

Cross-Linking Electrospun Type II Collagen Tissue Engineering Scaffolds with Carbodiimide in Ethanol

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ABSTRACT

In trying to assess the structural integrity of electrospun type II collagen scaffolds, a modified but new technique for cross-linking collagen has been developed. Carbodiimides have been previously used to cross-link collagen in gels and in lyophilized native tissue specimens but had not been used for electrospun mats until recently. This cross-linking agent, and in particular 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), is of extreme interest, especially for tissue-engineered scaffolds composed specifically of native polymers (e.g., collagen), because it is a zero-length cross-linking agent that has not been shown to cause any cytotoxic reactions. The unique aspect of the cross-linking protocol in this study involves the use of ethanol as the solvent for the cross-linking agent, because the pure collagen electrospun mats immediately disintegrate when placed in an aqueous solution. This study examines 2 concentrations of EDC with and without the addition of N-hydroxysuccinimide to the reaction (which has been shown to result in higher cross-linking yields in aqueous solutions) to test the hypothesis that the use of EDC in a nonaqueous solution will cross-link electrospun type II collagen fibrous matrices in a comparable manner to typical glutaraldehyde fixation protocols. The use of EDC is compared with the cross-linking effects of glutaraldehyde via mechanical testing (uniaxial tensile testing) and biochemical testing (analysis of the percentage of free amino groups). The stress-strain curves of the cross-linked samples demonstrated uniaxial tensile behavior more characteristic of native tissue than do the dry, untreated samples. The heated, 50% glutaraldehyde cross-linking protocol resulted in a mean peak stress of 0.76 MPa, a mean strain at break of 127.30%, and a mean tangential modulus of 0.89 MPa; mean values for the samples treated with the EDC protocols ranged from 0.35 to 0.60 MPa for peak stress, from 111.83 to 159.23% for strain at break, and from 0.57 to 0.92 MPa for tangential modulus. Low and high concentrations (20 mM and 200 mM, respectively) of EDC alone were comparable in extent of cross-linking (29% and 29%, respectively) to the heated 50% glutaraldehyde cross-linking protocol (30% cross-linked).

INTRODUCTION

COLLAGENOUS SCAFFOLDS (of various types of collagen) are of particular interest in tissue engineering, because these scaffolds mimic the biochemical and ultrastructural

properties of the native extracellular matrix (ECM) of tissues, which is known to influence cell behavior.^{1,2} Type II collagen is the predominant collagenous component of articular cartilage, and it is of interest for use in scaffolds for tissue engineering articular cartilage, a field in which the use

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of collagen matrices has already been investigated.^{3–5} As with the other collagenous scaffolds, which have been fabricated in numerous ways, particularly as gels and sponges,^{6–9} freeze-dried solutions of collagen^{10,11} and more recently as electrospun fibrous mats,^{12–19} the type II collagen scaffolds lack substantial strength upon hydration. Thus, the structures must be treated or fixed in some capacity to render sufficient antigenicity and mechanical integrity to the scaffolds.²⁰

Cross-linking can be used to tailor degradation rate and biomechanical characteristics (typically to match those characteristics of the tissue designated for regeneration), but it may compromise biocompatibility. The requirement for tissue engineering scaffolds to not cause any prolonged immune responses has led to research and development of fixation techniques that may prove more biocompatible than popular chemical cross-linking agents, including glutaraldehyde, formaldehyde, and epoxy compounds, which have been shown to be cytotoxic because of the reactive moieties that are covalently coupled between neighboring collagen fibrils.^{20–23} Glutaraldehyde, in particular, offers the advantages of being less expensive, reacting relatively quickly, cross-linking over varying distances, and reacting with a larger number of amino groups present in a protein molecule.²¹ It is the most commonly used and accepted agent for the cross-linking of biological tissues, but it has also been implicated in the development of calcification.^{21,24,25}

Our laboratory has previously shown that the electrospinning process facilitates the assembly of collagen molecules into fibrils that demonstrate the 67-nm characteristic D-period repeat pattern.¹² Unfortunately, the native intramolecular and intermolecular cross-links do not form to a significant degree, if at all, between the electrospun fibers. Herein lie several critical problems involved in the cross-linking of electrospun collagen. Upon hydration in any aqueous solution, the electrospun collagenous mats immediately shrink and disintegrate such that the scaffold breaks itself apart but does not dissolve. Thus, cross-linking is a necessity in order to maintain the structural integrity of the scaffold. However, typical protocols for cross-linking electrospun collagen have used glutaraldehyde, but even then, the scaffolds tend to gel (unless more-severe cross-linking protocols are followed) and lose their nanofibrous morphology, which is one of the most important features of the scaffold to begin with. To avoid this, our laboratory has previously electrospun blends of collagen with synthetic polymers,²⁶ which has proven successful in initial cell studies, but it is desirable to be able to work with collagen-only electrospun scaffolds for such applications as cartilage tissue engineering. Thus, a complementary, exogenous cross-linking agent is required if pure collagen electrospun scaffolds are to be used as tissue-engineering scaffolds.

Choice of cross-linking agent requires knowledge of the reactive group(s) present, typically an amino acid side chain, and appropriate ambient conditions (e.g., pH, temperature, solvent,) that do not negatively affect the protein.²⁰ In choosing a cross-linking agent for collagen, heterobifunctional

agents, which contain 2 different reactive groups that are able to directly link 2 different amino acid side chains,²⁷ are of interest in maximizing the extent of cross-linking. The great interest in heterobifunctional carbodiimides, in particular 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), is that these are zero-length cross-linking agents (i.e., the agent itself is not incorporated into the macromolecule). The protonated carbodiimide reacts with the carboxylic functional groups of aspartic and glutamic amino acids and forms the intermediate *O*-isoacylurea, which then undergoes nucleophilic attack by the amine functional groups of lysine and hydroxylysine amino acids on adjacent collagen fibrils. The cross-link, an iso-peptide bond (that mimics the natural peptide bonds in proteins), is formed, thereby bridging neighboring polypeptide chains, and a urea derivative is released that can be removed via rinsing.^{23,28} By modifying the ratio of EDC to collagen, the extent of cross-linking can be controlled.²⁰ The addition of N-hydroxysuccinimide (NHS) to the reaction with EDC has been shown to result in more efficient cross-linking by minimizing any rearrangement of the reaction intermediate.^{20,29} EDC has been used to cross-link reconstituted collagen fibrils (i.e., collagen molecules removed from tissues via acid or enzyme extraction that spontaneously form fibrils),^{30–32} insoluble collagen, and collagen fibers from intact tissues.^{20,28,33–35} EDC is suited for use with type II collagen from bovine cartilage because each of the 3 $\alpha 1(\text{II})$ chains that constitute the triple helical structure consists of 15 lysine, 23 hydroxylysine, 43 aspartic acid, and 87 glutamic acid residues per thousand residues per chain.³⁶

The present article describes a method of cross-linking electrospun type II collagen (highly purified) scaffolds using carbodiimide in an ethanol solution to effectively cross-link the collagen by creating iso-peptide interfibrillar bonds while inhibiting or minimizing the shrinkage of the collagenous mats. The hypothesis being tested is that the use of EDC in a nonaqueous solution will cross-link electrospun type II collagen fibrous matrices in a manner comparable with that of typical glutaraldehyde fixation protocols. The electrospinning fabrication technique (described in detail elsewhere^{12,37,38}) enables the creation of nonwoven matrices composed of fibers on the nano-scale level that more closely mimic native ECM. Thus, it is desirable to maintain this fiber morphology while preventing shrinkage and gelation. Ethanol has been used previously with collagenous materials without report of deleterious effects such as denaturation. Pieper *et al.* have investigated the cross-linking of lyophilized collagen matrices (extracted from bovine Achilles tendon) in the presence of 40% v/v ethanol to preserve the porosity of the collagen matrix; these matrices resulted in tensile strengths comparable with that of matrices cross-linked in aqueous EDC alone.^{30,31} Buttafoco *et al.* used EDC and NHS in a 2-morpho-linoethane sulfonic acid buffer in 70% v/v ethanol/water for cross-linking electrospun type I collagen (electrospun from hydrochloric acid with poly (ethylene oxide) and sodium chloride added). Ethanol has been used with glutaraldehyde in the treatment of aortic

cusps to minimize the effects of calcification.³⁹ Among the variety of techniques used to determine the effect of the cross-linking strategies,^{20,40,41} morphological characteristics, uniaxial tensile testing, and biochemical analysis were all investigated in this study to compare the effect of the EDC cross-linking method with that of glutaraldehyde cross-linking.

MATERIALS AND METHODS

Collagen preparation

Collagen type II was extracted from articular cartilage removed from all joints of fetal calf legs.⁴² Unless otherwise stated, all procedures were performed at 4°C. Guanidine (2.5 M final concentration) and Tris (0.025 M final concentration), pH 7.4, were added to a slurry of homogenized cartilage and stirred overnight. After 4 rinses with cold deionized water, the cartilaginous tissue was suspended in 0.5 M acetic acid, pH 2.8. Pepsin (2×, Sigma-Aldrich, St. Louis, MO) was added to the mixture at 1 g/L of solution and incubated overnight. The viscous supernatant containing solubilized collagen was adjusted to pH 7.4, and sodium chloride was added to a final concentration of 5 M while stirring slowly. The solution was incubated overnight at room temperature, after which the collagen precipitate was dissolved in 0.05 M Tris/0.2 M sodium chloride, pH 7.4. This solution was dialyzed (Spectrum Spectra/Por 4 RC Dialysis Membrane Tubing 12000–14000 MWCO, Fisher Scientific, Inc., Pittsburgh, PA) against a buffer solution containing 0.05 M Tris/0.2 M sodium chloride buffer, pH 7.4, and allowed to equilibrate overnight; this was repeated 2 additional times. Anion exchange microgranular diethylaminoethyl cellulose separation media, DE-52 (Whatman, Fisher Scientific, Inc.), was added to the collagen solution (at 100 g/L of solution) and incubated overnight. Sodium chloride (0.8 M final concentration) and acetic acid (0.1 M final concentration) were added to the supernatant and stirred overnight. The precipitated collagen was dissolved in 0.1 M acetic acid, and the resulting solution was dialyzed against a solution containing 0.01 M acetic acid overnight with stirring; this was repeated 6 additional times. Finally, the purified collagen solution was frozen and lyophilized until dry product was obtained.

Collagen purity and apparent molecular weight

Isolated (lyophilized) and electrospun collagen type II were dissolved in 50 mM of acetic acid solution at a concentration of 4 mg/mL. These solutions were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions for purity and apparent molecular weight determination. Briefly, 40 µg of purified or electrospun collagen was prepared by adding 1× SDS sample buffer and boiled for 3 min. The samples were

resolved using an 8% to 16% gradient SDS-PAGE (Criterion precast gel, Bio-Rad, Hercules, CA) and visualized using GelCode blue stain reagent (Pierce, Rockford, IL).

Electrospinning

The lyophilized collagen was dissolved in 1,1,1,3,3,3 hexafluoro-2-propanol (TCI America, Portland, OR) at a concentration of 60 mg/mL at room temperature. The collagen solution was electrospun to create randomly oriented, non-woven, fibrous mats. Figure 1 illustrates the electrospinning process. The apparatus used included a syringe pump (KD Scientific, Holliston, MA), a high voltage power supply (Spellman CZE1000R, Spellman High Voltage Electronics Corp., Hauppauge, NY), a syringe (Becton Dickinson, plastic, Franklin Lakes, NJ) as the reservoir for the polymer solution to which was attached an 18-gauge blunt-end needle, and a 303 stainless steel mandrel (2.0 cm × 7.6 cm × 0.5 cm) as the collection target. All electrospinning parameters were kept constant, including applied voltage (22 kV), distance between the needle and grounded mandrel (12.7 cm), solution dispensing rate (2 mL/h), translational speed (2 cm/s over a 7.3-cm distance), and rotational speed (500 rpm for random fiber orientation). Three mL of solution was electrospun for each mat.

Dry scaffold (untreated) characterization

Fiber morphology of the dry electrospun mat was viewed using scanning electron microscopy using a JEOL JSM-820 JE electron microscope (Japanese Electron Optical Ltd., Tokyo, Japan). A micrograph was taken and digitized using a Scanjet 5550c flatbed scanner (Hewlett-Packard Company, Palo Alto, CA) and then examined using UTHSCSA ImageTool 3.0 imaging software (NIH shareware, University of Texas Health Science Center at San Antonio) to measure fiber diameter. Means and standard deviations were determined from 60 fiber diameter measurements; calibration for

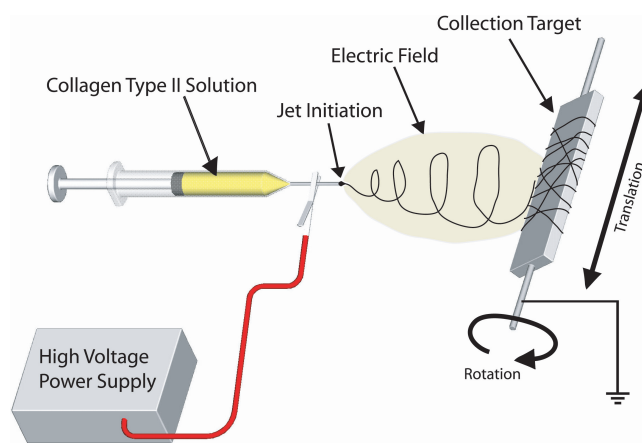


FIG. 1. Schematic of the electrospinning process to illustrate the basic phenomena and process components. Color images available online at www.liebertpub.com/ten.

each micrograph was made with the scale bar on the micrograph.

Mechanical properties of the mat were measured in the dry state to show handling properties. Uniaxial tensile testing of the mat on a MTS Bionix 200 mechanical testing system (MTS Systems Corp., Eden Prairie, MN), incorporating a 50 N load cell with an extension rate of 10.0 mm/min to failure, was performed on 3 “dog bone” shaped samples (2.67 mm wide at their narrowest width and a gage length of 7.49 mm) punched from the dry, untreated mat. The mechanical properties recorded include tangential modulus, peak stress, and strain at failure, which were calculated using the software TestWorks 4.0 (MTS Systems Corp., Eden Prairie, MN).

Cross-linking of collagen scaffolds

There were 6 cross-linking protocols compared in this study. Based on the apparent molecular weight of 150 kDa (which is consistent with the standard supplied by the University of Tennessee, Rheumatic Diseases Research Core Center, Collagen Core Center), as assessed using SDS-PAGE and shown in Figure 2, the molarity of the electrospun collagen solution was determined to be 0.4 mM. All of the carbodiimide cross-linking solutions were prepared by dissolving the solid cross-linking agent in pure ethanol (Fisher Scientific, Inc.) to the desired concentration. The first set of cross-linking reactions involved the treatment of EDC (Sigma-Aldrich Chemical Co.) alone at 50- or 500-fold molar excess (20 mM and 200 mM EDC, respectively). Two additional reactions were conducted that involved a combination of EDC and N-hydroxysuccinimide (NHS, Sigma-Aldrich Chemical Co.) cross-linking agents. The mats were treated with a constant 1:1 molar ratio of EDC to NHS, and similar to treatment with EDC alone, these reactions were

performed with 50- or 500-fold molar excess cross-linking compound. The remaining 2 cross-linking protocols (see below) involved the use of glutaraldehyde solution (Fisher Scientific, Inc.), a cross-linking agent commonly used in our laboratory.^{13,14}

For samples used in mechanical testing, 10 mL each of 20-mM EDC, 200-mM EDC, 20-mM EDC/20-mM NHS, and 200-mM EDC/NHS were prepared fresh. Electrospun mats of approximately 40 mg (with dimensions 4 cm length $\times$ 2 cm width $\times$ 0.02 cm thickness) were submerged in the EDC-only and EDC/NHS solutions and incubated with shaking for 18 h and 4 h, respectively. After cross-linking, the samples were placed in 0.1 M of sodium phosphate for 2 h to hydrolyze any unreacted *O*-isourea intermediates. Finally, all samples were rinsed and soaked in sterile phosphate buffered saline (PBS) for 2 h until mechanical testing was performed.

For the glutaraldehyde cross-linked samples to be used in mechanical testing, the 2 protocols used vapor fixation. Electrospun mats, approximately 40 mg in mass, were arranged in chambers in which glutaraldehyde solutions were placed such that the solutions would not contact the mats. For one mat, 300 μ L of 50% w/w glutaraldehyde was heated to 100°C, and the mat was exposed to the vapors for 30 min. The other mat was exposed to vapors of 3 mL of 3% w/w glutaraldehyde at room temperature for 18 h. Both samples were rinsed and soaked in sterile PBS for approximately 2 h until mechanical testing was performed.

Cross-linked, hydrated scaffold characterization

Environmental scanning electron microscopy (ESEM) was used to take images of the cross-linked mats in the hydrated state. Samples from all 6 cross-linking methods were imaged, as was a sample soaked in ethanol for 18 h (and then placed in water) and a dry sample with no treatment. The microscope used was a Philips XL30 ESEM TMP (FEI Company, Hillsboro, OR) purchased under National Science Foundation Award DBI-0098534 at the Scanning Electron Microscopy Laboratory, Department of Biology, Duke University. For improved image quality, the hydrated samples were blotted slightly to remove excess water such that the sample could be fixed to the sample holder. The grayscale of the digital images taken with the ESEM was inverted to allow for improved viewing of the topography of the mat surface.

Mechanical properties of the cross-linked mats were measured in the hydrated state (soaked in PBS for approximately 2 h at room temperature) to show behavior under approximate physiological conditions. Uniaxial tensile testing of these mats was performed as described above.

Degree of cross-linking of collagen scaffolds

To investigate the degree of cross-linking within the collagen mats, 2,4,6-trinitrobenzenesulfonic acid solution (TNBS-10% w/v in water, Research Organics, Inc., Cleveland, OH) was used to measure the percentage of free amino groups in a scaffold sample.^{43,44} Three circular discs

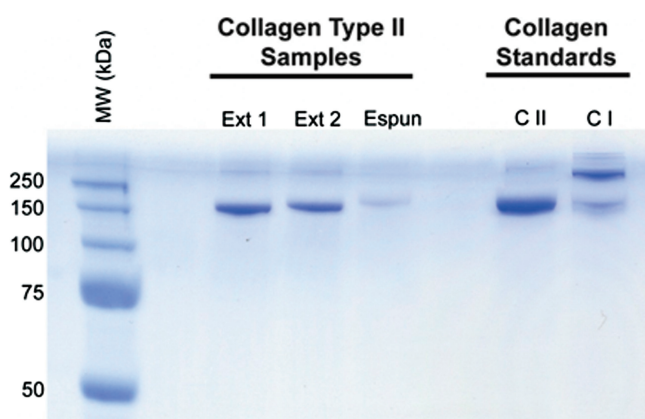


FIG. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the purified collagen from 2 different extractions (Ext 1 and Ext 2) and of electrospun collagen (Espun) was conducted to determine the apparent molecular weight and purity. The 150-kDa bands of Ext 1, Ext 2, and Espun are consistent with the molecular weight of the collagen type II standard (C II) and appear to be homogeneous. Color images available online at www.liebertpub.com/ten.

of 8.6 mm, each with a mass of approximately 1.3 mg, were punched from an electrospun mat from each cross-linking treatment and from the untreated scaffold; a total of 21 discs were prepared. The cross-linking treatments were performed as described above with the following exception; only 1 mL of EDC-containing solution was used for each disc. Each disc was then placed in a solution of 1.0 mL 4% (w/v) sodium bicarbonate and 1.0 mL freshly prepared 0.5% (v/v) TNBS in deionized water for 2 h at 40°C, after which 3.0 mL of 6-M HCl was added, and the temperature was increased to 60°C. After 1.5 h, the collagen samples solubilized, and the solution was diluted with 5.0 mL of deionized water. Three aliquots of 300 µL of each solution were placed in reservoirs of a 96-well plate, and absorbance (Abs) measurements at 345 nm were recorded using a SPECTRAmax PLUS³⁸⁴ microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA); thus, 3 measurements for each disc were recorded, so there were a total of 9 measurements for each cross-linking treatment. The degree of cross-linking was expressed as percentage loss in free amino groups after cross-linking and was calculated as follows:

$$\% \text{ Cross-linked} = 1 - \frac{\text{Abs}_c}{\text{mass}_c} \bigg/ \frac{\text{Abs}_{nc}}{\text{mass}_{nc}}$$

where *c* is the cross-linked sample and *nc* is the non-cross-linked sample.

Statistics

Unless otherwise stated, all statistical analysis was based on a Kruskal–Wallis one-way analysis of variance on ranks and a Tukey–Kramer pair-wise multiple comparison procedure ($\alpha = 0.05$) performed with the JMPIN 4.0.3 statistical software package (SAS Institute, Inc., Cary, NC).

RESULTS

Type II collagen was extracted from fetal calf legs and purified for use in this study. Figure 2 gives the results of the SDS-PAGE; the apparent molecular weight, determined to be 150 kDa, and the purity of the collagen samples are consistent with the type II collagen standard supplied by the University of Tennessee, Rheumatic Diseases Research Core Center, Collagen Core Center. The stained band for the electrospun sample is a result of a more-dilute solution loaded into the gel.

Figure 3 depicts the dry electrospun mat of type II collagen used in this study. The mean fiber diameter was determined to be 180 ± 69 nm. The handling characteristics of the electrospun mat, immediately removed from the mandrel, were such that the mat could be gently manipulated by hand without permanent deformation to the structure.

An electrospun type II collagen mat, when placed in an aqueous solution (after fabrication and before any fixation

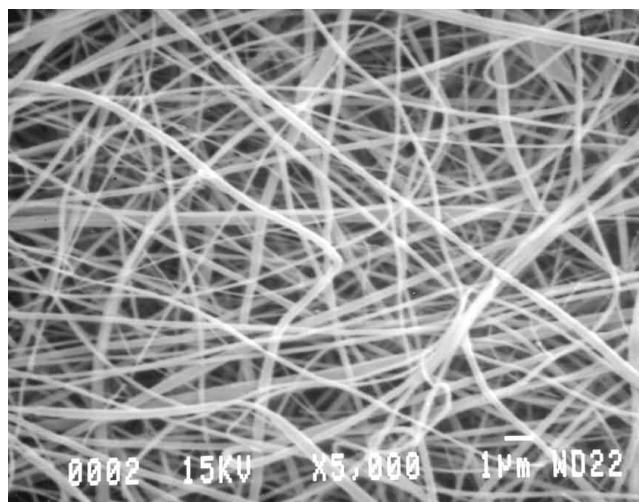


FIG. 3. Scanning electron micrograph of the dry type II collagen mat electrospun from 1,1,1,3,3,3 hexafluoro-2-propanol at a concentration of 60 mg/mL; mean fiber diameter is 180 ± 69 nm (scale bar is 1 µm).

treatments), immediately hydrates, shrinks, and disintegrates or pulls itself apart into fragments, although the mats do not appear to dissolve. This fragmentation presented a problem when attempting to cross-link the collagen mat, because EDC is typically used in an aqueous buffer at pH 5.5. In an attempt to prevent the shrinking and disintegration, several options involving alcohol solutions were investigated. Un-cross-linked mats first soaked in 70% isopropanol still shrank in size but did not disintegrate; when placed in a subsequent aqueous solution (not containing alcohol), the mat gelled and remained flat. In addition, a minimally glutaraldehyde vapor-fixed mat gelled when rinsed in an aqueous solution. An un-cross-linked mat can be placed in ethanol without any visible shrinkage of the mat, but when it was placed in a subsequent aqueous solution, the mat again gelled. Thus, EDC was dissolved in ethanol for the cross-linking protocols requiring EDC. There appeared to be some shrinkage of the mats cross-linked in EDC and EDC/NHS (particularly at the higher concentrations), as well as in 3% glutaraldehyde, but not to the extent of the 70% isopropanol.

Micrographs were taken of all cross-linked samples using an ESEM to visualize the morphology of the hydrated mat (fibrous versus gel). These micrographs are shown in Figure 4. For comparison, an electrospun type II collagen mat was soaked in ethanol for 18 h and then placed in water; this immediately gelled and appears as a smooth surface in the micrograph in Figure 4. The 20-mM EDC/NHS cross-linked sample showed a smoother, bunched appearance—not fibrous at all. All of the other cross-linked samples displayed a fibrous texture, although the sample cross-linked with 3% glutaraldehyde at room temperature for 18 h displayed a mixture of fibrous and gel characteristics.

Uniaxial tensile testing performed on the dry and cross-linked samples of electrospun mats indicated that a

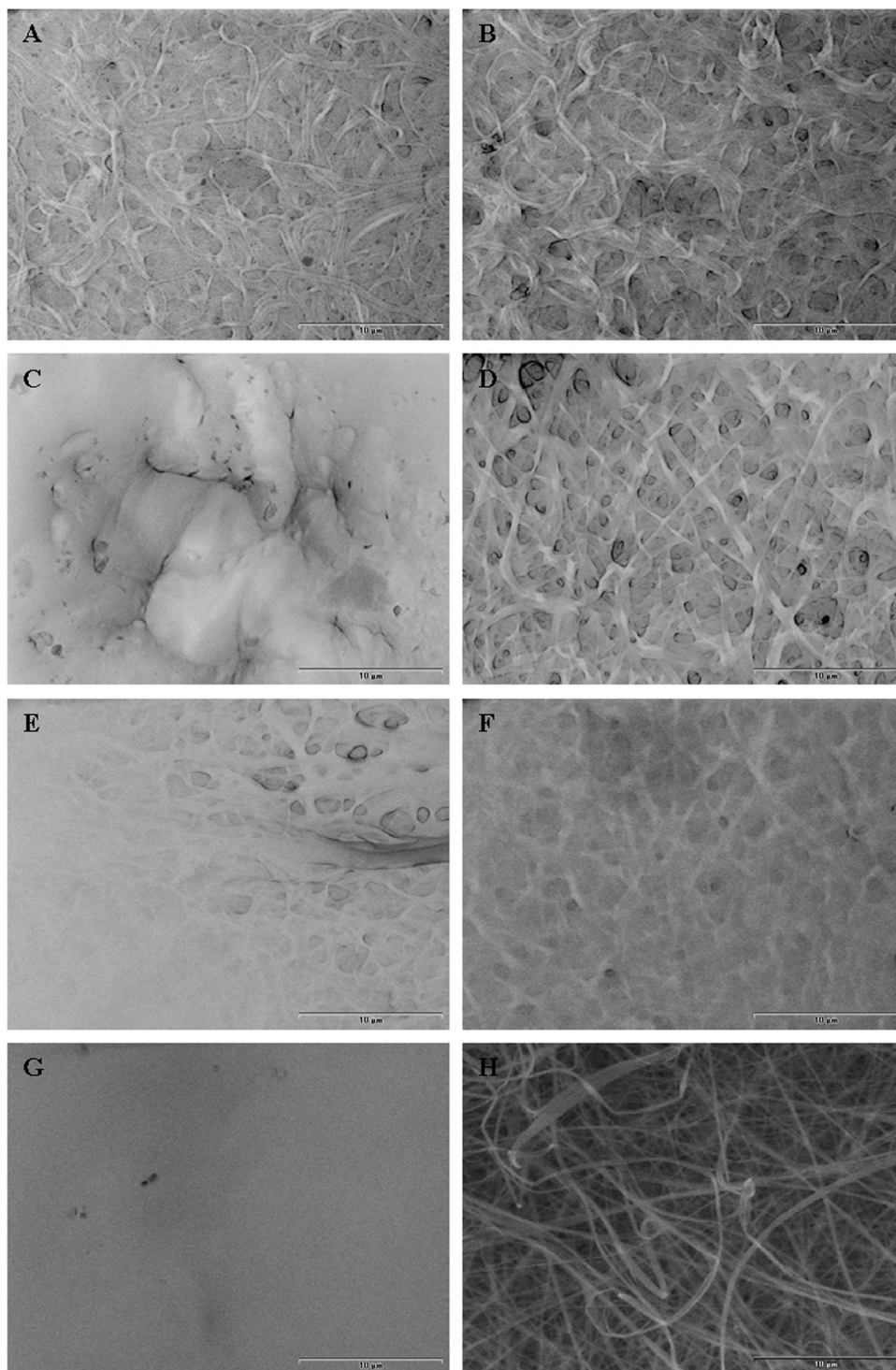


FIG. 4. Scanning electron microscopy micrographs: samples cross-linked with (A) 20-mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (B) 200-mM EDC (C) 20-mM EDC/N-hydroxysuccinimide (NHS) (D) 200-mM EDC/NHS (E) 3% glutaraldehyde (room temperature), (F) 50% glutaraldehyde (heated), (G) 100% ethanol-soaked, and (H) no treatment (dry); scale bar is 10 μ m.

statistically significant difference existed between the dry (no treatment) samples and all of the cross-linked samples for peak stress, strain at break, and tangential modulus. The mat soaked in ethanol for 18 h did not have sufficient mechanical strength to undergo tensile testing. Representative

plots of the stress-strain relationships of the dry samples and 2 cross-linked samples (1 in 20-mM EDC and 1 in heated 50% glutaraldehyde) are shown in Figure 5. The hydrated, cross-linked samples demonstrated uniaxial tensile behavior more characteristic of native tissue than did the dry samples.

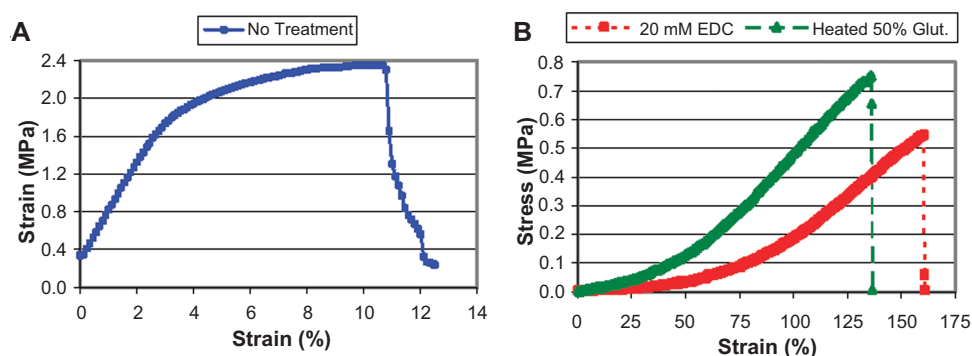


FIG. 5. Representative plots of stress-strain curves to demonstrate the difference in behavior of (A) dry samples and (B) hydrated samples cross-linked in 20-mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and in 50% glutaraldehyde (heated). Color images available online at www.liebertpub.com/ten.

Figures 6 and 7 show the results of peak stress and strain at break, respectively, for the dry (no treatment) and cross-linked mats. Table 1 lists the values of the mechanical properties (means and standard deviations) for each cross-linking treatment. The statistical analysis of the mechanical data is presented in Table 2; there are several relationships that should be noted. The samples cross-linked in heated 50% glutaraldehyde (for 30 min) demonstrated the greatest mean peak stress—this value was not significantly different from the mean peak stress values of the EDC-only cross-linked samples but was significantly different from those values of the samples from the other 3 cross-linking techniques. The samples cross-linked in 3% glutaraldehyde at room temperature (for 18 h) showed the lowest mean peak stress, which was significantly different from all other values for mean peak stress of the samples from other cross-linking techniques except the 200-mM EDC/NHS treatment. For strain at break, the 20-mM EDC groups had the greatest mean

strain, which was significantly greater than all other values for mean strain at break of the samples from other cross-linking groups except the 20-mM EDC/NHS treatment. The samples cross-linked in 3% glutaraldehyde at room temperature (for 18 h) also showed the lowest mean strain at break, which was significantly different from all other values for samples from all of the other cross-linking techniques. There were no significant differences between any of the values of tangential modulus for any of the cross-linked samples; thus, this plot is not shown, but the values are given in Table 1.

Also given in Table 1 are the results of the percentage cross-linking as determined according to the TNBS assay used to measure the percentage of free amino groups in the electrospun mat samples. Table 2 shows the statistical analysis for this assay (in the fourth subcolumn of each treatment heading). Of noteworthiness is the lack of significant difference between the heated 50% glutaraldehyde treatment

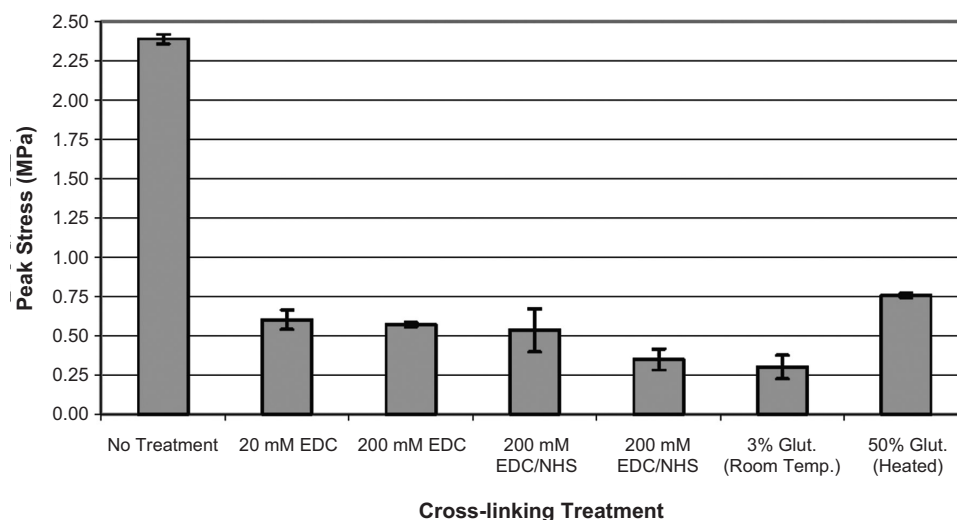


FIG. 6. Peak stress measurements for dry (no treatment) and cross-linked, hydrated, electrospun type II collagen mats.

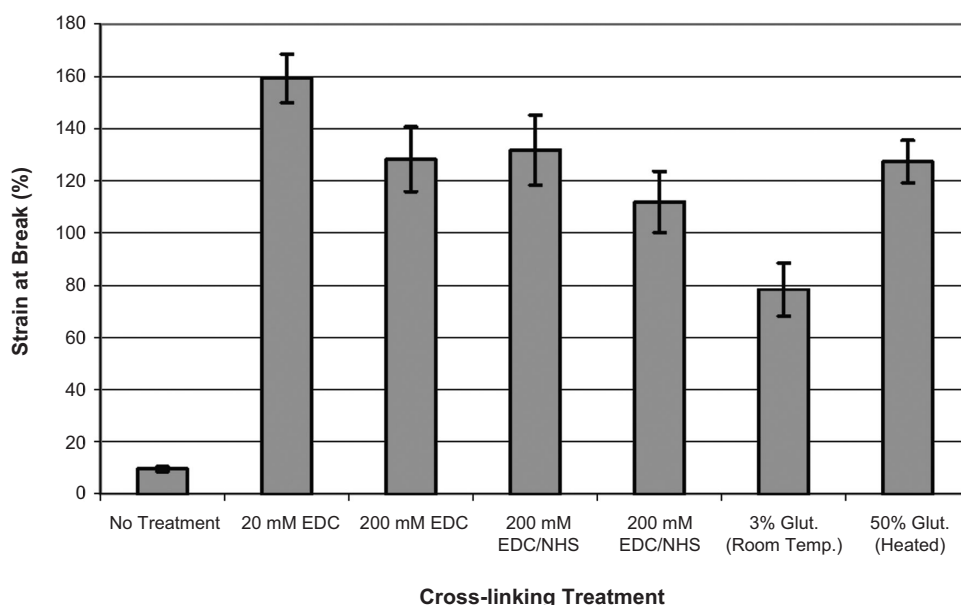


FIG. 7. Strain at break measurements for dry (no treatment) and cross-linked, hydrated, electrospun type II collagen mats.

and either of the EDC-only treatments, as well as the 200-mM EDC/NHS treatment. Additionally, the room temperature 3% glutaraldehyde treatment was significantly different from the other cross-linking techniques except those with NHS. The 200-mM EDC/NHS treated samples were not significantly different from any of the other cross-linking treatments. Finally, the 20-mM EDC and the 200-mM EDC treatments were not significantly different from each other, and the 20-mM EDC/NHS and 3% glutaraldehyde treatments were not significantly different from the control (no treatment).

DISCUSSION

Electrospun collagen fibers consist of reconstituted collagen fibrils (i.e., acid soluble collagen extracted from fetal calf cartilage) that are formed using the electrospinning fabrication process and possibly via the spontaneous organization of collagen molecules.⁴⁵ These electrospun colla-

gen fibers are identical in ultrastructure to native collagen; however, the fibers are fragile, possibly due to a lack of native cross-links, resulting in weakly bonded fragments of collagen molecules. Thus, the electrospun structures need further treatment to stabilize the material. Further cross-linking, beyond what little weak bonding may have spontaneously occurred, is necessary to work with an electrospun collagen scaffold in a hydrated state for tissue-engineering applications. The heterobifunctional reagent EDC, used with and without NHS, was used to introduce synthetic cross-links that form a bridge between 2 amino acids without imparting any other chemistry to the collagen molecule. Within the triple helical structure of collagen, there are a large number of opportunities for lysine interactions with aspartic acid and with glutamic acid residues; thus, cross-links created using EDC can be formed within an α chain, between α chains, between collagen molecules, or between collagen fibrils.³⁵ The unique aspect of the cross-linking protocol in this study involves the use of ethanol as the solvent for the cross-linking agent, because the pure colla-

TABLE 1. MECHANICAL PROPERTIES (GIVEN AS MEAN $\pm$ STANDARD DEVIATION) AND EXTENT OF CROSS-LINKING OF THE UNTREATED AND CROSS-LINKED MATS

Cross-linking Technique	Peak Stress (MPa)	Strain at Break (%)	Tangential Modulus (MPa)	% Cross-linked
Dry (No Treatment)	2.39 $\pm$ 0.03	9.53 $\pm$ 1.00	51.45 $\pm$ 1.65	0.0
20 mM EDC	0.60 $\pm$ 0.06	159.23 $\pm$ 9.35	0.73 $\pm$ 0.08	29 $\pm$ 5.2
200 mM EDC	0.57 $\pm$ 0.02	128.27 $\pm$ 12.46	0.92 $\pm$ 0.07	29 $\pm$ 11
20 mM EDC/NHS	0.54 $\pm$ 0.14	131.80 $\pm$ 13.44	0.75 $\pm$ 0.14	11 $\pm$ 8.4
200 mM EDC/NHS	0.35 $\pm$ 0.07	111.83 $\pm$ 11.75	0.57 $\pm$ 0.07	21 $\pm$ 14
3% Glut. (Room Temp.)	0.30 $\pm$ 0.08	78.27 $\pm$ 10.24	0.51 $\pm$ 0.09	11 $\pm$ 9.2
50% Glut. (Heated)	0.76 $\pm$ 0.01	127.30 $\pm$ 8.15	0.89 $\pm$ 0.06	30 $\pm$ 9.6

TABLE 2. STATISTICALLY SIGNIFICANT DIFFERENCES BETWEEN CROSS-LINK TREATMENT GROUPS DETERMINED FOR MECHANICAL PROPERTIES AND EXTENT OF CROSS-LINKING

	Dry (No Treatment)				20 mM EDC				200 mM EDC				20 mM EDC/NHS				200 mM EDC/NHS				3% Glut. (Room Temp.)				50% Glut. (Heated)			
Dry (No Treatment)					PS	SB	TM	%C	PS	SB	TM	%C	PS	SB	TM		PS	SB	TM	%C	PS	SB	TM		PS	SB	TM	%C
20 mM EDC	PS	SB	TM	%C						SB						%C	PS	SB			PS	SB		%C		SB		
200 mM EDC	PS	SB	TM	%C		SB										%C	PS				PS	SB		%C				
20 mM EDC/NHS	PS	SB	TM					%C				%C									PS	SB			PS			%C
200 mM EDC/NHS	PS	SB	TM	%C	PS	SB			PS													SB			PS			
3% Glut. (Room Temp.)	PS	SB	TM		PS	SB		%C	PS	SB		%C	PS	SB				SB							PS	SB		%C
50% Glut. (Heated)	PS	SB	TM	%C		SB							PS			%C	PS				PS	SB		%C				

The Table is Arranged as an Array of the Seven Cross-Linking Treatments; Each Treatment (Listed in the Top Row) has 4 Sub-Columns Under the Heading: PS is Peak Stress, SB is Strain at Break, TM is Tangential Modulus, and %C is Percent Cross-Linked. A Significant Difference is Indicated by an Entry in a Box.

gen electrospun mats immediately disintegrate when placed in an aqueous solution.

This is the first study that investigates the cross-linking of type II collagen-only electrospun mats using EDC as the cross-linking agent (i.e., the electrospun mats consisted only of type II collagen before and after the cross-linking procedures). Buttafoco *et al.* tested EDC and NHS in the cross-linking of electrospun type I collagen (electrospun from hydrochloric acid with poly(ethylene oxide) (PEO) and sodium chloride added). EDC and NHS in a 2-morpholinoethane sulfonic acid buffer in 70% v/v ethanol/water were used for cross-linking, and it was shown that the amount of free amino groups after cross-linking was reduced to approximately 30% of the original value and that the denaturation temperature increased.¹⁵ The cross-linking process resulted in the leaching of PEO from the fibers, and there were no longer any sodium chloride crystals, thereby leaving a collagen-only scaffold; Buttafoco *et al.* make the comment that the morphology of the fibers after cross-linking was not modified. However, scaffold morphology must have been modified because PEO had been added (at a weight ratio of collagen to PEO of 1:1) for the electrospinning to occur. No data or results were published, and no visual confirmation of the fibrous morphology of the cross-linked mats was performed in the hydrated state.

To avoid the disintegration or fragmentation of the electrospun structures, ethanol was used as the cross-linking solvent and the proton donor for the EDC reaction instead of a buffered solution. Ethanol molecules in concentrated solutions associate via hydrogen bonding, the energy of which is comparable with that of the hydrogen bonding of water.⁴⁶ Furthermore, the ethanol molecule has little steric hindrance and can interact with and hydrogen bond to chemical groups in tissues, including carbonyl oxygen, amine, and amide nitrogen (i.e., the O and N atoms in peptide bonds).^{46,47} Additionally, solvents of a lower dielectric constant (35 for ethanol, compared with 81 for water) reduce the dipolar

forces, resulting in a change in the bonding; the salt bridges (due to oppositely charged groups) of fibrous proteins in an aqueous solution will be converted to hydrogen bonds when immersed in alcohol solutions,^{46,47} which may be advantageous in maintaining the electrospun collagen mat structure. Further support for the better performance of the electrospun collagen mats immersed in ethanol than that of water is that ethanol molecules in concentrated solutions hydrogen bond to one another and can form polymeric chains,⁴⁶ which may help stabilize the electrospun collagenous structure. Also, the ethyl group of the solvent could interact through hydrophobic interactions with the collagen surface,^{48,49} the hydroxyl group is then available to interact with other ethanol molecules or other residues in the protein to again stabilize the electrospun collagenous mat structure. The critical issue involved in this study was not to provide better reactivity of groups but to provide the condition that maintained the proper structure of the mat. Ethanol provided that condition because it was not possible to use a buffered solution. EDC is carboxyl-reactive at a range of pH values from 4.7 to 6.0 in aqueous buffers; the intermediate then reacts with amine groups.^{28,31–35,50} However, for ethanol, a water-miscible nonaqueous solvent, the pH scale would be different (in fact, the scale is 19.1 units long and ranges from −4.2 to 14.9 with neutral pH at 9.55 using the water-based scale).⁵¹ Although the pH scales are not equivalent, measurements were taken of the ethanol solutions for comparison. Using a standard pH meter (with 4 M of potassium chloride saturated with silver chloride as the electrolyte), the measured “pH” of the EDC/ethanol solutions was about 6.4, and applying the error due to the liquid junction potential in ethanol solutions,⁵¹ the adjusted “pH” is approximately 7.6, which is considered acidic in ethanol and is consistent with a buffered pH to allow groups to react.

Glutaraldehyde reacts predominantly with primary amines in proteins.²⁰ As previously described, EDC reacts with the carboxyl groups of aspartic acid and glutamic acid and,

in turn, can interact with primary amines of lysine residues to form iso-peptide bonds. Thus, the TNBS biochemical assay is satisfactory in comparing the cross-linking techniques because it measures free amino groups. The results reveal that the samples cross-linked in EDC alone (at both concentrations) are as effective as the stronger concentration of glutaraldehyde in cross-linking the available amino groups. The 20-mM EDC/NHS cross-linked samples and the 3% glutaraldehyde cross-linked samples showed the least extent of cross-linking, with the 20-mM EDC/NHS cross-linked samples showing no statistical difference from the untreated samples.

Some shrinkage in overall dimensions of the electrospun mats was observed with the cross-linking treatments using the EDC and EDC/NHS systems. This shrinkage (12–33% decrease in dimensions) was more apparent with the higher concentrations of the cross-linking agents. This is probably due to the absorption of moisture by the agents during measurement of the solids before cross-linking and the subsequent reaction of the fibrous collagen to slight hydration.

Data from electron microscopy and the TNBS assay (extent of cross-linking) are supportive of one another and of the mechanical data for the cross-linked samples. Samples with greater fibrous texture and greater values of extent of cross-linking had greater values of peak stress, strain at break, and tangential modulus. There was one exception—the 20-mM EDC/NHS cross-linked samples. The lack of cross-linking, as measured using the TNBS assay, supports the gel-like appearance of the mat cross-linked with 20-mM EDC/NHS. To our surprise though, the mechanical data for these cross-

linked samples demonstrated high peak stress, strain at break, and tangential modulus values. This event raises the question of why we did not see the expected relationship between lower cross-linking and lower mechanical strength. It has been reported that the use of NHS improves the efficiency of cross-linking collagen in conjunction with EDC and thus improves the cross-link yields when used in aqueous solutions.^{29,35,52} The use of ethanol as a solvent may have inhibited this efficiency. A shorter time period of incubation (the EDC/NHS system was allowed to react for only 4 h) may significantly reduce cross-linking extent at lower concentrations of NHS. In aqueous solutions, a cross-linking dependency on the ratio of EDC to NHS has been shown.^{28,35} Additionally, there must be a critical ratio of EDC to protein that must be attained for cross-linking to proceed efficiently.²⁸ Both ratios play critical roles in the cross-linking performance, which is most likely dependent on the combined effect of these ratios. At the 20-mM EDC/NHS concentration, the threshold may not have been reached for cross-linking to proceed efficiently at an incubation time of 4 h. However, at the 200-mM EDC/NHS concentration, although the EDC-to-NHS ratio is the same, the EDC-to-protein ratio is greater, thereby meeting or exceeding the threshold. Furthermore, at the lower ratio, the NHS is not working to create a more stable bond. This could indicate that a molecule of ethanol undergoes esterification (possible but probably infrequent) or transesterification with collagen, thereby acting as a nucleophile, attacking the carboxyl groups of aspartate and glutamate; the amino acid side chain is modified, and in turn, the mechanical properties may be altered (Fig. 8). It is possible

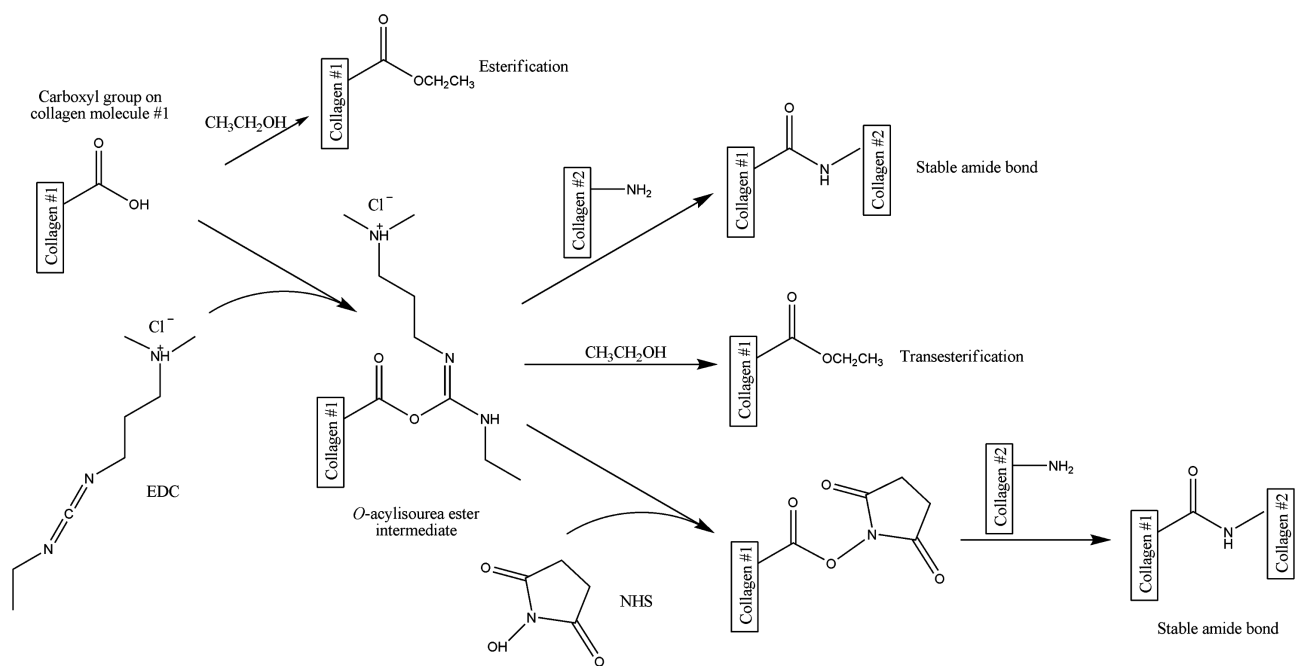


FIG. 8. Reactions of a carboxyl group on a collagen molecule with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride to result in esterification (possible but probably infrequent), transesterification, or a stable amide bond with another collagen molecule. This figure was adapted from ⁵⁰.

that stable NHS esters are created that do not proceed to the formation of amide bonds. After the reaction of EDC with glutamic or aspartic acids, the binding of NHS may sterically hinder the primary amines from reaching the necessary site within the microenvironment, thereby resulting in a covalently modified protein that will alter the mechanical properties. Several explanations have been proposed here, but further investigation is required.

The 2 EDC-only protocols resulted in structures comparable in morphology, extent of cross-linking, and mechanical properties with the higher-concentration, glutaraldehyde cross-linked structure. In an aqueous environment, the *O*-isoacylurea intermediate quickly undergoes hydrolysis; thus, as mentioned, NHS is added to stabilize the intermediate (prevent hydrolysis) and allow reaction with an amine group. However, the use of ethanol as the solvent may reduce or eliminate the hydrolysis of carbodiimide-activated derivatives, thereby improving the cross-link yields.

After cross-linking and hydration, all electrospun mats displayed behavior under uniaxial tension more like that of native tissue, namely articular cartilage. In native tissue, at small extensions, there is a nonlinear toe region in which collagen fibers are realigning, and at larger extensions, the aligned collagen fibers are stretched collectively, thereby contributing to the stiffness observed in the curve.⁵³ The same phenomenon occurs in the electrospun, nonwoven mats, in which the randomly oriented collagen fibers first align themselves at small extensions and are collectively strained at larger extensions. The stiffness of the structure as a whole depends on fiber diameter and orientation, number of fibers, and the strength of covalent cross-links and noncovalent bonds (i.e., type and amount). In healthy hyaline cartilage (the tissue as a whole including collagen fibers in the hydrated state), the tensile modulus ranges from 5 to 25 Mpa,^{53–55} peak stress has been determined to be between 1.3 and 4.4 Mpa,⁵⁵ and strain at break ranges between 9.2% and 25.9%.⁵⁵ All of the cross-linked, electrospun mats tested in this study demonstrated values of peak stress and modulus at least an order of magnitude less than those reported for cartilage tissue, and the strain at break values of the specimens tested were greater than that of cartilage tissue. Although these differences reflect variances between a simulated ECM alone versus whole tissue, it bodes well for the use of electrospun type II collagen mats in the tissue engineering of cartilage that, during tissue regeneration *in vitro*, the stiffness of the electrospun scaffold will not inhibit chondrocyte mobility, and the electrospun fibers will be free to be moved by the cells themselves.¹⁷ This will be investigated further in future work.

CONCLUSIONS

The major concerns in developing a protocol for the cross-linking of electrospun type II collagen mats for use in tissue-engineering applications (particularly cartilage tissue

engineering) are preventing any cytotoxic effects, imparting desirable mechanical properties, and maintaining the nanofibrous structure (rather than creating a gel). The desire for a pure collagen scaffold is to mimic the biochemical and ultrastructural properties of the native ECM of tissues, which is known to influence cell behavior. Additionally, a degradable scaffold may allow the cells to replace it with their own ECM, thereby allowing the mechanical strength of the regenerating tissue to take over as the device degrades in the body. In deciding what is most biomedically relevant for the collagenous electrospun structures to be used as tissue-engineering scaffolds, the use of EDC alone in an ethanol solvent, at a concentration of 20 mM, provided sufficiently greater cross-linking and mechanical strength than those of the typical glutaraldehyde protocols currently in practice that further investigation into this protocol is warranted. Future work will focus on degradation characteristics of and cellular interactions with these cross-linked electrospun scaffolds.

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REFERENCES

- Grinnell F. Cell–collagen interactions: overview. *Meth Enzymol* **82**, 499, 1982.
- Martins-Green M. Dynamics of cell-ECM interactions. In: Lanza RP, Langer R, Vacanti J, eds. *Principles of Tissue Engineering*. San Diego, CA: Academic Press, 2000, pp 33–55.
- Hunziker EB. Articular cartilage repair: basic science and clinical progress. A review of the current status and prospects. *Osteoarthritis Cartilage* **10**, 432, 2002.
- Temenoff JS, Mikos AG. Review: tissue engineering for regeneration of articular cartilage. *Biomaterials* **21**, 431, 2000.
- Nehrer S, Breinan HA, Ramappa A, *et al.* Chondrocyte-seeded collagen matrices implanted in a chondral defect in a canine model. *Biomaterials* **19**, 2313, 1998.
- Auger FA, Berthod F, Moulin W, Pouliot R, Germain L. Tissue-engineered skin substitutes: from *in vitro* constructs to *in vivo* applications. *Biotechnol Appl Biochem* **39**, 263, 2004.
- Wallace DG, Rosenblatt J. Collagen gel systems for sustained delivery and tissue engineering. *Adv Drug Delivery Rev* **55**, 1631, 2003.
- Nerem RM, Seliktar D. Vascular tissue engineering. *Annu Rev Biomed Eng* **3**, 225, 2001.
- Bell E. Organotypic and histiotypic models of engineered tissues. In: Lanza RP, Langer R, Vacanti J, eds. *Principles of Tissue Engineering*. San Diego, CA: Academic Press, 2000, pp 181–194.

10. Mueller SM, Shortkroff S, Schneider TO, Breinan HA, Yannas IV, Spector M. Meniscus cells seeded in type I and type II collagen-GAG matrices *in vitro*. *Biomaterials* **20**, 701, 1999.
11. Itoh H, Aso Y, Furuse M, Noishiki Y, Miyata T. A honeycomb collagen carrier for cell culture as a tissue engineering scaffold. *Artificial Organs* **25**, 213, 2001.
12. Matthews JA, Wnek GE, Simpson DG, Bowlin GL. Electrospinning of collagen nanofibers. *Biomacromolecules* **3**, 232, 2002.
13. Matthews JA, Boland ED, Wnek GE, Simpson DG, Bowlin GL. Electrospinning of collagen type II. A feasibility study. *J Bioactive Compatible Polymers* **18**, 125, 2003.
14. Shields KJ, Beckman MJ, Bowlin GL, Wayne JS. Mechanical properties and cellular proliferation of electrospun collagen type II. *Tissue Eng* **10**, 1510, 2004.
15. Buttafoco L, Kolkman NG, Engbers-Buijtenhuijs P, Poot AA, Dijkstra PJ, Vermes I, Feijen J. Electrospinning of collagen and elastin for tissue engineering applications. *Biomaterials* **27**, 724, 2006.
16. Kidoaki S, Kwon IK, Matsuda T. Mesoscopic spatial designs of nano- and microfiber meshes for tissue-engineering matrix and scaffold based on newly devised multilayering and mixing electrospinning techniques. *Biomaterials* **26**, 37, 2005.
17. Boland ED, Matthews JA, Pawlowski KJ, Simpson DG, Wnek GE, Bowlin GL. Electrospinning collagen and elastin: preliminary vascular tissue engineering. *Front Biosci* **9**, 1422, 2004.
18. Li MY, Mondrinos MJ, Gandhi MR, Ko FK, Weiss AS, Lelkes PI. Electrospun protein fibers as matrices for tissue engineering. *Biomaterials* **26**, 5999, 2005.
19. Rho KS, Jeong L, Lee G, *et al.* Electrospinning of collagen nanofibers. Effects on the behavior of normal human keratinocytes and early-stage wound healing. *Biomaterials* **27**, 1452, 2006.
20. Koob TJ. Collagen fixation. In: Wnek GE, Bowlin GL, eds. *Encyclopedia of Biomaterials and Biomedical Engineering*. New York: Marcel Dekker, 2004, pp 335–347.
21. Jayakrishnan A, Jameela SR. Glutaraldehyde as a fixative in bioprostheses and drug delivery matrices. *Biomaterials* **17**, 471, 1996.
22. Badylak SF. Modification of natural polymers: Collagen. In: Atala A, Lanza R, eds. *Methods of Tissue Engineering*. San Diego, CA: Academic Press, 2002, pp 505–514.
23. Khor E. Methods for the treatment of collagenous tissues for bioprostheses. *Biomaterials* **18**, 95, 1997.
24. Schmidt CE, Baier JM. Acellular vascular tissues: natural biomaterials for tissue repair and tissue engineering. *Biomaterials* **21**, 2215, 2000.
25. Simmons DM, Kearney JN. Evaluation of collagen cross-linking techniques for the stabilization of tissue matrices. *Biotechnol Appl Biochem* **17**, 23, 1993.
26. Barnes CP. Feasibility study of electrospun collagen type III. 8th Annual Meeting of Tissue Engineering Society International. Shanghai, China, 2005.
27. Tanzer ML. Cross-linking. In: Ramachandran GN, Reddi AH, eds. *Biochemistry of Collagen*. New York: Plenum Press, 1976, pp 137–162.
28. Olde Damink LHH, Dijkstra PJ, van Luyn MJA, van Wachem PB, Nieuwenhuis P, Feijen J. Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. *Biomaterials* **17**, 765, 1996.
29. Grabarek Z, Gergely J. Zero-length crosslinking procedure with the use of active esters. *Anal Biochem* **185**, 131, 1990.
30. Pieper JS, van der Kraan PM, Hafmans T, *et al.* Crosslinked type II collagen matrices: preparation, characterization, and potential for cartilage engineering. *Biomaterials* **23**, 3183, 2002.
31. Pieper JS, Oosterhof A, Dijkstra PJ, Veerkamp JH, van Kuppevelt TH. Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate. *Biomaterials* **20**, 847, 1999.
32. Angele P, Abke J, Kujat R, *et al.* Influence of different collagen species on physico-chemical properties of crosslinked collagen matrices. *Biomaterials* **25**, 2831, 2004.
33. Sung HW, Chang WH, Ma CY, Lee MH. Crosslinking of biological tissues using genipin and/or carbodiimide. *J Biomed Materials Res A* **64A**, 427, 2003.
34. Gratzel PF, Lee JM. Control of pH alters the type of crosslinking produced by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) treatment of acellular matrix vascular grafts. *J Biomed Mater Res* **58**, 172, 2001.
35. Lee JM, Edwards HHL, Pereira CA, Samii SI. Crosslinking of tissue-derived biomaterials in 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). *J Mater Sci Mater Med* **7**, 531, 1996.
36. Miller EJ, Lunde LG. Isolation and characterization of cyanogen bromide peptides from alpha-1 (ii) chain of bovine and human cartilage collagen. *Biochemistry* **12**, 3153, 1973.
37. Reneker DH, Chun I. Nanometre diameter fibres of polymer, produced by electrospinning. *Nanotechnology* **7**, 216, 1996.
38. Reneker DH, Yarin AL, Fong H, Koombhongse S. Bending instability of electrically charged liquid jets of polymer solutions in electrospinning. *J Appl Physics* **87**, 4531, 2000.
39. Vyavahare NR, Hirsch D, Lerner E, *et al.* Prevention of calcification of glutaraldehyde-crosslinked porcine aortic cusps by ethanol preincubation: Mechanistic studies of protein structure and water-biomaterial relationships. *J Biomed Mater Res* **40**, 577, 1998.
40. Sinz A. Chemical cross-linking and mass spectrometry for mapping three-dimensional structures of proteins and protein complexes. *J Mass Spectrometry* **38**, 1225, 2003.
41. Rajini KH, Usha R, Arumugam V, Sanjeevi R. Fracture behaviour of cross-linked collagen fibres. *J Mater Sci* **36**, 5589, 2001.
42. Brand DD. *Protocols: Acid Extraction of Fetal Bovine Skin Collagen*. Memphis, TN: University of Tennessee, 2005.
43. Sheu MT, Huang JC, Yeh GC, Ho HO. Characterization of collagen gel solutions and collagen matrices for cell culture. *Biomaterials* **22**, 1713, 2001.
44. Nagai N, Yunoki S, Suzuki T, Sakata M, Tajima K, Munekata M. Application of cross-linked salmon atelocollagen to the scaffold of human periodontal ligament cells. *J Biosci Bioeng* **97**, 389, 2004.
45. Yuan L, Veis A. Self-assembly of collagen molecules. *Biopolymers* **12**, 1437, 1973.
46. Puchtler H, Waldrop FS, Conner HM, Terry MS. Carnoy fixation—practical and theoretical considerations. *Histochemie* **16**, 361, 1968.
47. Gustavson KH. *The Internal Linking of the Collagen Molecule. The Chemistry and Reactivity of Collagen*. New York: Academic Press Inc., 1956, pp 133–154.

48. Usha R, Maheshwari R, Dhathathreyan A, Ramasami T. Structural influence of mono and polyhydric alcohols on the stabilization of collagen. *Colloids Surf B Biointerfaces* **48**, 101, 2006.
49. Lins L, Brasseur R. The hydrophobic effect in protein-folding. *FASEB J* **9**, 535, 1995.
50. Pierce Biotechnology. Thermo Fisher Scientific Inc. EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride), a water-soluble carbodiimide for rapid preparation of peptide conjugates. Available at www.piercenet.com/Objects/View.cfm?type=ProductFamily&ID=02030312, 2006.
51. Frant MS. How to Measure pH in Mixed and Nonaqueous Solutions. *Today's Chemist at Work* **4**, 39, 1995.
52. Staros JV, Wright RW, Swingle DM. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. *Anal Biochem* **156**, 220, 1986.
53. Mow V, Guo XE. Mechano-electrochemical properties of articular cartilage. Their inhomogeneities and anisotropies. *Annu Rev Biomed Engineering* **4**, 175, 2002.
54. Akizuki S, Mow VC, Muller F, Pita JC, Howell DS, Mancourt DH. Tensile properties of human knee-joint cartilage. 1. Influence of ionic conditions, weight bearing, and fibrillation on the tensile modulus. *J Orthop Res* **4**, 379, 1986.
55. Yamada H. Tensile properties of human hyaline cartilage. In: Evans FG, ed. *Strength of Biological Materials*. Huntington, NY: Robert E. Krieger Publishing Co., 1970, pp 80–81.

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