Responsive Hybrid Hydrogels with Volume Transitions Modulated by a Titin Immunoglobulin Module

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The I28 immunoglobulin (Ig)-like module of human cardiac titin, an elastic muscle protein, was used to cross-link acrylamide (AAm) copolymers into hybrid hydrogels. Cross-linking was accomplished through metal coordination bonding between terminal histidine tags (His tags) of the I28 module and metal-chelating nitrilotriacetic acid (NTA)-containing side chains on the copolymer. In solution, the β-sheet structure of the I28 module unfolded with a transition midpoint of about 58 °C as the temperature was elevated. Hydrogels cross-linked with the I28 module demonstrated positive temperature responsiveness; they swelled to 3 times their initial volume at temperatures above the melting temperature of the cross-links. Positive temperature responsiveness is unusual for synthetic hydrogels. The I28 hybrid hydrogels demonstrate that cross-linking synthetic polymers with natural, well-characterized protein modules is a practical strategy for creating new materials with unique environmental responsiveness predictably determined by the mechanical properties of the protein cross-links. These new materials may be useful for controlled chemical delivery.

INTRODUCTION

Stimuli-responsive hydrogels undergo volume transitions in response to physical or chemical changes in their environment. A remarkable number of hydrogels have been developed that are responsive to changes in pH, ionic strength, biochemicals, solvent, temperature, electric and magnetic fields, and light (*1*). The extensive interest in responsive hydrogels stems in large part from the broad range of their potential applications. Responsive hydrogels have been used as selective absorbents (*2*), as matrixes for immobilization of enzymes (*3*), as reservoirs for controlled release of chemicals and drugs (*4*), and as microactuators (*5*). The majority of responsive hydrogels have been created using conventional methods of chemical and physical cross-linking of a relatively small number of synthetic polymers, especially (methyl)-acrylates and their copolymers.

As an alternative approach to synthesizing stimuli-responsive hydrogels, we are interested in cross-linking synthetic water-soluble polymers with recombinant protein modules to create hybrid hydrogels with environmental responsiveness that has not been observed in purely synthetic hydrogels. The rationale behind this approach is that the unique properties of the protein module cross-links, like well-defined structures, consistent mechanical properties, and cooperative folding and unfolding, may allow hydrogels to be designed with new, more pronounced, or uniquely controlled responses to environmental stimuli. The defined structures and properties of the cross-links will allow the hydrogel response to be directly related to the structure of the cross-links. Via adjustment of the amino acid sequence of the cross-links, it should be possible to fine-tune the responsiveness of the hybrid hydrogel for a specific application, perhaps much more precisely than previously possible through conventional polymer synthesis. As an example of a hybrid hydrogel, we recently reported a thermally responsive hybrid hydrogel that was assembled by cross-linking a copolymer of *N*-(2-hydroxypropyl)methacrylamide (HPMA) with synthetic and natural coiled-coil domains (*6*). These hydrogels collapsed sharply to about 10% of their equilibrium volume at a temperature that corresponded to a temperature-induced conformational transition in the coiled-coil cross-links. Responsive hydrogels have also been cross-linked with intact antibody molecules (*7*).

In this paper, we describe a new hybrid hydrogel assembled by cross-linking an acrylamide copolymer with a single immunoglobulin (Ig)-like domain from the elastic muscle protein, titin. Titin is a gigantic, modular protein consisting of more than 200 Ig and fibronectin type III domains. Structurally, the repeating titin Ig domain is a sandwich of two antiparallel β-sheets held together by hydrophobic interactions between the β-sheets in the core of the domain and by hydrogen bonds between the β-strands forming the β-sheets (*8*). Lately, a number of research groups have used optical tweezers (*9*, *10*) or AFMs (*11*−*13*) to stretch individual molecules of native titin, or recombinant segments of titin containing defined numbers of titin Ig domains, to investigate the molecular basis of titin's elasticity. The most prominent feature of the force-extension profiles generated in these experiments was a sawtooth pattern of exponential increases in force, followed by stress relaxation at a force threshold of about 200 pN. The number of events in the pattern corresponded to the number of Ig domains and the approximately 30 nm periodicity corresponded to the length of a stretched-out Ig domain, providing evidence that the events were due to the sequential stress denaturation of individual Ig domains. Steered molecular

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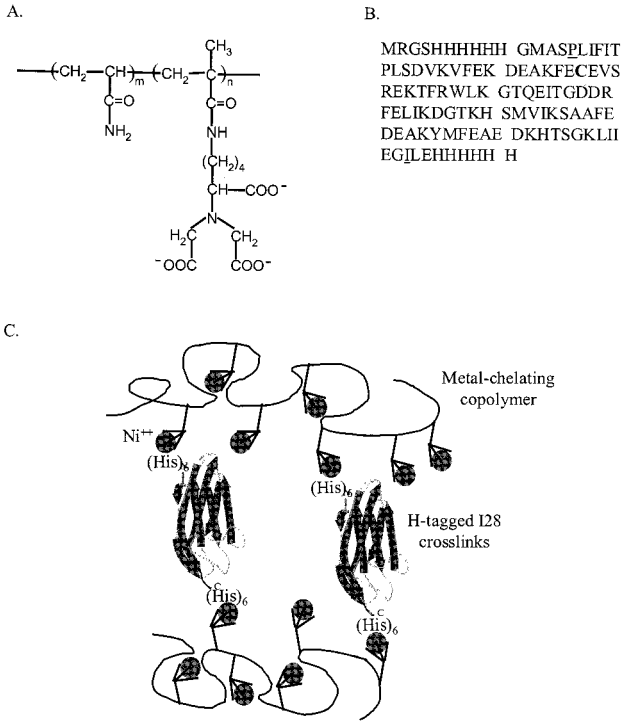

Figure 1. Assembly of the I28 cross-linked hydrogel. (A) Chemical structure of metal-chelating copolymer poly(AAm-*co*-MABNTA). The polymer was prepared by copolymerizing AAm with the metal-chelating comonomer MABNTA in Tris buffer (pH 8.9) under nitrogen, using ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylenediamine as the redox initiator. (B) Protein sequence of the recombinant titin I28 cross-linker H-I28-H. The protein contains His tags at both ends. Single-letter abbreviations for amino acids are used: M, methionine; R, arginine; G, glysine; S, serine; H, histidine; A, alanine; P, proline; L, leucine; I, isoleucine; F, phenylalanine; T, threonine; D, aspartic acid; V, valine; K, lysine; E, glutamic acid; C, cysteine; W, tryptophan; Q, glutamine; Y, tyrosine. The underlined amino acid residues mark the start or end of the human cardiac titin I28 segment. The recombinant protein module contains 111 amino acids with a predicted molecular mass of 12.2 kDa. (C) Structure of the hybrid hydrogel. The hydrogel was formed through metal complex formation by the pendant metal-chelating ligand, NTA-Ni^{2+}, on the polymer and the terminal His tags of the protein.

dynamic simulations suggested that a folded Ig domain when pulled from its ends resists stretching until hydrogen bonds between β-strands begin rupturing near a threshold force, which is followed by a sudden drop in force and complete unraveling of the domain structure (*14*).

Our objective was to create a hydrogel in which the chemical potential of the hydrogel polymer matrix generates the driving force stretching titin Ig modules from their ends. The titin Ig module, in turn, would control the swelling of the polymer matrix. The cooperative unfolding of the Ig module cross-links would lead to a unique hydrogel swelling profile in response to environmental conditions. Our strategy for assembling an Ig module cross-linked hydrogel is diagrammed in Figure 1. Cross-linking is through noncovalent, metal coordination bonds between pendant metal-chelating nitrilotriacetic acid (NTA) groups on the polymer side chain and terminal histidine residues (His tag) of the titin Ig module. This convenient protein binding scheme has been applied extensively in recombinant protein purification (*15*), in protein immobilization at interfaces (*16*, *17*), and recently in hybrid hydrogel assembly (*6*).

EXPERIMENTAL PROCEDURES

Construction of the pET24a-I28 Expression Plasmid. A segment of DNA encoding the I28 Ig domain of human cardiac titin was PCR amplified from a plasmid kindly provided by A. Pastore. The I28 PCR product was digested with *Nhe*I and *Xho*I (Promega) and ligated into a pET21a *Escherichia coli* expression vector (Novagen) that had been previously modified to add histidine tags at both the amino and carboxyl ends of the expressed protein. The construct was verified by restriction enzyme digestion analysis and DNA sequencing (Figure 1B). The double-His-tagged I28 insert was transferred to the kanamycin resistant pET24a plasmid.

Expression and Purification of I28. For expression, the pET24a-I28 plasmid was transformed into *E. coli* strain BL21(DE3) (Novagen, Inc.). Single colonies were selected and grown overnight at 37 °C in LB medium (5 mL) containing 50 μg/mL kanamycin. LB cultures (500 mL) with 50 μg/mL kanamycin were then inoculated with the overnight cultures and grown at 37 °C until the A_{600} reached approximately 0.8 (about 2 h). Expression was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C or for 6 h at 30 °C. The induced cells were pelleted (Beckman JA 10 rotor, 4500 rpm, 30 min) and stored at −70 °C.

To purify the double-His-tagged I28 (H-I28-H), the cell pellets were resuspended in binding buffer [10 mM Tris and 200 mM sodium chloride (pH 7.4)] containing 20 μg/mL lysozyme, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.1% Triton X-100. The cell suspension was sonicated on ice four times for 10 s using an ultrasonic demembranator (Fisher Scientific) to lyse the cells. After centrifugation at 4 °C, the clarified lysate was loaded onto a Ni-NTA (Qiagen, Inc.) column pre-equilibrated with binding buffer. The column was washed with binding buffer with 60 mM imidazole. H-I28-H was eluted with binding buffer with 300 mM imidazole. The H-I28-H fractions were combined and exchanged into 20 mM HEPES buffer with 150 mM NaCl (pH 7.4). The protein was concentrated, frozen in liquid nitrogen, and stored at −70 °C.

Sedimentation Equilibrium. H-I28-H samples were dialyzed into PBS buffer (pH 7.4) with 5 mM dithiothreitol (DTT). Three H-I28-H solutions with A_{280} values of 0.70, 0.35, and 0.18 were analyzed simultaneously in a Beckman Optima XL-A analytical ultracentrifuge with an An-60 Ti rotor. Centrifugation was carried out at 20 °C at three speeds, 25K, 32K, and 38K rpm. Protein sedimentation was assessed at 280 nm. Data analysis was performed using the nonlinear regression program NONLIN (*23*).

Circular Dichroism Spectroscopy. H-I28-H CD spectra were generated using a quartz rectangular cuvette with a path length of 0.1 cm and a volume of 300 μL in an Aviv 62DS CD spectrometer equipped with a thermoelectric temperature control system. Data were collected using the program Star3.0. Wavelength scans were performed at 25 °C, in 0.5 nm steps, and 5 s data averaging at each step. Scans for each sample were repeated at least five times and averaged. In the thermal stability experiments, the CD signal at 215 nm was monitored as the temperature was ramped from 30 to 90 °C in 1 °C increments. The sample was equilibrated for 2 min at each temperature, and the signal was averaged for 1.5 min.

Fluorescence Spectrometry. The fluorescence of the single tryptophan residue in H-I28-H was investigated using an ISS Grey multifrequency fluorometer with an

excitation wavelength of 293 nm. The fluorescence spectra of H-I28-H were determined under native conditions in PBS buffer [20 mM $NaPO_4$, 150 mM NaCl, and 5 mM DTT (pH 7.4)] and under denaturing conditions in PBS buffer with 5 M urea. For the denaturation experiment, spectra were recorded after 20 h at room temperature. The fluorescence spectra of H-I28-H cross-links in a hydrogel were collected at room temperature in PBS buffer and after heating the cuvette at 85 °C overnight.

Synthesis of a Metal-Chelating Acrylamide Copolymer [Poly(AAm-*co*-MABNTA)]. The metal-chelating monomer 2-methacrylamidobutyl nitrilotriacetic acid (MABNTA) was synthesized as described previously (*24*). The final product had a melting point of 104−107 °C, and a molecular mass of 329.7 Da determined by electrospray ionization mass spectrometry. ^{1}H NMR analysis (DMSO-d_6, 200 MHz): δ 1.33−1.59 [m, 6.9H, $CH_2(C\underline{H}_2)_3$], 1.82 (s, 3H, $C\underline{H}_3$), 3.05 (t, 1.7H, CO-NH-$C\underline{H}_2$), 3.40 [t, 1H, CH_2-$C\underline{H}$(COOH)-N], 3.53 [3.3H, N-($C\underline{H}_2$-$C\underline{O}O)_2$], 5.28, 5.60 (s, s, 2H, $C\underline{H}_2$=), 7.87 (1H, CO-N$\underline{H}$), 12.40 [s, 3H, (COO$\underline{H}$)$_3$].

Copolymerization (total monomer, 2.6 wt %) of acrylamide (AAm, Polysciences, Inc., chemzymes ultrapure, 86.5 mol %) and MABNTA (13.5 mol %) was carried out in Tris buffer (pH 8.9) at room temperature, under nitrogen for 24 h, using ammonium persulfate (Fisher Scientific, electrophoresis grade, 7 wt %) and *N*,*N*,*N*′,*N*′-tetramethylenediamine (Bio-Rad, electrophoresis grade) as the redox initiator. The copolymer was dialyzed against water and lyophilized. Weight and number-average molecular masses were measured by size exclusion chromatography on a fast protein liquid chromatography system equipped with a Superose 6 (10/30) column (Pharmacia, Piscataway, NJ) and a light-scattering detector (MiniDawn, Wyatt). The column was calibrated using poly(HPMA) standards with narrow polydispersity. The percentage of NTA side chains in the copolymer was determined by proton NMR (500 MHz) and by acid−base titration using a 10 mL ABU80 autoburet and a PHM84 research pH meter.

Assembly of I28 Cross-Linked Hydrogels. The metal-chelating acrylamide copolymer was charged with nickel by mixing the copolymer and nickel sulfate in 20 mM HEPES buffer (pH 7.4) and 150 mM NaCl at a 1:1 molar ratio of Ni^{2+} to NTA side chains. A gel formed initially before the mixture was thoroughly mixed, likely because of cross-linking between NTA side chains. With continued mixing, the gel completely dissolved. The pH of the solution was adjusted to 7.8 with NaOH. To assemble the hybrid hydrogels, the H-I28-H protein from a 110 mg/mL stock solution was mixed with the Ni-charged copolymer solution to give approximately 2 mol % protein cross-linking. As a control, the same amount of H-I28-H was mixed with the chelating copolymer in the absence of Ni^{2+}. A chemically cross-linked control was prepared by copolymerizing the same ratio of acrylamide and MABNTA comonomers with 2.5 mol % *N*,*N*′-methylenebisacrylamide (Polysciences, Inc., chemzymes ultrapure) under the same conditions used for preparing the titin cross-linked copolymer.

Temperature Responsiveness of I28 Cross-Linked Hydrogels. Small pieces of the hydrogels were immersed in PBS buffer [20 mM $NaPO_4$ and 150 mM NaCl (pH 7.4)] in a quartz cuvette with a jacket connected to a water bath to control the temperature. The gels were observed by brightfield microscopy (Nikon Eclipse E800) and recorded on videotape. The dimensions of the gel were measured from digitized images using Scion Image (Scion Corp.) software. The average rate of temperature increase was 2 °C/min, with the equilibration time being about 1 h at each recorded temperature. The temperature of the water bath was calibrated by measuring the temperature inside the cuvette with a thermocouple. The volume swelling ratio is the ratio of the hydrogel volume at an elevated temperature to the volume at 29 °C. Three samples were measured at each time.

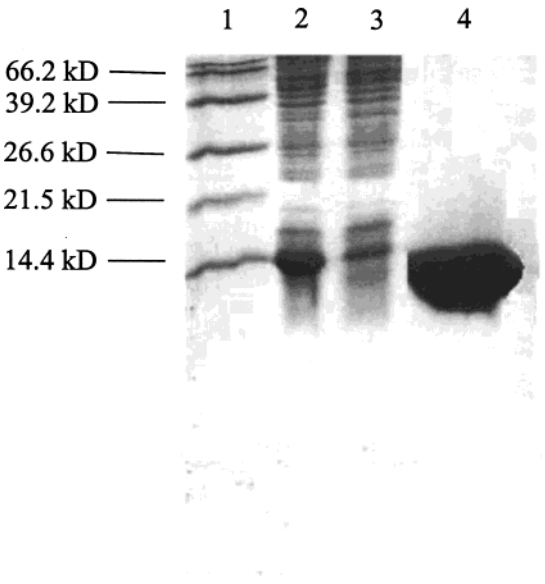

Figure 2. H-I28-H purification: lane 1, molecular mass markers; lane 2, soluble proteins from the cell lysate; lane 3, flow-through of the Ni-NTA agarose column; lane 4, purified H-I28-H.

RESULTS

Characterization of the H-I28-H Protein Module. The I28 Ig module from human cardiac titin was chosen because it was previously cloned, biochemically characterized, and had a reasonable melting temperature, 58 °C (*18*). During the subcloning process, His tags were added to both the carboxyl and amino termini of the I28 Ig module, which we term H-I28-H. The His-tagged Ig module was expressed efficiently in *E. coli* and was purified by metal affinity chromatography from *E. coli* lysates. By SDS−PAGE (Figure 2), the molecular mass of the Ig module was estimated to be about 12 kDa, in good agreement with the calculated molecular mass of 12.2 kDa based on the sequence. The native mass of H-I28-H was analyzed by sedimentation equilibrium in PBS buffer [20 mM phosphate, 150 mM NaCl, and 5 mM DTT (pH 7.4)]. The sedimentation data (not shown) were well-fit by an equation for a single ideal species with a molecular mass of 11.4 kDa. Comparison of the native molecular mass to the calculated molecular mass of 12.2 kDa suggested that H-I28-H was a stable, nonaggregating monomer in physiological conditions we used in our experiments.

The secondary structure and thermal stability of H-I28-H were investigated by CD spectroscopy. The CD spectrum of H-I28-H had a negative peak at about 213 nm (Figure 3A) characteristic of β-sheet structure (*19*), suggesting that the recombinant His-tagged I28 module was in its native conformation, which is comprised primarily of β-sheet. Thermal stability was investigated by monitoring the CD signal at 215 nm as the temperature was raised from 30 to 90 °C. In agreement with previously published stability studies of the I28 module (*18*), the H-I28-H module underwent thermal denaturation with a transition midpoint of about 58 °C (Figure 3B).

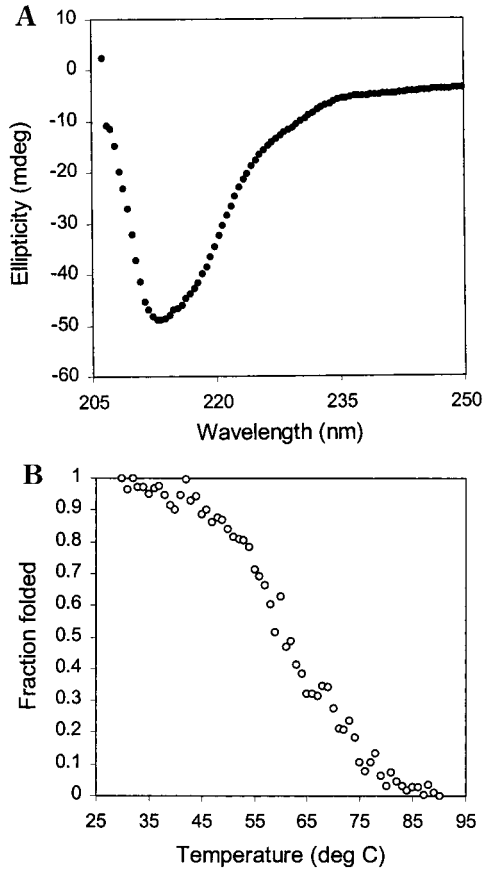

Figure 3. (A) CD spectra of H-I28-H at 25 °C in phosphate-buffered saline (pH 7.2). (B) Thermal denaturation of H-I28-H monitored by ellipticity at 215 nm.

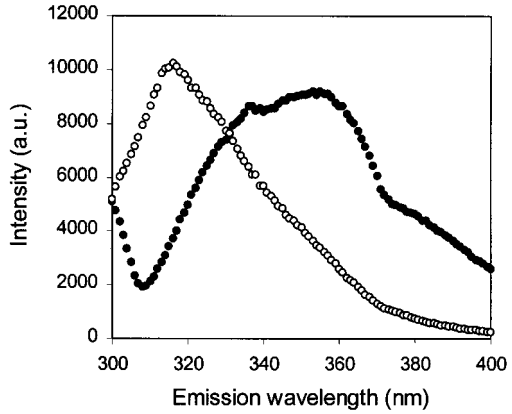

Figure 4. Intrinsic fluorescence of the single tryptophan residue of H-I28-H determined in the native state in phosphate-buffered saline (○) and after denaturation in phosphate-buffered saline and 5 M urea (●). The excitation wavelength was 293 nm.

The I28 module contains a single tryptophan (W) residue that is highly conserved in titin's Ig domains. From the structure of the titin Ig domains (*8*), the single W residue is known to be buried in the hydrophobic core between the two β-sheets of the Ig fold. Tryptophan residues that are fully exposed to water fluoresce maximally at about 350 nm, whereas Ws buried in a hydrophobic environment fluoresce at about 330 nm (*20*). Fluorescence spectrometry in the presence and absence of denaturant (Figure 4) confirmed that the W residue of H-I28-H was buried; in 5 M urea, the peak emission was at 350 nm, compared to peak emission at 317 nm in PBS.

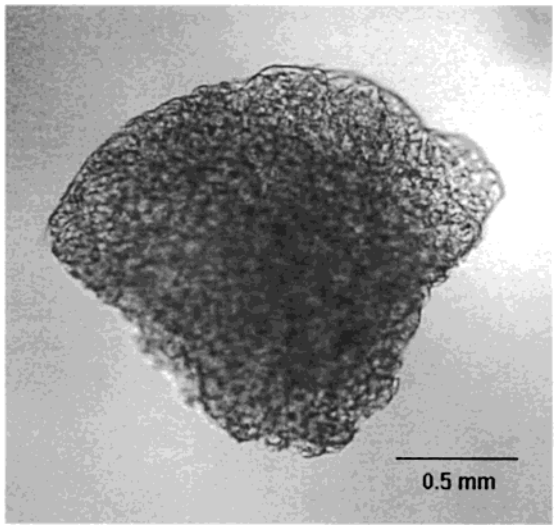

Figure 5. Photograph of a titin I28 cross-linked hydrogel. The thickness of the gel was less than 1 mm.

Characterization of the Metal-Chelating Acrylamide Copolymer [Poly(AAm-*co*-MABNTA)]. The chemical structure of the chelating MABNTA monomer was confirmed by ^{1}H NMR spectroscopy. After copolymerization with acrylamide, the copolymer number and weight-average molecular mass were 11 and 14 kDa, respectively. The percent of chelating monomers in the copolymer was determined by ^{1}H NMR and acid−base titration of the three COOH groups of the MABNTA units. During titration, a single neutral point was observed (not shown). If it is assumed that all three COOH groups in the MABNTA side chains were neutralized, the copolymer contained 10.0 mol % MABNTA. The same mol % of MABNTA was obtained by ^{1}H NMR.

Assembly of I28 Hybrid Hydrogels. When poly(AAm-*co*-MABNTA) chelated with a stoichiometric amount of Ni^{2+} was mixed with an H-I28-H solution at a cross-linking ratio of about 2 mol %, gels formed immediately. The I28 cross-linked hydrogels were translucent, macroscopically homogeneous, and relatively rigid (Figure 5). The gels remained intact for more than 30 days submerged in PBS buffer. In control experiments in which poly(AAm-*co*-MABNTA) was mixed with I28 in the absence of Ni^{2+}, no gels formed, confirming that gel formation occurred through metal coordination bonds. Additional control experiments were carried out in the absence of I28 and with substoichiometric amounts of Ni^{2+} to investigate gel formation by cross-linking between NTA side chains. When poly(AAm-*co*-MABNTA) chelated with a stoichiometric amount of Ni^{2+} was mixed with unchelated poly(AAm-*co*-MABNTA) at final NTA to Ni^{2+} ratios of 1.7:1 and 2.5:1, soft gels formed. However, these soft gels dissolved in excess PBS buffer, or when Ni^{2+} was added to raise the NTA to Ni^{2+} ratio toward 1:1. The control experiments suggest that the stable gel formation that we observed with Ni^{2+}-chelated poly(AAm-*co*-MABNTA) and H-I28-H was predominantly due to specific cross-linking between the His tags of H-I28-H and the NTA side chains of the copolymer.

Temperature Response of the Hybrid Hydrogels. The dimensions of the I28 cross-linked hydrogels were recorded as the hydrogels were heated under a video microscope. Via measurement of the distance between three triangular points on the gel, it appeared that the gel swelled isotropically (not shown). Therefore, the

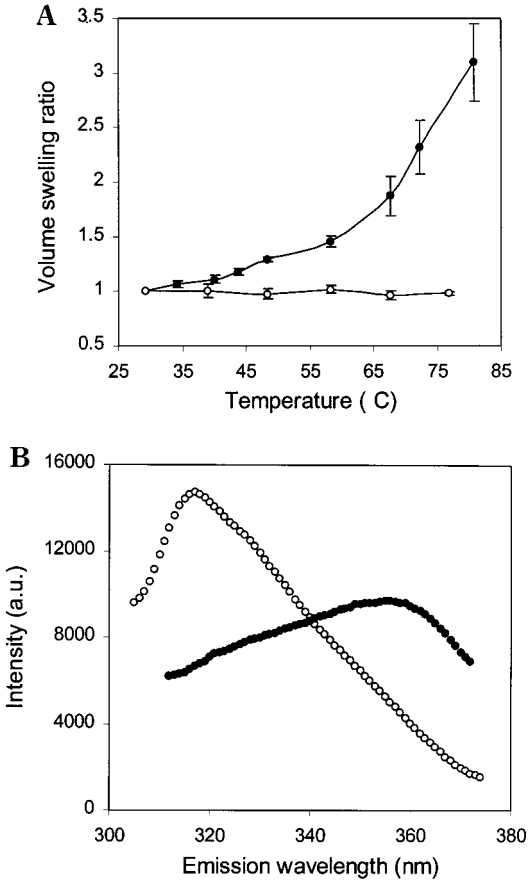

Figure 6. (A) Temperature responses of hydrogels in phosphate-buffered saline (pH 7.2). The volume swelling ratio is the ratio of hydrogel volume at an elevated temperature to the volume at 29 °C: (●) titin I28 cross-linked hydrogel (10 mol % NTA comonomer and 2 mol % protein cross-link) and (○) chemically cross-linked hydrogel (10 mol % NTA comonomer and 2.5 mol % BisAAm). The gels were heated in a jacketed quartz cuvette connected to a water bath. The average rate of temperature increase was 2 °C/min, with an equilibration time of about 1 h. The temperature of the water bath was calibrated by measuring the temperature inside the cuvette with a thermocouple. The dimensions of the gel were measured from digitized video images using Scion Image (Scion Corp.) software. (B) Intrinsic fluorescence of the H-I28-H cross-linked hydrogel at room temperature (○) and after heating the gel at 85 °C (●). The excitation wavelength was 293 nm.

volume swelling ratio,

$$Q = (L/L_0)^3 \quad (1)$$

, was calculated as where L/L_0 is the ratio of the length to the initial length. As the temperature was increased from 30 to 80 °C, the hybrid hydrogel swelled to about 3 times its initial equilibrium volume (Figure 6A). In contrast, a chemically cross-linked control gel, prepared with the same level of MABNTA (10 mol %) and a similar amount of cross-linking agent (2.5 mol %) as the hybrid gels, did not change in volume over the same temperature range. The temperature transition was not reversible since 12 h after returning the gel to room temperature the volume of the temperature-swelled hydrid hydrogel did not change.

The structure of the H-I28-H cross-links inside the hydrogel was investigated using the fluorescence of the I28 W residue as a reporter. At room temperature, the fluorescence maximum of the I28 cross-linked hydrogel was 317 nm (Figure 6B), corresponding to the fluorescence maximum of the protein in PBS buffer without denaturant (Figure 4), which suggested that the I28 cross-links were in their native structure. After the gel was heated at 85 °C, the fluorescence maximum shifted to 360 nm, corresponding to the denatured state of I28, providing evidence that gel swelling occurred due to the denaturation of the I28 cross-links.

DISCUSSION

The titin protein has unique viscoelastic properties that arise from the highly ordered molecular structure of the modules of which it is comprised. Our goal was to take advantage of the consistent structure and predictable mechanical properties of one of titin's well-characterized structural modules, an Ig module, to create a hydrogel with unique environmental responsiveness. We constructed a double-His-tagged single I28 Ig domain, which we produced in *E. coli*. The recombinant I28 domain was correctly folded into its native structure as judged by its molecular mass in solution, its secondary structure, its thermal stability, and intrinsic fluorescence spectra.

Hydrogels readily formed when the H-I28-H protein was mixed with the Ni^{2+}-chelated acrylamide copolymer. The metal coordination cross-linking strategy provides a convenient and effective method for assembling hybrid hydrogels. One of the major advantages of this strategy is that hydrogels are formed in aqueous buffer, at neutral pH, at ambient temperature, and in the absence of redox initiators (radicals) that can damage proteins. The metal coordination linkage between His tags and NTA is quite robust; the H-I28-H cross-linked gels were stable submerged in buffer for more than 30 days. One concern about the strategy could be the potential for Ni^{2+} cross-linking between NTA side chains. In control experiments, in which Ni^{2+} was added to the chelating copolymer with a substoichiometric ratio of Ni^{2+} to NTA side chains, soft gels formed at high polymer concentrations. These gels dissolved in excess buffer or as nickel sulfate was added. After the chelating polymer had been preloaded with a stoichiometric amount of Ni^{2+}, the NTA−NTA cross-links were minimized during hydrogel assembly and cross-linking most likely occurred predominantly through metal coordination between His tags and NTA.

The H-I28-H cross-linked gel responded to rising temperature by swelling isotropically more than 3-fold over the temperature range of 30−80 °C (Figure 6). The equilibrium volume increased significantly above 58 °C, the melting temperature of the H-I28-H protein determined by CD spectroscopy (Figure 3B), suggesting that the swelling was due to the thermally induced unfolding of the H-I28-H cross-links. The fluorescence maximum at 350 nm from the heated gel confirmed that the H-I28-H domain was unfolded in the temperature-swollen gels (Figure 6B). A chemically cross-linked gel of the chelating acrylamide copolymer did not change in volume over the same temperature range (Figure 6A). Swelling due to disruption of the metal coordination bonds at elevated temperatures can be ruled out by previously reported hybrid hydrogels that were cross-linked by metal coordination with a His-tagged coiled coil that had a melting temperature of >100 °C. These gels did not change in volume over the temperature range of 25−90 °C (*6*).

The positive temperature response of the I28 hybrid hydrogel is unusual. Most temperature-responsive hydrogels demonstrate negative thermal responses; they shrink at elevated temperatures. A common example is chemically cross-linked poly(*N*-isopropylacrylamide)

[p(NIPAAm)] which shrinks near the LCST of pNIPAAm due to hydrophobic interactions among its side chains that become stronger as the temperature increases. Hydrogels of chemically cross-linked polymers and copolymers of other *N*,*N*′-alkyl-substituted acrylamides, such as *N*-ethylacrylamide or *N*,*N*′-dimethylacrylamide, also have similar but less sharp negative thermosensitivity (*21*). An exception is poly(acrylic acid)-containing hydrogels, which swell with elevated temperature at low pH due to disruption of hydrogen bonding between acrylic acid and acrylamide side chains (*22*). The poly(acrylic acid) hydrogels are not temperature-responsive at pHs significantly greater than the pK_a of the carboxylic acid side chains, i.e., neutral pH. Therefore, the I28 cross-linked hydrogels may be unique in demonstrating a substantial positive temperature response at neutral pH.

In the single titin molecule stretching experiments, some Ig modules were observed to consistently unfold at lower forces than others (*11*). Likewise, biochemical stability studies with isolated titin Ig modules demonstrated that there can be dramatic variations in stability between modules (*18*). For example, the melting temperature of the I27 module was 72.6 °C, compared to 35.3 °C for the I11 module. The variable stability of homologous titin Ig modules suggests that small changes in amino acid sequence can have pronounced effects on module stability. It may be possible, therefore, to select natural Ig modules as cross-links to create hydrogels for a particular application that respond at a specified temperature. The temperature response could be adjusted over a wide range of temperatures. Furthermore, since the Ig module structure is known (*8*), it is likely that the thermal stability of the Ig modules can be adjusted in a predictable manner by altering a few key amino acids within the domain. By using multiple-Ig module cross-links, it may be possible to assemble hydrogels that undergo volume transitions at multiple temperatures.

In addition to its temperature response, we expect that the I28 cross-linked hybrid hydrogel may respond to other stimuli as well, like ionic strength. As the ionic strength is lowered, the osmotic pressure inside the polyanionic acrylamide copolymer gel will exert increasing force on the I28 cross-links. At a critical ionic strength, the swelling force exerted by the polymer matrix may exceed the threshold force needed to rupture the I28 structure, leading to the cooperative unraveling of the I28 modules. The sudden expansion of the hydrogel cross-links from 4 to ∼30 nm, as seen in the titin stretching experiments (*11*), could result in a pronounced step in the volume transition. The novel swelling profile in response to changes in temperature, and potentially other environmental conditions, along with the potential for adjusting the response over a wide range of environmental conditions, suggests that the I28 cross-linked hybrid hydrogels may have applications in controlled chemical delivery. The gels may be particularly useful for controlled delivery of biologically active proteins because of the mild hydrogel assembly conditions.

The Ig cross-linked hybrid hydrogels demonstrate that well-defined protein modules can be used to mechanically modulate the intrinsic response of a synthetic copolymer matrix in a predictable manner. Biology is a vast resource of predesigned, prefolded protein modules, with well-characterized structures and properties, which can be recombined and modified using the tools of molecular biology. Cross-linking synthetic water-soluble copolymers with recombinant protein modules offers a new approach and a new inventory of molecular materials with which to design responsive hydrogels for a wide variety of applications.

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