Reversible Hydrogels from Self-Assembling Genetically Engineered Protein Block Copolymers

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A series of triblock protein copolymers composed of a central water-soluble polyelectrolyte segment flanked by two coiled-coil domains was synthesized by genetic engineering methods. The copolymers self-assembled into reversible hydrogels in response to changes in temperature, pH, and the presence or absence of denaturating agent (guanidine hydrochloride, GdnHCl). Hydrogel formation was concentration-dependent, and the concentration needed for hydrogel formation correlated with the oligomerization state of the coiled-coil domains in the protein copolymers. The morphology of the hydrogels, as determined by scanning electron microscopy (SEM), indicated the presence of porous interconnected networks. The thermal stabilities and self-assembling properties of the protein copolymers were successfully controlled by manipulating the amino acid sequences of the coiled-coil domains. The stimuli responsiveness and reversibility of the hydrogel self-assembly suggest that these protein copolymers may have potential in biomedical applications.

Introduction

It has been several decades since hydrophilic polymer networks were proposed for biological uses.[1] Nowadays, hydrogels are widely used as biomaterials, including drug delivery systems and tissue engineering.[2−5] Among these, stimuli-responsive hydrogels are of extreme interests. These hydrogels can undergo volume and phase transitions induced by minor changes in environmental conditions, such as temperature,[6,7] pH,[8,9] solvent,[10] electric and magnetic fields,[11,12] light,[13] and biochemicals.[14,15] Numerous studies have been focused on chemically synthesized systems based on copolymers of *N*-isopropylacrylamide (temperature-sensitive), (meth)acrylic acids (pH-sensitive), and their derivatives. Stimuli-responsive two-component hybrid hydrogels have also been developed, with a hydrophilic synthetic polymer as one component and a molecule of biological origin as the other. The biological component is usually the determinant of the stimuli responsiveness in these hydrogels and could include oligopeptide sequences recognized by specific proteases,[16−18] full length native proteins such as concanavalin A that can associate with glucose,[19] antibodies that can form complexes with antigens,[20] and genetically engineered proteins containing coiled-coil domains[21,22] or immunoglobulin modules from the muscle protein titin.[23]

Protein-based polymers have also been used to prepare stimuli-responsive hydrogels.[24] Elastin-mimetic protein polymer or silk-elastinlike protein polymer-based hydrogels are the most frequently studied protein-based hydrogels.[25−30] These protein polymers are composed of repeating pentapeptide sequences, which are either elastin-like (e.g., VPGVG) and/or silk-like (e.g., GAGAGS). It has been shown that these protein polymers can self-assemble into hydrogels that are temperature- or pH-sensitive.[31] Petka et al.[32] have produced a 230 amino acid recombinant protein triblock copolymer, consisting of two terminal leucine zipper domains flanking a central polyelectrolyte segment, and have shown that their proteins undergo reversible gelation in response to changes in pH or temperature. Recombinant DNA technology has made it possible to design and synthesize these protein-based polymers with defined compositions, sequences, stereochemistry, and molecular weights.[33−35]

The coiled-coil is an attractive module for protein-based polymers. It is one of the basic folding patterns of native proteins, consisting of two or more α-helices winding together and forming a superhelix.[36] The primary structure of the coiled-coil motif is characterized by a repeated heptad sequence, $(abcdefg)_n$, where a and d positions are usually hydrophobic amino acid residues, whereas e and g positions are often charged amino acid residues. The hydrophobic interaction between the a and d residues and the electrostatic interaction between the e and g residues contribute to the stability of the coiled-coil structure. The distinctive association−dissociation and the specific spatial recognition of the coiled-coils make it an ideal candidate for physical cross-linkers in a protein-based polymer hydrogel.

In this report, we describe the design of a series of recombinant triblock (ABA, CBA, ABC, CBC) polypeptides, consisting of two terminal coiled-coil domains (blocks A or C) flanking a central water-soluble random coil segment (block B). The design is based on the hypothesis that self-assembly of such block copolymers occurs as a result of the balance between oligomerization of the helical ends and

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swelling of the central water-soluble polyelectrolyte segment. Consequently, temperature-/pH-responsiveness may be achieved by manipulating the amino acid sequences of the coiled-coil domains. The structure of the coiled-coil domains in the copolymer was either $(VSSLESK)_6$[37] (block A) or $(VSSLESK)_2-VSKLESK-KSKLESK-VSKLESK-VSSLESK$ (block C), while the random coil segment (block B) was an Ala−Gly-rich sequence $[(AG)_3PEG]_{10}$.[32] To evaluate the relationship between the structure of block copolymers and their properties, the structure of block A was modified (K residues replaced one V and three S) to produce block C. Lysine residues inserted into the structure of block C disturb the hydrophobic interaction at the helical interface and introduce electrostatic repulsion among the helices. The self-assembly of these copolymers into hydrogels and hydrogel properties were closely related to the association of the coiled-coil domains. This conclusion is supported by the reversible hydrogel formation in the presence or absence of a denaturant (GdnHCl) and by the observation that the hydrogel properties can be greatly influenced by tailoring the amino acid sequence of the coiled-coil domains.

Experimental Section

Abbreviations. AUC, analytical ultracentrifugation; BCA, bicinchoninic acid; CD, circular dichroism; G', storage modulus of elasticity; G'', loss modulus of elasticity; GSER, generalized Stokes−Einstein equation; GdnHCl, guanidine hydrochloride; IDL, Interactive Data Language; IPTG, isopropyl β-thiogalactoside; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy; MSD, mean-square-displacement; PAGE, polyacrylamide gel electrophoresis; PEG, poly(ethylene glycol); SE, sedimentation equilibrium; SEM, scanning electron microscopy.

Materials. Isopropyl β-thiogalactoside (IPTG) was from Sigma (St. Louis, MO). Ni−NTA agarose resin was from Qiagen (Santa Clarita, CA). Micro-BCA protein assay reagent kit used for protein concentration measurement was from Pierce (Rockford, IL).

Enzymes *Nde*I, *Nhe*I, *Spe*I, *Hin*dIII, *Hpa*I, *Eco*RI, and T4 DNA ligase were from New England Biolabs (Beverly, MA). Zero Blunt TOPO PCR cloning kit was from Invitrogen Life Technologies (Carlsbad, CA).

Escherichia coli strain DH5α was obtained from Gibco BRL, Life Technologies (Grand Island, NY). BL21(DE3)-pLysS was from Novagen (Madison, WI).

Surfactant-free yellow-green fluorescent amidine-modified and red fluorescent carboxylate-modified polystyrene latex beads (0.5 and 1.0 μm) were from Interfacial Dynamics Corporation (Portland, OR). PEG-coated polystyrene latex beads with red fluorescent were synthesized according to a published procedure.[38] CytosealTM 60 was from Richard-Allan Scientific (Kalamazoo, MI).

Construction of Expression Vectors for the Protein Polymers. The previously constructed expression vector pRSETB-HC[39] was first inserted with gene encoding block B at the restriction enzyme sites of *Nhe*I and *Spe*I (the gene encoding block B was from a plasmid as a kind gift from Dr. D. A. Tirrell, California Institute of Technology), which was then digested with *Nde*I/*Nhe*I. The fragment was ligated with the fragment from pRSETB-HC (digested with *Nde*I/*Hin*dIII) and an oligonucleotide encoding partial block A with *Nhe*I/*Hin*dIII overhangs, giving rise to the expression vector for polymer I.

To construct the expression vector for polymer II, a chemically synthesized oligonucleotide encoding block C, which included *Hpa*I and *Nhe*I enzyme sites, was first cloned into pCR-Blunt II-TOPO plasmid. It was then cleaved from the plasmid with *Hpa*I and *Nhe*I enzymes and ligated with the fragment from *Hpa*I/*Nhe*I-digested polymer I expression vector, resulting in the expression vector for polymer II.

The expression vectors for polymer III and polymer IV were constructed similarly to that for polymer II. Again, a chemically synthesized oligonucleotide encoding block C with *Spe*I and *Eco*RI enzyme sites was first cloned into pCR-Blunt II-TOPO plasmid. It was cut from the plasmid with *Spe*I and *Eco*RI and ligated with the fragment from *Spe*I/*Eco*RI-digested polymer I or polymer II expression vector, resulting in the expression vectors for polymers III and IV, respectively.

The structures of all vectors were verified by direct DNA sequencing.

Expression and Purification of the Protein Polymers. *E. coli* BL21(DE3)pLysS competent cells were transformed with the expression vectors separately. Cultures were grown at 37 °C in 1.5 L of LB medium containing 50 μg/mL kanamycin and 34 μg/mL chloramphenicol until the optical densities (OD_{600}) reached 1. Then IPTG was added to a final concentration of 0.4 mM. The protein synthesis was induced at 37 °C for 4 h before cells were harvested. The protein polymers were purified by immobilized metal affinity chromatography on Ni−NTA agarose resin.[40] Bacterial cell pellets were first resuspended in Tris buffer (20 mM Tris and 500 mM NaCl, pH 6.9). The cell suspensions were then sonicated and centrifuged at 15 000 rpm for 30 min at 4 °C. The supernatants were loaded onto Ni−NTA columns preequilibrated with Tris buffer. The columns were washed sequentially with Tris buffer containing increasing concentrations of imidazole. The eluted fractions were analyzed by SDS−polyacrylamide gel electrophoresis. The fractions containing the target protein polymers were loaded onto PD-10 columns containing Sephadex G-25, and eluted with 10 mM PBS buffer (10 mM phosphate and 150 mM NaCl, pH 7.4). The fractions were analyzed by SDS−PAGE, and the molecular weight and purity of the protein polymers were verified by use of MALDI-TOF-MS. Yields of polymer I, II, III, and IV were 29, 38, 29, and 38 mg/L of growth medium, respectively.

Circular Dichroic Spectroscopy. An Aviv 62DS CD spectrometer with a thermoelectric temperature control system was used. The measurements were carried out at 25 °C in 10 mM PBS buffer. The protein concentration used was 5 μM. Each sample was scanned from 200 to 250 nm with 1 nm/step. Data from three sequential scans were averaged, subtracted from the buffer spectrum. For thermal stability experiments, the CD signal at 222 nm was recorded when the temperature increased from 13 to 94 °C at 3 °C/step. For each step, the sample was equilibrated for 1.5

min followed by 30 s of data point averaging. The thermal stability of each protein polymer at different pH was also evaluated. The transition temperatures were determined from the first-order differentiation of the CD signal at 222 nm with respect to temperature.[41]

In the reversibility experiments, GdnHCl was added to the polymer IV PBS solution. This mixture was equally divided into two tubes, one of which was applied for dialysis against PBS buffer. After the dialysis, the concentrations of the samples were measured and adjusted to 5 μM before the measurement. Wavelength scans were performed for polymer IV solutions with GdnHCl and after dialysis.

Analytical Ultracentrifugation. Sedimentation equilibrium (SE) experiments were performed on a Beckman Optima XL-A analytical ultracentrifuge, with an AnTi60 rotor and six-channel, 12-mm-thick, charcoal-epon centerpieces. All of the protein polymers were in 10 mM PBS buffer, and three different loading concentrations of 5, 30, and 70 μM were used. Samples were centrifuged to equilibrium with rotor speed of 20 000 rpm at 20 °C and absorbance was recorded at 230 nm against 10 mM PBS reference buffer. Equilibrium was confirmed by the overlays between scans taken at 4-h intervals. A baseline scan at a nonabsorbing wavelength (360 nm) was taken once the equilibrium was attained. Data were analyzed by nonlinear least squares techniques and NONLIN analysis program.[42,43] The values of $\bar{v}$ for each protein were calculated from the amino acid sequence.[44]

Microrheology. Microrheology is a recently developed method for measuring the mechanical properties of a material by monitoring the motion of micrometer-sized tracer particles. Compared to conventional mechanical rheometers, which typically require a milliliter of sample, microrheology requires much less sample volume, usually less than 10 μL. In passive microrheology, there is no external driving force applied to the tracer particles; the intrinsic Brownian motion of the particles is used, driven by the thermal energy k_BT. The theoretical basis for passive microrheology is a generalized Stokes−Einstein relation (GSER) for materials with viscoelastic properties,[45,46] which can be presented in the following form:

$$\langle \Delta\tilde{r}^2(s)\rangle = \frac{dk_BT}{3\pi as\tilde{G}(s)}$$

where $\langle \Delta\tilde{r}^2(s)\rangle$ is the Laplace transform of the tracer particles' mean-squared displacement (MSD) $\langle [r(t+\tau) - r(t)]^2\rangle$, d is the dimensionality of the displacement vector (usually 2 in videomicroscopy), s is the Laplace frequency, a is the radius of the particles, and $\tilde{G}(s)$ is the Laplace representation of the complex modulus, which encompasses the storage (G') and loss (G'') moduli.

The preparation of the suspensions of tracer particles in polymer solutions was carried out as follows. Purified protein polymers were weighed and placed into plastic Eppendorf tubes. The appropriate amounts of solvent (deionized water or PBS buffer) and suspension of fluorescently labeled latex beads (radius 0.5 or 0.25 μm, yellow-green fluorescent amidine-modified microspheres, red fluorescent carboxylate-modified microspheres, or PEG-coated red fluorescent microspheres) were added to each tube. The samples were mixed thoroughly and left for more than 24 h to allow the formation of hydrogels. Before the measurement, the samples were sealed between a microscope slide and a no. 1.5 glass coverslip with Cytoseal 60. The Brownian motion of the embedded particles at particular experimental conditions, including concentration, temperature, and pH, was observed with an epifluorescence optical microscope (Nikon Eclipse E800) by use of a 100×, NA = 1.3, oil-immersion objective and a CCD camera (Dage-MTI, DC330) with exposure time of 2 ms. To avoid wall effects, the focus was at least 20 μm into the sample chambers. For each sample, 3000 images were recorded by use of StreamPix software (Norpix Inc.) at intervals of 33 ms. Images were analyzed with IDL image analysis software (Research Systems Inc., Boulder, CO) and the trajectories of the particles were extracted by use of algorithms developed and kindly provided by Crocker, Weeks, and co-workers.[47]

Scanning Electron Microscopy. The hydrogel samples were prepared similarly as in microrheology experiments. The purified protein polymers were weighed and placed in Eppendorf tubes. A particular amount of deionized water was added and mixed with the polymers thoroughly. The samples were left at room temperature for 24 h to allow the formation of hydrogel. After that, additional amount of deionized water was added into the hydrogel samples and was allowed to equilibrate for 3 days. Then the samples were shock-frozen in liquid nitrogen and quickly transferred to a freeze-drier to allow lyophilization. The dry samples were then carefully fractured and their interior morphologies were studied on a scanning electron microscope (Hitachi S-2460N SEM). Before SEM observation, specimens of the hydrogels were fixed on aluminum stubs and coated with gold for 40 s.

Reversibility of Polymer IV Hydrogel. Polymer IV hydrogel **(**10 wt %) was first prepared in deionized water and imaged. Then 0.5 M GdnHCl was added, and the hydrogel was completely dissolved. The 200 μL dissolved hydrogel solution was transferred to a diffusion cell, which was connected with another diffusion cell filled with 2.5 mL deionized water. The two diffusion cells were separated by a membrane with cutoff of 6000−8000 Da. This setup was placed on a horizontal shaker. The deionized water in one of the diffusion cells was exchanged twice a day for 10 days. The re-formed hydrogel was also imaged.

Size Measurement. Protein polymer samples were prepared at concentrations of 1, 2, and 3 mg/mL in the presence of 5 mM NaCl. The samples were filtered on a 0.45 μm membrane. Size measurements were carried out on Zetasizer (Malvern Instruments, Ltd., Southborough, MA). The results were averaged from 10 measurements for each sample.

Results and Discussion

Amino Acid Sequences of the Protein Polymers. Four protein-based triblock copolymers (ABA, CBA, ABC, and CBC) were synthesized by genetic engineering techniques. All copolymers contained a hydrophilic random coil block (block B) flanked by two coiled-coil domains (blocks A and/or C). The amino acid sequences of copolymers (poly-

Polymer I:

H_6VNADP[**(VSSLESK)$_6$**]ASYRDPMG[(AG)$_3$PEG]$_{10}$ARMPTSADP[**(VSSLESK)$_6$**]

Polymer II:

H_6VNADP[**(VSSLESK)$_2$-(VS*K*LESK)-(*KSK*LESK)-(VS*K*LESK)-(VSSLESK)**]-ASYRDPMG[(AG)$_3$PEG]$_{10}$ARMPTSADP[**(VSSLESK)$_6$**]

Polymer III:

H_6VNADP[**(VSSLESK)$_6$**]ASYRDPMG[(AG)$_3$PEG]$_{10}$ARMPTSADP[**(VSSLESK)$_2$-(VS*K*LESK)-(*KSK*LESK)-(VS*K*LESK)-(VSSLESK)**]

Polymer IV:

H_6VNADP[**(VSSLESK)$_2$-(VS*K*LESK)-(*KSK*LESK)-(VS*K*LESK)-(VSSLESK)**]-ASYRDPMG[(AG)$_3$PEG]$_{10}$ARMPTSADP[**(VSSLESK)$_2$-(VS*K*LESK)-(*KSK*LESK)-(VS*K*LESK)-(VSSLESK)**]

Figure 1. Amino acid sequences of protein polymers I−IV. Single-letter abbreviations for amino acid residues were used. Coiled-coil-forming sequences are shown in boldface type.

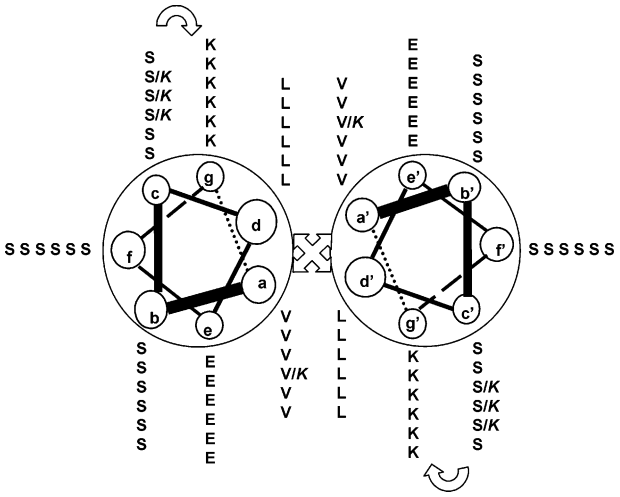

Figure 2. Helical-wheel diagram of the coiled-coil domains with A or C sequence.

mers I−IV) are shown in Figure 1. The random coil block B is an Ala−Gly-rich sequence $[(AG)_3PEG]_{10}$, which has been previously demonstrated to be water-soluble and not to form regular secondary structures.[32] The coiled-coil domain has either a block A or block C structure, where the A block sequence is $(VSSLESK)_6$,[37] while the C block sequence is $(VSSLESK)_2$−VSKLESK−KSKLESK−VSKLESK−VSSLESK. Proteins with the A block sequence have been demonstrated to have stable coiled-coil structures.[37] In our previous study, the genetically engineered coiled-coils containing proteins with the block A sequence were shown to have high thermal stabilities, with the midpoint transition temperature higher than 95 °C.[39] To decrease the thermal stability of the A block containing coiled-coil proteins, we modified its structure, forming the C block, in which Lys replaced Val in the a position of the fourth heptad and three additional Lys residues replaced Ser in the c positions of the third, fourth, and fifth heptads (indicated by boldface italic type in Figure 1). As shown in the helical-wheel diagram (Figure 2), the Lys residue at a position disturbs hydrophobic interactions at the hydrophobic interface, whereas Lys residues at the c positions introduce electrostatic repulsive forces between c and g residues. These

Table 1. Molecular Weights of Protein Block Copolymers[a]

polymer	structure	theoretical MW	experimental MW
polymer I	ABA	18 720.71	18 717.17
polymer II	CBA	18 873.01	18 874.88
polymer III	ABC	18 873.01	18 874.20
polymer IV	CBC	19 025.31	19 025.57

[a] See Figure 1 for composition.

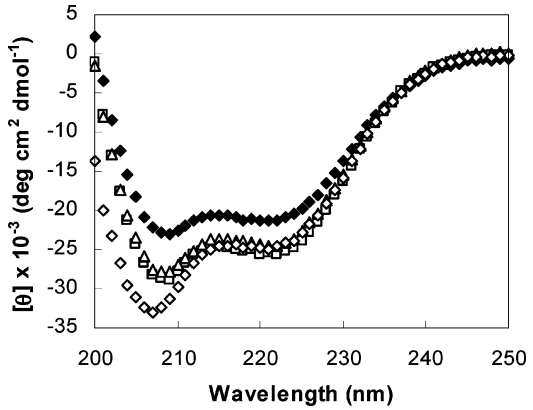

Figure 3. CD spectra of 5 μM protein polymer solutions in PBS (pH 7.4) buffer at 25 °C. (◆) Polymer I; (□) polymer II; (△) polymer III; (◇) polymer IV.

structural modifications should result in a decrease of the thermal stability of the coiled-coil association, and hence the association/dissociation of the coiled-coil domains may occur at reasonably low temperature. Furthermore, an increase in the pH responsiveness of the coiled-coil domains could also be expected. The four polypeptides in this study are the permutations t_HABA, t_HCBA, t_HABC, and t_HCBC, where t_H represents the histidine tag.

Characterization of the Protein Polymers. The molecular weight of the four polymers was determined by MALDI-TOF MS. The experimental values agree very well with the theoretical values, as indicated in Table 1.

The secondary structure was characterized by CD spectroscopy. All four polymers exhibited predominant α-helical structures, as indicated by the characteristic double negative peaks at 222 and 208 nm (Figure 3).

Thermal stabilities of the polymers were measured by monitoring the change of ellipticity at 222 nm with temperature (Figure 4). Polymer I showed extremely high thermal stability, as indicated by the incomplete unfolding curve. This result correlates well with the previous study where a coiled-coil domain containing block A sequence possessed a high degree of thermal stability.[39] Polymer IV, which contains two C blocks, was the least stable among the four polymers, with a midpoint transition temperature around 45 °C. Polymers II and III had similar unfolding processes with intermediate midpoint transition temperatures around 80 °C. These results correspond well with the predicted thermal stabilities from the sequence design, since the protein polymers with the block C sequence were expected to be less stable. The results obtained here indicated that the thermal stability of the coiled-coil containing proteins could be manipulated in a predictable way by substituting amino acids in the coiled-coil domain, which is in agreement with published data.[48] When the polymers were cooled to room temperature, their

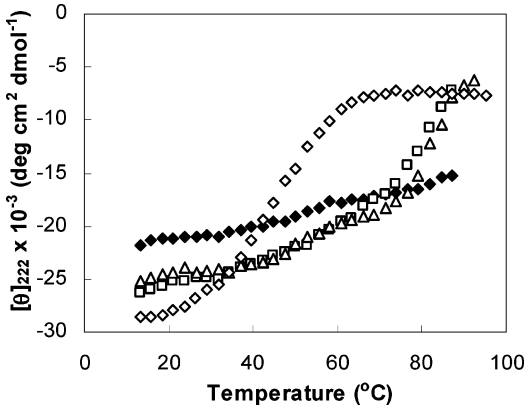

Figure 4. Temperature dependence of the secondary structures of the protein polymers. CD signal (ellipticity) at 222 nm as a function of temperature. (◆) Polymer I; (□) polymer II; (△) polymer III; (◇) polymer IV.

original ellipticity values recovered (data not shown), suggesting that the polymers' thermal unfolding was reversible.

Concentration Dependence of Gelation. To deduce the gelation behavior of the polymers, we used videomicroscopy[49] and multiple-particle-tracking microrheology,[45,50,51] which tracks the Brownian motion of embedded tracer particles inside a polymer solution or swollen hydrogel. The time- and ensemble-averaged mean-square displacement (MSD) of the tracer particles as a function of time, $\langle \Delta r^2(\tau) \rangle$, can be obtained from the particles' trajectories. Generally, in a viscous liquid, the particles diffuse freely, giving rise to a linear relationship between the MSD and lag time τ (resulting in slope 1 in a log−log plot). In an elastic medium, the movement of the particles is constrained, resulting a plateau in the MSD (slope 0 in a log−log plot).[45] A system is viscoelastic if the slope falls between 0 and 1 in a double-logarithmic plot of MSD versus lag time.

Since the surface chemistry of the embedded trace particles could influence the characterization of the biomaterials,[38,52] three different trace particles were used in this study, including positively charged amidine-modified particles, negatively charged carboxylate-modified particles, and neutral PEG-coated particles. The carboxylate-modified particles showed strong adhesion and aggregation with the protein polymers, especially polymer IV, which has more positively charged Lys residues; therefore, no useful results were obtained with the carboxylate-modified particles. This finding was not surprising, since there could be strong electrostatic attractions between the negatively charged particles and the positive charges in polymer IV, which interferes with the microrheological characterization.[51] Measurements with the amidine-modified particles and the PEG-coated particles did not reveal significant differences (data not shown). The results from both types of particles showed that all four polymers formed viscoelastic gels at sufficiently high concentrations (Figure 5). Polymer I was predominantly viscous at a concentration of 20 wt %, slightly viscoelastic at 30 wt %, and almost purely elastic at 35 wt %. Polymers II and III required lower concentrations to induce viscoelasticity. Both of them were still viscous at a concentration of 10 wt % and became increasingly viscoelastic at 20 and 25 wt %. Polymer IV exhibited viscoelasticity at the lowest concentration: it was viscous at 2 wt % but became strongly elastic at concentrations of 5 and 10 wt %.

Oligomerization States of Block Copolymers and Gelation Concentrations. To understand the differences in gelation thresholds among the four polymers, sedimentation equilibrium (SE) experiments were performed in PBS buffers at concentrations ranging from 5 to 70 μM. The AUC data indicated that polymer I was in a monomer−dimer equilibrium, Polymers II and III existed as dimers in solution, while polymer IV showed a dimer to tetramer association. These results were different from those of Wagschal et al., who demonstrated that when a Lys residue occupied the a position, an exclusively two-stranded monomer would be formed.[48] However, these authors used a 76-residue disulfide-bridged coiled-coil model in their study, whereas the polymers in this study contained 42-residue coiled-coils without any disulfide bridge. The difference between the results from these two studies is not unexpected, since the structure of the coiled-coils can be dramatically influenced by chain length and the existence of disulfide bridges.[53,54]

The concentrations required for gelation from the four protein polymers can be related to their oligomerization states (Table 2). In the same concentration range, polymer IV showed evidence of association from a dimer to a tetramer. As a result, polymer IV requires a much lower concentration to exhibit viscoelasticity. On the other hand, only monomer to dimer association was observed in solutions of polymer I, hence a much higher concentration is required to form a hydrogel. The relationship between oligomerization state and gelation concentration suggested that the hydrogel formation was related to the association of the coiled-coil domains.

Another interesting finding is that all of the polymers gelled at significantly higher concentrations in PBS buffer than in water, as shown in Figure 6. Polymer I was almost entirely viscous at 30 wt % in PBS and only slightly viscoelastic at 40 wt % in PBS, but it was quite elastic at 40 wt % in dH_2O. Similarly, polymers II and III were viscous at 30 wt % in PBS but more viscoelastic at 40 wt % in PBS and dH_2O. Polymer IV was quite elastic at 10 wt % in dH_2O but was only viscous at 10 wt % in PBS and more elastic at 20 wt % in PBS. It is known that, in the absence of electrolyte, the ionic pair Glu-Lys significantly stabilizes coiled-coil formation at neutral pH.[55,56] Since in all of the protein polymers (I−IV) Glu and Lys occupied the e and g positions in the coiled-coil domains, the coiled-coil formation was expected to be thermodynamically more favorable in dH_2O than in PBS buffer. As a result, the hydrogels formed at lower concentration in dH_2O than in PBS buffer.

It is known that triblock copolymers can form hydrogels through micelle packing.[57,58] To find out whether the hydrogels self-assembled from the protein polymers were from coiled-coil association or micelle packing, we performed size measurement for the four protein polymers at concentrations of 1, 2, and 3 mg/mL. None of the polymers showed micelle formation (data not shown), which suggested that the probability of micelle packing in our hydrogel systems was low, and the formation of the hydrogels was most probably due to the physical cross-linking by coiled-

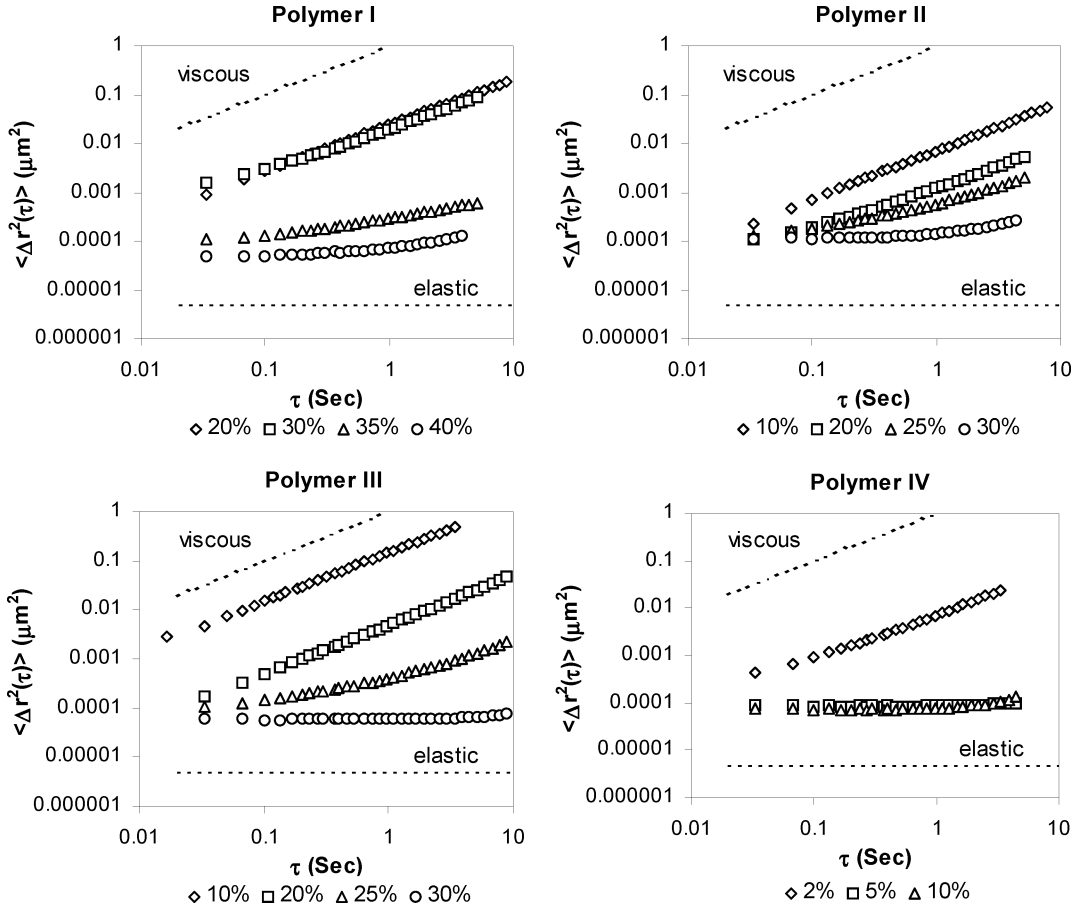

Figure 5. Mean-square-displacement as a function of lag time for 0.5 μm amidine-modified spheres in protein polymer water solutions.

Table 2. Relationship between Oligomerization State and Gelation Concentration[a]

polymer	oligomerization state	gelation concentration
polymer I	monomer−dimer equilibrium	35% in dH_2O, >40% in PBS
polymer II	dimer	25% in dH_2O, 40% in PBS
polymer III	dimer	25% in dH_2O, 40% in PBS
polymer IV	dimer−tetramer equilibrium	5% in dH_2O, 20% in PBS

[a] Analytical ultracentrifugation experiments were performed in 10 mM PBS buffer at 5, 30, and 70 μM loading concentrations. Data on gelation concentrations were obtained from microrheology measurements in deionized water and PBS. See Experimental Section for details. In a dimer, either only one of the coiled-coil domains or two coiled-coil domains of a polymer were involved in the intermolecular association.

coil association. The plausible structure of the hydrogels is shown in Figure 7. The structure is similar to the structure shown by Petka et al.[32] However, in contrast to the block copolymers of Petka et al.,[32] no terminal Cys residue was involved in our design. Consequently, no hexablock polymers could form prior to self-assembly.

A wide range in the gelation concentration for different protein-based hydrogel systems can be found in the literature.[32,59,60] Nowak et al.[59] synthesized amphiphilic diblock copolypeptides, which consisted of one highly hydrophilic block and one highly hydrophobic block (e.g., $K_{160}V_{40}$ or $K_{160}L_{40}$). They demonstrated that these diblock copolymers could form hydrogels at concentration as low as 0.25 wt %.[59] Using a triblock polypeptide similar to those used in this study, Petka et al.[32] found their polymer formed a hydrogel at the concentration of 5 wt %. This value is in agreement with the concentration needed to form a hydrogel observed in this study with polymer IV. Conticello and co-workers[60] report that elastin-mimetic triblock polypeptides formed hydrogels at 25 wt %.

These data suggest that the choice of the amino acid sequence in the protein polymers is as important as their secondary structures. Although the gelation concentrations observed in this study were relatively high, the results were comparable to data observed with similar structures. In addition, our study demonstrated that the self-assembly of the hydrogels can be mediated by the association of the coiled-coil domains. Consequently, the physical properties of the hydrogels can be directly related to that of the coiled-coil domains, such as their temperature or pH sensitivities. It was also demonstrated that the hydrogels' responsiveness to external stimuli could be tailored by the manipulation of the amino acid sequence of the coiled-coil domains in the protein polymers.

Morphology of the Hydrogels. To observe the microstructure of the hydrogels, scanning electron microscopy (SEM) was performed on each of the hydrogel samples. The samples were shock-frozen by use of liquid nitrogen followed by lyophilization. This procedure appears to have a minimal impact on the hydrogel structure.[61] An interconnected network structure was detected in all hydrogels (Figure 8), indicating that the hydrogels may not be formed from micelle packing but through physical cross-linking. The hydrogels self-assembled from polymers I, II, and III displayed denser

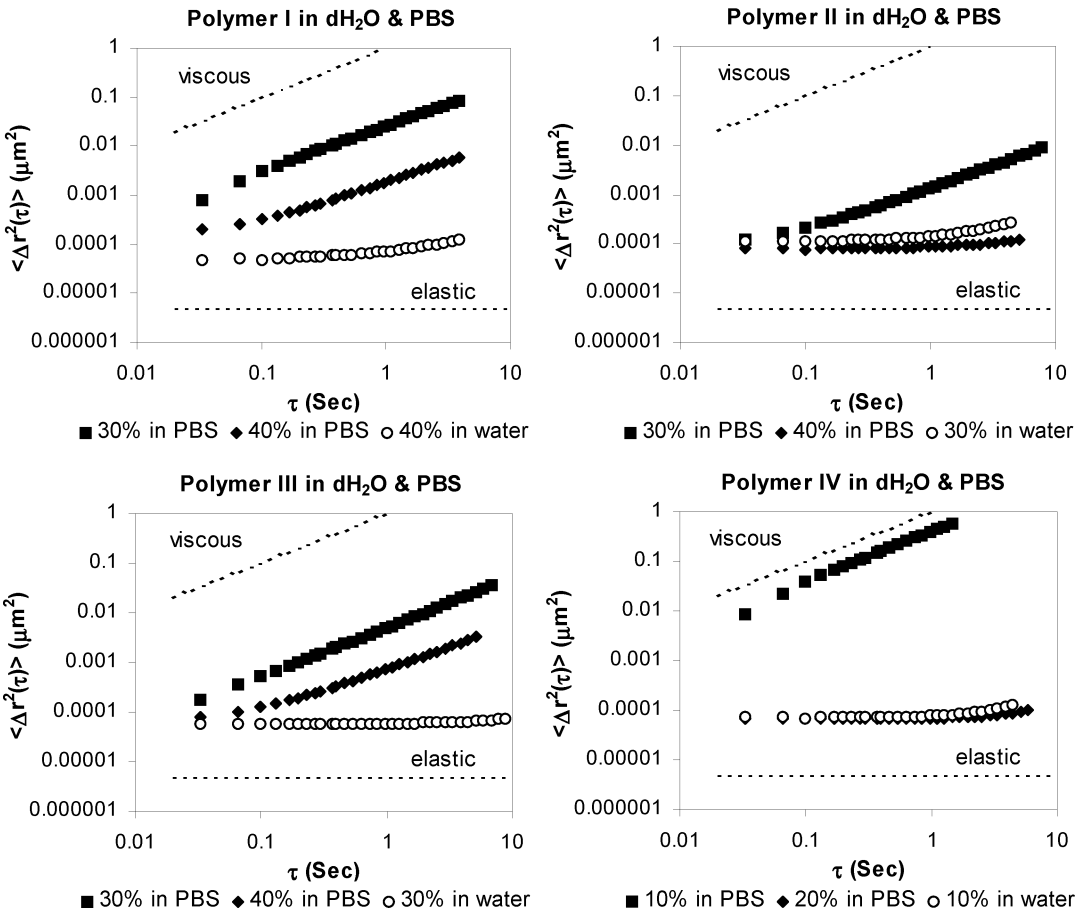

Figure 6. Comparison of hydrogel formation of polymers I−IV in water and 10 mM PBS buffer.

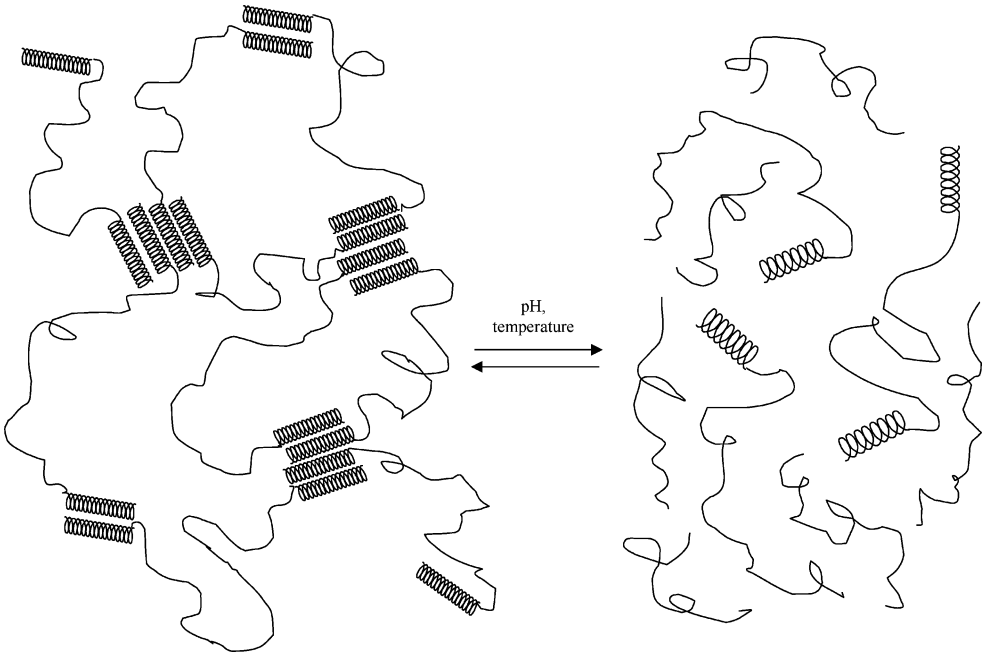

Figure 7. Proposed hydrogel formation from the triblock polymers. Hydrogels are formed through coiled-coil association. When there is a change in temperature or pH, the coiled-coils could dissociate or even lose the secondary structures, causing the loss of physical cross-linking forces inside the hydrogels, and the system becomes a viscous liquid eventually.

structures than polymer IV hydrogel, in agreement with the gelation concentrations detected by microrheology measurements.

pH Dependence of the Secondary Structure of Protein Polymers and pH Sensitivity of Polymer IV Hydrogel. The pH dependence of the secondary structures of the four polymers was measured by CD spectroscopy. Each sample was adjusted to the desired pH ranging from 1 to 11. The ellipticity value at 222 nm was monitored at each pH as a function of temperature. The secondary structures of the

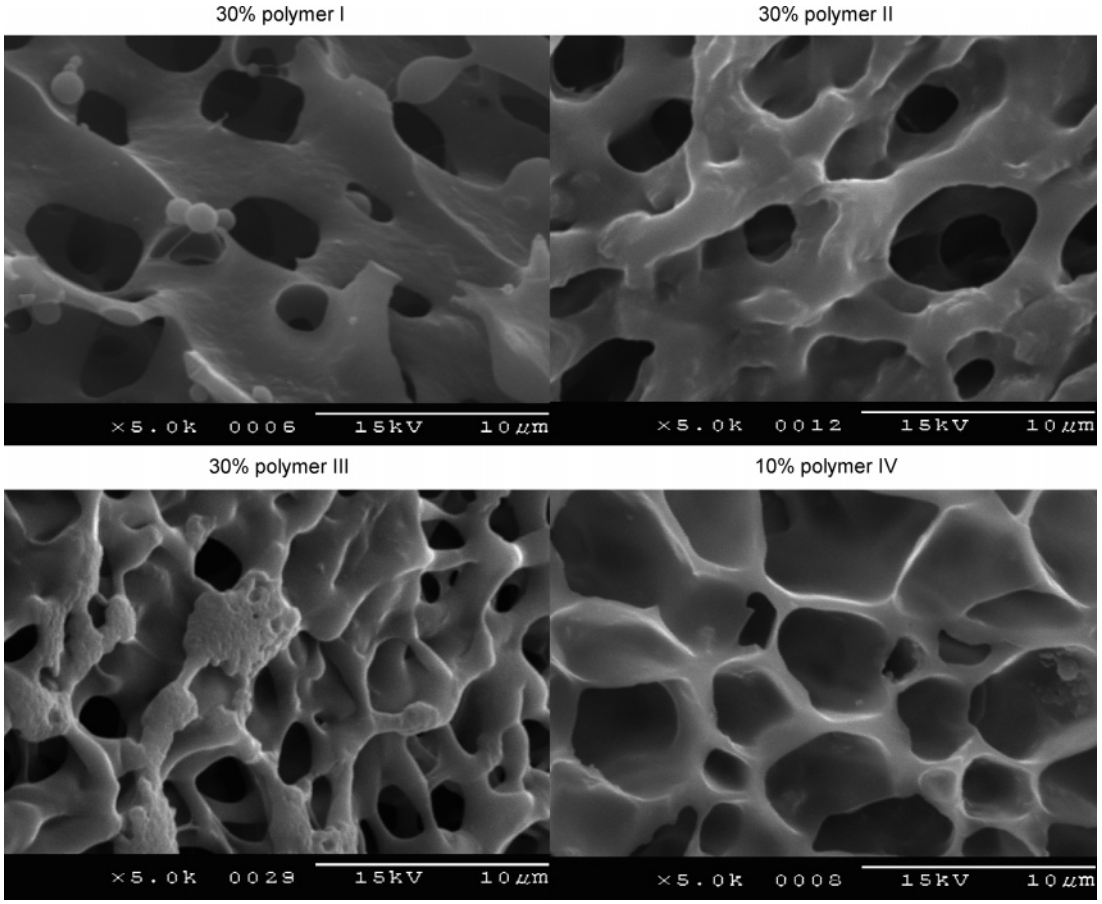

Figure 8. SEM images of the hydrogels.

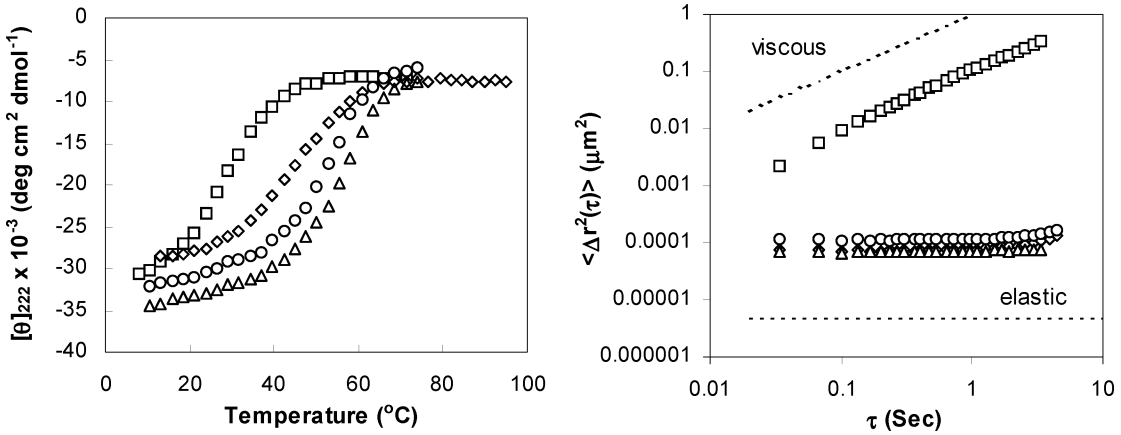

Figure 9. pH sensitivity of polymer IV. (Left panel) CD signal at 222 nm as a function of temperature. (Right panel) Mean-square-displacement as a function of lag time at different pH for 10 wt % polymer IV hydrogel in deionized water. (□) pH 2; (◇) pH 7; (△) pH 9; (○) pH 11.

polymers showed different patterns of pH dependence. Polymers I−III had more stable structures at neutral pH than at both acidic and basic conditions, while the secondary structure of polymer IV showed increasing stability from acidic pH to basic pH (Figure 9).

These results correlate well with the structures of the polymers. In polymer I, the coiled-coil blocks at both ends have the same A block structure, $(VSSLESK)_6$. At pH 7.4, there are electrostatic attractions between the negatively charged Glu and positively charged Lys (Figure 2), which stabilize the coiled-coil structure. On the other hand, at acidic pH (<4) or basic pH (>10), only one of these two residues will be charged. As a result, the stabilizing electrostatic attraction between two helices cannot be established, and hence the coiled-coil structure will be less stable when compared to the neutral condition.

In polymer IV, the coiled-coil blocks have the same C block structure, $(VSSLESK)_2$−VS**K**LESK-**K**S**K**LESK−VS**K**LESK−VSSLESK. At acidic pH (<4), only Lys residues are charged. The electrostatic attraction between e and g positions cannot be established. Also, there is an unfavorable electrostatic repulsion between c and g positions. In addition, the charged Lys residue at a position disrupts the hydrophobic interaction at the helical interface. As a result, the coiled-coil structure is largely destabilized. At pH 7.4, both Glu and Lys are charged. Although there are

unfavorable electrostatic repulsions at c and g positions and a hydrophobic interaction disrupting factor at the a position, the e and g positions have favorable electrostatic attractions, which make the coiled-coil structure more stable than under acidic conditions. At basic pH (>10), only Glu is charged. Although there is no favorable electrostatic attraction between e and g positions, there is no more disrupting factor at the hydrophobic interface or unfavorable electrostatic repulsion between c and g positions, resulting in an even more stable coiled-coil structure, since hydrophobic packing is the dominant determinant for coiled-coil stability.[36]

Polymers II and III contain blocks of both A and C sequences. The coiled-coil can be formed between an A/A pair, an A/C pair, or a C/C pair. When the coiled-coil associates between A/A or C/C pairs, the situation is similar to that of polymer I or IV. When coiled-coil forms between an A/C pair, only one helix contains Lys residue at a, c, and g positions. At neutral pH, the unfavorable electrostatic repulsion at c and g positions exists in only one helix, and only one Lys is present in the hydrophobic interface, so the overall destabilization effect is much weaker than that in polymer IV at identical pH. The favorable electrostatic attraction between negatively charged Glu and positively charged Lys residues largely contributes to the stability at neutral pH. At acidic pH, only Lys is charged. There is no stabilizing electrostatic attraction between e and g positions, while there is a weak electrostatic repulsion existing in one helix and a slight disturbing force at the hydrophobic interface. The resulting coiled-coil is hence less stable than under neutral condition. At basic pH, only Glu is charged. There is no stabilizing electrostatic attraction between e and g positions and also no unfavorable electrostatic repulsion and hydrophobic disturbing factor. Therefore, the coiled-coil is also less stable than at neutral pH. Overall, the secondary structures of polymers II and III have similar patterns as polymer I with respect to their pH dependence.

Next, we investigated the pH dependence of the self-assembled hydrogels. Polymer IV was chosen as a representative example. A 10 wt % polymer IV solution in deionized water was prepared at pH 2, 7, 9, and 11, and microrheological measurements were performed. As shown in Figure 8, the 10 wt % solution of polymer IV at pH 2 was still predominantly viscous, but it became more elastic at pH 7, 9, and 11. These results correlate well with the secondary structure obtained from CD spectra. At pH 2, the coiled-coil structure of polymer IV has the lowest thermal stability, the midpoint transition temperature being around 28 °C. Since the coiled-coil structure is not stable at pH 2, it cannot have a strong association and hence cannot form strong physical cross-links for a hydrogel. On the other hand, at pH 7, 9, and 11, the coiled-coil structure is much more stable, resulting in strong association between the coiled-coils, which can act as strong physical cross-links in the hydrogel.

Temperature Dependence of Secondary Structure Contributes to Temperature Sensitivity of the Polymer IV Hydrogel. The temperature dependence of the hydrogel from 10 wt % polymer IV in deionized water solution was evaluated by microrheology. Following an increase from room temperature to 55 °C, a change from strongly elastic to mostly viscous properties was observed, suggesting a loss of organization and disassembly of the three-dimensional structure of the hydrogel (Figure 10). As the sample was cooled from 55 °C to room temperature, the MSD vs lag time curve almost overlapped with the original one (before heating), suggesting a recovery of the self-assembled hydrogel structure of polymer IV. These results are in agreement with the thermal stability measurements of the secondary structure (see Figures 4 and 9) and support the hypothesis that the association/dissociation of the coiled-coil domains mediates the formation of hydrogel.

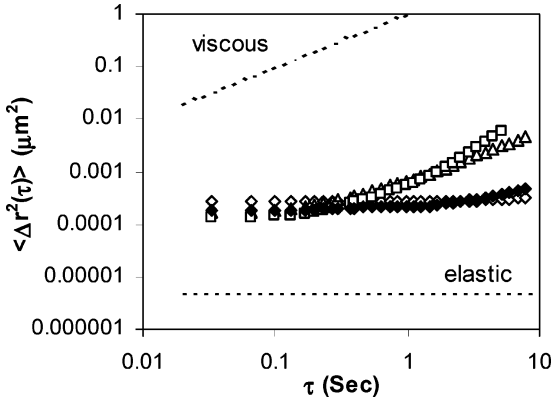

Figure 10. Temperature sensitivity of the self-assembly/disassembly of 10 wt % polymer IV hydrogel in deionized water. (◇) 27 °C; (△) 40 °C; (□) 55 °C; (◆) from 55 °C back to 27 °C.

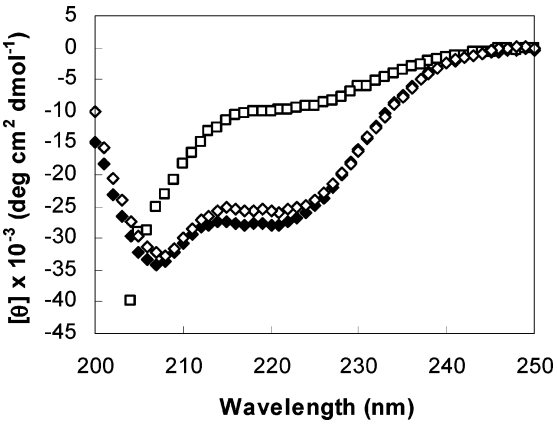

Figure 11. CD spectra of 5 μM polymer IV in the presence or absence of the denaturant GdnHCl. (◆) Polymer IV without the treatment of GdnHCl; (□) polymer IV treated with 0.5 M GdnHCl; (◇) polymer IV first treated with 0.5 M GdnHCl and then dialyzed to remove GdnHCl.

Reversibility of Secondary Structure of Polymer IV and of Self-Assembly of Polymer IV Hydrogel after Denaturation/Refolding of the Coiled-Coil Blocks. It is important to know if the secondary structure of coiled-coil domains in block copolymers will refold after the removal of the denaturating agent. To this end, CD measurements of polymer IV solutions were performed in the absence and presence and after removal of the denaturant, GdnHCl. As shown in Figure 11, the presence of 0.5 M GdnHCl in the solution caused the unfolding of polymer IV, resulting in a loss of the α-helical structure. However, when GdnHCl was removed by dialysis, polymer IV refolded back to its original structure. The α-helical structure recovered, as indicated by the two negative ellipticity values at 222 and 208 nm. The

CD spectrum almost completely overlapped with the one without GdnHCl treatment. This strongly suggests that the secondary structure of polymer IV is reversible. It can be unfolded upon denaturation and refolded after the removal of denaturant.

We also investigated the reversibility of the self-assembly of polymer IV into hydrogels. A hydrogel was self-assembled from 10 wt % polymer IV in deionized water solution. The hydrogel completely dissolved after the addition of 0.5 M GdnHCl. To remove the denaturant GdnHCl, the dissolved polymer IV solution was transferred to one compartment of a diffusion cell; the other compartment was filled with deionized water. After GdnHCl was completely removed, pieces of hydrogel were found re-formed on the diffusion membrane (data not shown), demonstrating the reversibility of hydrogel self-assembly from the solution of polymer IV. The data obtained bode well for the potential development of these hydrogels as advanced materials for biomedical applications.

Conclusions

Four triblock polypeptides (ABA, CBA, ABC, and CBC) containing different (A or C) terminal coiled-coil domains and a central random coil block (B) were synthesized by genetic engineering techniques. The thermal stabilities of the secondary (α-helical) structure of these protein polymers were successfully manipulated by tailoring the amino acid sequences in the coiled-coil domains. Hydrogels were self-assembled from these protein polymers. The formation of hydrogels was dependent on the protein concentrations, and the gelation concentrations correlated well with the oligomerization state of the coiled-coil domains. The self-assembly of hydrogels was responsive to the changes in temperature or pH and was reversible after denaturation. These physical properties were directly correlated to the structural properties of the coiled-coil domains, suggesting that the self-association, thermal stability, and pH sensitivity of the coiled-coils mediated self-assembly of the hydrogels. With further investigation, these protein polymers may be used in biomedical applications where stimuli responsiveness is required.

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References and Notes

(1) Wichterle, O.; Lím, D. *Nature* **1960**, *185*, 117.
(2) Peppas, N. A. *Curr. Opin. Colloid Interface Sci.* **1997**, *2*, 531.
(3) Hoffman, A. S. *Ann. N.Y. Acad. Sci.* **2001**, *944*, 62.
(4) Lee, K. Y.; Mooney, D. J. *Chem. Rev.* **2001**, *101*, 1869.
(5) Qiu, Y.; Park, K. *Adv. Drug Delivery Rev.* **2001**, *53*, 321.
(6) Chen, G.; Hoffman, A. S. *Nature* **1995**, *373*, 49.
(7) Yoshida, R.; Uchida, K.; Kaneko, Y.; Sakai, K.; Kikuchi, A.; Sakurai, Y.; Okano, T. *Nature* **1995**, *374*, 240.
(8) Brøndsted, H.; Kopeček, J. In *Polyelectrolyte Gels: Properties, Preparation, and Applications*; Harland, R. S., Prud'homme, R. K., Eds.; ACS Symposium Series; American Chemical Society: Washington, DC, 1992; Vol. 480, p 285.
(9) Kiser, P. F.; Wilson, G.; Needham, D. *Nature* **1998**, *394*, 459.
(10) Hirokawa, Y.; Tanaka, T. *J. Chem. Phys.* **1984**, *81*, 6379.
(11) Kwon, I. C.; Bae, Y. H.; Kim, S. W. *Nature* **1991**, *354*, 291.
(12) Osada, Y.; Okuzaki, H.; Hori, H. *Nature* **1992**, *355*, 242.
(13) Suzuki, A.; Tanaka, T. *Nature* **1990**, *346*, 245.
(14) Kokufata, E.; Zhang, Y.; Tanaka, T. *Nature* **1991**, *351*, 302.
(15) Kataoka, K.; Miyazaki, H.; Bunya, M.; Okano, T.; Sakurai, Y. *J. Am. Chem. Soc.* **1998**, *120*, 12694.
(16) Ulbrich, K.; Strohalm, J.; Kopeček, J. *Biomaterials* **1982**, *3*, 150.
(17) Šubr, V.; Duncan, R.; Kopeček, J. *J. Biomater. Sci., Polym. Ed.* **1990**, *1*, 261.
(18) West, J. L.; Hubbell, J. A. *Macromolecules* **1999**, *32*, 241.
(19) Obaidat, A. A.; Park, K. *Pharm. Res.* **1996**, *13*, 989.
(20) Miyata, T.; Asami, N.; Uragami, T. *Nature* **1999**, *399*, 766.
(21) Wang, C.; Stewart, R. J.; Kopeček, J. *Nature* **1999**, *397*, 417.
(22) Wang, C.; Kopeček, J.; Stewart, R. J. *Biomacromolecules* **2001**, *2*, 912.
(23) Chen, L.; Kopeček, J.; Stewart, R. J. *Bioconjugate Chem.* **2000**, *11*, 734.
(24) Kopeček, J. *Eur. J. Pharm. Sci.* **2003**, *20*, 1.
(25) Urry, D. W.; Pattanaik, A. *Ann. N.Y. Acad. Sci.* **1997**, *831*, 32.
(26) Lee, J.; Macosko, C. W.; Urry, D. W. *Biomacromolecules* **2001**, *2*, 170.
(27) Cappello, J.; Crissman, J. W.; Crissman, M.; Ferrari, F. A.; Textor, G.; Wallis, O.; Whitledge, J. R.; Zhou, X.; Burman, D.; Aukerman, L.; Stedronsky, E. R. *J. Controlled Release* **1998**, *53*, 105.
(28) Nagarsekar, A.; Crissman, J.; Crissman, M.; Ferrari, F.; Cappello, J.; Ghandehari, H. *Biomacromolecules* **2003**, *4*, 602.
(29) Wright, E. R.; Conticello, V. P. *Adv. Drug Delivery Rev.* **2002**, *54*, 1057.
(30) McMillan, R. A.; Caran, K. L.; Apkarian, R. P.; Conticello, V. P. *Macromolecules* **1999**, *32*, 9067.
(31) Nagarsekar, A.; Crissman, J.; Crissman, M.; Ferrari, F.; Cappello, J.; Ghandehari, H. *J. Biomed. Mater. Res.* **2002**, *62*, 195.
(32) Petka, W. A.; Harden, J. L.; McGrath, K. P.; Wirtz, D.; Tirrell, D. A. *Science* **1998**, *281*, 389.
(33) McGrath, K. P.; Tirrell, D. A.; Kawai, M.; Mason, T. L.; Fournier, M. J. *Biotechnol. Prog.* **1990**, *6*, 188.
(34) Cappello, J.; Crissman, J.; Dorman, M.; Mikolajczak, M.; Textor, G.; Marquet, M.; Ferrari, F. *Biotechnol. Prog.* **1990**, *6*, 198.
(35) Megeed, Z.; Cappello, J.; Ghandehari, H. *Adv. Drug Delivery Rev.* **2002**, *54*, 1075.
(36) Yu, Y. B. *Adv. Drug Delivery Rev.* **2002**, *54*, 1113.
(37) Graddis, T. J.; Myszka, D. G.; Chaiken, I. M. *Biochemistry* **1993**, *32*, 12664.
(38) Valentine, M. T.; Perlman, Z. E.; Gardel, M. L.; Shin, J. H.; Matsudaira, P.; Mitchison, T. J.; Weitz, D. A. *Biophys. J.* **2004**, *86*, 4004.
(39) Xu, C.; Joss, L.; Wang, C.; Pechar, M.; Kopeček, J. *Macromol. Biosci.* **2002**, *2*, 395.
(40) Hochuli, E. *Genet. Eng. (N.Y.)* **1990**, *12*, 87.
(41) Cantor, C. R.; Schimmel, P. R. *Biophysical Chemistry*; W. H. Freeman and Company: New York, 1980; Vol. 3.
(42) Yphantis, D. A. *Biochemistry* **1964**, *3*, 297.
(43) Johnson, M. L.; Correia, J. J.; Ypahantis, D. A.; Halvorson, H. R. *Biophys. J.* **1981**, *36*, 575.
(44) Laue, T. M.; Shah, B. D.; Ridgeway, T. M.; Pelletier, S. L. In *Ultracentrifugation in Biochemistry and Polymer Science*; Harding, S. E., Rowe, A. J., Horton, J. C., Eds.; The Royal Society of Chemistry: Cambridge, U.K., 1992; p 90.
(45) Mason, T. G.; Weitz, D. A. *Phys. Rev. Lett.* **1995**, *74*, 1250.
(46) Levine, A. J.; Lubensky, T. C. *Phys. Rev. Lett.* **2000**, *85*, 1774.
(47) Crocker, J. C.; Valentine, M. T.; Weeks, E. R.; Gisler, T.; Kaplan, P. D.; Yodh, A. G.; Weitz, D. A. *Phys. Rev. Lett.* **2000**, *85*, 888.
(48) Wagschal, K.; Tripet, B.; Lavigne, P.; Mant, C.; Hodges, R. S. *Protein Sci.* **1999**, *8*, 2312.
(49) Crocker, J. C.; Grier, D. G. *J. Colloid Interface Sci.* **1996**, *179*, 298.
(50) MacKintosh, F. C.; Schmidt, C. F. *Curr. Opin. Colloid Interface Sci.* **1999**, *4*, 300.
(51) Breedveld, V.; Pine, D. J. *J. Mater. Sci.* **2003**, *38*, 4461.
(52) Pakstis, L. M.; Ozbas, B.; Hales, K. D.; Nowak, A. P.; Deming, T. J.; Pochan, D. *Biomacromolecules* **2004**, *5*, 312.
(53) Su, J. Y.; Hodges, R. S.; Kay, C. M. *Biochemistry* **1994**, *33*, 15501.
(54) Litowski, J. R.; Hodges, R. S. *J. Pept. Res.* **2001**, *58*, 477.
(55) Yu, Y.; Monera, O. D.; Hodges, R. S.; Privalov, P. L. *J. Mol. Biol.* **1996**, *255*, 367.
(56) Yu, Y.; Monera, O. D.; Hodges, R. S.; Privalov, P. L. *Biophys. Chem.* **1996**, *59*, 299.
(57) Brown, W.; Schillen, K. *J. Phys. Chem.* **1992**, *96*, 6038.
(58) Mortensen, K.; Pedersen, J. S. *Macromolecules* **1993**, *26*, 805.

(59) Nowak, A. P.; Breedveld, V.; Pakstis, L.; Ozbas, B.; Pine, D. J.; Pochan, D.; Deming, T. J. *Nature* **2002**, *417*, 424.
(60) Wright, E. R.; McMillan, R. A.; Cooper, A.; Apkarian, R. P.; Conticello, V. P. *Adv. Funct. Mater.* **2002**, *12*, 149.
(61) McGrath, J. L.; Hartwig, J. H.; Kuo, S. C. *Biophys. J.* **2000**, *79*, 3258.

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