Engineered collagen–PEO nanofibers and fabrics

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Abstract—Type I collagen–PEO fibers and non-woven fiber networks were produced by the electrospinning of a weak acid solution of purified collagen at ambient temperature and pressure. As determined by high-resolution SEM and TEM, fiber morphology was influenced by solution viscosity, conductivity, and flow rate. Uniform fibers with a diameter range of 100–150 nm were produced from a 2-wt% solution of collagen–PEO at a flow rate of 100 μl min⁻¹. Ultimate tensile strength and elastic modulus of the resulting non-woven fabrics was dependent upon the chosen weight ratio of the collagen–PEO blend. ¹H NMR dipolar magnetization transfer analysis suggested that the superior mechanical properties, observed for collagen–PEO blends of weight ratio 1 : 1, were due to the maximization of intermolecular interactions between the PEO and collagen components. The process outlined herein provides a convenient, non-toxic, non-denaturing approach for the generation collagen-containing nanofibers and non-woven fabrics that have potential application in wound healing, tissue engineering, and as hemostatic agents.

Key words: Collagen; PEO; nanofibers; fabrics; tissue engineering.

INTRODUCTION

Collagen is a biodegradable, biocompatible, and non-immunogenic structural protein, which makes it a suitable compound for a variety of biomedical applications. For example, collagen has been used in cosmetic and urological surgery as an injectable compound for tissue augmentation [1]; in orthopedic surgery as an implantable matrix to promote bone growth [2–5]; and in plastic and general surgery as a topical agent for the treatment of both chronic non-healing wounds and burn injuries or as a template for tissue regeneration [6, 7]. The most abundant form of collagen isolated from adult connective tissues, such as skin, tendon, and bone,
is type I collagen. Characteristically, it is composed of two $\alpha 1$(I) chains and one $\alpha 2$(I) chain, each slightly more than 1000 amino acids long, that are organized as a triple helix and stabilized primarily by hydrogen bonds [8]. A single molecule of type I collagen has a molecular mass of 285 kD, a width of $\sim 14$ Å, and a length of $\sim 3000$ Å.

As a biomaterial, collagen has been predominantly used after processing into a dry powder or slurry, a hydrogel after solution phase cross-linking, or as a porous matrix with or without the addition of other components after freeze-drying. However, in native connective tissues, type I collagen molecules form fibrillar elements, twenty to several hundred nanometers in diameter that are organized into protein networks of varying architecture. Functionally, collagen fiber networks act to resist high strain deformation and in the process transmit forces, dissipate energy, and prevent premature tissue mechanical failure [9]. It is notable that these fiber networks constitute the principal structural elements of a variety of acellular bioprosthesis tissue substitutes, such as porcine heart valves and bovine artery heterografts, as well as other tissue derived matrices, including porcine subintestinal submucosa and bovine pericardium. Indeed, the versatility of collagen as a scaffold for tissue engineering applications is significantly enhanced when used as a native protein network. To date, attempts to reformulate tissue extracted native collagen into protein fiber networks and fabrics have been limited.

Traditional wet or dry spinning processes have been most commonly used to produce protein fibers. For example, Hirano et al. [10] described the production of chitosan–collagen fibers produced by wet spinning from an aqueous 2% acetic acid–methanol solution spun into an aqueous 5% ammonia solution containing 40–43% ammonium sulfate. As is characteristic of both wet and dry spinning methodologies, large fibers were produced with an average diameter of 36 $\mu$m. Likewise, Fofonoff and Bell [11] have reported a method for forming collagen fibers ($d > 100$ $\mu$m) by wet spinning from an aqueous 0.05% acetic acid (0.5 M) solution into a coagulating bath consisting of alkaline alginic acid/boric acid (pH 8–10), heated at 35°C. The collagen gel fiber is formed by polymerization when the acid in the collagen is neutralized upon contact with the neutralizing solution. Generated fibers are subsequently dehydrated in acetone and ethanol baths. An addition example is provided by Furukawa et al. [12] in which solubilized collagen is spun through a spinneret into a coagulating bath comprised of an aqueous solution of an inorganic salt, such as sodium sulfate, sodium chloride, ammonium sulfate, magnesium chloride, or aluminum sulfate.

As an alternate approach for fiber formation, we and others have recently investigated electrospinning as a mechanism for generating protein fibers with diameters in the submicron range [13–18]. In this technique, a polymer solution is subjected to an electric field that induces the accumulation of charge on the surface of a pendant drop. Mutual charge repulsion causes a force, which directly opposes that produced by surface tension. Above a critical value of electric field strength, the repulsive electric force exceeds the surface tension force and a charged jet of
solution is ejected. Subsequently, the jet splay into a series of fine filaments with a range of diameters on the order of several tens or hundreds of nanometers. Given the high surface area to volume ratio of the generated nanofibers, solvent evaporation occurs as a relatively efficient process even when operating with aqueous solutions at ambient temperature and pressure.

In this report, collagen–PEO nanofibers and non-woven fabrics were produced by an electrospinning technique. High-resolution SEM and TEM were used to assess the influence of PEO content, as well as solution viscosity, conductivity, and flow rate on fiber morphology and ultrastructure. In addition, uniaxial mechanical properties were defined in the dry state. In this regard, the mechanical properties of a polymer blend are directly influenced by the formation of phase-separated domains and, as a consequence, the extent of the polymer–polymer interface. Although several NMR techniques are available to study morphology, the use of dipolar magnetization transfer (i.e. spin diffusion) has been especially attractive since it provides quantitative estimates of domain sizes in multiphase polymer systems. Specifically, a magnetization gradient is created across a sample and then monitored to determine the time required for spin equilibration to occur. The equilibration time depends upon both domain sizes, as well as the spin diffusion coefficients associated with each phase. If the diffusion coefficients and the dimensionality of the diffusion process are known \textit{a priori}, then domain sizes can be extracted by fitting a simulated diffusion profile to the experimental data [22]. Characteristically, domain distances that can be observed using spin diffusion range from 2 to 100 nm and the versatility of this approach has been demonstrated in a variety of multiphase polymer systems [23–25]. In the current study, the spin diffusion technique provided a convenient tool to characterize the domain structure for varying collagen–PEO blends in the dry state and revealed that both ultimate tensile strength and Young’s modulus were related to the formation of an intimate PEO-collagen interface.

**METHODS**

*Materials*

Poly(ethylene oxide) (PEO) with a nominal molecular weight of 900 kD was obtained from Aldrich. Acid-soluble collagen was derived from tail tendons obtained from Sprague–Dawley rats weighing between 250 to 350 g using a protocol similar to that described by Silver and Trelstad [19]. Briefly, tendon fibers were extracted from rat tails using a wire stripper, immersed in 10 mM HCl (pH 2.0; ten fibers per 100 ml), and stirred for 4 h at room temperature. The soluble component was separated from the insoluble portion after centrifugation at 30 000g at 4°C for 30 min and then sequentially filtered through 0.65 and 0.45 μm filters (Millipore Corp., Bedford, MA, USA). NaCl was added to the filtrate so as to obtain a salt concentration of 0.7 M. The mixture was then allowed to stir for 1 h and the precipitate collected after a 1-h centrifugation at 30 000g and 4°C. The pellet was
allowed to dissolve overnight in 10 mM HCl (pH 2.0) and dialyzed against 20 mM phosphate buffer (disodium hydrogen phosphate at pH 7.4) for at least 8 h at room temperature. A second dialysis was then performed against a 20 mM phosphate buffer solution for at least 4 h at 4°C. The dialyze was centrifuged at 30,000 g at 4°C for 1 h and the pellet was then dialyzed overnight against against a solution of 10 mM HCl (pH 2.0) to obtain a collagen solution at a final concentration of 10 mg ml⁻¹. The solution was stored at 4°C.

Lyophilized collagen was obtained by dialyzing the collagen solution overnight against distilled, deionized water (18 MΩ cm, Continental), followed by lyophilization. Prior to use, lyophilized collagen was dissolved in 10 mM HCl at room temperature for 1 h. The identity and purity of the collagen samples was confirmed by polyacrylamide gel electrophoresis.

**Determination of viscosity and conductivity of collagen–PEO solutions**

Bulk viscosity was determined using silicone oil viscosity standards (Brookfield Engineering Laboratories, Inc.) and solution conductivity determined using a conductivity flow cell (Biorad).

**Preparation of collagen–PEO fibers**

Collagen–PEO solutions (1–2 wt%) were prepared in 10 mM HCl (pH 2.0) by mixing for 2 h at ambient temperature. With the aid of a syringe pump (Harvard Apparatus, Inc., Holliston, MA, USA), the solution was extruded at ambient temperature and pressure and at a defined flow rate through a positively charged metal blunt tipped needle (22G × 1.5 in.). Fibers were collected on a grounded aluminum plate located below the tip of the needle. A high voltage, low current power supply (ES30P/DDPM, Gamma High Voltage Research, Inc., Ormond Beach, FL, USA) was utilized to establish the electric potential gradient, which was varied between 0 and 30 kV, as indicated.

**Electron microscopy**

An in-lens field emission scanning electron microscope (ISI DS-130F Schottky Field Emission SEM) was used and operated at 5 kV. High-resolution topographic images at low (≈1000×), medium (30,000×), and high magnifications (≥100,000×) were digitally recorded with very short dwell times and without beam induced damage. For transmission electron microscopy (TEM) imaging, a JEOL 1210 TEM was utilized with an Oxford Light Element EDS and operated at 80 kV voltage. Fiber samples were deposited onto silicon chips and carbon-coated grids for scanning and transmission EM studies, respectively. Sample-containing silicon chips were subsequently mounted onto aluminum specimen stubs with silver paste, degassed for 30 min, and coated with a 1 nm chromium (Cr) film using a Denton DV-602 Turbo-pumped Magnetron Sputter System.
Nuclear magnetic resonance analysis

All solid-state NMR experiments were conducted at room temperature on a Bruker DSX 300 spectrometer operating at a $^1$H resonance frequency of 300 MHz in a Bruker double resonance MAS probehead. A standard cross-polarization (CP) pulse sequence was employed under conditions of magic angle spinning (MAS). A spinning speed of 5 kHz was employed. A TOSS sequence was used in conjunction with CP to provide a spectrum free of spinning sidebands [20]. A 4.5-μs $^1$H 90 deg pulse, a 1-ms contact time, a 9-μs $^{13}$C 180 deg pulse, and a 3-s recycle delay were employed with accumulation of 5000 to 16 000 scans for signal averaging. Direct polarization (DP) experiments were conducted with a recycle delay of 1 s, a $^{13}$C 90 deg pulse length of 4.5 μs, and a MAS rate of 5 kHz. For DP experiments, 1024 scans were acquired.

$^1$H dipolar magnetization transfer experiments were conducted under static conditions (without magic-angle sample spinning). Recycle delays of 5 s, $^1$H 90 deg pulse lengths of 4.5 μs, and $^1$H 180 deg pulse lengths of 9 μs were used. The timing diagram for the spin diffusion pulse sequence has been reported in detail elsewhere [21]. A dipolar filter selection sequence consisting of twelve 90 deg pulses separated by 10-μs delays was employed for twenty consecutive loops to establish the initial magnetization gradient. Thirty-two scans were accumulated for signal averaging and the spin diffusion time ($t_{\text{SD}}$) was incremented from 1 μs to 800 ms. Correction for spin-lattice relaxation during the spin diffusion time was achieved by repeating the experiment with the selection filter removed (# dipolar filter cycles, $n = 0$). The data acquired with and without the selection cycles were normalized with respect to the first time point and the spectral intensity corresponding to the mobile domain. The ratio of $I_{\text{PEO}}$ (with selection) to $I_{\text{PEO}}$ (without selection) provided the spin diffusion data as a function of $t_{\text{SD}}$.

In order to examine the chemical structure of the component selected using the dipolar filter, a CP sequence was appended to the dipolar filter sequence. For this experiment, a 3-s recycle delay, a 4.5-μs $^1$H 90 deg pulse, and a 1-ms contact time were used. The experiment was conducted under conditions of MAS with a spinning speed of 5 kHz and 10 000 scans were acquired for signal averaging.

Uniaxial stress–strain analysis of non-woven fabrics

Uniaxial tensile testing was performed on a Minimat 2000 (Miniature Materials Tester, Rheometric Scientific, Inc., NJ, USA). Dry collagen–PEO fabric samples were tested at an extension rate of 2 mm min$^{-1}$ and at an initial gage length of 8 mm. The maximum range of the load cell is 20 N. A total of six samples were analyzed. Samples thickness was determined by use of a profilometer (Tencor Alphastep 500, San Jose, CA, USA) at different points along the sample and an average sample thickness of 0.05 mm was obtained. The samples were cut at a width of 5 mm.
RESULTS AND DISCUSSION

Characterization of fiber morphology and topography by high-resolution SEM and TEM

PEO is non-toxic, chemically stable in acidic solution, and when of sufficient molecular weight is capable of forming electrospun fibers. Significantly, fibers could not be formed from a 1–2 wt% pure collagen solution, but were observed after the addition of PEO. High resolution SEM demonstrated unique morphological features as a function of the weight ratio of PEO to collagen, as well as solution conductivity and flow rate. Solution viscosity as related to PEO content and the effect of sodium chloride concentration on solution conductivity are summarized in Fig. 1. Increasing the concentration of PEO increased the yield of uniform fibers, while reducing bead formation (Fig. 2). Beads predominated at collagen–PEO weight ratios of 10 : 1 and 5 : 1, with only rare beads noted at a ratio of 1 : 1 and none observed at a collagen–PEO ratio of 1 : 2. Under this condition, uniform fibers were produced with diameters ranging between 50 and 150 nm. The formation of beads has been attributed to jet instability, which presumably is reduced upon increasing solution viscosity with the addition of high molecular weight PEO. Likewise, solution conductivity was also found to influence fiber formation (Fig. 3). When an electric field is applied to an electrolyte-containing aqueous polymer solution, 

![Figure 1.](image1.png)  
**Figure 1.** (A) Viscosity of collagen (2 wt%)–PEO solutions (34 mM NaCl) at varying PEO concentrations. (B) Conductivity of acid solubilized collagen solutions (2 wt%; pH=2.0) at varying NaCl concentrations.
field induced ion movement carries the solution along by an additional viscous drag force. This phenomenon enhances jet stability and, as a consequence, reduces bead formation. While fiber uniformity increased with the addition of sodium chloride, crystal formation was observed at high salt concentrations. Finally, we observed

Figure 2. SEM micrographs of fibers spun from 2 wt% acid solution (34 mM NaCl) at a flow rate of 100 μl min⁻¹ and at different collagen–PEO weight ratios: (A) 30 : 1, 50 000× magnification, (B) 10 : 1, 50 000× magnification, (C) 5 : 1, 50 000× magnification, (D) 2 : 1, 50 000× magnification, (E) 1 : 1, 20 000× magnification, (F) 1 : 2, 50 000× magnification.
that small changes in flow rate had a marked impact on fiber morphology (Fig. 4). Below 100 $\mu$l min$^{-1}$, bead formation became increasingly pronounced, while at flow rates above this level, the limited volatility of the polymer solution prevented adequate fiber drying under conditions of ambient temperature and pressure. TEM imaging of unstained samples confirmed HRSEM analysis and did not reveal any additional internal structural features (Fig. 5).

**Determination of phase structure of dry collagen–PEO blends by solid-state NMR**

The most important step in a spin diffusion experiment is the creation of a magnetization gradient, which is usually realized by determining an appropriate selection sequence. For multiphase systems that display a large gradient in mobility between constituent phases, a filter based on spin–spin relaxation ($T_2$) can generally be used to selectively retain the magnetization of the more mobile phase. In the polymer blends under consideration, PEO and collagen, have very different $T_g$s ($T_{g,\text{collagen}} \sim 125^\circ$C and $T_{g,\text{PEO}} \sim -65^\circ$C) and, hence, information about their domain
Figure 4. SEM micrographs of collagen–PEO ((1 : 1) (w/w), 34 mM NaCl) fibers spun from 2 wt% acid solution at different flow rates (μl min⁻¹): (A) 25; (B) 75; (C) 100; and (D) 150.

Figure 5. TEM micrographs of fibers spun from 2 wt% acid solution of collagen–PEO (NaCl 34 mM) at 1 : 1 (A) and 1 : 2 (B) collagen/PEO weight ratios.
sizes can be obtained via a mobility-based approach. Indeed, in the case of the collagen/PEO fabric the DP/MAS spectrum confirms that PEO is highly mobile when compared to collagen under dry conditions and at room temperature (Fig. 6). The $T_2$ filter, however, was replaced in the current investigation with a dipolar filter selection sequence since this approach provides greater tunability and overcomes spectral distortions produced by multi-quantum effects that are prevalent at short spin diffusion times [27].

Figure 7a shows the $^1$H spectra of an electrospun 1:2 collagen–PEO fabric before and after the application of the dipolar filter. The spectrum acquired before the application of the filter is a superposition of a broad (rigid) and a narrow (mobile) component, while after the application of the filter only the narrow component remains indicating that the magnetization associated with the rigid regions has been destroyed. In order to ascertain the chemical identity of the regions selected after filter application, a CP/MAS sequence was appended to the spin diffusion sequence. The $^{13}$C CP/MAS spectra are shown in Fig. 7b and confirm that the filter selectively

![Figure 6. $^{13}$C MAS spectra of collagen, PEO and a 1:2 collagen–PEO blended fabric. The CP spectrum of the blend appears to be a simple superposition of the CP spectrum of PEO (*) and collagen (C). The DP spectrum of the blend (DP discriminates against the more rigid components) shows that PEO is highly mobile in the sample when compared to collagen at the measuring temperature (24°C).}
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retains magnetization in the more mobile PEO regions. Thus, the chosen filter parameters establish an appropriate magnetization gradient in the sample. Similar results were obtained with a 1:1 fabric sample.

The spin diffusion data for 1:1 and 1:2 fabric blends are shown in Fig. 8 with the inset illustrating the initial time data. The spin diffusion curve for the 1:1 blend at short spin diffusion times is sigmoidal (c.f. inset in Fig. 8), whereas the curve for the 1:2 blend is linear. Characteristically, a sigmoidal curve is indicative of the presence of an interface since the magnetization in the source phase contacts the sink phase slower than expected due to the presence of the interface. The formation of an interface in collagen/PEO blends is not surprising since there exists significant potential for hydrogen bonding between the ether oxygen of PEO and the protons of the amino and hydroxyl groups in collagen. However, the nature and extent of hydrogen bonding, and as a consequence the presence an interface, will depend not only on the concentration of the polymer components in the blend, but also on whether the polymer chains are configured either in an amorphous or crystalline state.

If the PEO phase were comprised solely of amorphous chains then a predominant portion of this phase would become the source phase for spin diffusion subsequent to the selection sequence employed. However, the presence and extent of crystallinity precludes a certain fraction from being selected by the sequence. The dotted lines in Fig. 8 represents the theoretical or expected end value of spin diffusion. As expected, the theoretical value for the collagen/PEO 1:2 blend (0.74) is greater than the 1:1 blend (0.59) since more of the mobile phase is available for selection. However, it is evident for both blends that the experimental and theoretical values differ, which is likely attributable to the crystallization of PEO that

Figure 7. (a) $^1$H NMR spectrum of 1:2 collagen–PEO fabric is shown before and after the application of the dipolar filter. The dipolar filter eliminates the broad component of the spectrum and retains the narrow component. (b) $^{13}$C CP/MAS/TOSS spectra before and after application of the dipolar filter. After selection, only the PEO resonance is retained demonstrating that effective selection of mobile component has been achieved using the dipolar filter.
Figure 8. Spin diffusion data for 1:1 and 1:2 collagen–PEO fabrics. The initial portion of the curve, corresponding to times less than 9 ms, is shown in the inset. The data illustrates the presence of an interface for a 1:1 blend while showing no appreciable interface for a 1:2 blend. The dotted lines indicate the theoretical end point values for spin diffusion in case of 1:1 (●) and 1:2 (○) blends ($t_{sd}$ is the spin diffusion time in milliseconds and $I_o$ is signal intensity at $t_{sd} = 0$).

would limit the association of its ether oxygens with functional counterparts in the collagen phase. In fact, the DSC-estimated crystallinity for PEO was found to be 12% in the 1:1 blend and 36% in the 1:2 blend (data not shown). Thus, careful analysis of the spin diffusion data suggests that the loss of the interface observed for the 1:2 collagen/PEO blend (Fig. 8 inset) may be due to crystallization-induced phase separation.

In order to further validate the spin diffusion results, dynamic mechanical spectroscopy was conducted on collagen/PEO films (data not shown). While the glass transition of PEO is $\sim -65^\circ$C, in the 1:1 and 1:2 blends, glass transitions were $-36^\circ$C and $-61^\circ$C, respectively. The elevation of glass transition in the 1:1 blend is a clear indication of phase mixing across the interface, whereas the $T_g$ of the 1:2 blend occurs at almost the same temperature as that of pure PEO. Phase mixing in the 1:1 blend enhances the mechanical properties of the material by ameliorating stress transfer across the interface. Numerical estimate of the interface size can be obtained from the spin diffusion data. However, estimation of domain sizes from the spin diffusion data was not undertaken due to problems posed by crystallization of the source phase. This would require analysis of the spin diffusion data with the aid of three spin diffusion coefficients corresponding to the PEO (non-crystalline
phase), PEO (crystalline phase), and collagen (rigid phase). Although such analysis is possible it would not provide any new information about physical state of the blend.

**Uniaxial stress–strain analysis of collagen–PEO fabrics**

Non-woven fabrics were formed from fibers generated from a 2 wt% of type I collagen–PEO solution at a flow rate of 100 $\mu l \text{ml}^{-1}$ and uniaxial stress–strain properties characterized in the dry state. A pure PEO fabric sample had the lowest tensile strength of 90 kPa and a modulus of 7 MPa. The tensile strength and modulus of a 1:2 collagen–PEO blend were 270 kPa and 8 MPa, respectively. Maximum values were observed for the 1:1 blend with a tensile strength of 370 kPa and a modulus of 12 MPa. The ultimate tensile strength and the modulus are both higher for the 1:1 blend when compared to the 1:2 blend. In addition to composition, processing can also significantly influence morphology and hence mechanical behavior. Since the two non-woven fabric blends were prepared under identical conditions, the difference in the mechanical behavior of the blends cannot be attributed to effects of processing alone. For example, the fiber diameter in both cases are in the same range and for the processing conditions used in this study, average network pore sizes are 65–75 $\mu m$ (data not shown). Moreover, intrafibrillar failure (as opposed to failure at bond points) was observed to occur during tensile testing of the non-woven specimens. Such being the case, the difference in the mechanical properties of the blends can be ascribed to the observed differences in blend morphology. The tensile and NMR data taken together indicates that the superior mechanical properties observed for collagen–PEO blends of weight ratio 1:1, are likely due to the maximization of intermolecular interactions between the PEO and collagen components.

**CONCLUSIONS**

Type I collagen–PEO fibers and non-woven fiber networks were produced by the electrospinning of a weak acid solution of lyophilized collagen purified from rat tail tendon. Fibers were generated at ambient temperature and pressure with optimal fiber formation observed with use of an 18 kV electric field and a 15-cm distance between the spinneret and plate collector. Fiber morphology was influenced by solution viscosity, conductivity, and flow rate. As determined by high-resolution SEM and TEM, highly uniform fibers with a diameter range of 100–150 nm were produced from a 2 wt% solution of collagen–PEO (1:1 weight ratio, 34 mM NaCl) at a flow rate of 100 $\mu l \text{min}^{-1}$. The ultimate tensile strength of the resulting non-woven fabric was 370 kPa with an elastic modulus of 12 MPa.

Efforts to process collagen into man-made fibers has been limited and all approaches to date have been largely confined to the use of wet spinning methodologies. This approach involves the extrusion of a protein solution through a spinneret
into an acid-salt coagulating bath, which usually contains aqueous ammonium sulfate, acetic acid, isopropanol, or acetone. Further treatments in ethanol and acetone solutions are often required for fiber dehydration. Limitations of this approach include the use of biologically toxic solvent systems that preclude the fabrication in real time of hybrid protein–cell constructs, as well as conditions which likely induce significant conformational changes in native protein structure, including protein denaturation. Finally, wet spinning is largely confined to the generation of fibers that range from tens to hundreds of microns in diameter. In contrast, the process outlined herein provides a convenient, non-toxic, non-denaturing approach for the generation collagen-containing nanofibers and non-woven fabrics that have potential application in wound healing, tissue engineering, and as hemostatic agents.

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