2 PHYSICAL PROPERTIES OF ARTIFICIAL EXTRACELLULAR MATRIX PROTEIN FILMS PREPARED BY ISOCYANATE CROSSLINKING

2.1 Abstract

Artificial extracellular matrix proteins, genetically engineered from elastin- and fibronectin-derived repeating units, were crosslinked with hexamethylene diisocyanate in dimethylsulfoxide. The resulting hydrogel films were transparent, uniform, and highly extensible. Their tensile moduli depended on crosslinker concentration and spanned the range characteristic of native elastin. The water content of the films was low (~27%), but the temperature-dependent swelling behavior of the crosslinked materials was reminiscent of the lower critical solution temperature (LCST) property of the soluble polymers.

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2.2 Introduction

Artificial proteins, because their architectures can be precisely controlled, are potentially useful for a variety of biomaterials applications. Here we describe artificial extracellular matrix proteins to address the long-standing problem of high failure rates in small- and medium-diameter vascular grafts. Both the autologous and synthetic (polytetrafluoroethylene and polyethylene terephthalate) grafts currently used have unacceptably low long-term patency.[1,2] The primary causes of graft failure are thrombosis and intimal hyperplasia, the latter associated with a proliferation of smooth muscle cells in blood vessels that eventually blocks circulation.[3,4] Intimal hyperplasia is widely thought to be caused by the unnatural stresses induced by the mismatch in mechanical properties between the graft and the natural vessel,[4,5] or by the inability of the graft to sustain an endothelial cell monolayer, which is critical in the maintenance of vascular health.[6,7] Thus, recent efforts to develop vascular grafts have focused on compliance matching, and on promoting the growth of, or sustaining, a monolayer of endothelial cells on the graft surface.[1,8-10]

We and others have reported artificial proteins designed to mimic the essential mechanical and biological properties of the extracellular matrix.[11-13] In the proteins described here, elastin-based polypentapeptide sequences [$(VPGIG)_x$] provide elasticity, and periodic CS5 cell-binding domains, derived from fibronectin, impart biological function. Lysine residues were incorporated into the protein design to allow amine-specific crosslinking: one N-terminal and two C-terminal lysines were added.[12] The three artificial extracellular matrix (aECM) proteins examined in this work are designated by their molecular

weights (aE16 = 16,117; aE43 = 42,974; aE70 = 69,832); their sequences are listed below in Figure 2.1.[14]

MMASMTGGQQMGRKTMG[LD**"CS5"**G(VPGIG)$_{25}$VP]$_1$LEKAAKLE aE16

MMASMTGGQQMGRKTMG[LD**"CS5"**G(VPGIG)$_{25}$VP]$_3$LEKAAKLE aE43

MMASMTGGQQMGRKTMG[LD**"CS5"**G(VPGIG)$_{25}$VP]$_5$LEKAAKLE aE70

"CS5" = GEEIQIGHIPREDVDYHLYP

Figure 2.1 Amino acid sequences of artificial ECM proteins.

Elastin-like polypeptides $(VPGZG)_x$, where Z is any amino acid, in aqueous solution undergo an aggregative inverse temperature transition upon heating. This entropy-driven transition occurs at a "lower critical solution temperature" (LCST) unique to the polymer. Urry and coworkers studied these polymers in detail.[15,16] The $(VPGIG)_x$ motif was chosen here as the structural basis of the aECM proteins, because it results in proteins with sub-ambient LCST (12 to 16 ºC).[11,12] The sub-ambient LCST limits protein solubility at physiological temperatures and facilitates purification of proteins expressed in bacterial hosts.

The CS5 region, an element of the alternatively spliced type III connecting segment of fibronectin, contains the minimal cell-binding ligand REDV, which targets the $\alpha_4\beta_1$ integrin, a cell-surface receptor.[17] In particular, $\alpha_4\beta_1$ has been demonstrated to mediate endothelial cell adhesion to REDV peptides.[18] Peptides that contain the REDV sequence, when grafted to glycophase glass, have been shown to support the attachment and spreading of endothelial cells, but not of smooth muscle cells and platelets.[19] Artificial extracellular matrix proteins

constructed from alternating CS5 and $(VPGIG)_x$ domains, adsorbed on glass, also support endothelial cell attachment and spreading.[11]

Since the primary amine functional groups are located only at the ends of the protein, amine-selective reagents should be capable of crosslinking the proteins without disrupting the CS5 domains. Welsh et al. crosslinked these proteins with glutaraldehyde and found that the tensile moduli of the resulting films approached that of native elastin, and, as expected, varied inversely with protein molecular weight.[12] However, the results of endothelial cell culture on glutaraldehyde crosslinked films were variable,[20] perhaps because of non-specific interactions between the proteins and endothelial cells or because of the cytotoxicity of glutaraldehyde.[21,22]

Here, hexamethylene diisocyanate (HMDI) was employed as an alternative lysine-targeted crosslinker. HMDI has been employed previously in biomaterials fixation, and its cytotoxicity is considerably lower than that of glutaraldehyde.[22] Films prepared from aECM proteins with HMDI crosslinking were transparent, had low water contents, and were viscoelastic and highly extensible. The films retained the temperature-dependent swelling behavior of the component proteins. Their initial tensile moduli were observed to be similar to that of native elastin, and should be advantageous with respect to application in vascular grafts.

2.3 Experimental

2.3.1 Materials and methods.

Hexamethylene diisocyanate (HMDI) was purchased from Aldrich; dimethylsulfoxide (DMSO) was from Mallinckrodt. Amino acid analysis was performed at the University of California, Davis Molecular Structure Facility via HCl hydrolysis, cation exchange chromatography, and post-column ninhydrin derivatization for quantitation. Bacterial fermentations were performed in a New Brunswick Scientific BioFlo 3000 bioreactor. An Instron model 5542 tensile tester with a 5 N load cell and accompanying software was used to collect tensile data.

2.3.2 Protein expression and purification.

Details of gene construction and expression were reported previously.[12] Artificial genes encoding each of the three proteins were constructed in pET28 vectors; expression was controlled by a bacteriophage T7 promoter inducible by isopropyl β-thiogalactopyranoside (IPTG). Fermentations were performed at 37 °C under antibiotic selection; here, expression was induced by 2.5 mM IPTG when the OD_{600} reached ~8, and cells were harvested 2-4 hours later. The purification scheme, which takes advantage of the inverse temperature transition of the proteins, matches that of Welsh.[12] Briefly, the target proteins partitioned into the pellet of the whole-cell lysate after centrifugation (20,100 × g, 60 min, 24 °C), were resuspended in 4M urea and centrifuged (20,100 × g, 60 min, 4 °C) to remove non-protein cellular debris, dialyzed against water (5 days, 4 °C), and

separated from the precipitated proteins by centrifugation (20,100 × g, 60 min, 4 °C). The supernatant was decanted and warmed to 30 °C to aggregate the target protein, which was then separated by centrifugation (20,100 × g, 60 min, 30 °C), resuspended in cold water, and lyophilized. The purity of the proteins and uniformity of molecular weight was previously established[12] and confirmed here by SDS-PAGE. The yields of aE16, aE43, and aE70 were 1.6, 9.1, and 6.0 g, respectively, for 10 L fermentations.

2.3.3 Crosslinked films.

The protein of interest (500 mg) was dissolved in 5 mL of dimethylsulfoxide (DMSO); the appropriate amount of hexamethylene diisocyanate (12-39 mg) was added, and the solution was mixed rapidly with a pipet. The solution was poured into an open-faced, 6.3 × 6.3 cm, polished aluminum mold and reacted in a desiccator for 8 h. To remove the DMSO, the mold was placed on a hotplate at 50-60 °C overnight. The dried film was hydrated and rinsed with distilled water, removed from the mold, and stored in water at 4 °C. When hydrated, the films were transparent, had a uniform thickness of about 0.15 mm, and were characterized by a water content of about 27%. Smaller (1.0 × 1.5 mm) films, used to obtain results in Figures 2.3 and 2.5, were made in PTFE molds by the same procedure.

2.3.4 Uniaxial tensile testing.

Films were tested in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH adjusted to 7.40 with NaOH) using a specially designed sample chamber, in which the temperature was kept at 37 ± 1 °C by a water jacket. Rectangular test films were pressure-cut from the large cast films with a new razor blade to minimize nicks; extensions-to-break (E_b) were measured on ~2 × 18 mm samples, while moduli were measured on ~5 × 50 mm samples; all samples had a length-to-width aspect ratio of 8 or greater to minimize end effects.[23] Samples were equilibrated in the PBS bath for 30 min prior to testing. Extensions were performed at a rate of 10 % of the gauge length per minute (0.00167 / sec), a standard test rate for polymeric films.[23] Extension-to-break samples consistently broke at one of the grips, where stress concentration is highest; thus the reported E_b are artificially low.

2.3.5 Temperature-dependent length changes.

A film was mounted in the tensile tester, and the PBS bath temperature was controlled by a water jacket and monitored by a thermometer adjacent to the sample. As the temperature changed, the sample length was measured by manually adjusting the distance between the grips to keep the film taut but at zero-stress. Starting from room temperature, the sample was twice cycled between 2 °C and 50 °C; the data presented are from the second cycle. The cooling and heating rates, although not precisely controlled, were approximately 0.2 °C / min.

2.4 Results and discussion

2.4.1 Tensile properties of crosslinked films.

Films used for mechanical testing were made with isocyanate-to-primary amine ratios of 5.0 (5× HMDI) or greater; these films were uniformly insoluble, visually defect-free, and transparent both in the dried and hydrated states. At temperatures above the LCST, the films had water contents, based on weight fraction, of around 27% (Table 2.1). This water content is similar to the 32% reported for hydrated purified elastin at 36 °C.[24] Despite the low water content of these hydrogels, the water is essential to their elasticity; when dried, the films were brittle and their elastic moduli were roughly 1000 times higher than in the hydrated state. Dehydration of the films appeared completely reversible, as the water content was unchanged through five cycles of drying and wetting the films.

Table 2.1 Physical properties (37 °C) of aECM films crosslinked with HMDI. Standard deviations indicated, n = 3. 5× and 10× refer to the molar ratio of isocyanate to primary amines used in preparing the films.

Film	Weight fraction polymer	E (MPa)	G (MPa)	E_b (%)	Pred. M_c
aE16-5×	0.742 ± 0.012	0.71 ± 0.04	0.24 ± 0.01	340 ± 20	11,000
aE43-5×	0.731 ± 0.003	0.58 ± 0.02	0.19 ± 0.02	480 ± 40	13,000
aE70-5×	0.719 ± 0.005	0.40 ± 0.09	0.13 ± 0.03	570 ± 30	19,000
aE43-10×	0.752 ± 0.001	0.93 ± 0.11	0.31 ± 0.06	420 ± 30	8,000

Representative stress-strain curves for the three proteins crosslinked with isocyanate-to-primary amine ratios of 5:1 (designated "aEMW-5×") are shown in Figure 2.2. Each of the crosslinked films was highly extensible and did not break until well into the non-ideal, non-entropic range of the stress-strain curve. While the E_b are artificially low, owing to breakage at the sample ends, the films made from longer proteins were reproducibly more extensible to break. The effect of increasing the crosslinker concentration on aE43 was to stiffen the film and reduce the measured extension to break. A summary of the mechanical testing results is given in Table 2.1 above.

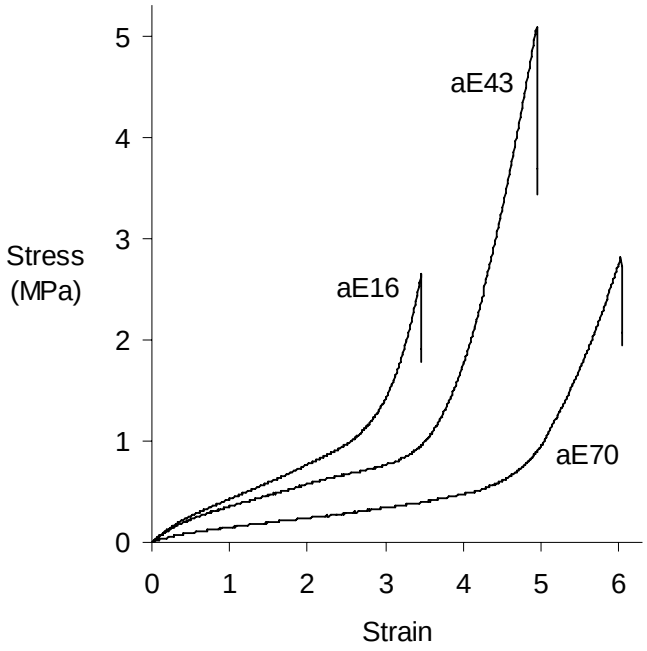

Figure 2.2 Representative tensile behavior of aE16-5×, aE43-5×, and aE70-5× films (each crosslinked with a 5:1 molar ratio of isocyanate to primary amines).

The stress-strain curves are typical of rubbers; at low strain (< 0.5) the slope decreases slightly with increasing strain, an effect associated with the statistical mechanics of chain deformation.[25] At the slow extension rate employed, 10% per minute (.00167 / sec), viscoelastic effects are minimal, and the films are considered to behave as ideal rubbers. This allows details about the molecular architecture to be extracted from the stress-strain behavior. In an ideal network, a plot of σ versus $(\lambda - 1/\lambda^2)$, where σ is the stress and λ is the elongation ratio, is linear. The slope is the shear modulus, G, which in ideal rubbers is equal to one third of the elastic modulus, E.[25] Here, the elastic moduli, E, were calculated from the linear portions of the stress-strain curves, at strain < 0.05, and the shear moduli, G, were obtained from plots of σ versus $(\lambda - 1/\lambda^2)$, up to strains of 0.25 ($\lambda = 1.25$). The shear modulus plots were indeed linear, and the resulting values of G were near one third the values of E, justifying the approximation of these materials as ideal. The elastic moduli span the range of natural elastic fibers, 0.3 to 0.6 MPa.[26]

From a statistical treatment of the ideal network, the shear modulus is inversely proportional to the average molecular weight between crosslinks, M_c, by $G = \rho RT/M_c$.[25] The ideal network treatment assumes Gaussian distribution statistics and freely jointed, non-interacting chains, whereas protein polymers are conformationally restricted and are characterized by strong inter- and intra-molecular interactions. In this case, however, the elastin units appear sufficiently mobile to resolve molecular entanglements at the test rate used, and the films behave nearly ideally. Values of M_c calculated from the equation above, then,

provide an estimate of the chain length between crosslinks. Since the lysine residues are only at the ends of the protein, the M_c should equal the molecular weight of the protein if only lysines react. To calculate ρ, the effective polymer density for each film, a protein density of 1.3 g/cm^3, commonly used for elastin,[27] and the measured water contents of the films were used. The resulting M_c, shown in Table 1, are considerably smaller than expected, especially for films made from high molecular weight proteins (aE70-5×) and high isocyanate ratios (aE43-10×). Slow-resolving entanglements may account for some of the discrepancies between the calculated M_c and the known molecular weight,[28] but the magnitude of the difference may imply that the side chains of residues other than lysine react with the isocyanate, forming additional crosslinks.

The minimum isocyanate-to-primary amine ratio required to crosslink the proteins into films was about 2.0 for aE43 and 3.0 for aE70. At and above these ratios of isocyanate, the dried protein films swelled, but were insoluble in good solvents for the protein such as cold water or DMSO. At slightly lower ratios, such as 1.5 for aE43 and 2.5 for aE70, films appeared to gel and were partially intact when hydrated, but were not completely insolubilized, suggesting partial crosslinking. Larger ratios of isocyanate are required to crosslink aE70, in which the reactive amines are more dilute and intramolecular crosslinking is thought to be more prevalent.

The concentration of diisocyanate used to crosslink the films strongly affected film stiffness. Various stoichiometric ratios of isocyanate to primary amine were used in crosslinking aE43 with HMDI, and the elastic moduli of the

resulting films are plotted in Figure 2.3. Film modulus increases with increasing isocyanate-to-amine ratio, from near-zero in minimally crosslinked films, through the 0.3-0.6 MPa range of native elastin. Above a ratio of ca. 10, the modulus appears to plateau. The fact that the modulus continues to increase at isocyanate:amine ratios greater than 1 may indicate non-productive (intramolecular) crosslinking. Furthermore, if amino acid side chains other than those of lysine react with HMDI, the calculated ratios of isocyanate to reactive groups would be artificially high.

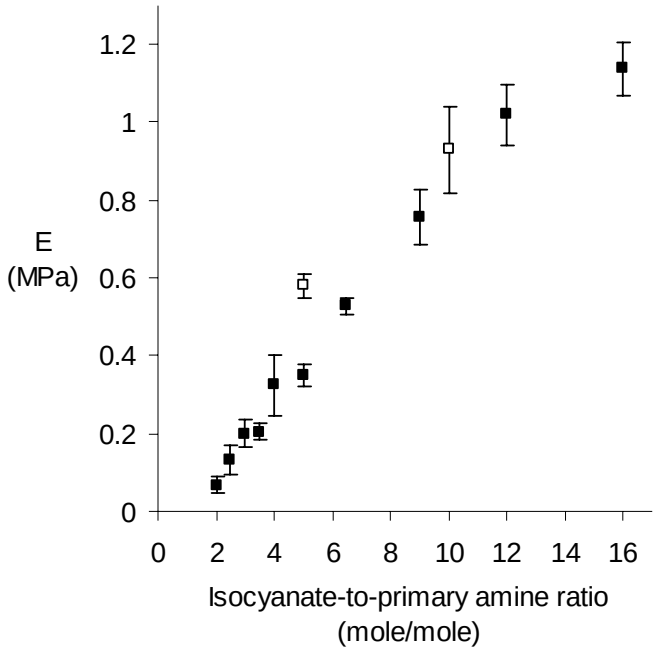

Figure 2.3 Elastic moduli of aE43 films crosslinked with various molar ratios of HMDI-to-protein. The open squares are data reported in Table 1 for large aE43 films cast in aluminum molds. The filled squares are data for films cast from smaller PTFE molds. Errors indicated as one standard deviation (n=3 films).

2.4.2 HMDI reactivity.

Amino acid analysis was used to probe the reactivity of the isocyanate toward side chains other than those of lysine. Table 2.2 indicates measured concentrations of selected amino acids before and after crosslinking for each of the three proteins; it also confirms the purity of the protein by comparing expected to measured compositions. The only significant change appears in the lysine fraction, indicating that at least 65% of lysine residues reacted with the crosslinker in each case. The other amino acid fractions, including those not shown, were experimentally indistinguishable before and after crosslinking. However, other isocyanate-side chain bonds that were formed during crosslinking may have been hydrolyzed to yield the original amino acids during the sample preparation.

Table 2.2. Amino acid analysis results comparing expected and measured mole fractions of selected amino acids in aECM proteins before and after HMDI crosslinking.

%	protein	expected	before XL	after 5×	after 10×
Arg	aE16	1.14	1.28	1.10	
	aE43	0.84	0.96	0.90	0.85
	aE70	0.77	0.93	0.92	
Asp[a]	aE16	1.71	2.08	2.10	
	aE43	1.89	2.18	2.16	2.05
	aE70	1.94	2.23	2.30	
Gly	aE16	32.6	30.3	31.6	
	aE43	34.3	32.1	31.5	32.5
	aE70	34.7	33.2	33.4	
His	aE16	1.14	1.18	1.18	
	aE43	1.26	1.21	1.23	1.30
	aE70	1.29	1.29	1.26	
Lys	aE16	1.71	1.95	**0.60**	
	aE43	0.63	0.75	**0.20**	**0.27**
	aE70	0.39	0.63	**0.14**	
Val	aE16	15.4	16.2	16.1	
	aE43	17.1	17.6	18.0	17.4
	aE70	17.4	17.6	17.5	

[a] In some preparations amino acid analysis indicated that acidic residues (Asp, Glu) partially react (< 30%)

The activity of HMDI toward lysine is apparent, but the specificity remains uncertain. In general, isocyanates may react with nucleophilic functional groups such as amines, alcohols, and protonated acids.[29] HMDI in particular was observed to react with the side-chains of backbone-protected lysine, cysteine, and histidine, and to a lesser extent tyrosine, in water,[30] but comparable studies in DMSO do not appear to have been reported. Reactions of HMDI with other residues, such as arginine or aspartic acid, are of particular concern because such reactions could alter the functionality of the CS5 cell-binding domain.

2.4.3 Unidimensional temperature swelling.

aE43-10× films swell considerably when cooled from physiological conditions to near 0 °C (Figure 2.4). The transition is centered near 10 °C, which is slightly lower than the reported LCST of 17 °C for aE70 in solution,[12] and coincides well with the LCSTs of 12 °C and 13 °C reported for similar aECM proteins lacking the lysine-containing terminal linkers[31] and with the value of 10 ± 5 °C predicted for $(VPGIG)_x$.[15] Thus, the film retains the thermal transition behavior of the soluble protein. In comparison with the transition of the protein, which at high concentrations occurs over a range of just a few degrees, the transition in the film is much broader. Apparently, crosslinking the film reduces the cooperativity of the transition, perhaps by trapping elastin-like segments of the protein in conformations that alter their tendency to undergo the phase change. Lee et al. also examined the temperature-dependent swelling behavior

of elastin-like materials, and observed a similarly broadened LCST transition in films crosslinked by γ-irradiation.[32,33]

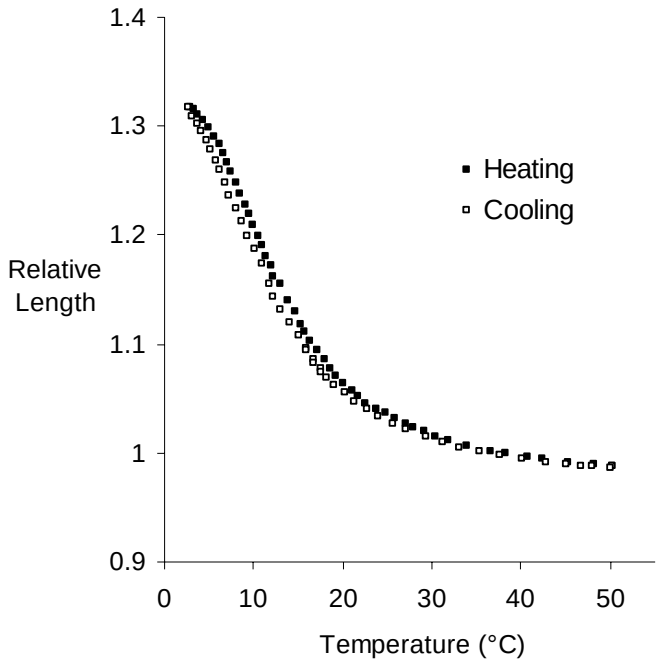

Figure 2.4 Temperature-dependent change in length of an aE43-10× film. Length plotted relative to the length at 37 °C. Hysteresis between the heating and cooling curves is minimal at the slow heating and cooling (0.2 °C / min) rates used.

The extent of swelling of aE43 and aE70 films, as the temperature is reduced from above to below the LCST (from 21 °C to 4 °C), decreased as the crosslinker concentration increased, as indicated in Figure 2.5. It is interesting to compare the length changes observed upon cooling of films in Figure 2.5, 94% for aE43-2× and 74% for aE70-3×, with the 63% increase in radius of gyration of a $(VPGVG)_{18}$ molecule (LCST = 27 ºC) between 42 and 10 ºC, calculated by Li et al. using molecular simulations of a single peptide chain surrounded by water

molecules.[34] Lee et al. observed a wide range of swelling extents upon traversing the LCST in films of $(VPGZG)_x$-based materials crosslinked chemically or with γ-irradiation; as in the present study, the degree of swelling was dependent on crosslink density.[32,33] At similar crosslink densities, however, they observed more extensive swelling than was seen here: for free-radical crosslinked $(VPGIG)_{260}$ with a calculated molecular weight between crosslinks (M_c) of 19,000, they observed a length change upon cooling from above to below the LCST of ~90% (deduced from weight swelling ratio data),[33] compared to the 46% change in length seen here for aE70-5× (M_c = 19,000).

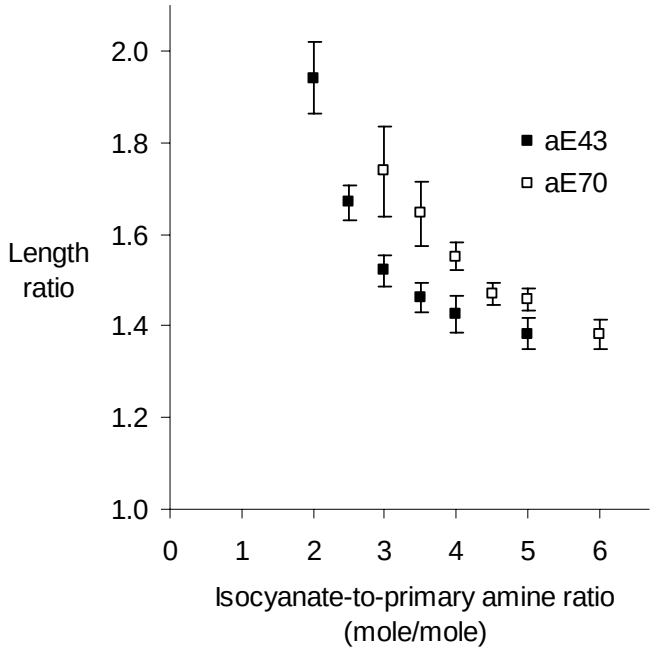

Figure 2.5 Dimensional changes from above to below the LCST in aE43 and aE70 films crosslinked with varying concentrations of HMDI. The length ratio refers to the length of the film in water at 4 °C divided by the length at 21 °C. Errors indicated as one standard deviation (n=4 films).

2.5 Conclusions

Insoluble, coherent, elastic films were made from artificial extracellular matrix proteins by crosslinking with hexamethylene diisocyanate in dimethylsulfoxide. The films were considerably more extensible than previously reported crosslinked elastin-like proteins, which is attributed to their uniformity and lack of defects. The elastic moduli of the films were larger than was observed for the same materials crosslinked with glutaraldehyde,[12] and matched the modulus of native elastin. Additionally, the moduli varied with both protein size and crosslinker concentration; thus, the elastic modulus of the films can be precisely controlled. Assessment of the reactivity of the isocyanate toward protein side chains was incomplete, but amino acid analysis confirmed a change in lysine concentrations after crosslinking. The unexpectedly high modulus of the films may indicate additional reactive side-chains. The engineering demands of the vascular graft warrant continuing characterization of the viscoelastic and biological properties of crosslinked aECM films. The transparency of the films makes them convenient substrates for cell culture studies, which are underway.

Acknowledgement: This work was supported by the National Institutes of Health (R01-HL59987) and the National Science Foundation (BES-9901648).

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