Published in final edited form as:

J Biomater Sci Polym Ed. 2009 ; 20(11): 1629–1644. doi:10.1163/156856208X386228.

A Permanent Change In Protein Mechanical Responses Can Be Produced By Thermally Induced Microdomain Mixing

Rory E. Sallach^{1,2}, Johannes Leisen³, Jeffrey M. Caves^{1,2}, Emily Fotovich^{1,2}, Robert P. Apkarian⁴, Vincent P. Conticello⁵, and Elliot L. Chaikof^{1,2,4,*}

¹Department of Surgery, Emory University, Atlanta, GA 30332

²Department of Biomedical Engineering, Emory University/Georgia Institute of Technology, Atlanta, GA 30332

³School of Polymer, Textile, and Fiber Engineering, Georgia Institute of Technology, Atlanta, GA 30322

⁴Integrated Microscopy & Microanalytical Facility, Emory University, Atlanta, GA 30332

⁵Department of Chemistry, Emory University, Atlanta, GA 30332

⁶School of Chemical and Biomolecular Engineering, Georgia Institute of Technology, Atlanta, GA 30322

Abstract

Electrospinning was employed to fabricate three dimensional fiber networks from a recombinant amphiphilic elastin-mimetic triblock protein polymer and the effects of moderate thermal conditioning (60°C, 4h) on network mechanical responses investigated. Significantly, while cryo-high resolution scanning electron microscopy (cryo-HRSEM) revealed that macroscopic and microscopic morphology of the network structure was unchanged, solid-state ¹H NMR spectroscopy demonstrated enhanced interphase mixing of hydrophobic and hydrophilic blocks. Significantly, thermal annealing triggered permanent changes in network swelling behavior (28.75 ± 2.80 non-annealed vs. 13.55 ± 1.39 annealed; p < 0.05) and uniaxial mechanical responses, including Young's modulus (0.170 ± 0.010 MPa non-annealed vs. 0.366 ± 0.05 MPa annealed; p < 0.05) and ultimate tensile strength (0.079 ± 0.008 MPa vs 0.119 ± 0.015 MPa; p < 0.05). To our knowledge, these investigations are the first to note that mechanical responses of protein polymers can be permanently altered through a temperature-induced change in microphase mixing.

Keywords

Protein polymers; thermal annealing; microphase separation; biomechanics; fiber network

Introduction

The fabrication of artificial organs and engineered living tissues is dependent on the ability to generate and assemble novel materials into appropriate 2-D or 3-D structures and to precisely tailor material-related properties to achieve a desired clinical response[1]. To that end, the development of artificial extracellular matrices is integral to fashioning microenvironments that are engineered for optimal mechanical and biological performance. It is likely that the

*Address correspondence to: Elliot L. Chaikof, M.D., Ph.D. 101 Woodruff Circle, Room 5105, Emory University, Atlanta, GA 30322, Phone (404) 727-8413, Fax: (404) 727-3660, E-mail: echaiko@emory.edu.

design of artificial matrices will require the emulation of many, if not all, of the morphological and physiologic features of native tissues. Indeed, as the ability to control the physical and biological properties of scaffolding materials improves, the quality of tissues thus formed is enhanced. In the late 1990s, our group was the first to demonstrate that electrospinning could be used to fabricate native and recombinant elastin-mimetic proteins and native collagen into non-woven fiber networks comprised of nanometer scale fibers that more closely mimic native scaffolds[2–6].

We have recently demonstrated that elastin-mimetic protein polymers can be genetically engineered with complex amphiphilic block sequences[7–10]. Notably, the segregation of the protein blocks into compositionally, structurally, and spatially distinct domains affords ordered structures on the nanometer-to-micrometer size range that have unique mechanical, chemical, and biological properties. The biosynthetic scheme for generating self-assembling recombinant proteins has been based upon a convergent strategy for assembling multiple blocks of concatemerized DNA cassettes by sequential ligation[11]. To date, this strategy has been used to design amphiphilic multiblock proteins (e.g., diblock, triblock, and tetrablocks) ranging from 100 to 200 kDa in molecular weight [7,8,12]. The protein sequences used to design these protein block copolymers were derived in part from a consideration of the primary structure of elastin. Specifically, we have synthesized and characterized a series of elastomeric triblock copolymers capable of virtual or physical crosslink formation[7–9,11,12]. Proteins were synthesized that incorporate identical hydrophobic endblocks with a repeat sequence of [(IPAVG)₄(VPAVG)], separated by a central hydrophilic block containing [(VPGVG)₂(VPGEG)(VPGVG)₂] repeats. While all elastin-mimetic polypeptides of sufficient size display an inverse transition temperature (T_i), coacervation of the hydrophilic block alone does not occur at temperatures as high as 75°C, when in dissolved in water at pH 7.4. In contrast, the hydrophobic endblocks have a T_i below 21°C (~13–21°C) and, as a consequence, recombinant triblock protein polymers reversibly self-assemble from concentrated aqueous solution at 37°C to form a stable, water-solvated, interlocking protein network [7–9,12].

The versatility of these materials has been displayed through the capacity to process triblock protein polymers into a variety of forms, including films, gels, and fiber networks [7–9,12, 13]. Recent two dimensional Fourier transform infrared (FTIR) spectroscopy studies have revealed a conformational transformation in the protein endblock above the inverse transition temperature from helix to sheet-like structures that tightly assemble into physical or virtual crosslinks [14].

In this report, electrospinning was employed to fabricate three dimensional fiber networks from a triblock protein polymer and the effects of thermal annealing on network mechanical responses investigated. Significantly, while macroscopic and microscopic morphology of the network structure was unchanged, thermal annealing triggered permanent changes associated with enhanced interphase mixing of both hydrophobic and hydrophilic blocks. As a consequence, permanent changes in network swelling behavior and mechanical responses were observed. To our knowledge, these investigations are the first to note that mechanical responses of protein polymers can be permanently altered through a temperature induced change in microphase mixing.

Materials and Methods

Synthesis of elastin-mimetic triblock copolymer (B9)

Synthetic methods used to produce the DNA inserts that encode the various block copolymers have been described previously [7,9,11]. Oligonucleotide cassettes encoding the elastomeric midblock (**A**) and plastic-like (**B**) endblocks were independently synthesized and inserted into the *Bam*H I/*Hin*D III sites within the polylinkers of pZErO-1 and pZErO-2, respectively.

Recombinant clones were isolated after propagation in *E. coli* strain Top10F' and double-stranded DNA sequence analysis verified the identity of the DNA inserts A and B. These clones were propagated in *E. coli* strain SCS110 in order to isolate preparative amounts of plasmid DNA. DNA monomers **A** and **B** were liberated from the respective plasmids via restriction digestion with *Bsp*M I and *Sex*A I, respectively. Self-ligation of each DNA cassette afforded a population of concatemers encoding repeats of the midblock and endblock sequences, respectively.

Concatemers derived from DNA monomer **B** were inserted into the *Bsp*M I site of the modified polylinker in plasmid pET-24a. A concatemer encoding sixteen repeats of the plastic-like sequence was isolated and identified via restriction cleavage with *Kpn* I and *Pst* I. Double-stranded DNA sequence analysis confirmed the integrity of the concatemer within the recombinant plasmid, which was labeled pPC. The concatemer was liberated from pPC via restriction cleavage with *Kpn* I and *Pst* I and purified via preparative agarose gel electrophoresis. Enzymatic ligation was used to join the concatemer cassette to the *Kpn* I/*Pst* I sites within another modified polylinker in plasmid pET-24a. Double stranded DNA sequence analysis confirmed the integrity of the concatemer within the recombinant plasmid, which was labeled pPN. The pair of recombinant plasmids pPN and pPC encoded the N-terminal and C-terminal domains, respectively, of the triblock polymer. Restriction cleavage of each plasmid with *Sex*A I and *Xma* I afforded two fragments, which were separated via preparative agarose gel electrophoresis. Enzymatic ligation of the fragment containing PN and the fragment containing PC afforded the plastic flanking sequences in a single contiguous reading frame within plasmid pET-24a. This plasmid was identified as pP2.

Plasmid pP2 was propagated in *E. coli* strain SCS110 and cleaved with restriction endonuclease *Sex*A I. A concatemer encoding the elastin sequence **A** was inserted into the compatible *Sex*A I site. The recombinant clone was analyzed by restriction digestion with *Kpn* I and *Pst* I, followed by agarose gel electrophoresis. The clone, p**B9** was isolated that contained 48 repeats of the elastic sequence A, and encoded the triblock **B9** protein.

Plasmid p**B9** was used to transform the *E. coli* expression strain BL21-Gold(DE3). Large-scale fermentation (3 L) was performed at 37°C in Terrific Broth (TB) medium supplemented with kanamycin (50 mg/mL). The fermentation cultures were incubated under antibiotic selection for 36–48 h at 37°C with agitation at 175 rpm in an orbital shaker. Cells were harvested via centrifugation at 4°C and 4000 g for 20 min and the cell pellet was resuspended in lysis buffer (150 mL; 100mM NaCl, 50mM Tris-HCl, pH 8.0) and stored at –80°C. The frozen cells were lysed by three freeze/thaw cycles. Lysozyme (1 mg/mL), protease inhibitor cocktail (5 mL), benzonase (25 U/mL), and MgCl₂ (1 mM) were added to the lysate and the mixture was incubated at 25°C for 30 min. The cell lysate was incubated for 12 h at 4°C and was centrifuged at 19,000g for 30 min at 4°C to pellet the cell debris. The target proteins were purified from the clarified cell lysate by three to five cycles of temperature-induced precipitation (4°C/37°C) from 500 mM NaCl solution. Dialysis and lyophilization afforded the protein as a fibrous solid with typical yields of 418 mg/L.

Fabrication of thermally annealed elastin-mimetic fiber networks

Previous work has established electrospinning as a useful technique for producing nonwoven protein-based fabrics [2–6]. In this study a 12 wt% solution of **B9** was prepared by dissolving lyophilized protein in 2,2,2 trifluoroethanol (TFE) at room temperature. The solution was extruded at ambient temperature and pressure using a syringe pump (Havard Apparatus, Inc) at a flow rate of 150 µL/min through a positively charged needle (18G x 4 in). A high voltage, low-current power supply (ES30P/DDPM, Gamma High Voltage Research, Inc) was used to generate an electric potential gradient at approximately 18 kV. Fibers were collected on a grounded stainless steel mandrel (d=3.18 mm for ring testing studies, d=6 mm for DMTA

studies) located 7–10 mm from the needle tip. The mandrel was subject to rotational and translational motion during the electrospinning process to create a nonwoven fabric conduit. The electrospun conduit was removed from the mandrel in the dehydrated state and used in subsequent experiments. In selected experiments, electrospun fabrics were thermally annealed at either 50 or 60°C in phosphate buffer saline (PBS) (pH 7.4) for four hours. Following annealing, samples were equilibrated in PBS at 37°C for 16–24 hours. For comparative analysis, non-annealed samples were incubated in PBS at 37°C for 16–24 hours.

Mechanical behavior of electrospun fiber networks

Uniaxial stress-strain properties measured with respect to the radial axis of the conduit—The electrospun conduit was sectioned into 3 mm length rings, hydrated in PBS at 37, 50, or 60 °C for four hours and stored at 37 °C in PBS for 16–24 hours prior to testing. Four reference beads (~300 µm) were attached to the surface of the ring, two on each wall. Following the placement of the beads, samples were loaded on two hooks in a ring testing apparatus, and strained to failure at a rate of 5 mm/min as force/displacement data were recorded through load cell signal acquisition and digital imaging, respectively [15,16]. Ultimate tensile strength was defined as the maximum stress withstood by samples with respect to the original cross-sectional area. The elastic modulus was determined by the slope of the region extending between 25–75% of the ultimate tensile strength.

Uniaxial stress-strain properties measured with respect to the longitudinal axis of the conduit—The electrospun conduit was cut longitudinally and pressed flat for 24 hours. The flattened tube was hydrated at either 37 or 60°C in phosphate buffered saline (PBS) for four hours and incubated at 37°C overnight. Samples were sectioned using a dog-bone shaped stainless steel die with gauge dimensions of 13 × 4.75 mm. Hydrated fabric thickness was measured by optical microscopy using a standard image analysis protocol. Mechanical characterization of the hydrated fiber networks was performed on a Dynamic Mechanical Thermal Analyzer DMTA V with a 15N load cell in the inverted orientation. Samples were immersed in a 37°C PBS bath and strained at a constant rate of 5 mm/min. Tensile tests were not conducted to failure as the travel distance of the DMTA drive shaft was 23 mm, which allowed for stretch to 70% of engineering strain. Thus, only the Young's modulus was obtained from this data set. To characterize ultimate tensile strength, a miniature materials tester, a Minimat 2000 (Rheometric Scientific) was used in tensile deformation mode at a rate of 5 mm/min. Dog-bone shaped samples, with gauge dimensions of 13 mm x 4.75 mm, were examined. Samples were tested under ambient conditions and were coated in thin layer of mineral oil prior to loading to limit evaporation during the test. Ultimate tensile strength and elastic modulus were determined, as described above.

Stress relaxation analysis—Dog-bone shaped samples were immersed in PBS at 37°C and strained at 5 mm/min to 64% strain. The evolution of stress over time was examined for times greater than 50 minutes.

Statistical analysis—The Student's two-tailed unpaired t-test was used to evaluate data sets collected from different constructs to assess batch-to-batch variation. Statistically significant differences were not observed among different samples prepared under identical annealing conditions (37, 50, 60°C). Additionally, Student's two-tailed unpaired t-test was used to establish statistical significance ($p < 0.05$) of mechanical properties measured for non-annealed (37°C) and thermally annealed (50 and 60°C) samples.

Equilibrium water content in B9 fiber networks

Fabric specimens measuring 7 × 7 mm were sectioned from an electrospun conduit. Specimens were either hydrated at 37°C or annealed at 60°C for four hours. In all cases, this treatment

was followed by a 24 hour incubation at 37°C. Fully hydrated weights were measured using a Mettler balance. Dry weights were measured after drying the samples for more than 48 hours under vacuum at room temperature. Equilibrium water content and equilibrium swelling ratios were determined according to the Equation (1) and Equation (2), respectively [17].

$$\text{Equilibrium Water Content} = \frac{\text{hydrated weight} - \text{dehydrated weight}}{\text{hydrated weight}} \quad (1)$$

$$\text{Equilibrium Swelling Ratio} = \frac{\text{hydrated weight}}{\text{dehydrated weight}} \quad (2)$$

Cryo-High Resolution Scanning Electron Microscopy (Cryo-HRSEM)

An electrospun conduit was sectioned and either incubated at 37°C or annealed at 60°C in ddH₂O for four hours followed by a second incubation at 37°C overnight. Small sections of hydrated fiber networks were applied to flat-bottom-well gold planchets and immediately plunge-frozen in liquid ethane at −183°C and stored in liquid nitrogen (LN₂). Frozen samples were transferred to a pre-chilled cryo-stage (< −170°C) placed into the Denton DV-602 Cr coater and allowed to equilibrate under vacuum at 10^{−7} torr. Freeze drying of the samples was performed by increasing the temperature to −85°C for 10 minutes. The specimens were sputter coated with 2 nm of chromium [18–21]. The chromium-coated specimen was transferred to the upper stage of the DS-130F field emission scanning electron microscope operated at 5 kV, warmed to between −120 and −110°C and allowed to equilibrate prior to imaging. Care was taken to avoid frost contamination during all transfers and processes [20]. Cryo-HRSEM images of low (1000x), medium (50,000x) and high (>100,000x) magnifications were digitally collected.

Solid-State ¹H NMR Spectroscopy

Electrospun **B9** fiber networks were hydrated in D₂O at 37°C for 16–24 hours prior to testing. D₂O was used instead of H₂O in order to reduce the intensity of the water peak, which otherwise would have dominated the spectrum such that the detection of the polymer component would be difficult. Excess fluid was carefully removed from these hydrated specimens prior to their placement in 4 mm MAS rotors. ¹H solid-state NMR experiments were performed on a Bruker DSX400 NMR spectrometer operating at a magnetic field of 9.4 T. Samples were investigated at 37°C (310°K) in a conventional CP-MAS probe at a spinning speed of 2 kHz. This provided ¹H spectra with adequate chemical shift resolution to distinguish the major chemical components. After an initial study, the sample was annealed by setting the temperature in the NMR probe to 60°C while maintaining MAS. After 4 hours the temperature was reduced back to 37°C and the sample was investigated using the identical protocol as previously used for non-annealed samples.

The mobility of individual sites in annealed and non-annealed samples was investigated using T₂-CPMG relaxation experiments under conditions of MAS. This experiment consisted of a 90° pulse at a length of 5 μs followed by a train of an increasing number of π-pulses at a length of 10 μs separated by a time interval of 200 μs [22].

For CPMG experiments studying the mobility of individual sites in the polymer (i.e. remaining water moieties and aliphatic components), a mono-exponential relaxation behavior was not found. Therefore, the relaxation could be fitted to equation (3), assuming a two-component relaxation mechanism using the software package IGOR Pro (Wavemetrics).

$$I(TE) = w_a \exp \left\{ -\frac{TE}{T_{2a}} \right\} + w_b \exp \left\{ -\frac{TE}{T_{2b}} \right\} + w_c \quad (3)$$

Results and Discussion

The fabrication of fibers and fiber networks from recombinant elastin-mimetic proteins serves as a foundation for the design of protein scaffolds for tissue engineering (Figure 1A) [5, 7]. We have previously noted that 2,2,2 trifluoroethanol (TFE) is a convenient solvent for room temperature electrospinning of elastin-mimetic protein polymers, as coacervation of concentrated protein solutions is not observed (Figure 1B and C) [9]. Prior investigations have also revealed that TFE promotes interphase mixing between both hydrophilic and hydrophobic blocks of **B9**, which under conditions of uniaxial mechanical testing increases Young's modulus and ultimate tensile strength [7].

Thermal annealing has been employed as a processing strategy to alter the microstructure of polymeric materials, most often to increase the crystallinity or to modulate microphase separation in order to improve material or application dependent properties, including strength or stiffness [23–25], hardness [23], viscoelastic properties [26,27], water absorptive properties [26,28], or photoluminescence [28]. In particular, thermal annealing for one hour at 160°C of polyvinyl alcohol (PVA) hydrogels resulted in increased creep resistance and decreased water content which was attributed to a thermally induced change in microstructure initiating a collapse in hydrogel pore structure [26,29,30]. Additionally, thermal annealing of poly (L-lactic acid) (PLLA) electrospun nanofibers in the dry state at 75°C for 24 hours resulted in a decrease in fiber diameter accompanied by enhanced stiffness, strength, creep resistance and decreased in water content. This was attributed to thermally induced microstructural changes involving growth of closely-packed crystallites [24].

Mechanical responses

Thermal annealing significantly altered the tensile properties protein polymer fiber networks with an increase in elastic modulus and tensile strength that was commensurate with the temperature at which annealing occurred (Figure 2, Table 1). Both elastic modulus and tensile strength increased after thermal treatment at 50°C, but the largest effect was noted after treatment at 60 °C. After sample annealing at 60°C for 4 hours, the Young's modulus at 37°C doubled from 0.170 ± 0.010 MPa to 0.366 ± 0.05 MPa, which is comparable to values measured for elastin in the arterial wall (~ 0.3 MPa) [31]. Likewise, tensile strength increased 30% from 0.079 ± 0.008 MPa to 0.119 ± 0.015 MPa. As these samples consisted of randomly oriented fiber networks, similar properties were observed in longitudinal and circumferential directions. The mechanical isotropy observed in the electrospun materials is consistent with the mechanical response of elastin within the aortic wall [32].

When deformation is held constant, a relaxation of the imposed tensile stress is observed. This phenomenon is a result of the disappearance of frictional forces, rearrangement of polymer chains, and possibly micro-damage to the protein. Stress relaxation of **B9** fiber networks reveals rapid relaxation of imposed tensile stress following deformation to 64% strain. The stress relaxation took place in the first two hundred seconds. At ten minutes, engineering stress had dropped approximately 45% and stabilized, which we speculate is related to both the structural re-orientation of anisotropic fibers in the direction of deformation along with conformational rearrangements of protein chains and network entanglements. Thermal annealing reduced stress relaxation, with rapid relaxation occurring in the first tens of seconds with stabilization of relaxed stress at 50 minutes (Figure 3C). A similar improvement in time dependent viscoelastic properties was observed upon thermal annealing polymeric systems such as

hydroxyapatite-reinforced polyethylene composites [33] and polyvinyl alcohol hydrogels [26]. Significant improvement in creep responses accompanied a thermally induced increase in crystallinity in these systems.

Equilibrium swelling ratio and water content

Temperature induced changes in mechanical properties were associated with a ~50% decrease in the equilibrium swelling ratio of annealed fiber networks (Table 2). An inverse relationship between material stiffness or strength and water content has been observed for biological [33–36] and polymeric materials [26,33]. Specifically, annealing synthetic polymers above their crystallization temperatures but below the melting temperature reveals an associated increase in stiffness and strength with a decrease in water content commensurate with thermal induced changes in morphology [24,33]. Specifically, it has been previously observed with polyvinyl alcohol hydrogels, that thermal annealing above the crystallization temperature but below the melting temperature decreases water content through a proposed collapse of pores within the hydrogel structure containing water molecules. In fact, recent investigations have sought to preserve pore spacing during annealing treatments by filling pores with poly(ethylene glycol) (PEG) to prevent collapse and have observed equilibrium water contents of 83% compared to 28% in gels processed without PEG [26,29,30].

Previous studies have reported that thermal annealing of polymeric materials alter mechanical response by inducing changes in morphology, i.e. pore structure or other macro effects [24, 26], molecular structure [24,27,28,37,38], or microphase separation [39]. Although the effect of thermal annealing on protein-based materials had not previously been investigated, we speculated that temperature induced alterations of mesoscopic and/or microscopic structure may have been responsible for the observed change in mechanical properties. Cryo-HRSEM and solid-state NMR spectroscopy were used to further define temperature-induced changes on protein polymer morphology, microphase separation of block structure, and secondary chain conformation.

Surface morphology

Cryo-HRSEM was employed to evaluate the morphology of hydrated electrospun fiber networks [18,21,40]. By raising the specimen stage temperature from -170 to -85°C , water was sublimated from the surface of the specimen. The surface topography of annealed and non-annealed samples revealed a similar porous microstructure (Figure 3A). Although reminiscent of the ‘open cell’ morphology observed for **B9** films cast from water at room temperature with pore sizes of 500 nm [7], pore sizes within the electrospun fibers were smaller, on the order of 100 nm. Surprisingly, these morphological features differed significantly from those previously observed for **B9** films cast from TFE, which were characterized by the presence 200 nm diameter fibers [7]. This observation highlights the potential impact of the duration of solvent evaporation on microstructure. Because the surface area is much greater for electrospun fibers than cast films, TFE solvent evaporation likely occurs within microseconds whereas TFE evaporation from cast films takes place over a period of hours. A comparative analysis of annealed and non-annealed networks revealed subtle differences in microstructure within the electrospun fibers (Figure 3B, C versus Figure 3D, E) including a change in pore size and texture of the polymer network. Specifically, annealed fiber networks displayed smaller pore sizes of ~50 nm compared to non-annealed samples that exhibited pore sizes of ~100 nm. Additionally, surface roughness observed in non-annealed samples decreases upon thermal annealing, possibly as a consequence of thermally induced interphase mixing of the hydrophobic and hydrophilic domains of **B9**.

Solid-state ^1H NMR spectroscopy of electrospun fiber networks

Solid-state ^1H -NMR spectroscopy was used to discern the structural differences of annealed and non-annealed electrospun **B9** fiber networks. NMR experiments conducted under magic angle spinning (MAS) revealed identical chemical structure for annealed and non-annealed **B9** fibers networks hydrated in D_2O (Figure 4). Indeed, the presence of similar OH peak integrals in both spectra, confirms that no water (D_2O) was lost as a result of sample annealing in the MAS rotors.

Relative molecular mobilities for both samples and the associated water component were quantitatively characterized by measuring T_2 spin-spin relaxation values through a Carr-Purcell-Meiboom-Gill (CPMG) experiment [22]. Specifically, the observed relaxation behavior was fit to the biexponential relaxation function where I is the measured peak intensity as a function of the relaxation delay (TE), w_a , w_b denote the respective amounts of spins with different relaxation times T_{2a} and T_{2b} . In some instances, it was necessary to assume a minor influence of a third component $w_c \neq 0$, which might be due to a relaxation component with a very long relaxation time or an offset of the detected baseline. Admittedly, a more complex mechanism than that described by equation (3) is likely. Such a mechanism would likely involve a distribution of correlation times caused by moieties in different hydration states. This, however, would require the introduction of additional parameters, which could not be determined unambiguously by fitting. Therefore, equation (3) provides a simple and effective method for quantitative comparison of the relaxation data observed for different samples.

These data suggests that annealed **B9** fiber networks exhibited lower values for T_{2a} and T_{2b} , which indicates decreased molecular motion when compared with their non-annealed counterparts (Figure 5, Table 3). Interestingly, as compared to our prior studies of hydrated **B9** films, hydrated electrospun fiber networks exhibit an intermediate mobility when compared to films cast either from TFE in which molecular mobility is most restricted or those cast from water at room temperature in which chain dynamics display the greatest degree of mobility [22]. Mobility has been correlated with microstructural phase separation phenomena. Previous reports note processing dependent differences in microstructure of water and TFE cast films. Water cast films displayed an open cell morphology, while those cast from TFE were characterized by highly aligned fiber networks. The open cell morphology is a consequence of hydrophobic aggregation of the plastic-like domains interconnected by the highly mobile elastin-like domains. TFE cast films composed of aligned fiber networks display an interpenetration of these domains. Lower values for T_{2a} and T_{2b} were exhibited by TFE cast films, which indicates decreased molecular motion, a consequence of decreased microphase separation of the elastic and plastic-like domains as compared to water cast films [7]. Similarly, the change observed in T_{2a} and T_{2b} values induced by thermal annealing is likely indicative of a thermal induced change in molecular structure resulting in a greater interpenetration of elastic and plastic-like domains. Significantly, the interpenetration of midblock and endblock polymer segments is not reversed upon lowering of sample temperature to 37°C , which suggests that the polymer chains remain kinetically trapped. We have previously demonstrated that solvent induced interphase mixing of the plastic-like hydrophobic domains and elastomeric hydrophilic domains of **B9** affords materials that show plastic deformation under stress with tensile properties resembling those of the plastic block alone [7]. Whether block mixing is induced by choice of solvents or by thermal treatment, these data suggest that the sustained load is largely carried by plastic-like $[(\text{IPAVG})_4(\text{VPAVG})]$ domains that extend over relatively long regions of the material.

Conclusions

In summary, thermal conditioning has a substantial impact on the macroscopic properties of a recombinant protein polymer designed as a multiblock thermoplastic elastomer. One

manifestation of this effect is an alteration of the mechanical responses of a protein fiber network. The data presented herein demonstrate that the thermally induced alteration in mechanical properties is a consequence of multiple phenomena occurring over nano- and micrometer length scales. Specifically, we observed a temperature triggered phase mixing of midblock and endblock regions with an increase in plastic-like material characteristics. In addition, a decrease in network pore size and equilibrium water swelling was noted after thermal annealing. Significantly, thermal annealing provides an additional modality for tunable control of a potential wide range of material properties that are dependent on the structural polymorphism displayed by protein-based multiblock copolymers.

REFERENCES

1. Chaikof EL, Matthew H, Kohn J, Mikos AG, Prestwich GD, Yip CM. *Ann N Y Acad Sci* 2002;961:96. [PubMed: 12081873]
2. Huang L, McMillan RA, Apkarian RP, Pourdeyhi B, Conticello VP, Chaikof EL. *Macromolecules* 2000;33:2989.
3. Huang L, Apkarian RP, Chaikof EL. *Scanning* 2001;23:372. [PubMed: 11770932]
4. Huang L, Nagapudi K, Apkarian RP, Chaikof EL. *J Biomater Sci Polym Ed* 2001;12:979. [PubMed: 11787524]
5. Nagapudi K, Huang L, McMillan RA, Brinkman W, Conticello VP, Chaikof EL. *Macromolecules* 2002;35:1730.
6. Tirrell DA. *Nature* 2004;430:837. [PubMed: 15318200]
7. Nagapudi K, Brinkman WT, Thomas BS, Wright ER, Conticello VP, Chaikof EL. *Macromolecules* 2005;38:345.
8. Wu X, Sallach R, Haller CA, Caves JA, Nagapudi K, Conticello VP, Levenston ME, Chaikof EL. *Biomacromolecules* 2005;6:3037. [PubMed: 16283724]
9. Nagapudi K, Brinkman WT, Thomas BS, Park JO, Srinivasarao M, Wright E, Conticello VP, Chaikof EL. *Biomaterials* 2005;26:4695. [PubMed: 15763249]
10. Sun XL, Haller CA, Wu X, Conticello VP, Chaikof EL. *J Proteome Res* 2005;4:2355. [PubMed: 16335985]
11. Wright ER, Conticello VP. *Adv Drug Deliv Rev* 2002;54:1057. [PubMed: 12384307]
12. Wu X, Sallach RE, Caves JM, Conticello VP, Chaikof EL. *Biomacromolecules* 2008;9:1787. [PubMed: 18558738]
13. Jordan SW, Haller CA, Sallach RE, Apkarian RP, Hanson SR, Chaikof EL. *Biomaterials* 2007;28:1191. [PubMed: 17087991]
14. Sallach RE, Wei M, Biswas N, Conticello VP, Lecommandoux S, Dluhy RA, Chaikof EL. *J Am Chem Soc* 2006;128:12014. [PubMed: 16953644]
15. Bergland JD, Mohseni MM, Nerem RM, Sambanis A. *Biomaterials* 2003;24:1241. [PubMed: 12527265]
16. Seliktar D, Black RA, Vito RP, Nerem RM. *Annals of Biomedical Engineering* 2000;28:351. [PubMed: 10870892]
17. Dinerman AA, Cappello J, Ghandehari H, Hoag SW. *Biomaterials* 2002;23:4203. [PubMed: 12194523]
18. Apkarian RP, Wright ER, Seredyuk VA, Eustis S, Lyon LA, Conticello VP, Menger FM. *Microsc Microanal* 2003;9:286. [PubMed: 12901763]
19. Apkarian RP. *Scanning Microsc* 1994;8:289. [PubMed: 7701300]
20. Apkarian RP, Caran KL, Robinson KA. *Microsc Microanal* 1999;5:197. [PubMed: 10383992]
21. Wright ER, Conticello VP, Apkarian RP. *Microsc Microanal* 2003;9:171. [PubMed: 12807669]
22. Meiboom S, Gill D. *Rev Sci Instrum* 1958;29:688.
23. Suwanprateeb J. *Polym Test* 2004;23:157.
24. Tan E, Lim C. *Nanotechnology* 2006;17:2649.
25. Tiemprateeb S, Hemachandra K, Suwanprateeb J. *Polym Test* 2000;19:329.

26. Bodugoz-Senturk H, Choi J, Oral E, Kung JH, Macias CE, Braithwaite G, Muratoglu OK. *Biomaterials* 2008;29:141. [PubMed: 17950839]
27. Suwanprateeb J, Tanner KE, Turner S, Bonfield W. *J Biomed Mater Res* 1998;39:16. [PubMed: 9429092]
28. Li G, Shrotriya V, Yao Y, Huang J, Yang Y. *J Mater Chem* 2007;17:3126.
29. Cha W, Hyon S-H, Ikada Y. *Macromol Chem Phys* 1993;194:2433.
30. Cha W, Hyon S-H, Oka M, Ikada Y. *Macromol Symp* 1996;109:115.
31. Mithieux SM, Rasko JEJ, Weiss AS. *Biomaterials* 2004;25:4921. [PubMed: 15109852]
32. Marsh JN, Takiuchi S, Lin SJ, Lanza GM, Wickline SA. *J Acoust Soc Am* 2004;115:2032. [PubMed: 15139613]
33. Yamaguchi I, Itoh S, Suzuki M, Sakane M, Osaka A, Tanaka J. *Biomaterials* 2003;24:2031. [PubMed: 12628822]
34. Berchane NS, Andrews MJ, Kerr S, Slater NK, Jebrail FF. *J Mater Sci Mater Med.* 2008
35. Lillie MA, Gosline JM. *Biopolymers* 1990;29:1147. [PubMed: 2369629]
36. Chalmers GW, Gosline JM, Lillie MA. *J Exp Biol* 1999;202:301. [PubMed: 9882642]
37. Park MJ, Nedoma AJ, Geissler PL, Balsara NP. *Macromolecules* 2008;41:2271.
38. Elabd YA, Napadensky E, Walker CW, Winey K. *Macromolecules* 2006;39:399.
39. Martin DJ, Meijs GF, Renwick GM, Gunatillake PA, McCarthy SJ. *J Appl Polym Sci* 1996;60:557.
40. Peters KR. *Scan Electron Microsc* 1985:1519. [PubMed: 4095499]

Figure 1. Fiber networks produced from an electrospun recombinant elastin-mimetic protein multiblock copolymer

(A) Cryo-HRSEM micrograph of fiber networks electrospun from at 12-wt% solution of **B9** in TFE. Samples were plunge frozen in liquid ethane (-183°C), freeze dried at -85°C , sputter coated with 2 nm of chromium, and imaged at -120°C and -110°C . Microstructure of hydrated fiber at 1000x. (B) A tubular conduit was fabricated from electrospun protein fibers using a mandrel as the collecting apparatus.

Figure 2. Effect of thermal annealing on the mechanical responses of electrospun protein fiber networks

Electrospun fabrics were thermally annealed at either 50 or 60°C in phosphate buffer saline (PBS) (pH 7.4) for four hours. Following annealing, samples were equilibrated in PBS at 37°C for 16–24 hours. For comparative analysis, non-annealed samples were incubated in PBS at 37°C for 16–24 hours. Thermal annealing increases elastic modulus (**A**) and ultimate tensile stress (**B**) of the fiber networks in a temperature dependent manner. Data is presented as mean $\pm$ standard error of 6–10 experimental sets. Elastic modulus was determined by linear regression of stress-strain curves ($R^2 > 0.9$) * $p \leq 0.05$. (**C**) Stress relaxation is increased by thermal pre-conditioning of protein fiber networks. Representative curves present data collected from samples subjected to 64% strain while immersed in PBS at 37°C. (**D**) Representative uniaxial stress-strain curves generated from ring testing of fiber networks strained at a rate of 2 mm/min in 37°C PBS.

Figure 3. Cryo-HRSEM micrographs of fiber networks electrospun from at 12-wt% solution of B9 in TFE and hydrated at 37 °C

Samples were plunge frozen in liquid ethane (-183°C), freeze dried at -85°C , sputter coated with 2 nm of chromium, and imaged at -120°C and -110°C . (A) Sectioned electrospun fiber (1000x). Microstructure of hydrated fiber at 150kx (B) and 50kx (C). Microstructure of thermally annealed fibers at 150kx (D) and 50kx (E). Annealed fibers display 50 nm pores as compared to non-treated fibers in which 100 nm pores were observed. A smoothing of surface topography was also observed after thermal treatment.

Figure 4. ^1H NMR MAS spectrum of B9 protein fiber networks

Thermal annealing at 60°C under conditions of MAS did not lead to any noticeable change of the spectrum. Identical intensity of the OH peak prior to or after temperature treatment suggests that thermal annealing is not associated with loss of water (D_2O).

Figure 5. NMR T_2 spin relaxation for protein polymer chains and associated water components for fully hydrated samples

Non-annealed **B9** fiber networks (water peaks at 4.67 ppm • and aliphatic peaks at 1.9 ppm ○).

Annealed **B9** fiber networks (water peaks at 4.67 ppm ■ and aliphatic peaks at 1.9 ppm □).

Table 1
Tensile Behavior of Electrospun Protein Fiber Networks

Treatment		Elastic Modulus (MPa)	Tensile Strength (MPa)
37°C Incubation	Circumferential	0.170 ± 0.010	0.079 ± 0.008
	Longitudinal	0.190 ± 0.026	0.108 ± 0.050
60°C Anneal	Circumferential	0.366 ± 0.050	0.119 ± 0.015
	Longitudinal	0.294 ± 0.030	0.111 ± 0.010

* Circumferential refers to mechanical property testing using a ring testing apparatus while longitudinal refers to property testing using DMTA and Minimat testing systems. Results stated as mean $\pm$ standard error.

Table 2
Swelling and Water Content Hydrated Fiber Networks

	Equilibrium Swelling Ratio	Equilibrium Water Content (%)
Non-Annealed B9 Fibers	28.75 ± 2.80	96.2 ± 0.4
Annealed B9 Fibers	$13.55 \pm 1.39^*$	94.4 ± 2.2

*
 $p < 0.05$

Table 3T₂ Relaxation Data for Hydrated Protein Fiber Networks

	w_a	T_{2a} [ms]	w_b	T_{2b} [ms]
Non-annealed				
Water	0.57	165 ± 92	0.43	17.2 ± 9.4
Methyl	0.50	19.2 ± 1.4	0.50	3.0 ± 0.4
Thermally annealed				
Water	0.61	65.0 ± 13	0.49	10.6 ± 4.2
Methyl	0.41	12.2 ± 1.4	0.59	1.7 ± 0.3