Published in final edited form as:

Biomaterials. 2010 November ; 31(32): 8121–8131. doi:10.1016/j.biomaterials.2010.07.044.

Biomaterials Derived from Silk-Tropoelastin Protein Systems

Xiao Hu¹, Xiuli Wang¹, Jelena Rnjak², Anthony S. Weiss², and David L. Kaplan^{1,*}

¹ Department of Biomedical Engineering, Tufts University, Medford, MA 02155, USA

² School of Molecular Biosciences, The University of Sydney, Sydney, NSW 2006, Australia

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% to ~97%, and elastic modulus between 2~9Mpa, 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.

Keywords

miscibility; silk; tropoelastin; elastin; polymer blends; DSC; FTIR; AFM; nano-indentation

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

© 2010 Elsevier Ltd. All rights reserved.

* Corresponding author: david.kaplan@tufts.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 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 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.

Experimental

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 Na₂CO₃ 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].

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.

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].

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 400-4000 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.

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.

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 :

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

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.

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 10^5 cells/cm² 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% CO₂. hMSCs were separated from hematopoietic stem cells (HSCs) on the basis of their adherence to tissue culture plastic; HSCs in suspension were removed after approximately 5 days of culture. hMSCs were expanded until confluence, with medium changes every 3–4 days. Cells at passage 2–4 were used for all experiments.

To prepare the silk-tropoelastin films for cell culture, the silk-tropoelastin blend solutions were directly cast into 24-well plates, and air dried for 2 days in a laminar flow hood. The resulting silk-tropoelastin films were approximately 50 μm thick. For cell attachment, the silk-tropoelastin films in 24-well plates were first sterilized in 70% ethanol for 2 h, and then preconditioned with cell culture medium overnight before cell plating. The short 70% ethanol treatment did not alter the structure of the films based on FTIR analysis. hMSCs were trypsinized and re-plated on silk-tropoelastin films at a density of 5,000 cells/well. Cell attachment and spreading on the films was observed after 15, 45 min and 90 min incubation in 5% CO_2 at 37°C, after being washed with the culture medium to remove those unattached cells. Images were captured by a phase contrast microscope with a TCS SP2 scanner (Leica Microsystems, Mannheim/Wetzlar, Germany).

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed to determine hMSCs proliferation on the silk-tropoelastin films. The silk-tropoelastin films were prepared and pretreated as described above. Then hMSCs (passage 2) were seeded onto the films at the density of 5,000 cells/well and cultured at 37°C and 5% CO_2 . After 3 and 5 days cultivation, MTT stock solution (5 mg/ml, Gibco Media, Invitrogen Corp., Carlsbad, CA) was added to the wells, which were incubated at 37°C for 4 h, and then the medium was aspirated and the converted dye was solubilized with DMSO (Sigma-Aldrich, St. Louis, MO). The dye solution was transferred into 96-well assay plates and a Beckman DU-600 spectrophotometer (Beckman Coulter Inc., USA) was used to measure the OD value at a wavelength of 570 nm with background subtraction at 650 nm.

Results and Discussion

DSC Analysis

To assess the miscibility of silk-tropoelastin mixtures and macrophase interactions, the thermal properties of the dried silk-tropoelastin blend films were examined by standard DSC and temperature modulated DSC (TMDSC). The interaction of silk with tropoelastin can be viewed as the interaction between a “solvent” (in this case the dominate polymer component in the blend) and a “solute” (the minor component) based on the Flory-Huggins's lattice model [39-43]. The key factor that governs the miscibility of the blend is the free energy of mixing [39-43]. If we consider the entropic contribution to the total free energy of mixing is negligible according to Flory-Huggins [39-43], then the enthalpic contributions in the protein blends system are directly related to its miscibility [39-43]. A negative enthalpic contribution can occur when the interaction between the protein components becomes significant, which will increase the miscibility of the blend system [39-43]. A homogeneous macrophase in the protein blend therefore can be obtained if specific favorable interactions exist between each protein component in the mixture [39-43]. One of the simplest interactions is hydrogen bonding between the different protein chains, invoking hydrogen-bonded beta-structure interactions between silk and tropoelastin. The clearest experimental evidence of a miscible blend is the occurrence of only one glass transition temperature (T_g) for the miscible protein system by DSC, which is normally located between the T_g s of the two individual protein components [40-43].

Figure 1(a) shows standard DSC scans of the silk-tropoelastin film samples: SE100 (pure silk), SE90, SE75, SE50, SE25, SE10, and SE0 (pure tropoelastin). All of these samples showed bound water peaks around 50~80°C, similar to the pure silk-bound water film (SE100), which has been intensively studied previously [16, 17]. The bound water peak shifted to a slightly lower temperature with increasing tropoelastin content, from 85°C in pure silk (SE100) to 53°C in pure tropoelastin (SE0). After the glass transition temperature regions of the silk-

tropoelastin samples around 170~200°C, a nonisothermal, endothermal peak that appeared in all sample scans and shifted to a higher level, from 213°C (SE100) to 230°C (SE0), can be assigned to the thermal transition/crystallization of the silk-transition blend [15, 16, 23, 28]. No separate individual transition peak for the silk or tropoelastin components was observed, which indicates that silk and tropoelastin were well blended to become a homogeneous system without macrophase separation. After this endothermal transition, a degradation peak from 265°C (SE100) to 310°C (SE0) was seen for silk-tropoelastin samples, which shifted to a higher temperature with increasing tropoelastin content. All silk-tropoelastin blend samples had a higher degradation temperature than for the pure silk sample (265°C), which indicates that the tropoelastin increases the thermal stability of silk protein after blending.

To investigate in detail the phase transition behavior of the silk-tropoelastin samples, temperature modulated DSC (TMDSC), a technique that can eliminate the non-reversing thermal phenomena of the sample (*e.g.*, water evaporation process, or physical aging/degradation process), was used to measure the reversing thermal properties of the silk-tropoelastin samples. Figure 1(b) shows the reversing heat capacity of the samples from -30°C to 260°C, which were measured with a 2°C/min heating rate, a modulation period of 60s and a temperature amplitude of 0.318K. All samples showed a curve around 25~70°C, with slight shift from 25°C in SE0 to 70°C in SE100. These downward shifting curves in the reversing heat capacity traces were due to the loss of bound water in the silk-tropoelastin-bound water system, which reduced the mass of the system and resulted in decreased heat capacity [16, 17, 27]. Only pure silk (SE100) showed a clear silk-bound water glass transition (upward shift) [16] before the water loss around 70°C, which may indicate that the bound water does not heavily affect the structural properties of blended silk-tropoelastin and pure tropoelastin samples. Above 140°C, the bound water was completely removed and the baseline of heat capacity became stable. A clear glass transition appeared for all samples after bound water removal. With the increase of tropoelastin content, the glass transition temperatures (T_g) of the blends increased gradually from 178°C (pure silk) to 190°C (pure tropoelastin), accompanied by stable heat capacity steps during the T_g , ΔC_p . The homogeneous single glass transition for the silk-tropoelastin blends indicate that a stable single silk-tropoelastin macrophase was formed for each sample during blending and drying, resulting in a fully miscible protein system with hydrogen bonds between the protein components. Therefore, miscible silk-tropoelastin systems were formed with these two protein components.

FTIR Analysis

To understand the impact of protein interactions in the silk-tropoelastin films, FTIR analysis was performed to measure the structures of these blends before and after methanol treatment. Figure 2(a), (b) show the FTIR absorbance spectra of the silk-tropoelastin samples (SE100, SE90, SE75, SE50, SE25, SE10, and SE0) before and after 20 min methanol treatments, for the region 1450-1750 cm^{-1} . The FTIR spectra of fibrous proteins such as pure silk fibroin have been well described [15]. Generally, the IR spectral region within 1700-1500 cm^{-1} is assigned to the peptide backbone of Amide I (1700-1600 cm^{-1}) and Amide II (1600-1500 cm^{-1}) absorptions. The Amide I region mainly comes from the C=O stretching vibration (>80%) [15, 16, 18], which directly reveals the secondary structure of the protein backbone, and is most commonly used for the quantitative analysis of different secondary structures in protein. In general, the region from 1600 to 1640 cm^{-1} is related to the intermolecular and intermolecular beta-sheet bands, which increase during silk crystallization [15, 18]. The region between 1640 and 1660 cm^{-1} includes contributions from random coils and alpha-helices. The remaining parts of the spectra from 1660 to 1690 cm^{-1} are dominated by vibrations from beta-turn structures, with some small bands from other structures, such as a possible beta-sheet peaks between 1690-1705 cm^{-1} [15]. The Amide II bands are mainly from the out-of-phase combination of the CN stretching and the NH in-plane bending vibrations in protein backbone

[15,16]. Amide II regions mix with the vibrational modes of protein side chain groups for solid samples, which can be used to analyze the change of the micro-environment and tertiary conformation of the proteins [16]. To quantify the percentage of the secondary structures in the silk-tropoelastin samples, a deconvolution method based on curve fitting spectra by FSD [15] was performed in the Amide I region. Figure 2(c) shows the FSD Amide I spectra of the pure tropoelastin protein (SE0) with curve fitting by single vibrational bands (in dashed lines). The peak position and their related secondary structures have been detailed in previous work [15-18] and assigned as side chains (S), beta-sheets (B), random coil (R), alpha helix/PPII (A), and turns (T) for tropoelastin. Table 1 summarizes the percentage of secondary structures for each untreated silk-tropoelastin sample with different component fractions. The beta sheets change after 20 min MeOH treatments. For the untreated original blends, pure tropoelastin had about 23.1% beta sheet, with 46.3% random coil and alpha helix/PPII (dominant), and 28.7% turns. These values are compatible to those derived from a Circular Dichroism study of tropoelastin in solution [1-4], where tropoelastin presented 30~40% primary beta sheet structure. With an increase in silk content in the untreated blends, the amount of beta sheet structure gradually decreased to 8.2% in the untreated pure silk, while random coil and alpha helix decreased to 36.5%, and turns increased to 53.5%. The amide II regions remained stable for all untreated samples. After 20 min methanol treatment, the beta-sheet crystal peak (around 1620 cm^{-1}) increased significantly with increasing content of silk in the silk-tropoelastin blends, as seen in the Amide I region of Figure 2(b). In the pure silk films, 45.6% beta-sheet crystal was induced. With an increase in tropoelastin, less beta sheet crystals formed, which reduced to 28.7% in sample SE10. However, even this small amount of beta-sheet crystal promoted the formation of stable protein networks between the silk and tropoelastin molecules, resulting in water-insoluble blend materials. The pure tropoelastin sample also had a small increase in beta sheet content (23.1% to 25.4%) after MeOH treatment, but insolubility was unstable after 7 days in aqueous solution. The amide II decreased significantly with less silk content, mostly due to side chain movements in the protein structure. Typically random coil and alpha-helix structures were converted to beta-sheets in the blends (Table 1), similar to the situation in pure silk protein films [15-18].

Mechanical Properties by Nano-indentation

Mechanical properties provide significant indicators for polymer blends and their interactions. With the change in mixture component fractions, structures can change and alter the mechanical properties. To determine the local elasticity of each blend silk-tropoelastin film, nano-indentation was used to apply forces in the nano-Newton range, and the resulting elasticity (resilience) and elastic modulus for each sample were determined at the nano-scale. The measurements were carried out in force calibration mode. Figure 3 shows force-distance relationships for SE100, SE75, SE50, SE25, and SE0, which demonstrated deflection of the cantilever tip as a function of the position of the blend film surfaces. To clearly demonstrate the difference in resilience, the deflection values were normalized to between 0-100 nm [30-32]. However, the calculation and fitting of the area-fraction based resilience values and the elastic (Young's) modulus for each sample were based on the original curve data without normalization.

The calculation of elasticity (resilience) has been discussed in detail for elastic resilin proteins in our previous studies [31]. The elasticity (resilience) calculated is defined as the energy recovered after removal of the stress, divided by the total energy of deformation. The energy of deformation/recovery is given by converting force displacement curves into force-penetration/retraction curves, and calculating the ratio of the areas under the penetration and retraction curves [30-32]. Figure 3 shows the resilience of each blend/pure sample, and the calculated results are listed in Table 1. Compared with the ~94% resilience of uncrystallized silk protein, pure tropoelastin has almost perfect resilience, around 97%, while all blend

samples have generally lower resilience than the pure silk or tropoelastin samples. For the tropoelastin dominated samples SE10 and SE25, and the silk dominated sample SE90, the resilience remains high, above 85%, which is superior to that of known highly-elastic synthetic rubbers such as polybutadiene (80%) [32]. In comparison, the SE75 sample had reduced resilience to around 79%, while the equal weight mixed SE50 sample had the lowest resilience at around 68%, indicating that the surface morphology of these two samples in the nano-scale could be significantly different from the pure protein samples. These data suggest that blending silk and tropoelastin in different ratios results in modification of surface mechanical features, and that these can be controlled by varying the composition of the blend.

In comparison, the elastic modulus of silk-tropoelastin blend samples was quantified based on the Hertz model [33-37], and shown in the last histogram of Fig. 3. The calculated values are listed in Table 1. The errors bars represent the distribution of the measured modulus in different areas of the sample. We focus on the impact of silk-tropoelastin blend ratios to the elastic modulus, therefore, differences during post-processing treatments or chemically crosslinking are not discussed [11,44]. The results show that all thin film samples have a similar elastic modulus, between 2~10 MPa. Notably, recombinant human tropoelastin displayed an elastic modulus between 5-10 MPa, near or above those of other elastin-like polypeptides [11] and native aortic elastin (~ 0.81 MPa) [11]. The addition of silk did not reduce the elastic modulus until the silk component became dominate at 90 wt%. This result is also comparable to engineered silk-elastin-like polypeptides [11], which have an elastic modulus around 1~4 MPa depending on post-processing treatment.

AFM analysis

To understand nanostructure differences for silk and tropoelastin blends the surface morphology of the silk-tropoelastin thin films (cast on the mica surface) were observed by AFM. Figure 4 shows the AFM topography/height (first column), phase (second column), and 3-D topography (third column) of the films consisting of pure silk (SE100), pure tropoelastin (SE0), and the blend systems with different protein compositions: SE90, SE75, SE50, SE25, and SE10. Both pure silk and pure tropoelastin films have homogeneous surface features without obvious nano/micro patterns, which was confirmed by the single color phase images in the second column of images. For pure silk, the average surface roughness was around 350 nm with root mean square (rms) amplitude 1.5 ± 0.2 nm, while pure tropoelastin had an average surface roughness around 160 nm with rms amplitude about 0.09 ± 0.03 nm, respectively. At 10 wt% tropoelastin (SE90), the film showed a homogeneous surface layer with some loose-distributed holes. The sizes of holes were around 20~50nm, with an average depth about 0.5 nm, and a clear nano phase separation was identified in the phase image. With increasing tropoelastin content (SE75) the film showed a surface layer with many densely distributed holes. The tropoelastin enriched cavities remained around 20~50 nm, but the number of these increased significantly compared with the SE90 film, and covered most of the surface (the insert $5 \times 5 \mu\text{m}$ image shows the macro-features of SE75). When the tropoelastin content was increased to 50 wt% (SE50), the surface of the films became pitted with homogenous nano-pores (the insert $5 \times 5 \mu\text{m}$ image shows the macro-features of SE50) whose diameters ranged from 15 to 35 nm. The depth of the pores was at least 2 nm on average, compared with the shallow depth of the nano-holes in the previous silk dominated samples. With a decrease in the silk component in the blend film, tropoelastin became the major component in sample SE25 and the size of the nano-pores increased to 50~90 nm with depth 3~7 nm, on average. However, the phase image indicated that the blend became more homogenous between silk and tropoelastin, with only small discontinuous phase domains observed, compared with the silk dominated blend samples. This observation could indicate that the tropoelastin reduced protein side chain energy boundaries between the silk-silk or silk-tropoelastin chains, as tropoelastin contains multiple hydrophilic regions along the molecule [1-4]. With a further increase in the

tropoelastin fraction to 90 wt%, silk proteins became the plasticizers (SE10) and a surprising micro-porous structure appeared with diameters around 0.1~0.4 μm . The inserted $10\times 10\ \mu\text{m}$ image shows the macro-features of the film surface, displaying sharp depths more than 7 nm on average. Additionally, many small nano-pores were present on the surface, but their depths were only 1~1.5 nm on average, and their size was less than 20 nm. These surface patterns for silk-tropoelastin blends are similar to the nano-porous patterns seen with the model system elastin-like [(VPGVG)₂(VPGE)(VPGVG)₂]₁₅ polypeptide [12]. An aperiodic pattern of nanopores appeared on the peptide film surface when it was deposited from basic solution. This type of film topology was explained by the higher polarity of the carboxyl side group in the glutamic acids of silk, compared with the rest of the protein domains. Under these conditions, the charged domains would avoid hydrophobic contact with their surroundings and then segregate from the hydrophobic surroundings giving rise to nanopores. For the silk-tropoelastin blends in the present study, hydrophobic silk protein side chains have different polarities than does tropoelastin protein in water. Therefore, even if the protein backbones were well mixed at the macrophase scale, the side chain groups with different polarities could still form variable micro-/nano- patterns during drying from aqueous solution. The size, depth, and density of these patterns are controllable by changing the fraction of silk or tropoelastin in the blends, which results in blend surface features that can be controlled (as in Figure 4).

Cell Interactions

Stem cell attachment and spreading were assessed to evaluate their compatibility with the biomaterials. hMSCs were utilized in the present study and their attachment on the surface of the different samples (SE100, SE75, SE50, SE25, SE0) was assessed at different time points. After 15 min incubation, the hMSCs started to attach to the surface of the blend samples (Figure 5). After 90 min, many hMSCs with elongated morphology were observed in the blend sample groups (SE50 and SE25). In contrast, less elongated cells were seen on the surfaces of the pure protein samples, and most of the attached cells displayed spherical morphology, which would lead to reduced viability. These differences in attachment suggest that the surface topologies of the blend samples, in which nano-scale porous were present, improved cell attachment. In addition, compared with the pure silk or tropoelastin groups, the highest cell numbers were obtained with SE10 and SE25, similar to those in the tissue culture plate (TCP) control group. Cell proliferation by days 2 and 6 was related to the ratio of the tropoelastin incorporated into the blend (Figure 6). With 10 wt% tropoelastin, cell numbers were two-fold higher than on pure silk after 5 days, while constructs with more than 25 wt% of tropoelastin tripled the cell numbers when compared to pure silk samples. This result is likely due to the cell interactive sequences in tropoelastin [1-4,45].

A schematic of the mechanisms of silk-tropoelastin system based on blending ratios is presented in Figure 7(a). The AFM morphology of the silk-tropoelastin system demonstrated how the protein chains interacted and formed different structures. By adding a small fraction of tropoelastin into silk (SE90), the tropoelastin proteins tend to microphase-separate and formed only monomers or discontinuous small aggregates in the silk “solvent” [39], due to the hydrophobic and hydrophilic interactions in the silk-tropoelastin protein chains. This phenomenon could be used as a method to generate tropoelastin nanoparticle/microspheres or nano-porous silk structures in future studies. With more tropoelastin added into the silk “solvent” (SE75, SE50), the tropoelastin “drops” start to connect with each other (SE50). The system transformed to a bi-continuous system (SE25) in which silk and tropoelastin chains interpenetrated to form a joint network. This transition indicates that silk-tropoelastin system could be used as interpenetrating polymer networks (IPN) in future biomedical material applications, such as for hydrogels. When silk became dominate (SE90), the interpenetrating polymer network gradually disappeared and eventually pure silk structures formed (SE100).

Figure 7(b) also presents a schematic of the corresponding cell growth and attachment on the silk-tropoelastin surfaces. For the pure silk or tropoelastin samples, especially the pure silk, the surface is generally smooth with low surface roughness. The cells cannot easily attach to the surface and grow (Figure 7(b) upper panel). However, with mixing of tropoelastin (Figure 7(b), lower panel), nano/micro scale porous structures formed on the surface with significantly higher roughness, which helps the cells easily attach on the surface, resulting in faster cell attachment and proliferation on the blend protein materials [13, 45-48]. Only a small fraction of tropoelastin (~10 wt%) was needed to double cell proliferation after 2 weeks in culture.

Conclusions

These silk-tropoelastin physical blends represent a new class of biomaterials. Semi-crystalline silk protein confers mechanical strength to tropoelastin and removes the need for chemical crosslinking by varying beta-sheet crystal content. Human tropoelastin confers elasticity and promotes cell interactions. These two proteins form biomaterial blends that support hMSC attachment and proliferation. As evidenced by TMDSC, silk forms hydrogen bonds and becomes miscible with tropoelastin at different blend ratios without macrophase separation. As evidenced by FTIR, the structures change incrementally with the blend ratio. All blended samples have sufficient mechanical elasticity at the nano-scale, and the mechanical differences are dependent on the blend ratio. During blending and drying, silk-tropoelastin systems form variable micro-/nano- scale porous surface patterned structures. Thus, direct blending of crystallizable silk proteins into tropoelastin appears to be an attractive strategy due to simplicity and control of the process with preservation of useful mechanical properties.

Acknowledgments

The authors thank the NIH P41 Tissue Engineering Resource Center (P41 EB002520), the Australian Research Council, the National Heart Foundation and the National Health and Medical Research Council for supporting this work.

References

1. Wise SG, Weiss AS. Tropoelastin. *Int J Biochem Cell Biol* 2009;41:494–497. [PubMed: 18468477]
2. Wise SG, Mithieux SM, Raftery MJ, Weiss AS. Specificity in the coacervation of tropoelastin: solvent exposed lysines. *J Struct Biol* 2005;149:273–281. [PubMed: 15721581]
3. Wise SG, Mithieux SM, Weiss AS. Engineered tropoelastin and elastin-based biomaterials. *Adv Protein Chem Struct Biol* 2009;78:1–24. [PubMed: 20663482]
4. Vrhovski B, Weiss AS. Biochemistry of tropoelastin. *Eur J Biochem* 1998;258:1–18. [PubMed: 9851686]
5. del Mercato LL, Maruccio G, Pompa PP, Lochicchio B, Tamburro AM, Cingolani R, et al. Amyloid-like fibrils in elastin-related polypeptides: structural characterization and elastic properties. *Biomacromolecules* 2008;9:796–803. [PubMed: 18257556]
6. Annabi N, Mithieux SM, Weiss AS, Dehghani F. Cross-linked open-pore elastic hydrogels based on tropoelastin, elastin and high pressure CO₂. *Biomaterials* 2010;31:1655–1665. [PubMed: 19969349]
7. Heinz A, Jung MC, Duca L, Sippl W, Taddese S, Ihling C, et al. Degradation of tropoelastin by matrix metalloproteinases-cleavage site specificities and release of matrikines. *FEBS J* 2010;277:1939–1956. [PubMed: 20345904]
8. Taddese S, Weiss AS, Jahreis G, Neubert RHH, Schmelzer CEH. In vitro degradation of human tropoelastin by MMP-12 and the generation of matrikines from domain 24. *Matrix Biol* 2009;28:84–91. [PubMed: 19144321]
9. Pepe A, Armenante MR, Bochicchio B, Tamburro AM. Formation of nanostructures by self-assembly of an elastin peptide. *Soft Matter* 2009;5:104–113.

10. Clarke AW, Arnsperg EC, Mithieux SM, Korkmaz E, Braet F, Weiss AS. Tropoelastin massively associates during coacervation to form quantized protein spheres. *Biochemistry* 2006;45:9989–9996. [PubMed: 16906757]
11. Teng WB, Cappello J, Wu XY. Recombinant silk-elastinlike protein polymer displays elasticity comparable to elastin. *Biomacromolecules* 2009;10:3028–3036. [PubMed: 19788307]
12. Reguera J, Fahmi A, Moriarty P, Girotti A, Rodriguez-Cabello JC. Nanopore formation by self-assembly of the model genetically engineered elastin-like polymer [(VPGVG)(2)(VPGE) (VPGVG)(2)](15). *J Am Chem Soc* 2004;126:13212–13213. [PubMed: 15479061]
13. Chung TW, Liu DZ, Wang SY, Wang SS. Enhancement of the growth of human endothelial cells by surface roughness at nanometer scale. *Biomaterials* 2003;24:4655–4661. [PubMed: 12951008]
14. Jin HJ, Kaplan DL. Mechanism of silk processing in insects and spiders. *Nature* 2003;424:1057–1061. [PubMed: 12944968]
15. Hu X, Kaplan D, Cebe P. Determining beta-sheet crystallinity in fibrous proteins by thermal analysis and infrared spectroscopy. *Macromolecules* 2006;39:6161–6170.
16. Hu X, Kaplan D, Cebe P. Dynamic protein-water relationships during beta-sheet formation. *Macromolecules* 2008;41:3939–3948.
17. Hu X, Kaplan D, Cebe P. Effect of water on the thermal properties of silk fibroin. *Thermochim Acta* 2007;461:137–144.
18. Hu X, Lu Q, Kaplan DL, Cebe P. Microphase separation controlled beta-sheet crystallization kinetics in fibrous proteins. *Macromolecules* 2009;42:2079–2087.
19. Lu Q, Hu X, Wang XQ, Kluge JA, Lu SZ, Cebe P, et al. Water-insoluble silk films with silk I structure. *Acta Biomater* 2010;6:1380–1387. [PubMed: 19874919]
20. Wang XY, Hu X, Daley A, Rabotyagova O, Cebe P, Kaplan DL. Nanolayer biomaterial coatings of silk fibroin for controlled release. *J Controlled Release* 2007;121:190–199.
21. Motta A, Maniglio D, Migliaresi C, Kim HJ, Wan XY, Hu X, et al. Silk fibroin processing and thrombogenic responses. *J Biomater Sci: Polym Ed* 2009;20:1875–1897. [PubMed: 19793445]
22. Lu Q, Wang XQ, Hu X, Cebe P, Omenetto F, Kaplan DL. Stabilization and release of enzymes from silk films. *Macromol Biosci* 2010;10:359–368. [PubMed: 20217856]
23. Lu Q, Zhang XH, Hu X, Kaplan DL. Green process to prepare silk fibroin/gelatin biomaterial scaffolds. *Macromol Biosci* 2010;10:289–298. [PubMed: 19924684]
24. Wang XQ, Yucel T, Lu Q, Hu X, Kaplan DL. Silk nanospheres and microspheres from silk/pva blend films for drug delivery. *Biomaterials* 2010;31:1025–1035. [PubMed: 19945157]
25. Lammel AS, Hu X, Park S, Kaplan DL, Scheibel TR. Controlling silk fibroin particle features for drug delivery. *Biomaterials* 2010;31:4583–4591. [PubMed: 20219241]
26. Omenetto FG, Kaplan DL. A new route for silk. *Nat Photonics* 2008;2:641–643.
27. Hu X, Kaplan D, Cebe P. Thermal analysis of protein-metallic ion systems. *J Therm Anal Calorim* 2009;96:827–834.
28. Chen H, Hu X, Cebe P. Thermal properties and phase transitions in blends of Nylon-6 with silk fibroin. *J Therm Anal Calorim* 2008;93:201–206.
29. Yucel T, Cebe P, Kaplan DL. Vortex-induced injectable silk fibroin hydrogels. *Biophys J* 2009;97:2044–2050. [PubMed: 19804736]
30. Huson MG, Maxwell JM. The measurement of resilience with a scanning probe microscope. *Polym Test* 2006;25:2–11.
31. Qin GK, Lapidot S, Numata K, Hu X, Meirovitch S, Dekel M, et al. Expression, cross-linking, and characterization of recombinant chitin binding resilin. *Biomacromolecules* 2009;10:3227–3234. [PubMed: 19928816]
32. Elvin CM, Carr AG, Huson MG, Maxwell JM, Pearson RD, Vuocolo T, et al. Synthesis and properties of crosslinked recombinant pro-resilin. *Nature* 2005;437:999–1002. [PubMed: 16222249]
33. Yamada K, Tsuboi Y, Itaya A. AFM observation of silk fibroin on mica substrates: morphologies reflecting the secondary structures. *Thin Solid Films* 2003;440:208–216.
34. Rotsch C, Jacobson K, Radmacher M. Dimensional and mechanical dynamics of active and stable edges in motile fibroblasts investigated by using atomic force microscopy. *Proc Natl Acad Sci USA* 1999;96:921–926. [PubMed: 9927669]

35. Domke J, Radmacher M. Measuring the elastic properties of thin polymer films with the atomic force microscope. *Langmuir* 1998;14:3320–3325.
36. Schafer A, Vehoff T, Glisovic A, Salditt T. Spider silk softening by water uptake: an AFM study. *Eur Biophys J* 2008;37:197–204. [PubMed: 17851658]
37. Domke J, Dannohl S, Parak WJ, Muller O, Aicher WK, Radmacher M. Substrate dependent differences in morphology and elasticity of living osteoblasts investigated by atomic force microscopy. *Colloids Surf B: Biointerfaces* 2000;19:367–379. [PubMed: 11064259]
38. Altman GH, Horan RL, Martin I, Farhadi J, Stark PR, Volloch V, et al. Cell differentiation by mechanical stress. *FASEB J* 2002;16:270–272. [PubMed: 11772952]
39. Flory, PJ. Principles of polymer chemistry. Cornell University Press; Ithaca, N.Y.: 1953.
40. Miwa Y, Usami K, Yamamoto K, Sakaguchi M, Sakai M, Shimada S. Direct detection of effective glass transitions in miscible polymer blends by temperature-modulated differential scanning calorimetry. *Macromolecules* 2005;38:2355–2361.
41. Feldstein MM, Roos A, Chevallier C, Creton C, Dormidontova EE. Relation of glass transition temperature to the hydrogen bonding degree and energy in poly(N-vinyl pyrrolidone) blends with hydroxyl-containing plasticizers: 3. Analysis of two glass transition temperatures featured for PVP solutions in liquid poly(ethylene glycol). *Polymer* 2003;44:1819–1834.
42. Pizzoli M, Scandola M, Ceccorulli G. Crystallization kinetics and morphology of poly(3-Hydroxybutyrate) cellulose ester blends. *Macromolecules* 1994;27:4755–4761.
43. Penning JP, Manley RSJ. Miscible blends of two crystalline polymers .1. phase behavior and miscibility in blends of poly(vinylidene fluoride) and poly(1,4-butylene adipate). *Macromolecules* 1996;29:77–83.
44. Lawrence BD, Wharram S, Kluge JA, Leisk GG, Omenetto FG, Rosenblatt MI, et al. Effect of hydration on silk film material properties. *Macromol Biosci* 2010;10:393–403. [PubMed: 20112237]
45. Nivison-Smith L, Rnjak J, Weiss AS. Synthetic human elastin microfibers: stable cross-linked tropoelastin and cell interactive constructs for tissue engineering applications. *Acta Biomater* 2010;6:354–359. [PubMed: 19671457]
46. Curtis A, Varde M. Control of cell behaviour: topographical factors. *J Nat Cancer Res Inst* 1964;33:15–26.
47. Folkman J, Moscona A. Role of cell shape in growth control. *Nature* 1978;273:345–349. [PubMed: 661946]
48. Miller C, Shanks H, Witt A, Rutkowski G, Mallapragada S. Oriented schwann cell growth on micropatterned biodegradable polymer substrates. *Biomaterials* 2001;22:1263–1269. [PubMed: 11336298]
49. Martin SL, Vrhovski B, Weiss AS. Total synthesis and expression in escherichia coli of a gene encoding human tropoelastin. *Gene* 1995;154:159–166. [PubMed: 7890158]
50. Wu WJ, Vrhovski B, Weiss AS. Glycosaminoglycans mediate the coacervation of human tropoelastin through dominant charge interactions involving lysine side chains. *J Biol Chem* 1999;274:21719–21724. [PubMed: 10419484]

Figure 1.

(a) Standard DSC scans of the silk-tropoelastin blends (SE100, SE90, SE75, SE50, SE25, SE10 and SE0). The samples were heated at $2^\circ\text{C}/\text{min}$ from -30°C to 340°C . (b) Reversing heat capacities of the silk-tropoelastin samples (SE100, SE90, SE75, SE50, SE25, SE10 and SE0) from -30°C to 340°C , measured by TMDSC with a $2^\circ\text{C}/\text{min}$ heating rate, a modulation period of 60 s and a temperature amplitude of 0.318°C .

Figure 2.

FTIR absorbance spectra of the silk-tropoelastin samples. (a) Untreated sample spectra in Amide I and II region, and (b) 20 min MeOH treated sample spectra in Amide I and II region: a. SE0; b. SE10; c. SE25; d. SE50; e. SE75; f. SE90; and g. SE100. (c) A curve fitting example of FSD Amide I spectra using the tropoelastin (SE0) sample. The fitted peaks are shown by dashed lines, and assigned as side chain (S), beta-sheet (B), random coil (R), alpha helix / PPII (A), and turn (T).

Figure 3. Elasticity (resilience) and elastic (Young's) modulus evaluation of silk-tropoelastin by AFM nano-indentation. Normalized force-distance curves were showed for sample SE100, SE75, SE50, SE25, SE0. Red denotes the tip approach to/into the film, and blue represents the tip as it is pulled away from the surface.

Figure 4.

AFM topography images (first column), phase images (second column), and 3-D topography images (third column) of the silk-tropoelastin films: (a) pure silk (SE100), (b) SE90, (c) SE75, (d) SE50, (e) SE25, (f) SE10, and (g) pure tropoelastin (SE0). The inserted images show the macro-features of the film surfaces. Black bar length: 200 nm, white bar length: 2 μ m.

Figure 5. hMSC cell attachment to the surface of the silk, tropoelastin, and the blend samples SE75, SE50, and SE25. Confocal microscopy images were recorded at 15, 45 and 90 min. White bar length: 200 μm.

Figure 6. hMSC cell proliferation after 2 and 5 days on the surfaces of the silk, tropoelastin, and all blend samples, with TCP as a control.

Figure 7.
(a) Schematic of mechanisms of silk-tropoelastin interactions based on blending ratio, and
(b) cell attachment and growth on silk-tropoelastin surfaces. (Sample SE100 (silk) is compared
with sample SE50 as an example.)

Table 1

Percentage of secondary structures (FTIR) and the calculated elasticity (nanoindentation) with change of silk content in silk-tropoelastin blends.

Silk-tropoelastin Sample	Silk fraction in Sample	Beta Sheet (B)	Alpha Helix/PPH Random Coils (A+R)	Turns (T)	Side Chains (S)	Elasticity (Resilience) Silk-tropoelastin blend (%)	Elastic (Young's) modulus of Silk-tropoelastin blend (MPa)
SE100	100	8.2/45.6*	36.5	53.5	1.8	94.1±2	2.11±0.12
SE90	90	9.2/43.3*	35.7	53.4	1.7	92.5±2	2.64±0.19
SE75	75	19.8/38.7*	36.0	42.6	1.6	78.9±4	6.43±0.61
SE50	50	21.2/32.6*	37.8	39.2	1.8	67.8±5	5.98±1.02
SE25	25	21.6/31.5*	37.6	39.2	1.6	88.7±4	6.01±0.55
SE10	10	21.8/28.7*	39.4	37.5	1.3	88.9±3	6.89±1.76
SE0	0	23.1/25.4*	46.3	28.7	1.9	97.2±2	7.18±2.61

All calculated secondary structure fraction numbers have same unit (wt%) with a ±2 wt% error bar;

* Beta-sheet percentages after 20 min methanol treatment.