

Assembly of an Artificial Protein Hydrogel through Leucine Zipper Aggregation and Disulfide Bond Formation

Wei Shen, Rob G. H. Lammertink, Jill K. Sakata, Julia A. Kornfield, and David A. Tirrell*

Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125

Received August 11, 2004; Revised Manuscript Received February 3, 2005

ABSTRACT: We present a strategy to stabilize artificial protein hydrogels through covalent bond formation following physical association of terminal leucine zipper domains. Artificial proteins consisting of two terminal leucine zipper domains and a random coil central domain form transient networks above a certain concentration, but the networks dissolve when placed in excess buffer. Engineering of a cysteine residue into each leucine zipper domain allows formation of disulfide bonds templated by leucine zipper aggregation. Circular dichroism spectra show that the zipper domains remain helical after cysteine residues and disulfide bonds are introduced. Asymmetric placement of the cysteine residues in the leucine zipper domains suppresses intramolecular disulfide bonds and creates linked “multichains” composed of ca. 9 protein chains on average, as determined by multiangle light scattering measurements. These “multichains” act as the building units of the physical network formed by leucine zipper aggregation. The increased valency of the building units stabilizes the hydrogels in open solutions, while the physical nature of their association allows the reversibility of gelation to be retained. The gel networks dissolve at pH 12.2, where the helicity of the leucine zipper domains is reduced by ca. 90%, and re-form upon acidification. The hydrogels show anisotropic swelling when anchored on aminated surfaces and may find applications in tissue engineering, controlled release, and microarray technologies on the basis of their stability, reversibility, and swelling behavior.

Introduction

Hydrogels have attracted interest over many years, in part for fundamental reasons and in part because of their potential for application in fields ranging from superabsorbents to tissue engineering.^{1–9} Hydrogels are chemically or physically cross-linked networks that absorb substantial amounts of aqueous solutions. They are especially interesting as candidate materials for tissue engineering because the high water content of the network can be matched to that of soft tissue.⁹ Hydrogels currently used in biomedical engineering are prepared from either natural or synthetic polymers.¹ Hydrogel-forming natural polymers include proteins such as collagen and gelatin and polysaccharides such as alginate and agarose. These materials exhibit useful properties,¹ but sources are limited, properties are variable, and concerns about viral contamination restrict the use of animal products in biomedical applications. Systematic control of structure and properties is also difficult. On the other hand, synthetic polymers address these shortcomings at the expense of specific biological functionality.

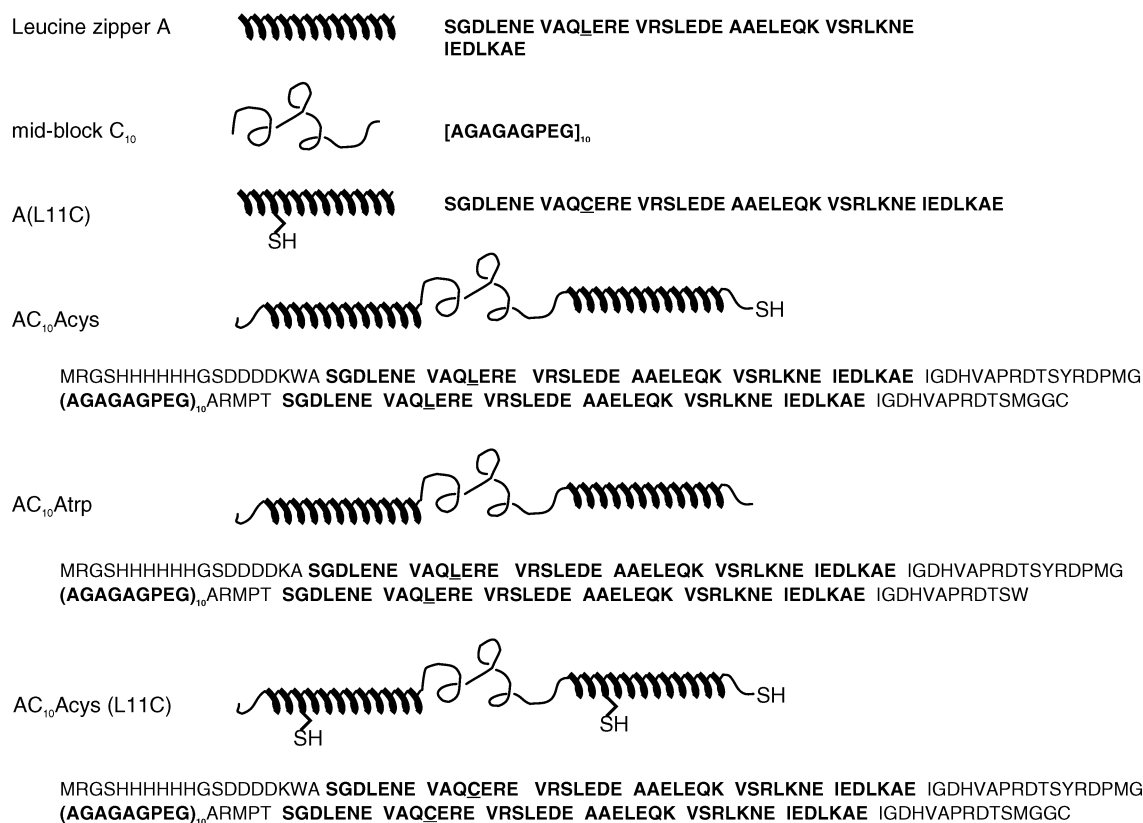
With genetically engineered biopolymers, it is possible to combine the advantages of both classes. Hydrogels constructed from genetically engineered self-assembling proteins have been prepared in our laboratory¹⁰ and by others.¹¹ The artificial proteins reported by our laboratory, here designated as AC₁₀A, are multidomain proteins consisting of two associative leucine zipper end-blocks (A) and a random coil midblock (C₁₀) (Scheme 1). Leucine zippers constitute a subcategory of coiled-coil domains found widely in nature and play critical

roles in functions ranging from muscle contraction¹² to transcriptional control.¹³ Coiled coils are characterized by heptad repeating units designated as *abcdefg*, where the *a* and *d* positions are occupied by hydrophobic residues such as leucine and the *e* and *g* positions are occupied by charged residues. These domains fold into amphiphilic α -helices, and hydrophobic interactions drive association into oligomeric clusters.¹⁴ Self-assembly of the leucine zipper domains of the AC₁₀A protein leads to a network, in which oligomer bundles serve as junction points.¹⁰ These hydrogels are reversible in response to pH and temperature. The physically associated network can be turned off under conditions of high pH or high temperature, where the leucine zipper domains are denatured.¹⁰ The hydrophilic mid-block lacks regular secondary structure and prevents precipitation of the chain under conditions that favor leucine zipper aggregation.

Artificial proteins such as AC₁₀A are readily prepared by biosynthetic methods. The amino acid sequence of interest is encoded into an artificial gene, and the protein is expressed in an appropriately transformed bacterial host. The flexibility of recombinant DNA technology allows systematic investigation of structure–property relationships and provides the potential for incorporating biological information—including cell binding domains and enzyme recognition sites—into engineered hydrogels. Since gelation is driven by self-assembly of protein domains and does not require chemical cross-linking reagents, cytotoxicity of these hydrogels is low. The low cytotoxicity of AC₁₀A was established by a WST-1 assay¹⁵ on 3T3 fibroblast cells cultured in the presence of AC₁₀A.¹⁶

AC₁₀A forms transient networks above a threshold concentration, as manifested by a plateau in the storage modulus in the high-frequency regime in dynamic

*Corresponding author: Tel (626) 395-3140; Fax (626) 793-8472; e-mail tirrell@caltech.edu.

Scheme 1. Schematic Representations and Amino Acid Sequences of Triblock Proteins and Their Constituent Domains


rheological tests.¹⁷ However, these hydrogels dissolve quickly when placed in open solutions near physiological pH. This behavior precludes their use in applications in which the gel is surrounded by excess water or tissue fluid.

Here we present a successful strategy to improve the stability of artificial leucine zipper hydrogels in open systems. We show that dissolution can be suppressed by judicious engineering of disulfide bonds between leucine zipper domains. The combined effects of leucine zipper self-assembly and disulfide bonding result in artificial protein hydrogels that are stable in open solutions near physiological pH and reversible in response to pH in closed systems.

Design Considerations. Dissolution of AC₁₀A hydrogels is a consequence of the structural and dynamic properties of leucine zipper aggregates and of the topological states of the artificial protein chains. The small aggregation number of leucine zipper assemblies,¹⁸ the constant exchange of the constituent peptides,¹⁹ and the tendency of the protein to form loops¹⁷ are all significant factors. Unlike the hydrophobic alkyl end groups of synthetic associative polymers,²⁰ the leucine zipper domains of AC₁₀A are characterized by small aggregation numbers. Kennedy¹⁸ found that such leucine zipper domains oligomerize into dimers and tetramers at micromolar concentrations; at the higher concentrations where hydrogels form, tetramers dominate. Oligomeric leucine zipper aggregates are known to undergo rapid strand exchange.^{19,21,22} The topological states of the protein chains are also critical. When the two end groups of a given chain participate in different network junctions, the midblock of the chain forms a bridge. When both ends participate in the same junction, the chain forms a loop and does not contribute to the

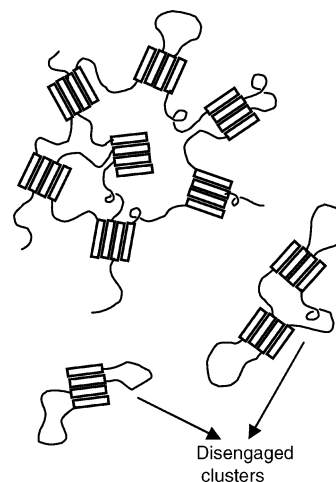


Figure 1. Disengaged clusters form constantly in AC₁₀A hydrogels, causing dissolution.

elasticity of the network. Rheological measurements show the storage moduli of AC₁₀A hydrogels in the examined concentration range (below 10%) are typically less than 15% of the values calculated on the basis of an ideal network assumption where 100% chains are elastically effective, suggesting that more than half the chains form loops.¹⁷ The predominance of loops and the small aggregation number of the end groups combine to yield a high probability of forming disengaged clusters as shown in Figure 1. Clusters at the surface diffuse into the surrounding medium, and end group exchange among different junction points ensures that disengaged clusters are regenerated, leading to relatively rapid dissolution of these physical gels in open systems.

Stabilizing the network in open solutions can be achieved by introducing covalent bonds, such as disul-

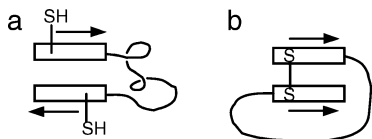


Figure 2. Rationale for asymmetric placement of the cysteine residue. (a) Looped configurations predominantly involve antiparallel association of leucine zipper end groups; asymmetric placement of the cysteine residue disfavors disulfide linkages in this configuration. (b) Only in the rare event of loop formation with parallel end groups would an intramolecular disulfide bond form.

fide bonds, between leucine zippers. Nature provides several examples of protein assemblies stabilized in analogous fashion. Type IV collagen forms a network through formation of twisted triple helices linked by multiple disulfide bonds.²³ Fibrous α -keratin in hair and wool is composed of self-assembled coiled coils, stabilized by disulfide bonds that contribute to the toughness and abrasion resistance of the structure.²⁴ Silk fibroin in *Lepidoptera* is composed of three different proteins that are linked by disulfide bonding and hydrophobic interactions.²⁵ In the systems examined in this work, disulfide bonds are engineered to suppress the generation of disengaged clusters by limiting intramolecular association and by increasing the valency of the building units of the physical gels.

To implement the design proposed above, several considerations must be taken into account. First, the cysteine residues in the leucine zipper domains must be placed such that they are buried in the hydrophobic core and brought into proximity for disulfide bond formation when the zippers assemble into oligomer bundles. Second, substitution of the residue at the *a* position or at the *d* position by a cysteine residue should cause minimal disruption of the secondary and tertiary structures of the leucine zipper domains. Zhou studied the positional effects of disulfide bonds on leucine zipper stability and found substitution at the *d* position to be most effective²⁶ for maintaining the secondary structure. Finally, among the six heptad repeats that constitute each coil domain, the specific heptad chosen to bear the newly introduced cysteine residue should favor formation of intermolecular disulfide bonds. The length of a coiled-coil domain with six heptad repeats is 65 Å,¹⁸ longer than the average dimension of the midblock. Dynamic light scattering measurements carried out in our laboratory revealed that the midblock chains have an average hydrodynamic diameter of 40 Å.²⁷ As a consequence, among looped configurations those with antiparallel association of the leucine zipper end groups are energetically favored over those with two parallel end groups, in which the midblock has to be stretched (Figure 2). Therefore, to prevent the cysteine residues in the leucine zipper domains from forming intramolecular disulfide bonds, the cysteine residue in each leucine zipper domain should be engineered asymmetrically. A similar strategy is used to control the orientation specificity of some natural leucine zipper domains: the GCN4 leucine zipper, which bears an asparagine residue positioned asymmetrically, adopts a parallel orientation that enables Asn residues to form interhelical hydrogen bonds; Asn \rightarrow Leu variants lose orientation specificity.^{14,28} On the basis of these considerations, a new triblock protein was designed in which the leucine residue at the *d* position of the second heptad repeat is substituted by a cysteine residue.

Experimental Section

Construction of Expression Vectors Encoding Artificial Proteins. Scheme 1 illustrates the sequences of the three triblock artificial proteins that will be discussed in this paper. Expression vectors pQE9AC₁₀Acys and pQE9AC₁₀Atrp were constructed previously by Petka.²⁹ pQE9AC₁₀Acys(L11C) was constructed from pQE9Acys²⁹ and pQE9C₁₀trp²⁹ through recombinant DNA manipulation. pQE9Acys(L11C), the L11C mutant of pQE9Acys, was made via the "QuickChange" (Stratagene, La Jolla, CA) procedure using the following oligonucleotides (the mutated codon for the 11th residue in the leucine zipper domain is italicized): 5' C GAA GTG GCC CAG TGC GAA AGG GAA GTT AG 3' (sense strand) and 5' CT AAC TTC CCT TTC GCA CTG GGC CAC TTC G 3' (antisense strand). The primers were synthesized at the Biopolymer Synthesis Center of the Beckman Institute at the California Institute of Technology. pQE9AC₁₀Acys(L11C) was constructed as follows: DNA segments encoding C₁₀ and A(L11C) were excised from pQE9C₁₀trp and pQE9Acys(L11C), respectively, by digestion with *Nhe*I and *Spe*I (New England Biolabs, Beverly, MA). The C₁₀ segment was ligated into the *Spe*I site of pQE9Acys(L11C) to yield pQE9AC₁₀cys(L11C). Digestion of pQE9AC₁₀cys(L11C) with *Spe*I followed by ligation with the A(L11C) fragment gave pQE9AC₁₀Acys(L11C). The sequence of pQE9AC₁₀Acys(L11C) was verified at the DNA sequencing core facility of the Beckman Institute at the California Institute of Technology.

Protein Synthesis and Purification. pQE9AC₁₀Acys, pQE9AC₁₀Atrp, and pQE9AC₁₀Acys(L11C) were each transformed into *Escherichia coli* strain SG13009, which carries the repressor plasmid pREP4 (Qiagen, Chatsworth, CA). Cultures of SG13009 pQE9AC₁₀Acys, SG13009 pQE9AC₁₀Atrp, and SG13009 pQE9AC₁₀Acys(L11C) cells were each grown at 37 °C in 1 L of Terrific Broth (TB) supplemented with 200 mg/L of ampicillin (Sigma, St. Louis, MO) and 50 mg/L of kanamycin (Sigma, St. Louis, MO). The culture was induced with 1.5 mM isopropyl-1- β -D-thiogalactoside (IPTG) (Calbiochem, Inc., San Diego, CA) when it reached an optical density (600 nm) of 0.7 to 1. Protein expression continued for 5 h; the optical density reached 1.4–2. Cells were harvested by centrifugation (5 min, 10000g); typical yields were ca. 5 g of wet cell mass per liter of cell culture. The cell pellet was resuspended in 8 M urea (pH 8.0) and frozen at –80 °C. The thawed lysate was centrifuged at 22100g for 20 min, and the supernatant was collected for purification. A 6 \times Histidine tag encoded in pQE9 vector allows the protein to be purified by affinity chromatography on a nickel nitrilotriacetic acid resin (Qiagen, Chatsworth, CA) following the denaturing protocol provided by Qiagen. To prevent thiol group oxidation and nonspecific disulfide bond formation during purification of AC₁₀Acys and AC₁₀Acys(L11C), 14 mM β -mercaptoethanol (Sigma, St. Louis, MO) was added to the washing and elution buffers. The eluted fractions were dialyzed against sterile deionized water for 3 days at room temperature, and the proteins were lyophilized. The average protein yield was 85 mg per liter of cell culture.

Mass Spectrometry. AC₁₀Acys and AC₁₀Acys(L11C) were digested by Lys-C, and the resulting proteolytic fragments were analyzed on an Applied Biosystems Voyager mass spectrometer. Purified AC₁₀Acys and AC₁₀Acys(L11C) were dissolved in digestion buffer (25 mM Tris-HCl, pH 8.5, 1 mM EDTA) at a concentration of 0.1 mg/mL. An AC₁₀Acys or AC₁₀Acys(L11C) solution (100 μ L) was mixed with 2 μ L of 0.1 μ g/ μ L Lys-C (Roche, Palo Alto, CA) and incubated at 37 °C for 6 h. The reaction was quenched by addition of trifluoroacetic acid (pH < 4.0). The sample was then purified on a ZipTipC₁₈ column (Millipore, Bedford, MA) and eluted with 10 μ L of elution buffer (50% acetonitrile, 50% water, 0.1% trifluoroacetic acid). The MALDI matrix α -cyano- β -hydroxycinnamic acid (4 μ L, 10 mg/mL in 50% CH₃CN) was added to 1 μ L of purified protein solution, and 0.5 μ L of the mixture was spotted on the sample plate and analyzed.

Free Thiol Titration. Free thiol concentrations in protein samples were determined by titration with Ellman's reagent³⁰ (5,5'-dithiobis(2-nitrobenzoic acid), DTNB) (Pierce, Rockford,

IL). A 10 mM DTNB solution was freshly prepared in 100 mM phosphate buffer or 8 M urea (pH 7.6), supplemented with 1 mM EDTA. Standard titration curves were generated with cysteine hydrochloride monohydrate (Sigma, St. Louis, MO) as the analyte. Each protein solution was mixed with the 10 mM DTNB solution in a volumetric ratio of 9:1, and the reaction mixture was incubated at room temperature for 15 min. The free thiol concentration in each protein sample was determined by measuring the absorbance at 412 nm.

Circular Dichroism Spectroscopy. CD spectra were recorded at room temperature on an Aviv 62DS spectropolarimeter (Lakewood, NJ). AC₁₀Atrp, AC₁₀Acys, and AC₁₀Acys(L11C) solutions (50 μ M) were prepared in 100 mM phosphate buffer by weighing at least 3 mg of each protein on a microbalance with an error less than 0.05 mg. Experiments were performed in a rectangular cell with path length of 1 mm. Spectra were scanned from 260 to 200 nm with points taken every 0.5 nm. Three scans were performed for each sample and averaged.

Release Studies. Red carboxylate-modified fluorescent polystyrene beads (1 μ m) (Molecular Probes, Eugene, OR) were embedded in 6.5% protein gels to a final concentration of 0.01%. Each gel was placed in a chamber filled with 100 mM phosphate buffer (pH 7.6). The volume ratio of buffer to gel was 40:1. The release of the beads and the stability of the gels were monitored using a Zeiss Axiovert 100 fluorescence microscope equipped with a 20 \times /0.45-NA objective. The illuminating beam for excitation went through a 535 nm band-pass filter, and the fluorescent images were collected through a 565 nm dichromatic splitter and a 610 nm band-pass filter.

Quantitative release rates were measured spectroscopically by using much smaller polystyrene beads (20 nm) that readily dispersed in the aqueous medium above hydrogel layers. For these experiments, hydrogel films with beads embedded were anchored on the interior bottom surfaces of cylindrical vials (12.8 mm diameter). The surfaces of the vials were aminated by cleaning with 98% sulfuric acid (EM Science, Gibbstown, NJ) and then reacting with a 3-(aminopropyl)triethoxysilane (Sigma-Aldrich, St. Louis, MO) solution (2%, in ethanol) for 30 min, followed by rinsing with ethanol and drying. A protein solution (120 μ L, 5.7%, pH 12.0) containing 0.02% red carboxylate-modified fluorescent polystyrene beads (20 nm, Molecular Probes) was added to the treated vial, followed by a pH adjustment with 6 N HCl to yield a pH value of 7.6. The solution gelled, and the final protein concentration was brought to 5.5% with buffer. The vial was placed at the center of a centrifuge holder and centrifuged at 1700*g* for 2 min, yielding a flat gel film of 12.8 mm diameter and 0.96 mm thickness. Phosphate buffer (4 mL, pH 7.6, 100 mM) was added into the vial 3 h later. The vial was then mounted on an Eppendorf Thermomixer (Brinkmann Instruments, Inc., Westbury, NY) and shaken at 300 rpm. Aliquots (130 μ L) of supernatant were taken at successive time points, and the fluorescence was measured on a fluorometer (Photon Technology International, Inc., Lawrenceville, NJ). The samples were excited at 575 nm, and the emission was scanned from 585 to 700 nm. Aliquots were returned into the vial after each measurement to maintain a constant volume of supernatant.

Particle Tracking. Red carboxylate-modified fluorescent polystyrene beads (0.5 μ m) (Molecular Probes, Eugene, OR) were embedded in protein solutions at a final concentration of 0.02%. Fluorescence imaging of the embedded beads was performed under epillumination by using a Zeiss Axiovert 100 microscope equipped with a 100 W mercury Oriel Q Arc lamp. The illuminating beam for excitation went through a 535 nm band-pass filter. The epilluminescence was collected by using a 40 \times /1.4-NA oil immersion Plan Neofluar (Zeiss) objective and imaged through a 565 nm dichromatic splitter and a 610 nm band-pass filter. Images were recorded by a Dage-MTI CCD-72 camera; the analog signal from the camera was converted to a digital movie by a JVC digital video recorder. Digitized movies were analyzed by using a software package written by Crocker and Grier.³¹ The software determines particle positions, connects particle positions to form trajectories, and determines mean-square displacement from those trajectories.

Multiangle Light Scattering. A 6% (w/v) AC₁₀Acys(L11C) hydrogel with a pH value of 7.6 was made in 100 mM phosphate buffer. The pH was adjusted to 12.2 12 h later, and the solution was converted to a viscous liquid. The solution was transferred into a volumetric flask, and the concentration was brought to 4.84×10^{-4} mg/L with 100 mM phosphate buffer (pH 11.0). To examine whether the concentration is low enough to allow determination of molecular weight from a Debye plot, a 2.42×10^{-4} mg/L solution was made by diluting the 4.84×10^{-4} mg/L solution. Both solutions were subjected to multiangle light scattering measurements on a DAWN EOS light scattering instrument (Wyatt Technology Corp., Santa Barbara, CA). The data were analyzed with Debye plots by using a dn/dc value of 0.185.³²

Rheological Oscillatory Shear Measurements. AC₁₀Atrp and AC₁₀Acys(L11C) solutions were prepared in phosphate buffer (13 mM NaH₂PO₄·H₂O, 87 mM Na₂HPO₄·7H₂O) with their pH values adjusted to 7.0. The solutions were centrifuged to remove entrapped bubbles before being loaded on an RFS III rheometer (TA Instruments, New Castle, DE). The temperature was controlled at 22.0 ± 0.1 °C by a peltier thermoelectric device. A parallel-plate geometry (0.5 mm gap and 8 mm diameter) was used for frequency sweep measurements from 100 to 1 rad/s under a 1% strain, which was confirmed to be within the linear viscoelastic regime on the basis of strain sweep tests. The edge of each sample was covered with mineral oil to minimize solvent evaporation.

Swelling Behavior. AC₁₀Acys(L11C) gel films were made by drying the protein solution (10 μ L, 5%, pH 11.0) in a circular reservoir (4.4 mm in diameter) on aminated or untreated glass slides. Rehydrating the dried pads at neutral pH yielded swollen hydrogel films. The lateral swelling ratio was measured by a micrometer (Mitutoyo). The vertical swelling ratio was measured by analyzing samples containing 0.02% fluorescent beads (0.5 μ m, Molecular Probes) on a confocal microscope (Zeiss LSM-Pascal) equipped with a 40 \times objective. Each sample was scanned in the XY plane, and the scanning plane varied in the Z direction with a resolution of 0.2 μ m. The thickness of the gel pad was determined by marking the first and the last Z stacks where the embedded fluorescent beads were observed. Dimensional changes in the lateral and vertical directions were also observed through a contact angle goniometer (Ramé-Hart Inc.). The degree of overall swelling was calculated on the basis of the changes in dimension or weight.

Micropatterning and AFM Imaging. Poly(dimethylsiloxane) (PDMS) stamps were prepared as described previously by Jackman et al.³³ A master consisting of cylindrical photoresist posts supported on a silicon wafer was prepared photolithographically. The posts were 30 μ m in diameter, with a center-to-center distance of 60 μ m. The height of the posts was controlled to be between 1 and 2 μ m. The PDMS (Sylgard 184, Dow Corning, Midland, MI) was cured against the master at 60 °C for 2 h and peeled away from the silicon wafer to yield a stamp.

An AC₁₀Acys(L11C) protein solution (2 μ L, pH 11.0, 5%) was placed on a aminated glass slide or silicon wafer. A PDMS stamp was placed on the top of the solution with its patterned surface facing down, so that the wells of the PDMS stamp were filled with protein solution and extra solution was squeezed out. After the protein solution dried, the PDMS stamp was peeled from the surface. Dry protein pads on the glass slide or silicon wafer were rehydrated with aqueous buffer (neutral pH). Micropatterned gel pads were soaked in excess buffer for 48 h, and the patterns were monitored with a Zeiss optical microscope. Images of the gel pads before and after rehydration were recorded in tapping mode on a Nanoscope III atomic force microscope (Digital Instruments, Inc.) to examine the changes in size and shape.

Results and Discussion

Protein Biosynthesis. AC₁₀Atrp, AC₁₀Acys, and AC₁₀Acys(L11C) were expressed in *E. coli*, purified, and analyzed by SDS-PAGE gel electrophoresis. To confirm

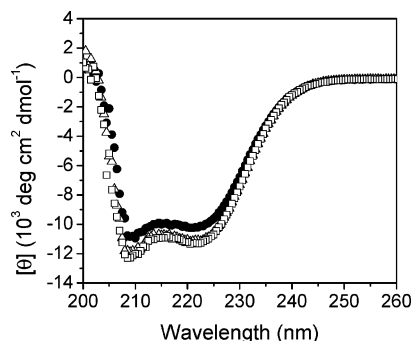


Figure 3. CD spectra of 50 μ M artificial protein solutions (pH 7.6, room temperature, 100 mM phosphate buffer): Δ , AC₁₀-Acys; \square , AC₁₀Atrp; \bullet , AC₁₀Acys(L11C).

that the 11th residue of the leucine zipper domain was mutated from a leucine in AC₁₀Acys into a cysteine in AC₁₀Acys(L11C), proteolytic fragments of purified AC₁₀-Acys and AC₁₀Acys(L11C) were prepared by digestion with Lys-C, followed by mass spectral analysis. A point mutation from *L* to *C* in the fragment WASGDLNEVAQL(−C)EREVRSLEDEAAELEQK yields an expected shift in mass from 3443.66 to 3433.58 Da. The observed fragments appear at 3443.76 and 3433.54 Da, respectively, consistent with a single *L* to *C* mutation.

Disulfide Bond Formation. Disulfide bond formation in 50 μ M AC₁₀Acys(L11C) solutions (100 mM phosphate buffer, pH 7.6, room temperature) was analyzed by titration with Ellman's reagent. About 90% of cysteine residues were oxidized within 3 h, indicating that no added oxidizing reagent is needed for disulfide bonds to form.

Secondary Structure. AC₁₀Atrp and AC₁₀Acys solutions showed almost identical helicity as evidenced by minima at 222 and 208 nm in their circular dichroism spectra. The molar ellipticity of AC₁₀Acys(L11C) at 222 nm was 92% of the values observed for AC₁₀Atrp and AC₁₀Acys (Figure 3), suggesting that the zipper domains remain helical after cysteine residues are incorporated and even after disulfide bonds form. Identical CD spectra were recorded for AC₁₀Acys(L11C) solutions under both oxidizing and reducing conditions, suggesting that it is substitution of the leucine residue by a cysteine residue, rather than disulfide bond formation, that causes this modest disruption of the secondary structure of the leucine zipper domains. This result also indicates little reaction between C-terminal and internal cysteine residues. Were such linkages significant, the secondary structure of the leucine zipper domain should be highly disrupted, but recoverable under reducing conditions. Fitting the data with a Ridge Regression Analysis (CONTIN) program³⁴ gives estimates of the percent of α -helix for AC₁₀Acys, AC₁₀Atrp, and AC₁₀Acys(L11C) of 34%, 36%, and 29%, respectively, while the predicted value for AC₁₀Acys and AC₁₀Atrp is 37% if one simply divides the number of amino acids in the putative helical region by the total number of amino acids in the chain. This comparison suggests that ca. 80% helicity is retained in the leucine zipper domains after cysteine residues and disulfide bonds are introduced.

Encapsulation and Release Behavior. AC₁₀Acys(L11C) hydrogels showed significantly improved stability compared to AC₁₀Atrp and AC₁₀Acys gels in open solutions, as shown by encapsulation and release of tracer particles. At pH 7.6, none of the fluorescent beads entrapped in AC₁₀Acys(L11C) gels were found to escape

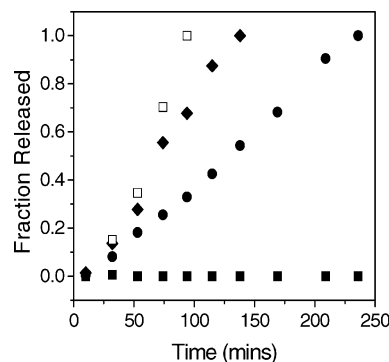


Figure 4. Release of embedded fluorescent beads (20 nm) from hydrogel films made from different proteins (5.5% w/v, pH 7.6, room temperature, 100 mM phosphate buffer): \blacksquare , AC₁₀-Acys(L11C); \bullet , AC₁₀Acys; \blacklozenge , AC₁₀Atrp; \square , AC₁₀Acys(L11C) in reducing buffer (10 mM TCEP).

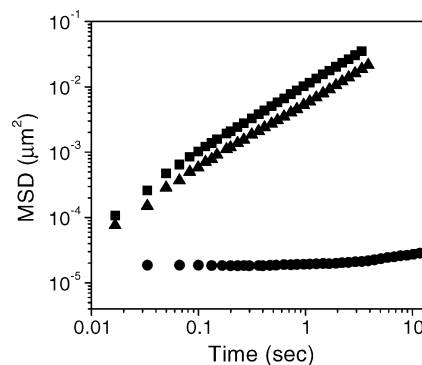


Figure 5. Mean-square displacement of beads (500 nm) embedded in 7.5% AC₁₀Acys(L11C) solutions (room temperature, 100 mM phosphate buffer): \blacksquare , pH 12.2; \bullet , pH adjusted from 12.2 to 7.2; \blacktriangle , pH adjusted from 12.2 to 7.2 and adjusted back to 12.2 3 h later.

into the surrounding buffer after 2 days. In contrast, AC₁₀Atrp and AC₁₀Acys hydrogels dissolved within several hours. Under sterile conditions, AC₁₀Acys(L11C) gels remain stable in open solutions of 100 mM phosphate buffer or Dulbecco's Modified Eagle's Medium (DMEM) for several weeks.

The release rates of embedded beads were quantified by measuring the fluorescence intensity of the surrounding buffer as a function of time. Under oxidizing conditions, no release from the AC₁₀Acys(L11C) gel was observed over several days. For AC₁₀Atrp and AC₁₀Acys gels, release was linear in time (Figure 4), suggesting that network failure occurred by surface erosion. When placed in reducing buffer (containing 10 mM TCEP), AC₁₀Acys(L11C) gels dissolved rapidly with release of embedded beads (Figure 4). Release from AC₁₀Acys(L11C) gels under reducing conditions was even faster than that from AC₁₀Atrp gels, probably because of the lower helicity and consequent weaker aggregation of the leucine zipper domains in AC₁₀Acys(L11C). This result confirms the important role of disulfide bonds in maintaining the stability of the AC₁₀Acys(L11C) network in open solutions.

Reversibility in Response to pH. Single particle tracking was used to examine the reversibility of gelation of AC₁₀Acys(L11C) in response to pH in closed systems. As shown in Figure 5, the mean-square displacement of tracer particles dispersed in a 7.5% AC₁₀Acys(L11C) solution at pH 12.2 increased linearly with time, indicating viscous behavior. When the pH was adjusted from 12.2 to 7.2, the mean-square dis-

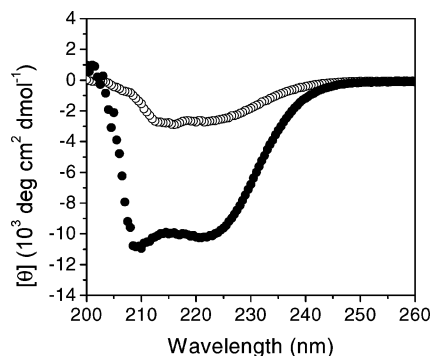


Figure 6. CD spectra of 50 μM AC₁₀Acys(L11C) solutions at different pH values (room temperature, 100 mM phosphate buffer): ●, pH 7.6; ○, pH 12.2. Percentages of α -helix at pH 7.6 and 12.2 are estimated as 29% and 3%, respectively, by a Ridge Regression Analysis (CONTIN) program.³⁴

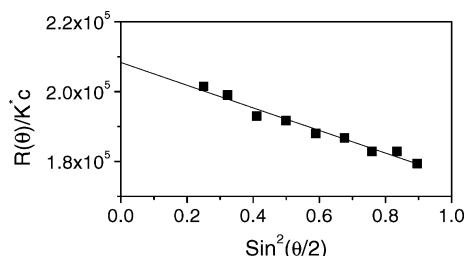


Figure 7. Debye plot of multiangle light scattering signals from a 2.42×10^{-4} mg/L solution (room temperature, pH = 11.0) reveals that the average molecular weight of the clusters disassembled from an AC₁₀Acys(L11C) hydrogel at pH 12.2 is ca. 207 300. This corresponds to ca. 9 chains in each cluster. (The molecular weight of each AC₁₀Acys(L11C) chain is 22 433.)

placement remained essentially constant on a time scale of seconds, consistent with formation of an elastic gel. Viscous behavior was instantly recovered when the pH was returned to 12.2 (Figure 5). As long as the pH was kept at 12.2 only for a short period of time (too short to allow alkaline cleavage of disulfide bonds to become significant³⁵), the viscous protein solution gelled again upon acidification. The resulting gel remained stable when placed in open solutions.

Although alkaline cleavage of disulfide bonds can occur at pH 12.2, only about 1% of the cleavage takes place within 1 h.³⁵ Therefore, the instant gel-to-sol transition observed upon raising the pH is attributed to the loss of helical secondary structure in the leucine zipper domains as determined by CD analysis (Figure 6). This suggests that leucine zipper self-assembly is indispensable in maintaining an AC₁₀Acys(L11C) network even after disulfide bonds form.

According to Miller–Macosko theory,^{36,37} a network can form from homopolymerization of trifunctional monomers when half of the functional groups have been consumed by intermolecular coupling. Although each AC₁₀Acys(L11C) chain carries three cysteine thiols and more than half form disulfide bonds under the conditions examined here, particle tracking and simple inspection show that the network dissolves when the leucine zipper domains are denatured. Multiangle light scattering measurements (Figure 7) revealed that, upon leucine zipper denaturation at high pH, the disassembled species in the solution are composed of ca. 9 triblock protein chains on average, suggesting that disulfide bonds create predominantly linear linkages rather than trifunctional covalent network junctions.

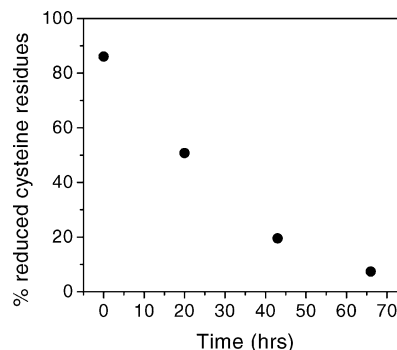


Figure 8. Percentage of reduced cysteine residues in a 6% w/v AC₁₀Acys(L11C) solution in 8 M urea as a function of time (pH 7.6, room temperature). The dry protein was pretreated so that the leucine zipper domains were denatured, and the majority of the cysteine residues were reduced.

Were such junctions significant, the network structure would be retained after the leucine zippers were denatured. The predominance of linear linkages is most likely due to the redundancy of the disulfide bonds formed from C-terminal cysteine residues.

“Multichains” with predominantly linear linkages created by disulfide bonds act as the building units of AC₁₀Acys(L11C) physical networks. These building units bear many sites of physical association through leucine zipper aggregation. The increased valency of the building units improves the stability of AC₁₀Acys(L11C) hydrogels, while the noncovalent nature of the junctions imparts reversibility to the network. Formation of “multichains” is a consequence of rationally engineered cysteine residues that are positioned in the leucine zipper domains asymmetrically. This design limits intramolecular disulfide bonds and enables chain extension.

We examined directly the possibility that oxidation of cysteine residues in AC₁₀Acys(L11C) could lead to a network in the absence of leucine zipper self-assembly at a concentration of 6% w/v, at which AC₁₀Acys(L11C) solutions form hydrogels under native conditions. A sample of AC₁₀Acys(L11C) with denatured leucine zipper domains and reduced cysteine residues was prepared by dissolving the purified protein (1 mM) in 8 M urea containing 20 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), followed by HPLC separation and lyophilization. A solution of the resulting protein was prepared in 8 M urea (pH 7.6) at a concentration of 6% w/v, and the free thiol concentration was titrated at successive time points. As shown in Figure 8, disulfide bond formation was substantially retarded in the absence of the template provided by leucine zipper self-assembly. In solutions containing properly folded zipper domains, 90% of cysteine residues are oxidized after 3 h of exposure to ambient air. In urea solutions, however, 90% oxidation requires ca. 60 h. Significantly, urea solutions of AC₁₀Acys(L11C) remained fluid even after oxidation, indicating that disulfide bonds alone do not lead to a network in the absence of leucine zipper self-assembly at 6% w/v. The most likely explanation for the absence of gelation is the formation of a significant number of intramolecular disulfide bonds in the random coil protein molecules in the absence of secondary structure.

Storage Modulus. AC₁₀Acys(L11C) hydrogels exhibited higher storage moduli than AC₁₀Atrp hydrogels at the same concentration (Figure 9). The storage modulus of the AC₁₀Acys(L11C) hydrogel increased by ca. 30%

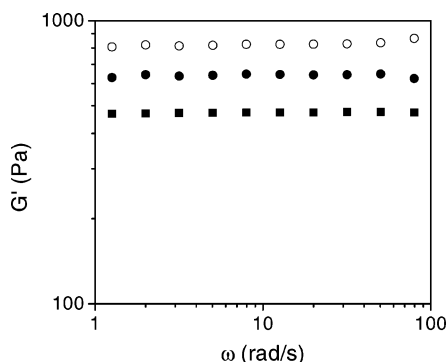


Figure 9. Storage moduli of protein hydrogels as determined by rheological oscillatory frequency sweep tests (7% w/v, 100 mM phosphate buffer, pH 7.0, 22 °C, 1% strain): ■, AC₁₀Atrp; ●, ○, AC₁₀Acys(L11C) (closed symbols, measured immediately after preparation of the gel; open symbols, measured 1 h later).

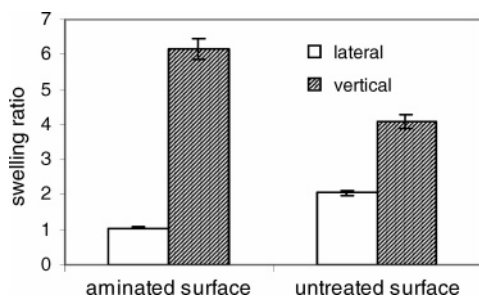


Figure 10. Lateral and vertical swelling ratios of AC₁₀Acys(L11C) gels on aminated and untreated surfaces. (The diameter to thickness aspect ratio of the dry protein pad is ca. 150.)

in the first hour after preparation, while that of the AC₁₀Atrp hydrogel remained constant. These differences likely reflect the consequences of placing cysteine residues asymmetrically in the leucine zipper domains. This design allows intramolecular, antiparallel leucine zipper dimers to undergo strand exchange until they form intermolecular coiled coils fastened by disulfide bonds. Reduction in the number of intramolecular loops in the AC₁₀Acys(L11C) hydrogel leads to a higher storage modulus.

Swelling Behavior. AC₁₀Acys(L11C) gel pads anchored on aminated glass surfaces undergo anisotropic swelling, which was observed through the microscope of a contact angle goniometer. Quantitative measurements by confocal microscopy revealed that the lateral swelling ratio was very slight (close to 1) while the vertical swelling ratio was as high as 6 (Figure 10). When AC₁₀Acys(L11C) gel pads were made on untreated glass surfaces, they detached from the surface upon

hydration. The lateral swelling ratio of unconstrained gels was approximately twice that of anchored gels (Figure 10). This suggests that the amine groups on the treated surface play an important role in anchoring the gel pad and suppressing lateral swelling. Since the midblock of AC₁₀Acys(L11C) carries 10 glutamic acid residues and is therefore negatively charged at pH 7.6, anchoring of the gels is likely to occur through electrostatic attraction.

The overall swelling ratio of an unconstrained AC₁₀Acys(L11C) gel pad in 100 mM phosphate buffer (pH 7.6) is 18.9 ± 2.3 , as calculated on the basis of the change in weight. Calculation on the basis of dimensional change reveals an overall swelling ratio of 17.2 ± 2.6 , reasonably consistent with the value obtained from gravimetric measurements. Although the vertical swelling of an unconstrained gel is less than that of an anchored gel, the overall swelling of the unconstrained gel (about 18) is much greater than that of the anchored gel (about 6). This accords with prior studies of anisotropic swelling of synthetic gels constrained on substrates.^{38,39} For constrained gels, the lateral stress imposed by the substrate complements the elastic force in balancing the osmotic pressure and thereby reduces the overall degree of swelling.

Micropatterning. The reversibility of gelation in response to pH in closed systems, the stability of the gels in open solutions, and the anisotropic swelling of the gels on aminated surfaces allowed us to generate stable AC₁₀Acys(L11C) hydrogel micropatterns by soft lithography. Protein solutions of low viscosity were prepared at high pH and then subjected to soft lithographic micropatterning as described in the Experimental Section. Rehydration of the dried protein at neutral pH resulted in micropatterned gel pads. The diameter of the gel pads was consistent with that of the wells of the stamp (30 μm) and remained constant when the gel pads were placed in excess buffer for 48 h, as examined by light microscopy. Further examination by AFM showed no obvious change in shape within 24 h (Figure 11). Retention of the original micropatterns is facilitated by electrostatic attraction between the gel pads and the aminated surface, which not only immobilizes the gel pads but also suppresses their swelling in the lateral direction. The ability of the hydrogel pads to retain shape and registration is important for their potential application in array technologies.

Conclusions

Artificial protein hydrogels were assembled through the combined effects of leucine zipper self-assembly and

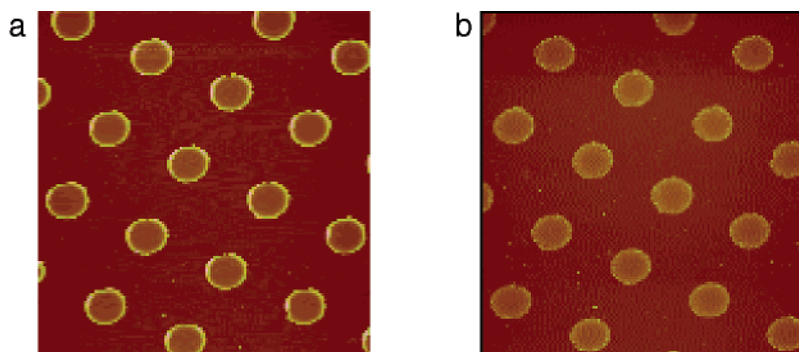


Figure 11. Atomic force images of micropatterned AC₁₀Acys(L11C) gel pads acquired in tapping mode (100 μm × 100 μm). (a) before rehydration; (b) after 24 h of rehydration in excess water. The image was recorded after the gel pads were redried. No obvious loss of material or change in shape was observed.

disulfide bonding. These hydrogels retain their integrity in open solutions for several weeks and exhibit reversibility of gelation in response to pH in closed systems. Gel pads made on aminated surfaces undergo anisotropic swelling, with lateral swelling significantly suppressed. The reversibility, stability, and anisotropic swelling allow the generation of stable hydrogel micro-patterns through soft lithography. These hydrogels show promise with respect to application in controlled release and microarray technologies, and their potential as scaffold matrices for tissue engineering is being addressed by ongoing studies.

Acknowledgment. The authors acknowledge the NSF Center for the Science and Engineering of Materials for financial support.

References and Notes

- Lee, K. Y.; Mooney, D. J. *Chem. Rev.* **2001**, *101*, 1869–1879.
- Gutowska, A.; Jeong, B.; Jasionowski, M. *Anatomical Record* **2001**, *263*, 342–349.
- Delgado, M.; Spanka, C.; Kerwin, L. D.; Wentworth, P.; Janda, K. M. *Biomacromolecules* **2002**, *3*, 262–271.
- Halstenberg, S.; Panitch, A.; Rizzi, S.; Hall, H.; Hubbell, J. A. *Biomacromolecules* **2002**, *3*, 710–723.
- Hisano, N.; Morikawa, N.; Iwata, H.; Ikada, Y. *J. Biomed. Mater. Res.* **1998**, *40*, 115–123.
- Jen, A. C.; Wake, M. C.; Mikos, A. G. *Biotechnol. Bioeng.* **1996**, *50*, 357–364.
- Risbud, M.; Endres, M.; Ringe, J.; Bhonde, R.; Sittinger, M. *J. Biomed. Mater. Res.* **2001**, *56*, 120–127.
- Stile, R. A.; Healy, K. E. *Biomacromolecules* **2001**, *2*, 185–194.
- Martens, P. J.; Bryant, S. J.; Anseth, K. S. *Biomacromolecules* **2003**, *4*, 283–292.
- Petka, W. A.; Harden, J. L.; McGrath, K. P.; Wirtz, D.; Tirrell, D. A. *Science* **1998**, *281*, 389–392.
- Wang, C.; Stewart, R. J.; Kopecek, J. *Nature (London)* **1999**, *397*, 417–420.
- Surks, H. K.; Richards, C. T.; Mendelsohn, M. E. *J. Biol. Chem.* **2003**, *278*, 51484–51493.
- O'Shea, E. K.; Rutkowski, R.; Stafford, W. F.; Kim, P. S. *Science* **1989**, *245*, 646–648.
- O'Shea, E. K.; Klemm, J. D.; Kim, P. S.; Alber, T. *Science* **1991**, *254*, 539–544.
- Ishiyama, M.; Shiga, M.; Sasamoto, K.; Mizoguchi, M.; He, P. G. *Chem. Pharm. Bull.* **1993**, *41*, 1118–1122.
- 3T3 fibroblast cells suspended in DMEM medium (supplemented with 10% donor calf serum and 1% PenStrep) were seeded at a density of 30 000 cells/cm² in 96-well plates. AC₁₀-Atrp was added into the medium to concentrations up to 4.3 mg/mL. WST-1 assays carried out after 24 h of cell culture revealed that the cells cultured in the presence of AC₁₀-Atrp had the same viability as control cells.
- AC₁₀A solutions (5–9% w/v) were made in phosphate buffer (13 mM NaH₂PO₄·H₂O, 87 mM Na₂HPO₄·7H₂O), and the pH values were adjusted to the range between 7.0 and 8.0. Oscillation frequency sweep tests (from 100 to 0.001 rad/s) were performed at 1% strain on an RFS III rheometer (TA Instruments, New Castle, DE) with a cone-and-plate geometry (0.04 rad cone angle and 25 mm diameter). The storage modulus levels off between 1 and 100 rad/s under all conditions examined (22 °C) with values less than 13% of those calculated on the basis of an ideal network assumption where 100% chains are elastically effective, suggesting the predominance of loops.
- Kennedy, S. B. Ph.D. Dissertation, University of Massachusetts—Amherst, Amherst, MA, 2001.
- Fluorescein maleimide was site-specifically labeled to the cysteine residue engineered at the end of the leucine zipper A (with a 14-amino acid linker between the zipper domain and the cysteine residue). Fluorescence is self-quenched in this labeled protein solution because of leucine zipper self-assembly. Mixing a labeled leucine zipper solution with an unlabeled leucine zipper solution in a stopped-flow spectrometer led to fluorescence de-quenching, suggesting the strand exchange among leucine zipper aggregates. Details will be reported separately.
- Xu, B.; Yekta, A.; Li, L.; Masoumi, Z.; Winnik, M. A. *Colloids Surf., A* **1996**, *112*, 239–250.
- Chao, H. M.; Houston, M. E.; Grothe, S.; Kay, C. M.; O'ConnorMcCourt, M.; Irvin, R. T.; Hodges, R. S. *Biochemistry* **1996**, *35*, 12175–12185.
- Wendt, H.; Berger, C.; Baici, A.; Thomas, R. M.; Bosshard, H. R. *Biochemistry* **1995**, *34*, 4097–4107.
- Kuhn, K.; Glanville, R. W.; Babel, W.; Qian, R. Q.; Dieringer, H.; Voss, T.; Siebold, B.; Oberbaumer, I.; Schwarz, U.; Yamada, Y. *Ann. N.Y. Acad. Sci.* **1985**, *460*, 14–24.
- Yu, J. L.; Yu, D. W.; Checkla, D. M.; Freedberg, I. M.; Bertolino, A. P. *J. Invest. Dermatol.* **1993**, *101*, S56–S59.
- Inoue, S.; Tanaka, K.; Arisaka, F.; Kimura, S.; Ohtomo, K.; Mizuno, S. *J. Biol. Chem.* **2000**, *275*, 40517–40528.
- Zhou, N. E.; Kay, C. M.; Hodges, R. S. *Biochemistry* **1993**, *32*, 3178–3187.
- A polypeptide (C₁₀) with the amino acid sequence of the midblock of AC₁₀A was synthesized. C₁₀ solutions (140 μM, pH 10.0) were filtered through 0.02 μM filters and subjected to dynamic light scattering measurements on BI-9000AT (Brookhaven) and WyattQELS (Wyatt Technology) digital autocorrelators. The data were analyzed with software (based on the CONTIN algorithm) provided by Brookhaven.
- Lumb, K. J.; Kim, P. S. *Biochemistry* **1995**, *34*, 8642–8648.
- Petka, W. A. Ph.D. Dissertation, University of Massachusetts—Amherst, Amherst, MA, 1997.
- Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Methods Enzymol.* **1983**, *91*, 49–60.
- Crocker, J. C.; Grier, D. G. *J. Colloid Interface Sci.* **1996**, *179*, 298–310.
- Huglin, M. B. *Light Scattering from Polymer Solutions*; Academic Press: London, 1972.
- Jackman, R. J.; Duffy, D. C.; Ostuni, E.; Willmore, N. D.; Whitesides, G. M. *Anal. Chem.* **1998**, *70*, 2280–2287.
- Provencher, S. W.; Glockner, J. *Biochemistry* **1981**, *20*, 33–37.
- Kuramitsu, S.; Hamaguchi, K. *J. Biochemistry* **1979**, *18*, 443–456.
- Miller, D. R.; Macosko, C. W. *Macromolecules* **1976**, *9*, 206–211.
- Macosko, C. W.; Miller, D. R. *Macromolecules* **1976**, *9*, 199–206.
- Tanaka, T.; Sun, S. T.; Hirokawa, Y.; Katayama, S.; Kucera, J.; Hirose, Y.; Amiya, T. *Nature (London)* **1987**, *325*, 796–798.
- Harmon, M. E.; Tang, M.; Frank, C. W. *Polymer* **2003**, *44*, 4547–4556.

MA048348S