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Biomaterials derived from silk–tropoelastin protein systems

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ABSTRACT

A structural protein blend system based on silkworm silk fibroin and recombinant human tropoelastin is described. Silk fibroin, a semicrystalline fibrous protein with beta-sheet crystals provides mechanical strength and controllable biodegradation, while tropoelastin, a noncrystallizable elastic protein provides elasticity. Differential scanning calorimetry (DSC) and temperature modulated DSC (TMDSC) indicated that silk becomes miscible with tropoelastin at different blend ratios, without macrophase separation. Fourier transform infrared spectroscopy (FTIR) revealed secondary structural changes of the blend system (beta-sheet content) before and after methanol treatment. Atomic Force Microscopy (AFM) nano-indentation demonstrated that blending silk and tropoelastin at different ratios resulted in modification of mechanical features, with resilience from ~68%–~97%, and elastic modulus between 2 and 9 Mpa, depending on the ratio of the two polymers. Some of these values are close to those of native aortic elastin or elastin-like polypeptides. Significantly, during blending and drying silk–tropoelastin form micro- and nano-scale porous morphologies which promote human mesenchymal stem cell attachment and proliferation. These blends offer a new protein biomaterial system for cell support and tailored biomaterial properties to match mechanical needs.

1. Introduction

Tropoelastin is the soluble precursor of elastin. The tropoelastin monomer self-assembles into coacervates on cell surfaces and cross-links to form elastin networks through the action of lysyl oxidase [1–10]. Elastin networks can form elastic fibers which serve as extracellular matrix assemblies to provide elasticity and resilience in most human tissues, such as dermis and blood vessels [1–10]. Tropoelastin is difficult to isolate from native tissues, even when animals have been fed on a copper deficient diet or treated with a chemical inhibitor to prevent or reduce the crosslinking from lysyl oxidase [1–4]. Therefore, full-length (60 kDa) human tropoelastin was genetically engineered to provide an alternative source of the pure protein [1–4]. The hydrophobic domains are rich in non-polar amino acid residues glycine, valine and proline, and typically form repeat motifs, while the hydrophilic domains contain a high content of lysine and alanine and are involved in elastin crosslinking [1–4]. No crystal structure has been obtained from full-length tropoelastin, although many secondary structures have been revealed, including a low alpha helix content, compact beta-turns, poly-proline II, and disordered structures [1–4].

Tropoelastin is a useful option for protein-based biomaterials, since it is found in human tissues and is elastomeric [1–10]. The protein could be used to replace elastin-rich tissues in the skin dermis and vasculature [1–10], to provide elasticity and confer improved cellular interactions and tissue regeneration [1–10]. However, a disadvantage of elastin-based biomaterials is the inadequate mechanical rigidity for robust, longer term physical support during tissue regeneration, such as tendons and ligaments, blood vessels and many other load bearing tissue applications [1–4,7,8]. To address this need to broaden the utility of tropoelastin biomaterials, tropoelastin can be combined with other crystallizable peptide domains, through genetic engineering, to provide new protein polymers such as silk–elastin copolymers [11,12]. However, these biosynthesized constructs can be challenging to produce in high yield, retention of the structural, cell signaling and mechanical properties of the tropoelastin can be problematic, and interference with associations and lysine crosslinking are also an issue [1–4]. An alternative strategy is to generate stable matrix materials by physically blending tropoelastin with other crystallizable biomaterials. Physical blending is a relatively simple yet useful approach to generate new polymer materials. According to polymer chain interaction theory, polymer blends with good miscibility can improve the physical and chemical properties of the original polymer materials even if one of the components contributes only a small fraction to the mixture. In addition to the change in

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properties of the individual polymer components, the mixed blends may also offer new surface morphologies with improved interfacial adhesion [13].

Silk fibroin protein is a fibrous protein synthesized by *Bombyx mori* silkworms. Silks have been widely used in textile manufacturing as well as in biomedical materials [14]. Silks combine extensibility and high tensile strength, and their toughness is similar to or higher than many synthetic high performance polymer fibers [14–18]. In addition to their impressive mechanical properties, silks are relatively stable slow degrading materials that are FDA approved and offer morphologic flexibility, resulting in recent applications in many areas of biomaterial science, tissue engineering, drug release, and bio-optics [19–29]. Silk fibroin protein consists of a high molecular weight heavy chain (~370 kDa) and a light chain (~26 kDa) linked by a disulfide bond, twelve highly repetitive hydrophobic domains (mainly GAGAGS segments) in the heavy chain, and forms anti-parallel beta-sheet crystals [14–18]. The transformation from the solution state to beta-sheet crystalline state can be induced with methanol, heat, low pH, vortexing or sonication, among other modes [14–29]. With this high content of beta-sheet crystals, silk can form into high packing density material for biomedical applications, where proteolytic degradation can take hours to years depending on the crystalline content [19–22]. These features suggest that silk would be a suitable protein to combine with tropoelastin for biomedical materials, as both protein polymers complement each other in terms of material features, yet both are offer biocompatibility and degradability.

In the present study the objective was to explore the features of silk–tropoelastin blends to determine the influence of interactions among the two proteins on material properties. These silk–tropoelastin physical blends are a new class of biomaterials that combine elasticity and mechanical robustness, while supporting cell interactions.

2. Experimental

2.1. Materials

Sequencing grade trypsin, chymotrypsin from bovine pancreas, human neutrophil elastase, and endoproteinase Lys-C were purchased from Calbiochem (EMD Biosciences Inc., San Diego, CA). All other reagents were of analytical grade. Silk fibroin preparation has been reported previously [14,15]. Briefly, *B. mori* silkworm cocoons (obtained from Japan) were boiled in a 0.02 M Na_2CO_3 solution to extract the glue-like sericin proteins [14]. The remaining silk fibroin was dissolved in a 9.3 M LiBr solution at 60 °C for 4–6 h and then dialyzed with distilled water using dialysis cassettes for 2 days. After centrifugation and filtration to remove insoluble residues, a 6 wt% silk fibroin aqueous solution was obtained. For the tropoelastin protein, recombinant human tropoelastin corresponding to amino acid residues 27–724 of GenBank entry AAC98394 (gi 182020) was expressed and purified as previously described [1–4,49,50].

2.2. Blend preparations

The tropoelastin was first slowly dissolved in distilled water at 4 °C to form a 1.0 wt% tropoelastin aqueous solution, and then slowly mixed with 1 wt% silk aqueous solution using a pipette to avoid protein aggregation during mixing. The final solutions obtained were based on a mass ratio of silk:tropoelastin = 90:10 (SE90), 75:25 (SE75), 50:50 (SE50), 25:75 (SE25), 10:90 (SE10), with pure silk (SE100) and pure tropoelastin (SE0) used as controls. The blend solutions were diluted with ion-free distilled water or immediately cast on to glass dish to form films, and kept at 4 °C before measurement to avoid structural changes in the samples.

2.3. Differential scanning calorimetry

The dried silk/tropoelastin blends (each about 5 mg) were encapsulated in Al pans and heated in a TA Instruments (New Castle, DE) Q100 DSC, with purged dry nitrogen gas flow (50 mL/min), and equipped with a refrigerated cooling system. The instrument was calibrated with indium for heat flow and temperature. Standard mode DSC measurements were performed at a heating rate of 2 K/min. Temperature modulated differential scanning calorimetry (TMDSC) measurements were also performed at a heating rate of 2 K/min with a modulation period of 60 s and temperature amplitude of 0.318 K. Aluminum and sapphire reference standards were used for calibration of the heat capacity. The heat capacity measurements consisted of three runs, as described in our earlier work [15–18]. In TMDSC, the "reversing heat capacity", which represents a reversed heat effect within the temperature range of the modulation, can be then measured and calculated [15–17].

2.4. FTIR

Fourier Transform Infrared spectroscopy (FTIR) analysis of silk/tropoelastin blends was performed with a Jasco (Japan) FT/IR-6200 Spectrometer, equipped with a deuterated triglycine sulfate detector and a multiple reflection, horizontal MIRacle ATR attachment (using a Ge crystal, from Pike Tech. (Madison, WI)). The instrument was continuously purged by nitrogen gas to eliminate the spectral contributions of atmospheric water vapor. For each measurement, 128 scans were co-added with resolution 4 cm^{-1}, and the wave numbers ranged from 400 to 4000 cm^{-1}. Fourier Self-Deconvolution (FSD) of the infrared spectra covering the Amide I region (1595–1705 cm^{-1}) was performed with Opus 5.0 software from Bruker Optics Corp. (Billerica MA), as we have described previously [15]. FSD is a common signal-processing tool that allows resolution of several overlapping bands [15]. Using a high pass filter, the broad and indistinct Amide I bands (C=O stretching bonds in protein backbones) can be narrowed synthetically to provide a deconvoluted spectrum with better peak resolution [15]. The deconvoluted spectra are better suited for subsequent Gaussian curve fitting [15]. The deconvoluted Amide I spectra were area-normalized, and the relative areas of the single bands were used to determine the fraction of the secondary structural elements in the blends.

2.5. Atomic force microscopy (AFM)

AFM imaging was performed in tapping mode on a Dimension 3100 Scanning Probe Microscope with Nanoscope III and IV controllers (Digital Instruments, Santa Barbara, CA) and equipped with rotated tapping mode etched silicon probes (RTESP; Nanodevices, Santa Barbara, CA). silk–tropoelastin blends with a concentration of 0.2 wt% were cast on mica surfaces to form thin films (~200 nm) for analysis.

2.6. Nano-indentation

Measurements of elasticity (resilience) on the silk–tropoelastin blend samples were conducted using AFM operated in force mode. Force–distance curves for each sample were measured at least 5 times in each different region, and the calculated elasticity was averaged and standard deviation determined. Elasticity (resilience) is a measure of the ability of a material to deform and recover and AFM is used to measure the modulus or stiffness of materials with resolution of nanometers, similar to conventional compression tests [30–32].

The elastic response of a sample indented by an AFM tip can also be described by the Hertz model [33–37]. The Hertz model predicts the following relation between indentation depth δ and the loading force F:

$$F = (2/\pi)\left[E/\left(1-\nu^2\right)\right]\delta^2\tan(\alpha)$$

where E is the elastic (Young's) modulus, ν is the Poisson ratio, α is the half-opening angle of the cylindrical cone for the AFM tip. On an infinitely stiff sample, the cantilever deflection d is equal to the movement of the piezo detector in the z direction, where $d = z$. In the case of most soft samples such as proteins or synthetic polymers, an indentation δ leads to a smaller deflection $d = z - \delta$, resulting in a flatter force curve with a smaller slope. According to Hooke's law [33–37], the loading force F will be given by the deflection of the cantilever multiplied by the force constant k of the cantilever: $F = kd = k(z - \delta)$. Because the protein network obeys rubber elasticity, we assumed a Poisson ratio of 0.5. A value of 3.152 N/m was adopted as the force constant, confirmed from its resonant frequency, 20° as the half-opening angle of the cone, corresponding to the manufacturer's specifications. The deflection d and the sample height z were estimated from the measured force curves, and the above equations and parameters were used for fitting the measured data to obtain a value for the elastic modulus E. The values were averaged based on measurements of 20 points on the surface of each sample. The stiff mica substrate surface served as a stiff control, with an elastic modulus ~160 GPa.

2.7. Human mesenchymal stem cells (hMSCs)

hMSCs were obtained from bone marrow aspirates from Cambrex Bio Science Walkersville Inc. (Walkersville, MD) from a 25-year old healthy male [38]. Whole bone marrow aspirates were plated at a density of 10 μL of aspirate/cm^2 in tissue culture flasks in high-glucose Dulbecco's Modified Eagle Medium (H-DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% non-essential amino acids, and 1 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, CA). Cells were maintained in a humidified incubator at 37 °C with 5% CO_2. hMSCs were separated from hematopoietic stem cells (HSCs) on the basis of their adherence to tissue culture plastic; HSCs in suspension were removed after