Effect of physical crosslinking methods on collagen-fiber durability in proteolytic solutions

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We previously demonstrated that ultraviolet (UV) or dehydrothermal (DHT) crosslinking partially denatured fibers extruded from an insoluble type I collagen dispersion. In this study denaturation effects were evaluated by measuring collagen-fiber sensitivity to trypsin. Shrinkage-temperature measurements and sensitivity to collagenase served as indices of crosslinking. UV or DHT crosslinking increased the collagen-fiber shrinkage temperature, resistance to degradation in collagenase, and durability under load in collagenase. However, in trypsin solutions, solubility was significantly increased for UV (~11%) or DHT (~15%) crosslinked fibers compared with uncrosslinked fibers (~4%). Size-exclusion chromatography indicated that no intact collagen α-chains were present in the soluble fraction of fibers exposed to trypsin (MW <1 kD). Interestingly, UV-crosslinked collagen fibers remained intact an order of magnitude longer (4840 ± 739 min) than DHT-crosslinked (473 ± 39 min) or uncrosslinked (108 ± 53 min) fibers when placed under load in trypsin solutions. These data indicate that mechanical loading during incubation in a trypsin solution measures denaturation effects not detected by the trypsin-solubility assay. Our results suggest that DHT-crosslinked collagen fibers should not be used as load-bearing implants. UV-crosslinked fibers may retain more native structure and should exhibit greater resistance to nonspecific proteases in vivo.

INTRODUCTION

Crosslinking significantly influences the strength and resorption of collagen-based implants. Dehydrothermal (DHT) and ultraviolet (UV) crosslinking may be advantageous for tissue-engineering applications since they do not introduce potentially cytotoxic chemicals. Our laboratory is developing high-strength, resorbable collagen fibers for anterior cruciate ligament (ACL) reconstruction and other tissue-engineering applications. We recently demonstrated that both DHT and UV crosslinking provide high strength (~50 MPa) but partially denature the collagen fibers. Denaturation was detected by determining fiber solubility in pepsin or acetic acid and demonstrating that the soluble fraction had a molecular weight far below that of a collagen α-chain. We did not demonstrate the extent to which the denaturation seen in the solubilized material was indicative of denaturation in the insoluble fraction.

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The ideal resorbable implant for ACL reconstruction would provide high strength and degrade gradually while transferring mechanical loads to neoligament tissue. Thus, denaturation during crosslinking must be minimized to avoid rapid degradation by nonspecific proteases in vivo. The objective of the present study was to assess denaturation effects in both the soluble and insoluble fractions of the collagen by evaluating fiber solubility and durability under load in trypsin solutions in vitro. Crosslinking was assessed by isometric shrinkage-temperature measurements and sensitivity to collagenase in vitro.

MATERIALS AND METHODS

Characterization of collagen

Insoluble collagen paste derived from bovine corium was obtained from Devro, Inc. (Somerville, New Jersey). Amino-acid analysis was conducted according to a previously described method. Evaluation of collagen types in the sample was also performed as described previously using α₁(I)-CB2 and α₁(III)-CB4 as
marker cyanogen-bromide peptides for types I and III collagens, respectively. These analyses indicated that the starting material consisted essentially of pure collagen composed predominantly of type I collagen and containing less than 3% type III collagen.

Fabrication of collagen fibers

A 1% (w/v) collagen dispersion at pH 3 was blended at low speed for 1 min every 10 min for 30 min in a Waring blender and degassed by centrifugation at 5000 rpm for 15 min. A syringe pump (Sage Instruments, Boston, MA, model 341B) was used to extrude the dispersion through polyethylene tubing (i.d. 860 μm) at a rate of 1 mL/min into fiber formation buffer at pH 7.5 and 37°C. This buffer was composed of 135 mM NaCl, 30 mM TES (N-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid) and 30 mM sodium phosphate dibasic heptahydrate. The extruded collagen fibers (20 cm length, 75 μm diameter) were rinsed in isopropanol (16 h) and then distilled water at room temperature for at least 30 min prior to testing.

Crosslinking of collagen fibers

Fibers were either left untreated, crosslinked with UV irradiation (254 nm), or crosslinked with DHT treatment. UV irradiation was applied by placing dry collagen fibers on a sheet of aluminum foil in an UV-crosslinking chamber (Stratagene Stratlinker™, LaJolla, CA, model 2400). Fibers were exposed to a bank of five 15-W UV bulbs for 30 min. The distance between the light source and the fibers was 6 in. DHT crosslinking was achieved by placing dry collagen fibers on a sheet of aluminum foil in a vacuum oven (Napco, Chicago, IL, model 5851) and applying a vacuum of approximately 0.1 μm. One hour later the temperature was increased at a rate of 30°C/h until the temperature reached 110°C. These conditions were maintained for 72 h. The oven was allowed to cool to room temperature prior to releasing the vacuum.

Shrinkage-temperature measurements

Isometric measurements of collagen-fiber shrinkage during heating were made with an Instron (Canton, MA) model 4201 materials tester (n = 5 per group). Thirty collagen fibers, each 7 cm long, were bundled in parallel and the ends were epoxied (cyanoacrylate adhesive) to vellum paper for ease of handling and to prevent failure at the grip. Fiber bundles were immersed in a bath of phosphate-buffered saline (PBS) at room temperature for at least 30 min prior to testing. A preload of 5 g was applied and the bundle was held at constant length throughout testing. The temperature of the immersion bath was increased at a rate of 2°C/min and the load versus temperature was continuously recorded. The temperature at which the load began to rise (onset temperature), the peak load generated (mN), and the temperature corresponding to the peak load (shrinkage temperature) were determined.

Solubility in collagenase and trypsin solutions

Solubility of collagen fibers in collagenase and trypsin solutions was measured (n = 3 per group) to estimate the extent of crosslinking and denaturation, respectively. Solubility in collagenase was measured by incubating a 5-mg sample of collagen fiber in 5 mL of a 0.1M Tris-base, 0.25M CaCl₂ - 2 H₂O solution (pH 7.4) containing 125 U of collagenase (type IA, Sigma Chemical Company, St. Louis, MO) at 37°C for 3 h. Solubility in trypsin was measured by incubating a 5-mg sample of collagen fiber in 5 mL of a 0.1M ammonium bicarbonate solution (pH 7.5) containing 5000 units of trypsin (type XII, Sigma Chemical Company) at 37°C for 72 h. Control samples contained buffer only. The samples were then centrifuged at 5000 rpm for 15 min to separate insoluble and soluble components of the collagen fibers. The proportion of collagen solubilized was calculated from the hydroxyproline content of the supernatant following hydrolysis. The molecular weight of solubilized products was evaluated by size-exclusion chromatography.

Durability of collagen fibers under load in solutions of collagenase and trypsin

The durability of collagen fibers under static load was measured in solutions of collagenase and trypsin (n = 8 per group). Twenty collagen fibers, each 5 cm long, were bundled in parallel and epoxied (cyanoacrylate adhesive) at both ends to vellum paper so that 3 cm of the bundle was exposed. A 5-g weight was attached to the bottom of each bundle, then suspended upright in a 50-mL tube containing 40 mL of enzyme solution prepared as described above. Tubes were then placed in a water bath set at 37°C. Fresh enzyme solutions were added every 48 h. To serve as a control one bundle from each group was placed in buffer only. The time required for bundles to rupture (when the weight fell to bottom of tube) was recorded.

Statistical analyses

Analysis of variance (ANOVA) was performed using StatGraphics software to determine the effects of crosslinking method (uncrosslinked, UV, DHT) on shrinkage temperature, solubility in enzymes, and dur-
rability of collagen fibers under load in enzyme solutions. Differences between individual groups were considered significant for $p < 0.05$.

**RESULTS**

**Shrinkage-temperature measurements**

The isometric load versus temperature curves for collagen fibers varied as a function of crosslinking method (Fig. 1). The onset temperature for DHT-crosslinked collagen fibers was significantly less than that of uncrosslinked or UV-crosslinked collagen fibers (Table I). The shrinkage temperature of UV or DHT-crosslinked collagen fibers was significantly greater than for uncrosslinked fibers. The peak load generated by DHT-crosslinked collagen fibers was significantly greater than uncrosslinked fibers or UV-crosslinked collagen fibers.

**Solubility in trypsin and collagenase solutions**

Collagen-fiber solubility in collagenase and trypsin solutions is presented in Table I. Both UV- and DHT-crosslinked fibers were significantly less soluble in collagenase solutions than were uncrosslinked fibers. In trypsin solutions, however, DHT-crosslinked fibers were significantly more soluble than were uncrosslinked fibers. Solubility of collagen fibers in buffer solutions alone ranged from 0% to 4%. Size-exclusion chromatograms of the material solubilized during incubation in either enzyme solution indicated that no intact collagen $\alpha$-chains were present and that the degradation products had MW $< 1$ kD (Fig. 2).

**Durability of collagen fibers under load in solutions of collagenase and trypsin**

When placed under load in solutions of collagenase, DHT-crosslinked fibers remained intact significantly longer than did UV-crosslinked fibers; fibers from both crosslinked groups remained intact longer than did uncrosslinked fibers (Table I). When placed under load in solutions of trypsin, UV-crosslinked fibers remained intact 10 times longer than did DHT-crosslinked fibers; both remained intact significantly longer than did uncrosslinked fibers. Collagen fibers remained intact for the duration of the experiment (5

![Figure 1](attachment:image.png)
TABLE I
Effect of Crosslinking on Isometric Shrinkage Profiles of Collagen Fibers and Solubility and Durability of Collagen Fibers in Solutions of Trypsin and Bacterial Collagenase

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Uncrosslinked</th>
<th>DHT-Crosslinked</th>
<th>UV-Crosslinked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isometric shrinkage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onset temp (°C)</td>
<td>45.6 ± 0.548</td>
<td>41.0 ± 3.81</td>
<td>47.8 ± 1.30</td>
</tr>
<tr>
<td>Shrink temp (°C)</td>
<td>67.7 ± 5.90</td>
<td>79.4 ± 1.14</td>
<td>76.2 ± 1.79</td>
</tr>
<tr>
<td>Peak load (mN)</td>
<td>250 ± 123</td>
<td>662 ± 45.9</td>
<td>515 ± 53.0</td>
</tr>
<tr>
<td>Fiber solubility (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In collagenase</td>
<td>19.7 ± 2.6</td>
<td>6.5 ± 2.7</td>
<td>5.8 ± 2.2</td>
</tr>
<tr>
<td>In trypsin</td>
<td>4.6 ± 1.4</td>
<td>14.7 ± 5.9</td>
<td>11 ± 1.4</td>
</tr>
<tr>
<td>Fiber durability (min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In collagenase</td>
<td>24 ± 7.6</td>
<td>359 ± 139</td>
<td>208 ± 43</td>
</tr>
<tr>
<td>In trypsin</td>
<td>108 ± 53</td>
<td>473 ± 39</td>
<td>4840 ± 739</td>
</tr>
</tbody>
</table>

All values represent mean ± one standard deviation.

days) when placed under load in buffer solutions without enzymes.

DISCUSSION

UV and DHT crosslinking increased the shrinkage temperature of collagen fibers and decreased their solubility in collagenase solutions. The lower onset temperatures and increased shrinkage temperatures and peak loads generated by DHT-crosslinked fibers are consistent with the theory that more crosslinks can form during DHT treatment (condensation reactions between carboxyl and amino groups) compared with UV irradiation (bonds formed between free radicals generated on aromatic amino-acid residues). These

Figure 2. (A) Size-exclusion chromatogram of a type I collagen preparation extracted from 0.5M acetic acid according to Miller and Rhodes. Patten of α, β, γ, and high-molecular-weight (HMW) components characteristic of the denaturation products derived from a native, intact type I collagen molecule are shown. (B) Typical chromatogram of material solubilized during incubation in either trypsin or collagenase. Actual sample shown was crosslinked by UV irradiation and placed in trypsin for 72 h. The presence of low-molecular-weight (LMW) peaks indicates fragmentation of collagen α-chains.
crosslinks probably decrease collagen fiber solubility in collagenase solutions by sterically hindering access to active sites. Bacterial collagenase (Clostridium histolyticum) is capable of cleaving more than 100 sites on each α-chain (the cleavage site is indicated by the dotted line in $-{X-\text{Gly}}-{Pro}-{Y-}$). The greater number of crosslinks provided by DHT-crosslinking would account for greater durability of samples exposed to collagenase solutions.

Denaturation effects of these crosslinking methods were assessed by determining the solubility and durability of collagen fibers in trypsin solutions. Trypsin, a serine protease, cleaves peptide bonds on the carboxyl side of lysine and arginine residues in the short telopeptide domains of collagen molecules. However, these bonds are resistant to cleavage within the lengthy triple-helical domain of collagen molecules and become susceptible to cleavage only after denaturation occurs. Size-exclusion chromatograms of the material solubilized during incubation in trypsin solutions indicated that no intact collagen α-chains were present. Visual inspection of assays (Fig. 3) suggested that DHT-crosslinked fibers were completely solubilized in trypsin. However, hydroxyproline measurements indicated that the amount of collagen actually solubilized was only 15%, consistent with results of Gorham et al. It is likely that microscopic complexes of denatured collagen crosslinked to native collagen are liberated from collagen fibers during incubation in trypsin solutions. These complexes form a small, filmlike pellet during centrifugation and are not accounted for in hydroxyproline assays of the supernatant.

The durability of UV-crosslinked fibers under load in solutions of trypsin was 10 times greater than that of DHT-crosslinked fibers. These data imply that the trypsin-insoluble fraction of DHT-crosslinked collagen fibers is more denatured than that of UV-crosslinked fibers. As a result DHT-crosslinked collagen may degrade more rapidly in vivo than does UV-crosslinked collagen. In fact, Gorham et al. have shown that collagen sponges crosslinked with a similar DHT treatment had relatively high solubility in trypsin and resorbed more rapidly than did uncrosslinked collagen sponges when implanted intramuscularly in rats.

We have demonstrated that application of mechanical loads during incubation in a solution of trypsin is a useful means of measuring denaturation effects that are not detected by trypsin-solubility assays. Similar information could be obtained by measuring the mechanical properties of fibers incubated under load in a solution of trypsin at predetermined time periods. Olde Damink et al. have conducted similar collagen-strength–retention assays utilizing bacterial collagenase. However, since bacterial collagenase does not distinguish between native and denatured regions of the molecule, these assays are more indicative of crosslinking than of denaturation. We are currently developing a model in which collagen fibers can be placed under dynamic loads in proteolytic solutions to more closely mimic a load-bearing implant site. In addition, implantation studies are underway to determine whether these in vitro results can predict in vivo performance.

**CONCLUSIONS**

The durability of collagen fibers in proteolytic solutions is dependent on both the degree of crosslinking and the extent of denaturation. We suggest that in vitro measurements of the strength retention of collagen fibers be made after incubation under load in separate solutions of collagenase and trypsin. UV irradiation appears to be a useful method of crosslinking collagen fibers for ligament reconstruction and other tissue-engineering applications since denaturation effects are minimal, high strength is achieved in 30 min or less, exogenous chemicals are not introduced, and crosslinking can be performed “on-line” in a fiber-production process. In contrast, DHT treatment appears to have a significant denaturation effect and we do not recommend its use for load-bearing, collagen-based implants.

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**References**


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