LETTER

Insoluble Synthetic Polypeptide Mats from Aqueous Solution by Electrospinning

Dhan B. Khadka[†] and Donald T. Haynie[*,†,‡]

Center for Integrated Functional Materials and Department of Physics, University of South Florida, 4202 East Fowler Avenue, Tampa, Florida 33620, United States

ABSTRACT Water-insoluble nanofiber mats of synthetic polypeptides of defined composition have been prepared by a process involving electrospinning from aqueous solution. L-ornithine is a physiological amino acid. Fibers of poly(L-ornithine) (PLO) were produced at feedstock concentrations above 20% w/v. Applied voltage and needle-to-collector distance were crucial for nanofiber formation. Attractive fibers were obtained at 35−40% w/v. Fiber diameter and mat morphology have been characterized by electron microscopy. Polymer cross-linking with glutaraldehyde (GTA) vapor rendered fiber mats water-insoluble. The study has yielded two advances on previous work in the area: avoidance of an animal source of peptides and avoidance of inorganic solvent.

KEYWORDS: aqueous solution • biomaterials • cross-linking • electrospinning • fiber mat • nanofiber • polypeptide • protein

INTRODUCTION

Electrospinning is a versatile means of fabricating continuous, ultrafine, indefinitely long fibers of nanometer diameter from polymers in solution (1−3). The structure, mechanical stability, chemical or biochemical functionality, and other properties of the fibers can be controlled (4, 5). Nonwoven textile mats, oriented fibrous bundles, and three-dimensional structured scaffolds with a large surface area and high porosity can be formed (6−8). Electrospun nanofibers are being studied for a variety of human purposes in different areas of science and technology.

In medicine and biotechnology, current or envisioned applications of insoluble or slowly degrading electrospun fibers include scaffolds for cell and tissue culture, drug delivery depots, medical implant coatings, wound dressings, dental applications, antimicrobial delivery vehicles, protective coatings for clothing, and biomimetic actuators and sensors (9−22). (For reviews with a biomedical focus, see refs 12, 16, 17, and 20−22.) Many biopolymers, modified biopolymers, and blends of biopolymers with synthetic polymers have been used for electrospinning (23). Soluble or solubilized proteins are widely considered promising for nanofiber production. To date, however, protein-based fiber production has involved organic solvents, animal source materials, or nonbiological polymer blends—all problematic for product development and medical regulatory approval processes.

A variety of proteins have been used to develop applications of electrospun fibers in drug-delivery and nanomedicine (24). For example, Huang et al. found in 2001 that collagen could be electrospun from solution in the presence of poly(ethylene oxide) (25, 26). Wnek et al. then electrospun human and bovine plasma fibrinogen from 9:1 hexafluoroisopropanol:modified Eagle's medium and minimum essential medium (Earle's salt) (27). Xie and Hsieh electrospun a mixture of casein and poly(ethylene oxide) (28). Bowlin and co-workers then showed electrospinning of collagen dissolved in hexafluoroisopropanol (29, 30), and Ramakrishna and co-workers electrospun gelatin, a complex mixture of proteins and other biological macromolecules, in 2,2,2-trifluoroethanol, producing bead-free fibers (31, 32). In 2005, gelatin was electrospun from 49:1 formamide:water (33). Chen et al. electrospun a composite fibrous mat of chitosan/collagen dissolved in hexafluoroisopropanol/trifluoroacetic acid in 2007 (34). Dror et al. (35) then electrospun bovine serum albumin from toluene. In all these cases, the proteins were from an animal source.

Other sources of peptides are important for key reasons. Polypeptides can be produced at the industrial scale by well-established chemical synthetic methods and procedures involving microorganisms. A practically unlimited number of different polypeptide sequences can be made by these methods, not only ones occurring in nature, even if just the 20 usual amino acids are considered. The biochemical functionality or general utility of polypeptides thus produced can be controlled to a remarkable extent, especially if no complex polymer folding process is required. Peptide-based materials can be cross-linked in different ways, including disulfide bond formation, enabling a further degree of control over aspects of mechanical properties. Ideally, it would be possible to electrospin peptides of any desired amino acid sequence. Nevertheless, to the best of our knowledge, there is no prior report on electrospinning fibers of synthetic polypeptides of defined composition, much less from water in the absence of nonbiological organic polymers and organic cosolvents.

* Corresponding author. E-mail: dhaynie@cas.usf.edu. Fax: (813) 974.5813. Tel.: (813) 974-7793.

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† Department of Physics, University of South Florida.

‡ Center for Integrated Functional Materials, University of South Florida.

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We have tested whether synthetic peptides of defined composition could be used to electrospin fibers, whether water could be used as the solvent in the absence of organic solvents and nonbiological polyelectrolytes, and whether the resulting fibers were water-insoluble or could readily be made insoluble. We were motivated by the knowledge that the avoidance of animal source materials, organic solvents and nonbiological polymers would be advantageous for the development of applications of peptide-based nanofibers in biomedicine and biotechnology. Here, we present initial results on poly(L-ornithine), a polymer of amino acids relevant to the urea cycle and to biotechnology.

MATERIALS AND METHODS

Lyophilized PLO hydrobromide (152 kDa by viscometric analysis), GTA (25% w/v in water) and indium tin oxide (ITO)-coated poly(ethyleneterephthalate) (60 Ω/in^2) were from Sigma-Aldrich (USA). Syringe needles for electrospinning were from Jensen Global (USA). A Glassman (USA) PS/FX20P15.0−11 20 kV power supply was used to generate electrical potential.

PLO was dissolved in deionized (DI) water in the range 10−50% w/v. Peptide solution was taken up into a 1 mL syringe outfitted with a blunt-tip metal needle capillary of inner diameter 0.6 mm. A copper wire connected the needle tip to the positive pole of the power supply; the ITO-coated collector was grounded. All peptide solution samples were studied at ambient temperature, pressure and humidity. Preliminary visualization of the nanofibers was done with a Zeiss Axiovert 200 inverted microscope (Germany) equipped with a Roper Scientific MicroMAX System CCD camera (USA). More detailed nanofiber images were obtained with a JEOL JSM-6390LV scanning electron microscope (Japan).

PLO nanofibers on an ITO substrate were cross-linked in situ. Samples were inverted and affixed to the top of a 25% GTA vapor-filled chamber consisting of 3 mL of GTA in a 40 mm diameter uncapped Petri dish in an 80 mm-diameter Petri dish. The larger dish was covered, sealed with parafilm, and maintained for a defined time interval at ambient temperature. GTA was selected for the study because it is readily available, inexpensive, and known to be an effective cross-linker of proteins in other contexts, notably, cell biology; formulation development could involve a different cross-linking method.

RESULTS

Experiments showed that PLO nanofibers could be spun from an aqueous solution. For PLO as prepared in this work, however, fibers were obtained only when the polymer concentration was at least 20% w/v for an applied voltage of 5−20 kV and a nozzle-to-electrically grounded collector distance of 5−15 cm; the electric field was $\sim 1 \times 10^5$ V m^{-1}. The optimal values for PLO fiber production suggested by the present work are 9 kV and 10 cm.

Fibers produced at 20, 25, or 30% w/v PLO contained beads, perhaps because of limited polymer entanglement. At 35% and 40%, by contrast, the fibers were long, continuous, essentially bead-free, and suitable for mat production

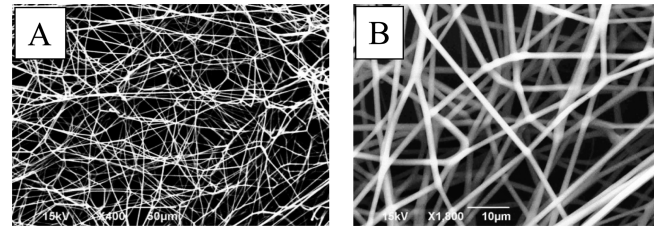

FIGURE 1. Electrospun mats of fibrous polypeptides. Visualization was by scanning electron microscopy: (A) 200× magnification, scale bar = 50 μm; (B) 1200× magnification, scale bar, 10 μm. The feedstock was 40% w/v PLO in water.

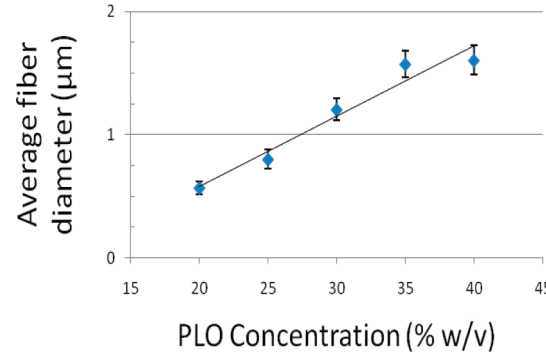

FIGURE 2. Variation in peptide fiber diameter. All electrospinning process variables besides PLO concentration were held constant. Average fiber diameter increases approximately linearly with PLO concentration. Error bars represent standard deviation of 10 measurements.

(see Figure 1). Less promising fibers were obtained at 45%, and none at 50%. Solution viscosity increased as PLO concentration increased. The influence of polymer concentration, viscosity, and other electrospinning parameters on fiber production broadly resembles results obtained with other synthetic and natural polymers (27, 36).

The underlying causes of the dependence of fiber formation on PLO concentration are not entirely clear at the present stage of research. As in other cases, however, spinnability it is likely to reflect the complex interplay of chain entanglement, solution viscosity and other process variables that vary with polymer concentration. Determination of the rate of water evaporation during electrospinning and the amount of water present in the fiber mats was beyond the scope of this initial study.

Fiber diameter varied approximately linearly with concentration when the needle gauge and applied voltage were held constant (Figure 2), consistent with the results of others (27, 36). The ability to control fiber diameter will allow for flexibility in the design and fabrication of nanofibers for different applications.

The solubility of PLO fibers was tested. Fibers as spun dissolved readily in aqueous solution at any pH and were sensitive to high humidity. It was therefore attempted to cross-link the fibers with the vapor of GTA solution. A single GTA molecule can cross-link two polypeptides by reacting with a free amino group on each of the polymer chains (37, 38).

The GTA cross-linking procedure, which was used here to establish proof of principle, resulted in slight shrinkage

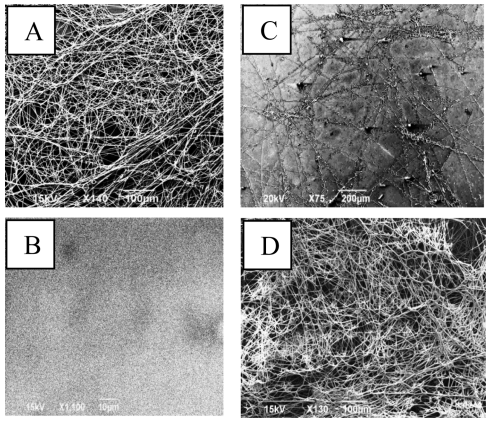

FIGURE 3. Insolubility of fibrous peptide mats upon cross-linking in situ on the collector. Feedstock was 40% w/v PTO in water. Analysis was by scanning electron microscopy. (A) Unmodified control. Scale bar, 100 μm. (B) No cross-linking, 1 min immersion in DI water, 1 h drying. Scale bar, 10 μm. (C) 15 min GTA vapor cross-linking, 1 h immersion in DI water, 1 h drying. Scale bar, 200 μm. (D) 6 h GTA vapor cross-linking, 48 h immersion in DI water, 1 h drying. Scale bar, 100 μm.

and discoloration of the fiber membrane (39). Extent of cross-linking was assayed qualitatively by immersion of a fibrous mat in aqueous solution at different pH values for different time periods (Figure 3). A cross-linking time of 6 h or more gave mats that were essentially insoluble; 1 h of cross-linking gave limited solubility. The data suggest that control over the cross-linking process will potentially be useful controlling mechanical, chemical, and biological properties of fiber mats.

DISCUSSION

In this work, we were more concerned with polymer provenance and structure and proof of specific principles than physiological function, exhaustive characterization or formulation development per se. The data show that nanofibers of PLO, a synthetic polypeptide of defined composition, can be prepared by electrospinning from aqueous solution. To the best of our knowledge, there is no other report in the scientific literature on electrospun nanofibers made of synthetic polypeptides, much less polypeptide nanofibers spun from aqueous solution. Moreover, the data show that neither organic solvents nor nonbiological organic polymers were required to achieve the outcome. Furthermore, it was found that water-insoluble PLO nanofibers could be prepared by at least one simple chemical cross-linking procedure. Taken together, the data support the view that PLO can serve as an exemplar of synthetic peptide spinnability and insoluble peptide fiber mat production. The significance of the results is now discussed with regard to polymers, solvents, and nanofibers.

Electrospun nanofibers are being studied for a variety of human purposes in different areas of science and technology (1−8). In medicine and biotechnology, for instance, envisaged applications of these nanofibers range from scaffolds for cell and tissue culture to drug delivery depots to medical implant coatings and beyond (9−22). The value of materials made of the fibers will probably depend on the degree of control one can exercise over the rate of degradation under conditions of interest. To date, most nanofiber-based materials for biomedical applications have been made of nonbiological polymers (21, 22).

Proteins are considered advantageous for the development of electrospun biomaterials (24−35). Biodegradable, absorbable, and environmentally benign, proteins encode potentially useful biochemical information in a completely natural way; the molecules can be purified relatively inexpensively, at least in some cases; and collectively proteins display a remarkable range of functional properties under mild solution conditions. Certain proteins exhibit extraordinary mechanical properties, for example, wool, spider silk and silkworm silk (39−41). Sequence motifs in other proteins play an indispensible role in specific molecular recognition, for instance, the RGD sequence of fibronectin in integrin-based cell adhesion (42). For these reasons and others, proteins have been objects of considerable interest for nanofiber production, especially for applications in biotechnology and medicine.

The development of protein-based nanofibers for use in vivo has unfortunately been limited in several key ways. These include the use of organic solvents or non-natural organic polymers to achieve spinnability, and the need to extract desired proteins from an animal source (24−35). It is apparently necessary to denature proteins to achieve appropriate chain entanglement or solution viscosity for fibril formation by electrospinning, and protein denaturation in the absence of aggregation often requires a strong chemical denaturant. Many organic solvents are toxic in small amounts, non-natural organic polymers may be toxic or undesirable for medical applications for other reasons, and proteins purified from an animal source may contain transmissible pathogens. These conditions may present significant hurdles for product function, quality assurance and product regulation (43). Some nonbiological polymers cause a severe immune response or are poorly absorbed (e.g., ref 44). For such reasons, there has been increasing interest in synthetic polypeptides for some years. Ideally, at least for many applications, it will be possible to electrospin nanofibers made of synthetic functional peptides from aqueous solution containing no organic solvent and no nonbiological synthetic organic polymers. Polypeptide cross-linking could be achieved by disulfide bond formation, as in many secreted proteins, hair, and other peptide-based biomaterials (45).

What makes a peptide structure appropriate for electrospinning under the ideal conditions discussed in this work? The structure of the ornithine side chain is shown in Figure 4A. The three methylene groups are hydrophobic in nature. The δ-amino group titrates above pH 10. It is hardly obvious from this information and the data presented above, however, which other peptide sequences will be spinnable from a completely aqueous feedstock in the absence of nonbiological polymers. For although ornithine closely resembles lysine (Figure 4B), which has just one

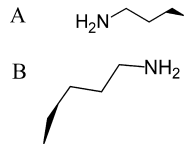

FIGURE 4. Side chain structure: (A) ornithine, (B) lysine. Ornithine has one fewer methylene groups. The amino group is positively charged at neutral pH. The carbon atom on the opposite end of the side chain is the α-carbon in each case.

additional methylene group in the side chain and a ε-amino group, and the linear charge density of both polymers is ca. +1 per residue at neutral pH (46), we have not yet succeeded in finding conditions that support fibril formation from poly(L-lysine) dissolved in water. The balance of charge and hydrophobic surface per unit length, in combination with solvent polarity, counterion concentration, charge screening, degree of polymerization, polymer concentration, nozzle diameter, and electric field strength, evidently combine in a way that supports fiber spinning more readily with PLO than poly(L-lysine). It may eventually be possible to prepare fibers from poly(L-lysine) under some as yet unknown conditions. In any case, it would be unduly speculative, we believe, to say at this point what determines the spinnability of PLO in aqueous solution. The present result can nevertheless be assumed to suggest that polypeptides having an amino acid composition other than 100% L-ornithine will show similar behavior.

As to ornithine itself, related reports from the scientific literature are, we believe, worth mentioning here. Thanos et al., for instance, have described how the biochemical stability of alginate-PLO microcapsules depends on the site of transplantation (47). Yamamoto and Hirata have used organic cross-linking agents to study the hydrogel-like properties of cross-linked PLO (48). The physical model of axonal elongation described by O'Toole et al. involves surfaces coated with PLO and laminin, an extracellular matrix protein (49). Finally, the amino acid L-ornithine is a key component of the urea cycle, the main role of which is biosynthesis of L-arginine, which is one of the 20 usual amino acids (50). These facts may suggest possible uses of nanofiber mats made of electrospun PLO.

CONCLUSIONS

The present results show that electrospun fibers can be made from at least one synthetic polypeptide of defined composition dissolved in an aqueous solution containing no organic solvent or nonbiological organic polymer. The ability to control the solubility of the resulting peptide nanofibers by a simple chemical cross-linking method has also been demonstrated. Current widespread interest in utilizing solubilized proteins in electrospinning suggests that the ability to electrospin synthetic polypeptides of defined composition could be important for the development of applications of electrospun materials, perhaps most in medicine and biotechnology

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