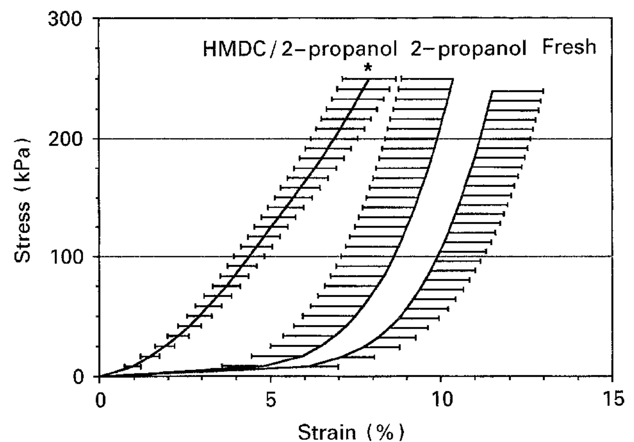

Figure 6 Treatment of pericardium with 2-propanol alone did not alter the stress-strain curve significantly—although a slight shift to lower strain was seen. Treatment with HMDC in 2-propanol resulted in a very different stress-strain response: a nearly linear curve with lower overall strain (differences significant below 200 kPa; effect of treatment significant with $p < 0.05$). The large extensions normally seen under low loads in fresh tissue were not seen in the fixed material. In addition, the HMDC treated tissue was significantly less stiff at 100 kPa (effect of treatment significant with $p < 0.004$). This change is strong evidence for a permanent change in the collagen conformation enforced by crosslinking.

TABLE I Comparison of the plastic deformation and fracture properties of the three treatment groups

Treatment group	Plastic deformation (%)[a]	UTS (MPa)	% strain at fracture[a]	Tissue modulus (MPa)	Tangential modulus (at 100 kPa) (MPa)
Fresh	4.0 ± 0.8	6.8 ± 2.0	28 ± 2	31 ± 9	7.7 ± 1.1
2-propanol	4.6 ± 0.9^{b}	4.4 ± 0.4	26 ± 3^{b}	29 ± 3	6.9 ± 0.7^{b}
5% HMDC/2-propanol	1.0 ± 0.1^{c}	2.1 ± 1.0^{c}	39 ± 5^{c}	34 ± 6	3.9 ± 0.5^{c}

[a] indicates effect of treatment significant at $p < 0.05$ (strain at fracture) or $p < 0.005$ (plastic deformation)
[b] indicates difference with HMDC/2-propanol treated tissue at the p-values stated above
[c] indicates difference with fresh tissue at the p-values stated above

crosslinking was reached with the highest concentration of reagent used (5% versus 0.05 or 0.5%). Here, the crosslinking reaction was nearly as rapid as that seen with aqueous glutaraldehyde, reaching completion in less than 2 h. The final denaturation temperature achieved (83 ± 0.3 °C) was not quite equal to the 85.8 ± 0.1 °C values typically seen in our experiments with glutaraldehyde [21].

Since the long-chain diisocyanate HMDC is highly unstable in water, some means is clearly needed to enhance stability and promote adequate penetration into water-rich tissues. Olde Damink and colleagues [8, 10–14] have chosen to use surfactants (Tween 80, Tween 20 and Emulvin W) to suspend the HMDC, resulting in micelles of HMDC within the aqueous environment. With this technique, they have been able to achieve significant crosslinking. Nonetheless, questions remain associated with the unknown effects of the detergents; e.g. potential for molecular extraction or alteration of protein charge and conformation due to residual binding of surfactant. Polar/non-polar detergents may have associated with them the risk of opening up non-polar proteins such as elastin and increasing their susceptibility to enzymes like elastases [16]. These questions are especially important to investigators currently experimenting with detergent extractions for preparation of acellular matrix materials [22, 23]. Olde Damink's [8] surfactant experiments were carried out on highly processed dermal collagen; the present experiments, however, have dealt with intact tissues. We have therefore taken a different approach: dissolving the HMDC in a short chain, secondary alcohol which offered both good tissue penetration and low reactivity with the reagent itself.

The effects of the alcohol itself appear slight but real. Exposure of bovine pericardium to pure 2-propanol for a period of 4 h produced nearly reversible planar shrinkage, thinning, and stiffening due to dehydration. After 2 h of rehydration, most properties returned to pre-treatment values; however, the residual thinning, and slight increases in elasticity and enzymatic resistance suggest that subtle changes in the collagen structure did occur. These changes may reflect changes in secondary, tertiary or quaternary structure resulting in: (i) crosslinking due to increased proximity during dehydration [24, 25]; and/or (ii) alteration of the side-chain chemistry of the collagen itself [26]. Some fat extraction, solvent binding, or reaction with collagen or other molecules may also have occurred. Since we have suggested that the use of surfactants to improve crosslinking must be approached with caution, it would seem equally prudent to better understand the effects of solvent exposure on collagen, elastin, and other molecular species. We also note that, in our experiments, it is likely that residual HMDC remained in the tissue after processing since a prolonged alcohol wash was not used to remove alcohol-soluble residue before rehydrating the tissue; however, since we were not concerned with cytotoxic responses in the present experiments, this was not deemed problematic. For *in vivo* use, a longer wash period would clearly be favourable.

The triple-helical conformation of collagen is determined, in part, by the polar/non-polar interactions between its primary amino acid structure and its usual aqueous environment. In solutions with different polarities (dielectric constants), the polar/non-polar interaction is altered and the conformation of the collagen is likely changed significantly. The mechanical effects of crosslinking by HMDC in 2-propanol strongly support the hypothesis that the conformational changes which occurred during the 2 h alcohol pretreatment were largely locked in place during crosslinking. It is possible that the crosslinks which were able to form in this new conformation were different from those which would have formed in the aqueous environment. Dehydration and conformational changes may both have brought additional pairs of amino groups into sufficient proximity for crosslinking to occur; other pairs may have been separated and crosslinking inhibited. It is similarly intriguing to question how much of the increase in enzymatic resistance with HMDC/2-propanol treatment was due to (i) crosslink-related reduction of solubility of lysed fragments, versus (ii) masking of sites of lysis due to the combination of conformational changes and crosslinking. It is also possible that free diisocyanate groups or pendant, singly-reacted HMDC molecules may have affected enzyme reactions. Greater understanding and control of these phenomena remain important objectives.

The mechanical results of crosslinking after solvent-induced conformation changes were particularly interesting since the resulting material exhibited opposing behaviours at low versus high stress levels. This difference is suggestive of a potentially important role for interfibrillar crosslinking in determining the mechanical behaviour of the HMDC/2-propanol-treated samples at low stress levels. These samples showed: (i) loss of initial extension under low stress, yet exhibited (ii) lower stiffness at 100 kPa. We may hypothesize that the collagen network is initially restrained from collapse or reorientation toward the direction of stress: thus explaining the reduced initial extensibility. The reduced reorientation also produces lower apparent stiffness at 100 kPa since less recruitment of fibres toward the direction of stress has occurred in these samples in comparison with the less restrained fresh or 2-propanol samples. Interfibrillar crosslinking is the best candidate to restrain fibre rearrangement and may have been facilitated by the dehydration step which brought the necessary reactive groups into approximation. Given HMDC's low propensity to self-polymerize under these conditions [29, 30], the approximation of reactive groups may be particularly important here. The formation of interfibrillar crosslinks would also reduce freedom of shearing between fibre layers consistent with the observed gross stiffening of the material. Plastic strain due to relative fibre motion may also have been reduced by this mechanism.

Further, the fall in tensile strength is likely indicative of stress concentrations produced during loading of a material crosslinked in a collapsed fibre configuration and with a reduced ability to reorient fibres

and reduce local stress [19, 27, 28]. By contrast, the increased strain at fracture for the HMDC group may be explained by increased collagen crimp after crosslinking which subsequently collapses under high stress levels [18]. This observation is consistent with the dimensional shrinkage seen in glutaraldehyde crosslinking by Trowbridge and co-workers and calculated by Lee *et al.* [31, 18].

While it is clear that the mechanical, thermal, and biochemical properties achieved here were far from optimal, they illustrate the principle that modulation of mechanical behaviour in bioprosthetic materials is possible through crosslinking in solvent environments of differing polarities. Furthermore, the distinctly different mechanical properties observed at high and low stress levels highlight the need to carefully consider the specific device requirements when selecting materials for design purposes. The implementation of solvent mediated crosslinking will be further explored in two studies to be published elsewhere.

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