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A Synthetic Polypeptide Electrospun Biomaterial

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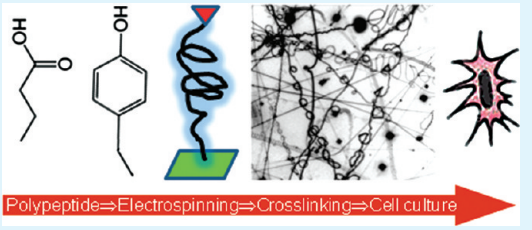
Supporting Information

ABSTRACT: Fiber mats of a synthetic anionic copolypeptide of L-glutamic acid and L-tyrosine (PLEY) have been produced by electrospinning, and physical, chemical, and biological properties of the fibers have been characterized in vitro. Fibers were obtained from polymer dissolved in water at concentrations of 20−60% (w/v) but not below this range. Applied voltage and spinneret-collector distance were also found to influence polymer spinnability. Oriented fibers were obtained by changing the geometry of the collector. Fiber diameter was measured by scanning electron microscopy (SEM). A common chemical reagent was used to cross-link polymers postspinning. Fiber solubility in aqueous solution varied as a function of cross-linking time. Cationic polypeptides labeled with a fluorescent dye became noncovalently associated with cross-linked fibers, enabling visualization by fluorescence microscopy. Spectroscopy provided information on polymer chain conformation in solution and in fibers. Degradation of cross-linked fibers by different proteases has been demonstrated. Fibroblasts were cultured on cross-linked fiber mats to test basic cytocompatibility. Synthetic polypeptide fiber mats may be useful in applications in medicine, biotechnology, and other areas.



KEYWORDS: biocompatibility, cross-linking, electrospinning, fiber scaffold, functionalization, polypeptide

INTRODUCTION

Considerable effort has gone into the development of biodegradable, biofunctional, and biocompatible nanostructured materials.[1−3] Electrospinning is a simple and versatile method of fabricating continuous nanometer-to-micrometer-diameter fibers from polymers in solution.[4−6] Nonwoven textile mats, oriented fibrous bundles, and three-dimensional scaffolds can be made by electrospinning. The structure, chemical and mechanical stability, functionality, and other properties of electrospun fibers can be tailored to specific applications. A variety of applications of these materials are envisioned.

In medicine and biotechnology, applications of electrospun nanofibers include tissue engineering scaffolds, implant coatings, wound dressings, dental coatings, enzyme immobilization and antimicrobial materials, chemical and biological protective clothing and biomimetic actuators and sensors.[4−14] It has been noted that the large surface area and high porosity of the fibers mimic key features of the extracellular matrix,[13,15] a biological structure that plays an important role in the attachment, migration, proliferation and other aspects of cell behavior in vivo.[16] Surface area and porosity are also important for the dissolution of entrapped solute particles and solvent evaporation; fiber mats could be useful in drug delivery.[7−10,17]

Many biopolymers, modified biopolymers, and blends of biopolymers and synthetic organic polymers have been electrospun.[5,13,17−19] Soluble or solubilized proteins are widely considered promising for fiber production.[4,5,13,20] To date, however, protein-based fiber production has relied on extraction of proteins from an animal or a plant source, solubilization of proteins in organic solvents, or addition of non-natural organic polymers to the protein solution feedstock—all potentially problematic for regulatory approval or consumer acceptability.

Earlier, we showed that the synthetic cationic peptide poly(L-ornithine) (PLO) was not only spinnable but spinnable from water, and we provided an introductory technical description of the fibers.[21] PLO spinnability was surprising because poly(L-lysine) (PLL), which is very closely related in structure, is apparently not spinnable under comparable conditions. Neither lysine nor ornithine has an aromatic group in its side chain. Here, we describe the electrospinning of the synthetic anionic polypeptide PLEY from water. Carboxylic acid groups and aromatic groups are present in the side chains. Data are provided on physical, chemical, and biological properties of the resulting fibers: the relationship of peptide concentration to spinnability and fiber diameter, fiber cross-linking and solubility, electrostatic properties of fibers, polymer structure in solution and in fibers, fiber degradation by proteases and cell-biocompatibility in vitro. Figure 1 illustrates polymer structure, electrospinning, and cross-linking.

MATERIALS AND METHODS

PLEY [(L-Glu, L-Tyr) 4:1 or poly(L-Glu_4-*co*-L-Tyr_1); E = Glu, Y = Tyr in single-letter code], 20−50 kDa by viscometry, was from Sigma-Aldrich (USA). Information on the choice of polymer is provided in the Discussion. Indium tin oxide-coated polyethylene terephthalate, 60 Ω/in^2 surface resistivity (ITO-PET), was from Sigma-Aldrich. This substrate material is particularly useful for fiber collection because it is both

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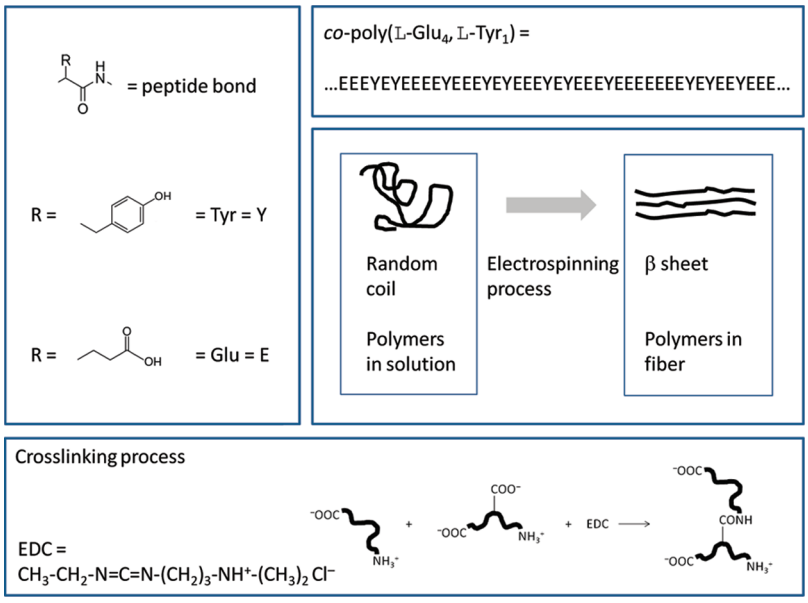

Figure 1. Illustration of polymer structure, electrospinning, and cross-linking.

conductive and semitransparent in the visible range. One mL plastic syringes were from Fisher (USA). Blunt-tip metal needles of inner diameter 0.6 mm were from Jensen Global (USA). A PS/FX20P15−11 Glassman High-Voltage Inc. (USA) power supply was used to create jets of polymer feedstock. The water used in all experiments had a resistivity of 18.2 MΩ cm.

Lyophilized PLEY was dissolved in deionized water at 60% (w/v), a concentration close to the solubility limit, and serially diluted with water. Fibers were spun from the polymer feedstock in a syringe; a blunt-tip needle served as the spinneret. The feedstock flow rate was otherwise determined by solution viscosity and gravity. A copper wire connected the cathode of the voltage source to the needle tip. The collector − ITO-PET unless indicated otherwise − was connected to the same ground as the anode of the power supply. The applied voltage and the spinneret-to-collector distance were varied from 7 to 20 kV and 5 to 15 cm, respectively. All fiber production was done at ambient temperature, pressure and humidity. Fibers were produced when conditions supported the formation of a Taylor cone and a jet of polymer solution. The solvent in the jet evaporated as it sped toward the collector, leaving mostly dehydrated fibers. Further dehydration was achieved by the gentle flow of air across the fibers on the collector. Oriented fibers were produced by connecting the power supply ground to a parallel plate collector, assembled from two 5 cm long copper electrodes separated by a distance of 2 cm. All other conditions were the same as for fibers spun onto a planar collector.

Preliminary visualization of fibers on planar collectors was done with a dissecting microscope and a Zeiss Axiovert 200 M inverted microscope (Germany). The latter was equipped with an incandescent source, a mercury vapor source, a filter set and a Roper Scientific MicroMAX System CCD camera (USA). Higher resolution images were obtained with a JEOL JSM-6390LV scanning electron microscope (SEM, Japan). The accelerating potential was 15−30 kV. Samples were metalized with a 10-nm layer of gold. Fiber diameter was quantified by a minimum of 20 measurements of fibers visualized by SEM.

PLEY fibers were chemically cross-linked by submersing samples on 4 cm × 4 cm ITO-PET substrates in 20 mL of 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Thermo Scientific, USA) in 90% ethanol/10% water at ambient temperature.[22] This water-soluble reagent, which is common in biochemistry and indeed in protein electrospinning, activates carboxyl groups for spontaneous reaction with primary amines. There are in every PLEY molecule one carboxyl group per glutamic acid side chain, one carboxylic acid group at the carboxyl terminus of the polymer chain and one amino group at the amino terminus. (see the reaction scheme in the Supporting Information.) The duration of the cross-linking reaction was 0−6 h. Cross-linked samples were rinsed extensively with water prior to further analysis or cell culture.

Cross-linked fibers were visualized by SEM as described above or by fluorescence microscopy following adsorption of dye-conjugated peptides. Fiber samples on 2 cm ×2 cm ITO-PET were fully immersed for 1 h in 2 mg/mL fluorescein isothiocyanate (FITC)-PLL (Sigma) in water or 5 mg/mL FITC (Sigma) in water and then rinsed with water. Samples and controls were then analyzed with a fluorescence microscope equipped with a fluorescein filter set.

PLEY structure was analyzed by circular dichroism spectroscopy (CD) and Fourier-transform infrared spectroscopy (FTIR). An Aviv 215 CD instrument (Aviv Biomedical, Inc., USA) was used to obtain far-UV dichroic spectra of PLEY dissolved in water. Fifteen scans were averaged for measurement in the 180−260 nm range at a rate of 1 nm s^{-1}, a step size of 1 nm, a path length of 0.1 cm and a bandwidth of 1 nm. Minor data averaging was done to obtain the final spectrum. A Jasco FT/IR 4100 spectrometer (Japan) outfitted with a Horizon^{TM} multiple-reflection attenuated total reflection (ATR) accessory with a ZnSe crystal (Harrick Scientific Products, Inc., USA) was used to analyze PLEY films and fibers. ZnSe transparency is approximately independent of wavelength in the range 1200−4000 cm^{-1}. Samples were analyzed in situ as polymer deposited directly from solution or as fiber mats on ITO-PET, before and after cross-linking. All spectra were acquired as 256 scan averages at a resolution of 4 cm^{-1}.

Enzymatic degradation of cross-linked fiber mats was tested with two protease species. Lyophilized Glu-C endoproteinase (Thermo Scientific) was reconstituted at a concentration of 0.2% (w/v) in 50 mM ammonium bicarbonate, pH 8.0, and successive 10-fold dilutions were prepared with the same buffer. Lyophilized protease XIV (Sigma) was reconstituted at 2% (w/v) in phosphate-buffer saline (PBS), and successive 10-fold dilutions were prepared with the same buffer. ITO-PET substrates covered with cross-linked fiber mats were divided into 6 equal areas, 2 cm ×2 cm each. Twenty μL of enzyme solution or buffer was then deposited on the corresponding sector of fiber mat and incubated at 37 °C for 0−5 h. The resulting samples were rinsed with deionized water, dried and analyzed by SEM as described above.

Cytocompatibility of cross-linked PLEY fibers has been tested with normal human dermal fibroblasts (NHDFs). These cells, which have a normal phenotype, play an important role in wound healing in vivo. Such considerations are relevant to possible biomedical applications of electrospun biomaterials. Cells were maintained at a subconfluent density in Fibroblast Basal Medium with Fibroblast Growth Medium-2 (Lonza, USA) and passaged every 72−96 h. ITO and electrospun fiber mats on ITO-PET were rinsed in 70% ethanol and then PBS prior to cell seeding. Cells cultured on tissue-culture polystyrene were rinsed in Hanks' balanced salt solution, released from the substrate by treatment with 0.25% trypsin in 2.21 mM EDTA (Mediatech, USA), centrifuged and resuspended in culture medium. Approximately 10,000 cells in 200 μL were seeded onto ITO-PET or fiber-coated ITO-PET and then incubated at 37 °C and 5% CO_2. The culture medium was changed every 48 h. Samples and controls were then analyzed by phase contrast microscopy with the Zeiss Axiovert 200 M instrument mentioned above.

RESULTS

PLEY was not spinnable at concentrations below 20% (w/v). Fibers produced in the 20−35% concentration range contained beads; the fibers were not smooth and continuous. At 50−60%, fibers were continuous, long and suitable for mat production. Less attractive, bead-containing fibers were obtained with 40−45% PLEY (see the Supporting Information). The spinneret-collector distance and the applied voltage were tested at 3 or more values in the 5−15 cm and 7−20 kV range for each polymer concentration; the electric field was $\sim 1 \times 10^3$ V m^{-1}. The most attractive fibers were obtained at 50−55% PLEY, 12 kV, and 10 cm. Table 1 shows the main electrospinning process variables considered in this study and the corresponding apparent optimal values for fiber mat production.

Figure 2 presents typical SEM images of fibers electrospun at 55% PLEY. Panel A shows a nonwoven fiber mat on a planar ITO collector at different magnifications; Panel B, aligned fibers obtained with the parallel-plate collector described above. In both cases, the fibers are smooth and bead-free. The average fiber diameter, determined by analysis of SEM images, was 670 nm-9.10 μm, depending on polymer concentration (Figure 3). In general, the variance in fiber diameter increased as the mean value increased. At 55%, the average diameter was 7 ± 1 μm.

PLEY fiber solubility has been tested at different pH values, above and below the pK_a of glutamic acid. This amino acid accounts for 4 in 5 of all residues of PLEY (see Materials and Methods). Side chain ionization was considered relevant because it strongly influences polymer solubility. In the absence of cross-linking, fibers were sensitive to water throughout the tested range, pH 2 (below the pK_a of Glu) to pH 12 (above the pK_a of Tyr), and at high humidity (Figure 4). Cross-linking was achieved by immersing fiber samples in EDC in ethanol/water at room temperature for defined periods of time. Resistance of fiber mats to dissolution in water was determined after cross-linking. Six hours of cross-linking was sufficient for essentially complete fiber mat insolubility. The results of the cross-linking and solubility tests are summarized in Table 2.

PLL-FITC has been used to visualize fiber mats by fluorescence microscopy. PLL-FITC (Figure 5A) but almost no free FITC (Figure 5B) became bound to PLEY molecules in fiber mats during incubation and remained bound following extensive

Table 1. Processing Parameters for Electrospinning of PLEY

quantity	range tested	apparent optimal value
polymer concentration (w/v %)	10−60	55
spinneret-collector distance (cm)	5−15	10
applied voltage (kV)	7−20	12

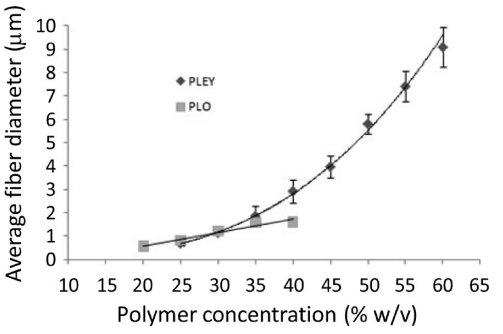

Figure 3. Fiber diameter as a function of polymer feedstock concentration. Diamonds, PLEY. Squares, PLO. Applied voltage and spinneret-collector distance were held constant. The values were 10 cm and 12 kV for PLEY and 10 cm and 10 kV for PLO. Each data point represents the average of 20 independent measurements. The error bars represent standard deviations. The PLO data are from ref 21.

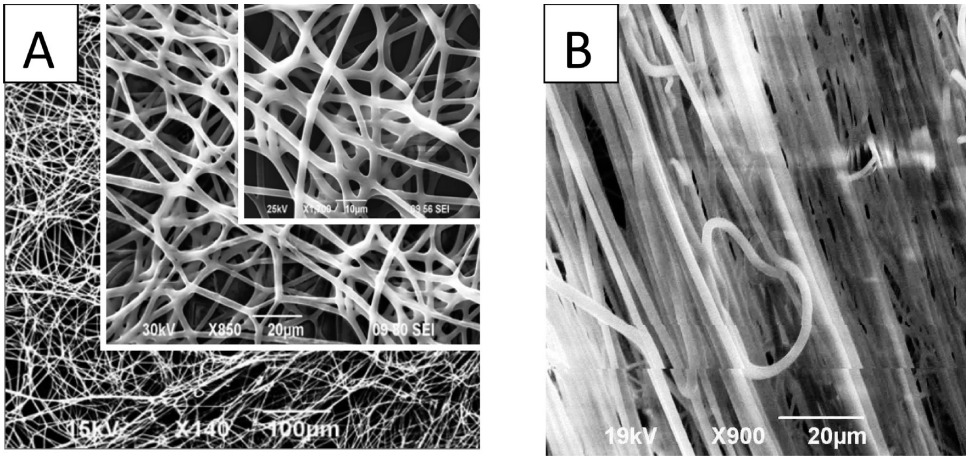

Figure 2. SEM micrographs of PLEY fibers electrospun at 55% (w/v). (A) Randomly oriented fibers at 140× and 15 kV (100 μm scale bar), 850× and 30 kV (large inset, 20 μm scale bar) and 1700× and 25 kV (small inset, 10 μm scale bar). (B) Aligned fibers at 900× and 19 kV (20 μm scale bar).

rinsing with water. The binding process resulted in essentially no change in fiber diameter. Any FITC that became bound to ITO-PET did so at an approximately uniform surface density (Figure 5C). Fibers on ITO could not be visualized by fluorescence microscopy in the absence of a dye (Figure 5D).

CD has been used to gain information on PLEY structure in solution. The data, presented in Supporting Information, show that the preferred backbone conformation in aqueous solution was a random coil. FTIR-ATR has been used to demonstrate fiber cross-linking and assess PLEY structure in fibers before and after cross-linking. The spectra of the polymer cast from solution and in fibers before cross-linking showed only relatively minor differences with regard to shape of the absorption envelope (Figure 6). Cross-linking and rinsing led to a sharp decrease in line broadening, especially in the amide I region (1600−1700 cm^{-1}). The amount of water bound to polymer molecules was nominally the same for all spectra.

The susceptibility of PLEY fibers to proteolysis has been tested. Fibers were incubated with reconstituted Glu-C protease or protease XIV for defined time periods. Degradation was evident in all fiber mats exposed to protease. Fragmentation of individual fibers increased with time (Figure 7). Fibers were almost completely degraded within 5 h by 0.2% (w/v) Glu-C or 2% (w/v) protease XIV. 20−25% of the fibers remained in 0.02% Glu-C and 0.2% protease XIV after the same amount of time; 40−45% of the fibers remained in 0.002% Glu-C and 0.02% protease XIV. The protease XIV SEM data, which closely resemble the Glu-C data, are provided in Supporting Information.

Biocompatibility testing of PLEY fibers has been initiated. NHDF cells were seeded onto cross-linked fibers or onto control surfaces for introductory assessment of adhesion, morphology and toxicity. Cells became well spread within 24 h and grew to confluence within 72 h on tissue culture polystyrene, ITO-PET and cross-linked PLEY fiber-coated ITO-PET (Figure 8). Randomly oriented fibers were spaced on the order of micrometers to tens of micrometers. Fiber diameter varied from hundreds of nanometers (dark fibers) to micrometers (bright fibers). Cells displayed apparently normal adhesion and morphology on fiber-coated ITO by light microscopy.

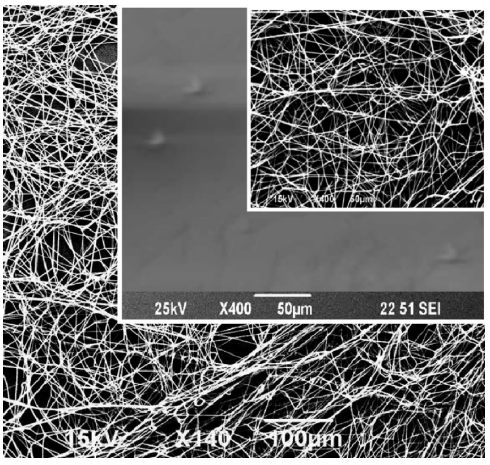

Figure 4. Solubility of fiber mats on ITO-PET before and after cross-linking. As-spun fiber mat; 15 kV, 140×; 100 μm scale bar. Large inset, fiber mat following immersion in water for 1 min and drying for 1 h; 25 kV, 400×; 50 μm scale bar. Small inset, fiber mat following cross-linking with EDC for 4 h, immersion in water for 2 days and drying for 3 h; 15 kV, 400×; 50 μm scale bar.

Table 2. Result of Fiber Mat Cross-Linking and Solubility Tests[a]

cross-linking time (h)	dissolution time (h)	result
0.25	0.25	~100%
0.5	0.5	<75% dissolution
1.0	1.0	<25% dissolution
6.0	12, 24, 48	<5% dissolution

[a] The duration of fiber mat exposure to crosslinking reagent and to water are given.

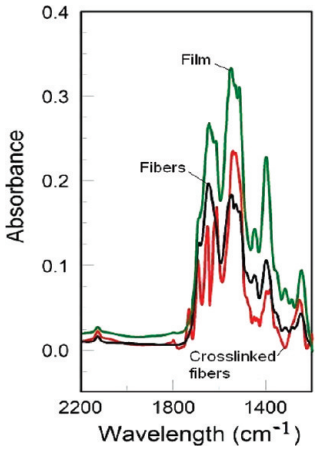

Figure 6. In situ FTIR spectra of an as-cast film and electrospun fibers of PLEY on ITO-PET before and after cross-linking. The spectra were obtained in ATR mode.

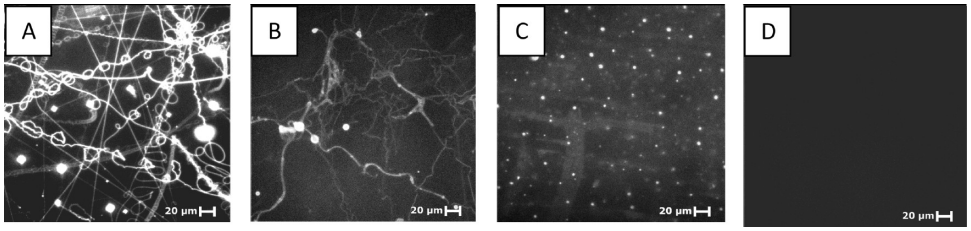

Figure 5. Fluorescence micrographs of cross-linked PLEY fibers on ITO-PET electrospun at 50% (w/v). The applied voltage was 10 kV, the spinneret-collector distance 9 cm. (A) Sample incubated with 2 mg/mL FITC-PLL for 1 h and rinsed with water. (B) Sample incubated with 5 mg/mL FITC for 1 h and rinsed with water. (C) Sample incubated with 5 mg/mL FITC for 1 h and rinsed with water. (D) Sample rinsed with deionized water. All micrographs were obtained with a 10× objective lens. The scale bar is 20 μm in each case.

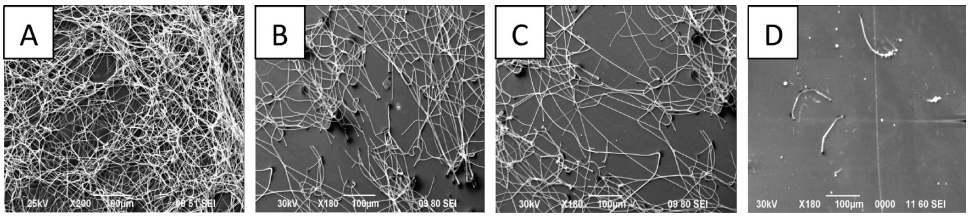

Figure 7. SEM micrographs of cross-linked electrospun 55% PLEY fibers following proteolytic digestion for 5 h at 37 °C. (A) 0, (B) 0.002, (C) 0.02, and (D) 0.2% (w/v) Glu-C protease in 50 mM ammonium bicarbonate buffer, pH 8. (A) 200×, all others, 180×. The accelerating potential was 25 kV or 30 kV. The scale bar is 100 μm in each case. See also the Supporting Information.

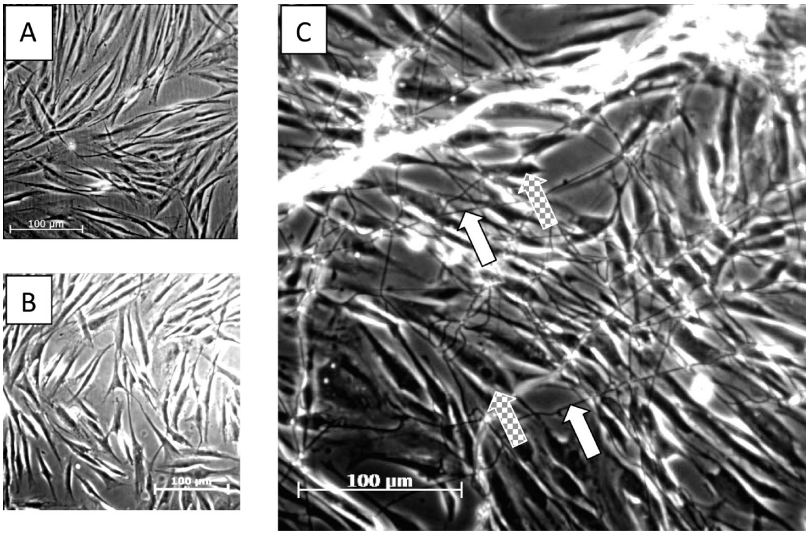

Figure 8. Phase contrast light micrographs of NHDFs culture in vitro. The substrate was (A) tissue culture polystyrene, (B) ITO-PET, or (C) EDC-cross-linked electrospun 55% PLEY on ITO-PET. A 10× objective was used. White arrows highlight specific fibers, shaded arrows specific cells. The scale bar is 100 μm in each case.

DISCUSSION

Every protein in nature comprises at least one polypeptide chain. What we mean by polypeptide in this paper is a certain kind of polymer of human design, whether the polymer is made by a chemical method or a biological approach. The purpose of this ad hoc definition is to achieve clarity of meaning. Dozens of electrospinning studies on proteins have appeared in the scientific literature;[13] there are apparently only five published electrospinning studies on polypeptides to date. A brief summary of the peptide papers follows. Minato et al. have investigated poly(γ-benzyl-L-glutamate), a synthetic homopolymer of a non-natural amino acid that resembles glutamic acid, one of the 20 usual amino acids.[23] This polypeptide, which is insoluble in water, was spinnable from dichloromethane and trifluoroacetic acid. We have found that fibers of PLO but not PLL could be electrospun from water.[21] This was surprising because the ornithine and lysine side chains differ by a single methylene group. Ornithine, a natural but unusual amino acid, plays a role in the urea cycle and the biosynthesis of arginine, another of the 20 usual amino acids.[24] Ner et al. have found that a synthetic 84 kDa elastin-like peptide can be electrospun from water.[25] The structure of this polymer was $(S_2E_3E_kE_4S_2)_{13}$, where S = GAGAGS, E = GVGVP, and E_k = GVGKP (G = glycine, A = alanine, S = serine, V = valine, P = proline, and K = lysine all used amino acids). Huang et al. have found that a similarly large but more practical elastin-like peptide was spinnable from water.[26] Having a mass of 81 kDa, $[(VPGVG)_4VPGKG]_{39}$ was produced by a recombinant DNA method in bacteria. Nivison-Smith et al. (2010) have recently electrospun synthetic human tropoelastin fibers dissolved in hexafluoroisopropanol.[27] The fibers were then cross-linked with di-isohexanecyanate or glutaraldehyde and rinsed extensively with graded dilutions of isopropanol in water or phosphate-buffered saline, respectively. To date, none of the published polypeptide electrospinning studies except the last one has included biological characterization of electrospun fibers.

In the present work, electrospinning was used to produce fibers of synthetic PLEY. The selection of this polymer may seem arbitrary. It was not. All chiral amino acids in proteins, that is, all amino acids other than glycine, are levororotary — in all known organisms. PLEY consisted of L-amino acids only. The mere demonstration of PLEY electrospinning may seem insignificant. It is not. To date, few polypeptides of any sequence have been proved spinnable — under any conditions, let alone from water. Only a handful of different proteins have thus far been spinnable — collagen, silkworm silk, a few others — and usually only after solubilization in a toxic organic solvent.[13] PLEY, by contrast, is spinnable from water. Aromatic side chains play an important role in nucleating protein folding and forming the hydrophobic core of the native state of globular proteins.[28] No other spinnable polypeptide (on the definition employed here)

has side chains with aromatic rings. The exception is poly(γ-benzyl-L-glutamate), which is insoluble in water. PLEY contains tyrosine, the side chain of which has an aromatic ring, and it is soluble in and spinnable from water.

The spinnability of PLEY, though significant on its own, should nevertheless be considered in a broader context. PLO was spinnable at a concentration of 20−40% (see Figure 3). The PLEY molecules of the present work had an approximate average mass of 35 kDa, and continuous fibers were obtained at a concentration of 55% (w/v). The volume occupied per polymer molecule at this concentration is about 105 nm^3, or a cube with sides of about 4.7 nm. The average contour length of the PLEY molecules was about 18-fold greater. Hemoglobin, by contrast, with a molecular mass of 68 kDa and a partial molar volume of 0.74, occupies a volume of about 85 nm^3 in the folded, organic crystal-like native state.[29] The density of amino acid residues in 55% (w/v) PLEY is then about 2.4-fold smaller than in native hemoglobin. That is, PLEY chains were solvated at 55% (w/v), but the polymer concentration was still high enough for continuous formation of bead-free fibers. Interchain hydrophobic interactions may have played a significant role in fiber formation. In PLO fibers, by contrast, interchain hydrophobic interactions are improbable: the side chain consists of three methylene groups and, at the distal end, an amino group. Ionizable and having a nominal pK_a of 10.75, the amino group will be charged with high probability at neutral pH.[30] PLO fibers are therefore more likely to be stabilized by ionic interactions involving counterions than are PLEY fibers.

Continuous spinning of PLEY resulted in porous scaffolds (Figure 2A). It has been suggested that geometrical features of such structures, which resemble some features of the ECM, could provide advantages for the attachment, proliferation, maturation and activation of cells in vitro relative to solid films cast from the same polymer in solution.[15] Further advantages could potentially be realized by spinning fibers from ECM proteins or synthetic polypeptides. PLEY scaffolds may therefore be useful for achieving biomedical aims. It was also possible to produce aligned fibers of PLEY (Figure 2B). In specific tissue engineering applications, such as controlling the guidance and alignment of nerve cells, tendons, and ligaments, a common approach has been to prepare aligned rather than randomly oriented fibers. Aligned fibrous scaffolds can provide topographic guidance to cells.[31]

Fibers spun from 20 to 35% (w/v) PLEY contained beads, similar to fibers in a report by Reneker et al. (1999). This may be due to an insufficient concentration of polymer for uniform chain entanglement during solvent evaporation.[32] The optimal conditions for spinning were 55% polymer, 12 kV applied voltage, and 10 cm spinneret-to-collector distance (Table 1). Fiber diameter varied with PLEY concentration (Figure 3), as with other synthetic and natural polymers.[33,34] The trend was nonlinear, as with other polymers.[35] For PLO, by contrast, fiber diameter varied approximately linearly with polymer concentration, at least in the spinnable range.[21] The ability to control fiber structure and diameter could be useful in the design and fabrication of fibers for different applications.

PLEY fibers readily dissolved in water in the absence of cross-linking, reflecting the solubility of the polymer prior to spinning. For biomedical applications, which will generally require immersion in an aqueous medium, the fibers must be cross-linked. Here, EDC was employed for the purpose, as in protein electrospinning studies.[22] An advantage of EDC, a so-called zero-length cross-linker, is that it does not become incorporated into the macromolecule (see reaction scheme in the Supporting Information). This decreases the potential that cross-linking will lead to cytotoxic effects.[36] Fiber dissolution during the cross-linking step was limited by applying EDC to fibers in ethanol/water (see Materials and Methods). Excess EDC was removed by rinsing after cross-linking. Any residual EDC had no obvious effect on cell attachment or proliferation in culture (Figure 7C).

Biodegradable materials are becoming increasingly attractive for surgery: with increasing practicality, they can diminish the need for subsequent removal of an implanted device.[1,3,37,38] Biodegradable products will decompose naturally. The experiments described here have shown that Glu-C protease and protease XIV digested PLEY fibers over time. These proteases, from *Staph. aureus* and *Strep. greiseus*, respectively, were selected for study because they are known to recognize glutamic acid. Specifically, Glu-C, a serine protease, and protease XIV, a mixture of at least three proteolytic activities, cleave peptide bonds at the C-terminus of glutamic acid residues. The susceptibility of a polypeptide fiber mat to proteolysis will presumably be a tunable function of polymer structure and cross-linking.

Charge properties of the fibers were further revealed by a simple binding experiment. PLL-FITC (Figure 5A) but not FITC (Figure 5B) became appreciably associated with cross-linked PLEY fiber mats and remained bound after extensive rinsing with deionized water. This provides indirect evidence that the EDC cross-linking process did not result in the consumption of all available carboxyl groups in the fibers. In fact, the ester intermediate is unstable, so that if it does not react with an amino group to form an amide bond, it will be hydrolyzed, yielding the original side-chain carboxyl group as a product. Lysine side chains in PLL will have bound glutamate side chains in the fibers, as in electrostatic layer-by-layer assembly;[39] hydrophobic interactions between side chains too may have contributed to the binding free energy. The resulting increase in fiber diameter was in any case too small to be visualized at the resolution of the micrographs in Figure 4; under the conditions of the experiment, polyelectrolyte layer-by-layer assembly is a self-liming process, resulting in a layer of PLL-FITC with a thickness on the order of nanometers.[40] To the extent that PLL-FITC models an active small molecule conjugated to a carrier peptide, the approach outlined here provides a thermostable means of "functionalizing" an ECM-like scaffold made of PLEY. The physical adsorption of molecules onto insoluble but degradable fibers could be useful for biomedical applications of polypeptide fibers, for example, delivery of biologics to targeted organs and or suppression of tumors.[41]

FTIR is widely used to obtain information on the structural properties of polymers, including peptides and proteins. The amide I, amide II and amide III bands are key ones in peptide structure analysis.[42] The amide I band (1600−1700 cm^{-1}) is mainly due to C═O stretching and directly related to polymer backbone conformation. Amide II resonances (1500−1600 cm^{-1}), which are mostly attributable to N−H bending and C−N stretching, are not especially sensitive to peptide backbone conformation. Amide III (1200−1450 cm^{-1}) resonances depend on side chain structure and hydrogen bonding. These bands can therefore shift with changes in backbone conformation.

The most significant resonances displayed by the cast peptide film in the 1200−1700 cm^{-1} range were at 1240, 1395, 1512−1550, 1614, 1641, and 1687 cm^{-1} (Figure 6). These resonances are tentatively assigned to β sheet, COO^- symmetric stretch, β sheet and NH_3^+, β sheet, random coil, and β turn, respectively. The peak right at 1512 cm^{-1} appears to be due to the ring−OH vibration in the tyrosine side chain. An absorption

envelope encompassing perhaps several resonances stretches across the entire amide I range, complicating analysis of secondary structure. Essentially the same resonances are seen in the fiber spectrum before cross-linking, though the band at 1614 cm^{-1}, which is probably due to β sheet, seems significantly less prominent.

Cross-linking fibers increased the sharpness of many absorption bands and, in several cases, resulted in a shift of resonant frequency. If the fibrous material is rinsed well, the EDC cross-linking reagents do not contribute to the FTIR spectrum; the only new bond is an amide formed between the distal ends of carboxylic acid side chains and N-terminal amino groups (see the Supporting Information). The most prominent bands were at 1251, 1375, 1540, 1611, 1650, 1697, and 1729 cm^{-1}. The respective tentative assignments are to β sheet, CH_2 in cross-linked glutamic acid side chains, β sheet and NH_3^+, β sheet, random coil, β turn and COOH. It is unclear why the last resonance appears in the cross-linked fiber data but not the other spectra; this resonance may be due to the reaction of phenol, which is present in the tyrosine side chain, with carbodiimide. The decreased line broadening is presumably a reflection of increased polymer rigidity, which will tend to limit the accessible conformations of polymers and the range of vibrational frequencies at which absorption can occur. In summary, the FTIR spectra provide indirect but nonetheless clear evidence of polymer cross-linking, and they suggest that some fraction of polymers adopted a β-sheet conformation in the cross-linked fibers.

Various investigators have studied the incorporation of protein-based electrospun fibers into different biomaterials.[5,11,43] Proteins are in general biodegradable, biocompatible and environmentally benign. To date, though, most protein electrospinning has required either organic solvent for polymer solubilization or non-natural polymers for electrospinning or fiber stability.[13] Such materials may be toxic or undesirable for reasons of cost, manufacture logistics, quality assurance, product regulation, or patient acceptability. Some nonbiological polymers are known to cause severe immune responses.[44] Others, including polymers approved for various purposes by the U.S. Food and Drug Administration, are nonimmunogenic but yield breakdown products that influence the pH of the surrounding physiological environment, possibly resulting in tissue necrosis. Most proteins of interest in electrospinning have been obtained from an animal source. There is increasing resistance from regulatory agencies, however, to approve medical products containing animal source material. Some problematic aspects of biomaterials development may be overcome by the use of synthetic polypeptides of defined composition that are soluble in water.

A first step toward showing that a peptide-based material will be suitable for some types of biomaterials applications is cytocompatibility validation. Here, NHDFs displayed apparently normal adhesion, morphology and proliferation on PLEY fiber mats (Figure 8C). These fibers or similar ones may therefore be useful in various applications in biotechnology or medicine. For example, reduction of xenogenic reactions, achieved by use a synthetic polypeptide, could be critical to the success of reintroducing cells or regrafting tissues in the body. No animal product, organic solvents or nonbiological synthetic organic polymers were used to fabricate PLEY fibers of this study.

CONCLUSION

The data presented here show that fibers of the synthetic copolypeptide PLEY can be prepared by electrospinning from aqueous solution under certain conditions. Important, neither organic solvents nor nonbiological organic polymers were needed for polymer solubilization or spinning. Fiber diameter varied with polymer feedstock concentration. Fibers became water-insoluble after cross-linking. PLEY was largely unstructured in solution but appears to have adopted a combination of β sheet and random coil in fibers. Charge properties enabled rapid and stable functionalization of cross-linked fibers in an aqueous medium by physical association with oppositely charged peptides. Proteases degraded fibers over time. Cross-linked fibers were compatible with cell attachment in vitro. The results seem promising for applications of fiber mats in tissue engineering, drug delivery, surface modification of medical implant devices and wound dressings.

ASSOCIATED CONTENT

Supporting Information. A reaction scheme for the cross-linking process; a far-UV circular dichroism spectrum of PLEY; a fluorescence micrograph of bead-containing fibers; scanning electron micrographs of protease XIV digestion of fibers; FTIR spectra of films, fibers, and cross-linked fibers in the range 1200−4000 cm^{-1} (PDF). This material is available free of charge via the Internet at http://pubs.acs.org/.

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