Effects of carbodiimide crosslinking conditions on the physical properties of laminated intestinal submucosa

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Abstract: Functional tissue engineering of load-bearing repair tissues requires the design and production of biomaterials that provide a remodelable scaffold for host infiltration and tissue regeneration while maintaining the repair function throughout the remodeling process. Layered constructs have been fabricated from chemically and mechanically cleaned porcine intestinal collagen using ethyl-3-(3-dimethylamino) propyl carbodiimide (EDC) and an acetone solvent. By varying the concentration of the crosslinker from 1 to 10 mM and the solvent from 0 to 90% acetone, the strength, stiffness, maximum strain, thermal stability, lamina- nation strength, and suture retention strength can be adjusted. These parameters have either functional importance or the potential to modify the remodeling kinetics, or they have both. This study investigates the interdependence of these parameters, the specific effects that variations in concentration can achieve, and how the two crosslinking variables interact. The results demonstrate that there is substantial latitude in the design of these constructs by these straightforward crosslinking modifications. These data provide the basis for studying the in vivo response to crosslinking conditions that will supply the requisite strength while still allowing host cell infiltration and remodeling. © 2001 John Wiley & Sons, Inc. J Biomed Mater Res 56: 101–108, 2001

Key words: repair biomaterial; collagen; crosslinking; mechanical properties; multilayer composite

INTRODUCTION

The goal of functional tissue engineering is to provide a temporary remodelable tissue scaffold for host infiltration and tissue regeneration, that is, to engineer a biomaterial that allows cells from the host to degrade the biomaterial while generating new, site-appropriate permanent tissue. The material must provide the immediate mechanical requirements of the repair and maintain function throughout the remodeling process.

Due to their low intrinsic antigenicity, collagen-based biomaterials have become increasingly prevalent in biomedical applications, such as tendon, ligament, and abdominal repair. The complex organization of native connective tissues such as pericardium, dura, skin, and fascia often can provide the strength needed for surgical repair. One such collagenous tissue, the small intestinal submucosa (SIS), appears to possess the requisite biocompatibility and strength for use in a variety of applications and has the potential to be remodeled by the host. Applications include tendon, dura, bladder, abdominal wall, and vascular conduits.

As with other native-tissue scaffolds, there are fundamental problems with using native SIS as an “off the shelf” surgical repair material. The native tissue is cellular, and manually stripping the muscular and mucosal layers of the intestine from the SIS yields a highly variable base material. We have developed a method for generating an acellular intestinal collagen layer (ICL) from the porcine submucosa without substantially altering the native collagen structure or natural strength of the material. The ICL material is mechanically and chemically cleaned to provide a uniform material with no cells or cellular debris (<0.1 ng/mL of DNA, <0.7%) and low glycosaminoglycan content (<0.6%). After processing, the ICL is approximately 80 μm thick and is 99% type I collagen.

Collagen constructs designed for long-term clinical use generally require crosslinking to improve their resistance to proteolytic degradation and to enhance their mechanical properties. Glutaraldehyde, the most widely used bioprosthetic tissue fixative, sterilizes, decreases antigenicity, and reduces susceptibility to biodegradation. However, glutaraldehyde is associated with calcification and has been implicated in local cytotoxicity. These drawbacks have led to the development of various treatments to be used in.
conjunction with glutaraldehyde and to the investigation of several alternative nonaldehyde crosslinking methods. Carbodiimides are promising alternative crosslinking agents in that they contain no potentially cytotoxic residuals and do not remain active. The nontoxic urea derivative released when the crosslink is generated is easily rinsed from the collagen, leaving no residual chemicals. Previous studies have shown that ethyl-3(3-dimethylamino) propyl carbodiimide (EDC) used in conjunction with acetone can modify the mechanical properties of braided and woven collagen constructs and evoke a range of in vivo responses, depending on the fixation time. Utilizing EDC and acetone, we have developed processes to layer multiple pieces of ICL with strong interlayer bonding to form a stable composite. One example, GraftPatch™ (Organogenesis Inc. Canton, Massachusetts), is a surgical repair material consisting of six layers of ICL that has been cleared by the FDA for marketing in the United States.

The utility of an ICL laminate as a surgical repair material has been demonstrated in a rabbit abdominal-wall hernia model. Histologic analysis of patches cleared by the FDA for marketing in the United States. The present study using a modified Ernest Bitterling Model M34 (Nottingham, England) under a constant flow of water. The remaining submucosal collagen layer was slit longitudinally between the lymphatic tags and cut into 15-cm lengths that then were processed to remove any residual cellular debris. The chemical cleaning of the intestinal collagen layer is a proprietary process involving a series of washes in EDTA and NaCl at specific pH ranges. Once the process is complete, the ICL was stored at −80°C until use.

**MATERIALS AND METHODS**

**ICL preparation**

ICL was prepared from the intestines of swine (≥200 kg) obtained from a closed herd (Parsons Farm, Hadley, Massachusetts). The mesenteric layer manually was removed from the small intestine prior to mechanical stripping of the mucosal and membranous layers. It was processed mechanically through three series of rollers on a modified Ernest Bitterling Model M34 (Nottingham, England) under a constant flow of water. The remaining submucosal collagen layer was slit longitudinally between the lymphatic tags and cut into 15-cm lengths that then were processed to remove any residual cellular debris. The chemical cleaning of the intestinal collagen layer is a proprietary process involving a series of washes in EDTA and NaCl at specific pH ranges. Once the process is complete, the ICL was stored at −80°C until use.

**Laminate construction**

The ICL constructs were formed by layering individual sheets of ICL on top of each other with the serosal (abluminal) side of the ICL facing up and drying them overnight in a laminar flow hood. GraftPatch™ is a six-layer laminate; however, the number of layers of ICL can be altered depending on the application. In this study, two-layer constructs were used for laminar and sutured testing and suture retention tests.

The layered constructs were crosslinked chemically using ethyl-3(3-dimethylamino) propyl carbodiimide (EDC; JBL Scientific, San Luis Obispo, California) in deionized water or in an acetone solvent. Crosslinking was allowed to proceed for at least 8 h. In this study, nine different crosslinking conditions were tested: 1, 7, and 10 mM of EDC in combination with 0, 50 or 90% v/v acetone solvent. After crosslinking, the constructs were sterilized in a solution of 0.1% peracetic acid for at least 8 h and rinsed three times with deionized water. The constructs then were sealed hermetically in foil bags and sterilized by gamma irradiation at a dose of 25–35 kGy.
Physical test protocols

Thermal stability

The temperature at which melting of the matrix began ($T_{\text{onset}}$) was used in this study as a measure of thermal stability. $T_{\text{onset}}$ was measured with and without gamma irradiation to distinguish between the effects of crosslinking and the effects of gamma irradiation. For each crosslinking condition, 5–10-mg samples ($n = 3$) were cut from the ICL construct, placed in a 40-μL aluminum crucible, and heated from 35° to 100°C at a rate of 10°C/min using a differential scanning calorimeter (model DSC12E, Mettler Toledo Co., Hightstown, New Jersey). Subsequently, $T_{\text{onset}}$ was determined from the thermogram using the DSC-Mettler Toledo TA 89A analysis software (version 4.01).

Suture retention

For all mechanical tests an ICL construct from each crosslinking condition was cut into test samples ($n = 6$) of the appropriate dimension and tested on a Mini Bionix 858 servo-hydraulic materials testing system (MTS Systems Corp., Minneapolis, Minnesota). All samples were kept hydrated throughout the testing protocols.

The suture retention strength was determined using a rectangular test sample 12.5 mm in width by 50 mm in length. The sample was held in the lower grip and threaded with a 5.0 Surgilene suture (Davis and Geck, Danbury Connecticut) 2 mm from its edge. The two ends of the suture were attached to the upper grip and pulled at a constant rate of 125 mm/min. The suture retention strength was defined as the peak force obtained during this procedure.

Lamination strength

Two-layer ICL constructs were used to determine the lamination strength of different process conditions using a standard protocol for adhesive testing (ASTM D1876-95). A one-inch region of separation between the layers was maintained to facilitate mechanical testing. This region was attained by placing a 1-inch wide strip of plastic across the first layer of ICL at one end before adding the second layer of ICL. The samples then were processed as any other ICL multilayer construct. Samples (24.5 mm wide and greater than 90 mm long) were cut from the laminates from each crosslinking condition. The plastic was removed and the nonlaminated sections of the construct were loaded into the two grips on the MTS. The layers then were pulled apart for 4 cm at a constant velocity of 0.5 cm/s. The lamination strength was defined as the average force per unit width applied during this test.

Tensile strength, stiffness, and peak strain

The stiffness, strength, and extensibility of the ICL constructs were tested using strips that were 12.5 cm wide and long enough to provide a gauge length of 28 mm after being loaded in the MTS grips. The samples were pulled to failure in uniaxial tension at a constant strain rate of 0.013 s$^{-1}$. The direction of loading was always parallel to the circumference of the intestine. The stiffness of the construct was defined as the maximum slope of the stress–strain curve in the high modulus region, or the maximum tangent modulus (MTM). The ultimate tensile strength (UTS) was calculated as the peak force per unit width. The peak strain ($\varepsilon_{\text{max}}$) was defined as the actuator travel at failure divided by the initial gauge length. Strengths rather than stresses were calculated because the patch is intended for use in medical applications where functional strength is important regardless of the thickness of the material.

Statistical analysis

For each parameter (e.g., UTS, $T_{\text{onset}}$) a two-way ANOVA was performed to determine the effects of different levels of both EDC and acetone and their interactions. A Type III regression approach was used so that each effect was determined after accounting for all other effects, and the significance level was set at $p < 0.05$. This method did not compare each of the nine groups independently. The ANOVA evaluated whether: (1) differences between the means of the acetone levels are significant; (2) differences between the means of the EDC levels are significant; and (3) those differences are interdependent (termed an interaction). Wherever main effects showed significance in the ANOVA, Newman–Keuls’ post-hoc comparisons were performed to determine groupings within that factor. In reporting the results, all values cited comparing levels in the text are the level means as those are the values compared in the ANOVA. However, for clarity and completeness the data also are presented with all nine groups individually tabulated in both table form, with mean and standard deviations, and graphically for straightforward visual interpretation. Note that it is not statistically valid nor is it meaningful to arbitrarily compare any two of the groups in this study.

The independence of the different parameters tested was evaluated using Pearson’s product-moment correlations between parameters with significance set at $p < 0.05$. For thermal stability, $T_{\text{onset}}$ values were compared between gamma irradiated and nonirradiated for each condition using a paired Student’s $t$ test with significance set at $p < 0.05$. All statistical analyses were performed on Statistica™ (StatSoft, Inc., Tulsa, Oklahoma).

RESULTS

All results are reported in Table I as means ± standard deviation values. The EDC and acetone both significantly affected thermal stability before gamma irradiation (Fig. 1). In this assay we found a 1°C change in $T_{\text{onset}}$ to be physically meaningful and highly repeatable. The lowest level of EDC crosslinking (1 mM) increased $T_{\text{onset}}$ significantly by 2.3°C from the mean
value of native tissue (68.6°C). Higher concentrations (7 and 10 mM) increased $T_{\text{onset}}$ significantly to 72.2°C and 74.1°C, respectively. Increasing acetone concentrations caused a less dramatic but significant increase in $T_{\text{onset}}$ from 71.8°C with no acetone to 73.8°C with 90% acetone. The interaction between EDC and acetone also was significant: increasing acetone concentration from 50 to 90% caused a significantly greater increase in the $T_{\text{onset}}$ of the 1-mM EDC condition compared to the increase induced at higher EDC concentrations.

Gamma irradiation significantly reduced $T_{\text{onset}}$ in every case. For comparison, uncrosslinked ICL undergoes a reduction in $T_{\text{onset}}$ of 8.1°C from 68.6 ± 1.7°C ($n = 3$) to 60.5 ± 1.0°C ($n = 5$; Student’s $t$ test, $p < 0.05$). The degree of stability of crosslinks to gamma irradiation can be seen by evaluating the magnitude of that reduction ($\Delta T_{\text{onset}}$), a smaller reduction in $T_{\text{onset}}$ indicating more stable crosslinks (Table I). Stability was reduced when 7 mM of EDC was used and stability increased with 90% acetone. While increasing acetone levels provide more stability with 7 and 10 mM of EDC, the trend is reversed for 1 mM of EDC (Fig. 1). After gamma irradiation there also is a significant increase in the final thermal stability with increased acetone (at each level) and increasing EDC from 1 mM. A significant interaction effect also was found because the increase in thermal stability with acetone level is more apparent at higher EDC levels.

Lamination strength was significantly affected only by acetone, which caused an increase from 17.0 N/m to 50.9 N/m when it was increased from 50 to 90% (Fig. 2). In contrast, suture retention strength was affected only by EDC levels. At 10 mM of EDC there was a significant reduction of 18% in the suture retention, from 11.9 N and 11.4 N for 1 and 7 mM of EDC, respectively.

### Table I

<table>
<thead>
<tr>
<th>Crosslinker Condition</th>
<th>UT$S_{\text{bef}}$ (N/cm) ($n = 6$)</th>
<th>MTM$S_{\text{bef}}$ (N/cm) ($n = 6$)</th>
<th>$v_{\text{max}}$ ($N$) ($n = 6$)</th>
<th>Suture Retention$^b$ (N) ($n = 6$)</th>
<th>Lamination Strength$^a$ (N/m) ($n = 6$)</th>
<th>$T_{\text{onset}, \text{Pre Gamma}}$ (°C) ($n = 6$)</th>
<th>$T_{\text{onset}, \text{Post Gamma}}$ (°C) ($n = 6$)</th>
<th>$\Delta T_{\text{onset}}$ (°C)</th>
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<tr>
<td>1/0</td>
<td>13.3 ± 4.1</td>
<td>30.5 ± 16.4</td>
<td>0.72 ± 0.1</td>
<td>10.9 ± 2.8</td>
<td>8.1 ± 2.1</td>
<td>68.7 ± 0.3</td>
<td>64.0 ± 0.2</td>
<td>4.7</td>
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<td>1/50</td>
<td>35.2 ± 15.7</td>
<td>107.7 ± 50.0</td>
<td>0.54 ± 0.04</td>
<td>12.3 ± 3.2</td>
<td>22.7 ± 7.6</td>
<td>70.7 ± 1.0</td>
<td>64.6 ± 0.4</td>
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<td>1/90</td>
<td>25.9 ± 2.3</td>
<td>70.0 ± 5.4</td>
<td>0.62 ± 0.06</td>
<td>12.6 ± 1.5</td>
<td>38.1 ± 21.3</td>
<td>73.2 ± 0.7</td>
<td>66.4 ± 0.3</td>
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<td>7/0</td>
<td>22.1 ± 3.3</td>
<td>44.5 ± 10.7</td>
<td>0.80 ± 0.08</td>
<td>12.6 ± 2.9</td>
<td>10.9 ± 4.8</td>
<td>72.6 ± 0.5</td>
<td>64.0 ± 0.4</td>
<td>8.6</td>
</tr>
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<td>7/50</td>
<td>34.3 ± 4.7</td>
<td>99.0 ± 26.7</td>
<td>0.54 ± 0.09</td>
<td>11.1 ± 3.2</td>
<td>13.7 ± 8.7</td>
<td>73.0 ± 0.5</td>
<td>66.1 ± 0.1</td>
<td>6.9</td>
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<td>7/90</td>
<td>26.9 ± 1.8</td>
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<td>10.6 ± 2.2</td>
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<td>10/0</td>
<td>37.0 ± 7.6</td>
<td>86.7 ± 30.0</td>
<td>0.62 ± 0.1</td>
<td>10.0 ± 1.8</td>
<td>12.8 ± 4.1</td>
<td>74.1 ± 1.4</td>
<td>64.3 ± 0.7</td>
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<td>10/50</td>
<td>28.3 ± 10.8</td>
<td>87.7 ± 28.5</td>
<td>0.51 ± 0.07</td>
<td>8.7 ± 3.7</td>
<td>19.8 ± 9.1</td>
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<td>10/90</td>
<td>42.3 ± 8.2</td>
<td>146.0 ± 27.8</td>
<td>0.38 ± 0.02</td>
<td>7.6 ± 1.9</td>
<td>52.1 ± 21.0</td>
<td>74.3 ± 0.7</td>
<td>69.3 ± 0.5</td>
<td>5.0</td>
</tr>
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</table>

$^a$EDC significantly different between each condition, $p < 0.05$; $^b$1 mM EDC significantly different between 7 and 10 mM, $p < 0.05$; $^c$1 and 7 mM significantly different between 10 mM, $p < 0.05$; $^d$Acetone significantly different between each concentration, $p < 0.05$; $^e$0 acetone significantly different from 50 and 90%, $p < 0.05$; $^f$Significant interaction between EDC and acetone, $p < 0.05$; means ± standard deviations.

Figure 1. Effect of EDC and acetone on $T_{\text{onset}}$ before gamma irradiation (dark) and after gamma irradiation (light). Acetone and EDC levels significantly affected the $T_{\text{onset}}$ with meaningful interaction both before and after gamma irradiation. The reduction in $T_{\text{onset}}$ caused by gamma irradiation was significant in every condition ($p < 0.05$).

Figure 2. Effect of EDC and acetone on lamination strength. A statistical difference, $p < 0.05$, is marked with an asterisk. Brackets signify a grouping based on Neuman–Keuls post-hoc analysis, $p < 0.05$. 

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tively, to 8.8 N for 10 mM of EDC crosslinking (Fig. 3). Despite this reduction, the suture retention strength of all constructs was more than adequate for implantation, which generally is accepted to require no more than 2 N.

EDC and acetone both caused an increase in strength (UTS) and stiffness (MTM) [Fig. 4(a,b)]. Specifically, 10 mM of EDC significantly increased UTS and MTM compared to 1 and 7 mM of EDC. Both 50 and 90% acetone increased strength and stiffness significantly compared with crosslinking without acetone. Values ranged from 30.5 N/cm (MTM) and 13.3 N/cm (UTS) for 1 mM of EDC without acetone to 146.1 N/cm (MTM) and 42.3 N/cm (UTS) for 10 mM of EDC with 90% acetone. Except for 1 mM of EDC with no acetone, all conditions yielded UTS values that exceeded the cited minimum requirement of 16 N/cm for human hernia repair.25 There was a significant interaction between the two factors manifest in the reduction of both MTM and UTS at 90% acetone compared to 50% for 1 and 7 mM of EDC while at 10 mM of EDC, MTM and UTS increased.

Laminates crosslinked with 10 mM of EDC had significantly higher peak strain than 1 and 7 mM of EDC while the use of acetone at both 50 and 90% significantly reduced the peak strain compared with crosslinking without acetone [Fig. 4(c)]. Peak strain values ranged from 0.38 to 0.80 for 10 mM of EDC in 90% acetone and 7 mM of EDC with no acetone, respectively.

Statistically significant and potentially physically meaningful correlations were found between a number of the parameters tested. Lamination strength after gamma irradiation correlated positively with $T_{onset}$ before gamma irradiation ($R^2 = 0.815$). Stiffness was positively correlated with strength ($R^2 = 0.870$), and both stiffness and strength were negatively correlated with peak strain, $R^2 = 0.789$ and $R^2 = 0.509$, respectively. Also correlated with peak strain were lamination strength ($R^2 = 0.471$, negative), $T_{onset}$ before gamma irradiation ($R^2 = 0.676$, negative), and suture

**Figure 3.** Effect of EDC and acetone on suture retention. A statistical difference, $p < 0.05$, is marked with an asterisk. Brackets signify a grouping based on Neuman–Keuls post-hoc analysis, $p < 0.05$.

**Figure 4.** Effect of EDC and acetone on (a) ultimate tensile strength, (b) maximum tangent modulus, and (c) peak strain. A statistical difference, $p < 0.05$, is marked with an asterisk. Brackets signify a grouping based on Neuman–Keuls post-hoc analysis, $p < 0.05$. 

with peak strain, $R^2 = 0.789$ and $R^2 = 0.509$, respectively.
retention ($R^2 = 0.454$, positive). After gamma irradiation, $T_{	ext{onset}}$ was significantly correlated with strength ($R^2 = 0.497$).

DISCUSSION AND CONCLUSIONS

In this study we have characterized the effects that crosslinking conditions have on some important physical properties of ICL laminates. The results indicate the degree to which those properties can be modified and their interdependence within the framework of the current crosslinking process. Strength and stiffness can be modified, with the UTS increasing more than threefold, from 13.3 N/cm to 42.3 N/cm, and the MTM being increased almost fivefold, from 30.5 N/cm to 146 N/cm. The maximum strain in these constructs ranges from 0.5 to 0.8. Also, the onset of denaturation in gamma-irradiated laminates can be shifted as much as 5.3°C, with a range from 64.0°C to 69.5°C. The laminate strength, a property unique to this type of tissue-engineered material, can range between 8.1 N/m and 63.1 N/m. The results clearly indicate that some key physical parameters implicated in the biologic outcome of such constructs can be manipulated substantially by modifying the crosslinking conditions.

It is important to note that this study describes a subset of the potential constructs that can be produced using the ICL. While this study was limited to variations in acetone concentration at pH 7.9, other solvents and conditions may allow even more flexibility in construct design. EDC exposure time is another area that was not explored in this study but that has been shown to affect the mechanical properties of collagen constructs. Also, a different number of layers or a change in their orientation obviously would produce constructs with substantially different mechanical properties.

The results also address the interdependency of the effects induced by changes in crosslinking, which is important when considering the manipulation of those properties in a specific manner. The thermal stability of the construct after gamma irradiation can be increased by increasing EDC concentrations without necessarily altering the lamination strength. The lamination strength, in turn, can be modulated independently of tensile strength and stiffness. However, for these constructs, UTS and MTM cannot be independently modulated because they correlate highly and show the same significant variations among the different crosslinking conditions. The MTM, although defined in a way that minimizes the sample-to-sample variation, still shows a significant degree of variability even within groups. This may have contributed to our inability to distinguish between the relative modula-

The degree of matrix crosslinking is an important parameter in tissue remodeling kinetics. Differential scanning calorimetry has been used effectively to evaluate intermolecular crosslinking of the collagen triple helix by evaluating the thermal stability (or melting temperature) of collagenous tissues. The thermal stability of a collagenous material has been shown to correspond to resistance to biodegradation both in vitro and in vivo. As expected, the onset temperature (of denaturation) of our laminates increased with increasing levels of both EDC and acetone. However, gamma irradiation subsequently decreased the onset temperature in a manner dependent on the initial crosslinking conditions (Fig. 1). Changing the crosslinking may modify the rate of resorption of the construct. For example, slower degradation of mechanically loaded prostheses may be achieved by using more acetone and EDC together.

Thermal stability is not a complete indicator of biodegradability in collagenous biomaterials. Other factors in the material construction and chemical modification also may play key roles in the remodeling process. For example, migration of cells between the layers of the ICL laminates may play an important role in increasing the rate of remodeling of the inner layers. In an earlier study, cells were able to infiltrate between the layers of lightly crosslinked constructs due to the low lamination strength (8.1 N/m) while few cells were observed between the layers of the highly crosslinked construct (lamination strength 19.8 N/m).

In a previous study investigating laminated structures of small intestinal submucosa without chemical modification, the adhesion strength between the layers was not reported. Our experience has been that without chemical crosslinking the lamination strength is insufficient for surgical use. The laminates tested in this study were dehydrated during construction to physically crosslink the adjacent collagen layers. The EDC, previously successfully used to crosslink collagen fibers and engineered tissues, then was used to strengthen the constructs and increase the bond strength between the layers. The acetone, an organic solvent, was used to improve the efficiency of EDC crosslinking.

We found that acetone played a second, unexpected role in the lamination process. By dehydrating the tissue, it appears that acetone brought the collagen fibers in the separate layers closer together, which facilitated interlayer bonding. This is indicated by the nonlinear effect that increasing acetone concentration has on lamination strength. Almost a threefold increase in lamination strength, from 17 N/m to 50 N/m, was achieved when the acetone level was raised from 50 to 90% while there was no detectable increase in lamina-
tion strength with 50% acetone compared with no acetone. This effect enhances our potential ability to decrease bioresorption by tightly laminating the construct in a way that limits cellular infiltration.

Although the strength of the construct will change in vivo, the properties at the time of implantation are likely to influence the strength of the construct as remodeling occurs. Also, it is important to verify that the construct has the strength required at the outset for the targeted application. Unlike lamination strength, which is a function of interlayer bonds, the uniaxial strength and stiffness are determined predominantly by the fibrous arrangement within each layer and the orientation of the specimen. In this study each of the laminate’s six layers was oriented such that all applied forces were in the direction parallel to the original intestine’s circumference. After processing, the two major collagen fiber populations of the ICL were oriented at approximately ±30° to the loaded axis. As the material was pulled, the fibers reoriented towards the direction of stretch.32,33 As a result, crosslinking between adjacent fibers may impede reorientation of the collagen bundles, decreasing strain while intrahelical crosslinks may increase the strength of individual fibers. In this complex structure, MTM and UTS were not, a priori, expected to correlate, nor was it clear what effect crosslinking would have. In fact, while increases in both strength and stiffness with EDC concentration have been observed previously in dermal collagen,27 decreases in these parameters also have been observed.26,28 The results show that EDC increased both strength and stiffness of the ICL laminate, and the addition of acetone provided additional strength and stiffness, presumably due to an increase in crosslinking efficiency.

The data indicate that with regard to stiffness and strength, acetone optimally may increase EDC crosslinking efficiency at 50% v/v. But, at 90% acetone, the dehydration that aids in interlayer crosslinking may hinder the penetration of the EDC such that lower concentrations no longer are as effective. A similar limitation in effective crosslinking has been described in glutaraldehyde crosslinking at high concentrations. This reportedly is caused by the initial crosslinking of the outer surface of the tissue, which then effectively blocks the diffusion of the crosslinker to the inside.34,35

The strength and stiffness of the constructs increased with both EDC and acetone concentration but did not significantly correlate with lamination strength. The effect of crosslinking conditions on lamination strength was most pronounced at the highest percentage of acetone (90%), where the lamination strength was increased substantially without a concomitant increase in tensile strength. Strength and stiffness also did not correlate with thermal stability. It follows that constructs could be designed to degrade quickly yet have a high initial strength and stiffness. It is of interest to note that despite the lack of correlation between MTM and UTS and lamination strength, the peak strain was reduced with increasing lamination strength. This suggests that the interlayer crosslinks limit the ability of fibers to reorient without affecting the inherent stiffness or strength of the fibers themselves.

Still, despite the mechanical stability of these interlayer crosslinks, their thermal and irradiation stability seem to be low as the correlation between lamination strength and Tonset is lost after gamma irradiation. As mentioned previously, cells can infiltrate the graft and degrade the matrix from within if they are able to gain access between layers. Since thermal stability and lamination strength were not shown to correlate in this study, the relative importance of biostability and cellular access can be investigated in future studies where those parameters are changed independently.

This information, taken together, can aid in the design of materials for specific applications. For the design of a remodelable hernia repair material, it appears that having both low lamination strength and low thermal stability are advantageous. This can be achieved by lowering both the EDC and the acetone. Further in vivo studies will be needed to determine what remodeling rates can be achieved without compromising the implant’s function. If strength is compromised too quickly, then more EDC with less acetone may be required.

In other high and repetitive loading applications, such as tendon, it may be beneficial to slow the degradation process. Then cellular infiltration could be discouraged, with high interlayer bond strength attained using high levels of acetone. This also would provide the necessary increased strength. Follow-up animal studies could be used to modify thermal stability to achieve the ideal resorption rate by changing the EDC concentration.

The goal in biomaterial design is to have the ability to tailor the material properties to provide the desired remodeling rates so as not to compromise the functional properties of the host tissue. The present study shows that altering concentrations of EDC and acetone can modify the physical properties of the laminate. These data provide the basis for choosing a crosslinking condition that will supply the requisite strength while still allowing host-cell infiltration and long-term remodeling and replacement of the implant.

Long-term in vivo studies will be needed to evaluate mechanical strength throughout the remodeling process. In such studies the construct properties should be correlated with histologic and mechanical measures of the remodeled construct in vivo. At that point, an empirical understanding of the extent to which crosslinking conditions alter the initial physical properties will guide the design of better biomaterials. De-
sign of the biomaterials of the future must be based on critical parameters that include both remodeling rates and physical requirements determined from animal models.

References