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Engineering hybrid polymer-protein super-aligned nanofibers via rotary jet spinning☆

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ABSTRACT

Cellular microenvironments are important in coaxing cells to behave collectively as functional, structured tissues. Important cues in this microenvironment are the chemical, mechanical and spatial arrangement of the supporting matrix in the extracellular space. In engineered tissues, synthetic scaffolding provides many of these microenvironmental cues. Key requirements are that synthetic scaffolds should recapitulate the native three-dimensional (3D) hierarchical fibrillar structure, possess biomimetic surface properties and demonstrate mechanical integrity, and in some tissues, anisotropy. Electrospinning is a popular technique used to fabricate anisotropic nanofiber scaffolds. However, it suffers from relatively low production rates and poor control of fiber alignment without substantial modifications to the fiber collector mechanism. Additionally, many biomaterials are not amenable for fabrication via high-voltage electrospinning methods. Hence, we reasoned that we could utilize rotary jet spinning (RJS) to fabricate highly aligned hybrid protein-polymer with tunable chemical and physical properties. In this study, we engineered highly aligned nanofiber constructs with robust fiber alignment from blends of the proteins collagen and gelatin, and the polymer poly-ε-caprolactone via RJS and electrospinning. RJS-spun fibers retain greater protein content on the surface and are also fabricated at a higher production rate compared to those fabricated via electrospinning. We measured increased fiber diameter and viscosity, and decreasing fiber alignment as protein content increased in RJS hybrid fibers. RJS nanofiber constructs also demonstrate highly anisotropic mechanical properties mimicking several biological tissue types. We demonstrate the bio-functionality of RJS scaffold fibers by testing their ability to support cell growth and maturation with a variety of cell types. Our highly anisotropic RJS fibers are therefore able to support cellular alignment, maturation and self-organization. The hybrid nanofiber constructs fabricated by RJS therefore have the potential to be used as scaffold material for a wide variety of biological tissues and organs, as an alternative to electrospinning.

1. Introduction

Therapeutic tissue engineering aims to produce synthetic tissues and organs to replace diseased and dying tissues and organs in the body [1]. An important component of engineered tissues is the scaffold that provides structural support for *ex vivo* or *in vivo* cell adhesion and tissue regeneration [2,3]. In addition to long-term biocompatibility, the ideal scaffold should effectively mimic the hierarchical three-dimensional architecture of native tissues [4].

Building a fiber-based scaffold from proteins will provide for a native, biomimetic environment that promotes cell attachment, maturation, differentiation and proliferation [4]; however, protein materials are not always chemically or mechanically robust enough

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for manufacturing and use [5–7]. Chemical crosslinking can stabilize protein structure and prevent hydrolysis under physiological conditions, but often promotes inflammation, calcification and tissue rejection [8,9]. Recent work has focused on utilizing protein-polymer hybrid composites as scaffold materials with tunable properties, the polymer component imparting mechanical strength, forming the structural backbone of the scaffold [6,10]. These reports on protein-polymer hybrids utilized electrospinning, which despite its versatility, has poor control over fiber orientation and relatively low production rates using high voltages [11–14]. Additionally, some materials are not easily fabricated into nanofibrous structures using conventional electrospinning methods due to high curing temperature [15] and low solution viscosity [16]. Thus, we need an alternative method for reproducible fabrication of hybrid nanofiber scaffolds that is able to overcome these limitations of electrospinning.

We hypothesized that we could synthesize highly anisotropic protein-polymer hybrid nanofibers by modifying the rotary jet spinning (RJS) method for producing nanofibers [11,17]. RJS is a technique that utilizes high-speed rotating polymer solution jets to extrude three-dimensional nanofiber structures with highly aligned nanofibers. Poly-ε-caprolactone (PCL), a biodegradable polyester with low glass transition temperature that degrades via hydrolysis of its ester linkages in physiological conditions, was utilized as the synthetic component [18,19]. However, PCL-based scaffolds lack the ability to interact with cells and to support cell adhesion and migration. Collagen, the most abundant structural protein found in mammals [20], or its hydrolyzed form, gelatin, made up the protein components of the hybrid material. Similar polymer/protein hybrid electrospun scaffolds have shown promise as tissue engineering materials demonstrating favorable mechanical properties and cellular adhesion [21–23]. However, electrospun fibers lack a high degree of fiber alignment and have relatively low production rates (~1 ml/h). Our study objective was therefore to engineer protein-polymer biohybrid aligned nanofibers via RJS and characterize their physical, chemical and biomimetic properties. We also highlight key physical and chemical differences in electrospun and RJS fabricated biohybrid nanofibers.

2. Materials and methods

2.1. Polymer materials

PCL (Mn 70,000–90,000; Sigma–Aldrich, St. Louis, MO), gelatin Type A (~300 Bloom; Sigma–Aldrich) and solvent 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP; Sigma–Aldrich) were used as received without further modifications. Collagen Type I (Sigma–Aldrich) was obtained as a 4 mg/mL solution in 20 mM acetic acid and lyophilized using standardized protocols before use.

2.2. Fabrication of protein-polymer hybrid SANF scaffolds via rotary jet spinning

PCL, PCL-collagen and PCL-gelatin fibers with different compositions were fabricated using rotary jet spinning (RJS). Briefly, PCL/collagen (75:25), PCL/gelatin (75:25, 50:50, 25:75) and PCL were dissolved in HFIP at various mass ratios at 6% total dopant concentration (w/v). Pure protein SANF scaffolds were not fabricated in this study due to their instability in an aqueous environment without glutaraldehyde fixation. This solution was injected into the rotating reservoir (~30,000 rpm) of the RJS system and fibers were collected proximal to the high-speed rotating reservoir. The scaffolds were spun for approximately 2 min for each condition and dried overnight in a desiccator to remove excess solvent prior to use.

2.3. Fabrication of protein-polymer hybrid scaffolds via electrospinning

PCL/collagen (75:25) was dissolved in HFIP at 6% total concentration (w/v). The solution was placed in a 1 mL syringe with a stainless steel needle (23.5 gauge), attached to a syringe pump (100 Series, Kd Scientific, USA) at a flow rate of 0.08 mL/h. A grounded copper plate was placed on 10 cm from the end of the needle and 14 kV of electrical potential was applied by high-voltage supplier (HV30, NanoNC, Korea) to generate the required potential difference between the tip of the needle to collector to produce fibers.

2.4. Scanning electron microscopy and physical characterization

Fiber diameter and alignment were characterized using a field emission scanning electron microscope (FESEM; Carl Zeiss, Dresden, Germany). Briefly, bare fiber samples were removed from the collector, mounted on sample stubs and sputter coated with Pt/Pd (Denton Vacuum, Moorestown, NJ). Samples that were seeded with cells were first fixed in a 2.5% (v/v) solution of glutaraldehyde in 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA) for 2 h. The samples were then washed 3 times in buffer and dehydrated with increasing concentrations of absolute ethanol (50%, 75%, 90% (2×), 100% (2×)). In place of critical point drying that can induce artifacts in biological samples [24], specimens were dried in hexamethyldisilazane (Electron Microscopy Sciences) via serial exchange from the ethanol (100:0, 50:50, 0:100) and sputter coated as before. Images were acquired and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). A total of 100–300 fibers were analyzed (5 random fields of view per sample) to determine the fiber diameter. At least 8 fields of view were analyzed per sample for fiber alignment/orientation. Fiber alignment was characterized using a parameter termed orientation order parameter (OOP). The OOP was developed for the study of organization of liquid crystals [25] and adapted for biological applications [26], and was computed from the pixel-based orientation vectors of the fiber images (Supplementary Fig. 1). The parameter value ranges from zero in isotropic systems to one in perfectly aligned systems.

2.5. Viscosity measurements

Rheological measurements were made on freshly prepared PCL and PCL/gelatin solutions of varying ratios (Supplementary Fig. 1). Solutions were loaded into the viscometer (Model AR-G2, TA instruments, New Castle, DE) fitted with a standard-size 40 mm cone and plate geometry. Viscosities were measured under steady state shear rate from 0.1 to 1000 s^{-1}. Experimental viscosity versus polymer concentration curve is best fitted by a polynomial function for small concentrations of polymer and by an exponential function at higher concentrations.

2.6. Chemical characterization

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy of PCL, PCL/collagen and PCL/gelatin scaffolds was obtained on a Nicolet ECO 1000 spectrometer system (Pittsburgh, PA) over a range of 4000–600 cm^{-1} at a resolution of 1 cm^{-1}. X-Ray Diffraction (XRD) was measured on a Scintag XDS 2000 (Cupertino, CA) instrument with Cu Ka radiation (1.54 nm wavelength) at 0.25° increments using fibers deposited on quartz substrate to examine the crystal structure of SANF scaffolds. Differential Scanning Calorimetry (DSC) was measured on a TA Q2000 instrument (New Castle, DE) to analyze polymer and hybrid melting and recrystallization temperatures. Fourier Transform Infrared spectroscopy imaging (imaging IR) of PCL, PCL/collagen, and collagen scaffolds was obtained on a Varian 640 FTIR spectrometer and a Varian 620 imaging microscope (Palo Alto, CA) equipped with a liquid nitrogen cooled 16 × 16 pixel focal plane array (FPA) detector. Imaging IR data were collected at a resolution of 4 cm^{-1} over a range of 4000–900 cm^{-1} using 64× scanning; scaffold samples were mounted on gold coated wafers (Sigma–Aldrich). Polymer/protein hybrid electrospun and RJS scaffolds were soaked in distilled water. Every 24 h, a portion of soaked fibers were taken out of water and dried in the oven at 37 °C for 12 h before being imaged by FTIR to quantify the time rate of change of protein composition on the fiber surface.

2.7. Mechanical characterization

Mechanical characterization of the SANF scaffolds was performed using an Instron 5542 mechanical tester (Norwood, MA). Scaffolds were cut into a rectangular shape (5 × 15 mm) and inserted into the grips within a phosphate buffered saline bath heated to 37 °C. Specimens were allowed to equilibrate in the bath under no tension for 15 min, were preconditioned to 3% of original specimen length for 20 cycles, and pulled to failure at 1 mm/min extension rate. At least four specimens from each hybrid composition were tested. Young's modulus (E) was calculated based on the tangent of the stress-strain curve at 0% strain. Ultimate tensile strength (UTS) was also reported.

2.8. Biodegradation analysis [27,28]

Primary neonatal rat fibroblasts were seeded on Transwell® membrane plates (BD Biosciences, San Jose, CA) within 6-well plates that contained PCL, PCL/collagen and PCL/gelatin SANF scaffolds (Supplemental Fig. 3A). Samples were extracted at 0, 2, 7, 14, and 28 days, dried and had their weight measured. Samples were also processed for ATR-FTIR analysis as before. PCL/collagen samples at 0 and 56 days were also processed for SEM analysis to measure fiber diameter, as outlined earlier.

2.9. Cell culture

Neonatal rat ventricular cardiomyocytes, fibroblasts and cortical neurons were isolated from 2-day old Sprague–Dawley rats as previously described [29,30]. Valve interstitial cells (VICs) were a kind gift from the laboratories of Drs. Robert A. Levine and Joyce Bischoff (Massachusetts General Hospital and Children's Hospital Boston, Boston, MA). All procedures were approved by the Harvard Animal Care and Use