Electrostatically Controlled Hydrogelation of Oligopeptides and Protein Entrapment

Sivakumar Ramachandran,[†] Peter Flynn,[‡] Yiider Tseng,[§] and Y. Bruce Yu*[,†,||]

Departments of Pharmaceutics & Pharmaceutical Chemistry, Bioengineering, and Chemistry, University of Utah, Salt Lake City, Utah 84112, and Department of Chemical Engineering, University of Florida, Gainesville, Florida 32611

Received June 16, 2005. Revised Manuscript Received October 3, 2005

Peptide-based hydrogels have gained interest for biomedical applications as a result of their biodegradability and bioresorbability. For in vivo applications, these hydrogels should be able to easily assemble in a controlled manner and should possess compatibility with entrapped biomolecules. Mutually attractive but self-repulsive oligopeptides were designed to achieve electrostatically controlled assembly. These peptide modules assembled into a hydrogel network upon changing pH or ionic strength or mixing of oppositely charged modules. Mixing-induced hydrogels are particularly attractive as they can be easily assembled by simple mixing of peptide solutions prior to application. Another advantage of mixing-induced gelation is that it preserves the pH and ionic strength of the original peptide solutions. The compatibility of these hydrogels with entrapped biomolecules (molecular biocompatibility) was confirmed using high-resolution, $^{1}H-^{15}N$ heteronuclear NMR spectroscopy. These novel biomaterials are highly elastic (revealed in dynamic rheological measurements), have fibrillar morphology, and are able to entrap and preserve proteins in their native form. These properties make them a good candidate for biomedical applications such as tissue engineering and drug delivery.

Introduction

Biomaterials based on protein or peptide self-assembly are ubiquitous both in nature, such as the formation of collagen in the extracellular matrix (ECM)[1] and of fibrin in blood clotting,[2] and in engineering, such as the formation of β-sheet peptide tapes.[3] In both nature and engineering, the control of the assembling process is crucial. The failure to exert control over protein self-assembly is most notably exemplified by the in vivo formation of protein aggregates which are associated with numerous disease states, such as Alzheimer's disease and prion diseases.[4] To exert such a control, various stimuli-sensitive, peptide-based biomaterials that respond to changes in environmental conditions such as pH,[5,6] ionic strength,[7,8] and temperature[9−11] have been explored. From a biomedical application standpoint, a potential problem for stimuli-triggered material assembly is that the pH, temperature, or ionic strength of the material will inevitably change and such changes may not be compatible with physiological conditions. To overcome this problem, we used a modular design approach in which material assembly is achieved by mixing two peptide modules (mixing-induced material assembly). Mixing-induced material assembly can preserve the pH, ionic strength, and temperature of a biological or pharmaceutical sample and, hence, is more likely to be biocompatible. In our previous study, we showed that this modular design approach can produce peptide-based hydrogels with novel material properties, such as rapid shear responsiveness.[12] In this study, we will demonstrate that the modular design also enables stimuli-triggered material assembly. In other words, each individual peptide module can, without being mixed with another module, assemble into a hydrogel in response to changes in its environment. However, we will also demonstrate that, compared to stimuli-triggered gelation, mixing-induced gelation is better at preserving the native conformation of biomolecules entrapped in the hydrogels. Such molecular biocompatibility is an important requirement for materials intended for biomedical applications.

* To whom correspondence should be addressed. E-mail: yby1@utah.edu.
[†] Department of Pharmaceutics & Pharmaceutical Chemistry, University of Utah.
[‡] Department of Chemistry, University of Utah.
[§] Department of Chemical Engineering, University of Florida.
[||] Department of Bioengineering, University of Utah.

(1) Forgacs, G.; Newman, S. A.; Hinner, B.; Maier, C. W.; Sackmann, E. *Biophys. J.* **2003**, *84*, 1272−1280.
(2) Ryan, E. A.; Mockros, L. F.; Weisel, J. W.; Lorand, L. *Biophys. J.* **1999**, *77* (5), 2813−2826.
(3) Aggeli, A.; Bell, M.; Boden, N.; Keen, J. N.; Knowles, P. F.; McLeish, T. C.; Pitkeathly, M.; Radford, S. E. *Nature* **1997**, *386* (6622), 259−262.
(4) Dobson, C. M. *Philos. Trans. R. Soc. London, Ser. B* **2001**, *356* (1406), 133−145.
(5) Schneider, J. P.; Pochan, D. J.; Ozbas, B.; Rajagopal, K.; Pakstis, L.; Kretsinger, J. *J. Am. Chem. Soc.* **2002**, *124* (50), 15030−15037.
(6) Petka, W. A.; Harden, J. L.; McGrath, K. P.; Wirtz, D.; Tirrell, D. A. *Science* **1998**, *281* (5375), 389−392.
(7) Ozbas, B.; Kretsinger, J.; Rajagopal, K.; Schneider, J. P.; Pochan, D. J. *Macromolecules* **2004**, *37* (19), 7331−7337.
(8) Caplan, M. R.; Schwartzfarb, E. M.; Zhang, S.; Kamm, R. D.; Lauffenburger, D. A. *Biomaterials* **2002**, *23* (1), 219−227.
(9) Pochan, D. J.; Schneider, J. P.; Kretsinger, J.; Ozbas, B.; Rajagopal, K.; Haines, L. *J. Am. Chem. Soc.* **2003**, *125* (39), 11802−11803.
(10) Jeong, B.; Bae, Y. H.; Lee, D. S.; Kim, S. W. *Nature* **1997**, *388* (6645), 860−862.
(11) Wang, C.; Stewart, R. J.; Kopecek, J. *Nature* **1999**, *397* (6718), 417−420.
(12) Ramachandran, S.; Tseng, Y.; Yu, Y. B. *Biomacromolecules* **2005**, *6* (3), 1316−1321.

10.1021/cm0513076 CCC: $30.25
Published on Web 11/24/2005

Table 1. Sequences of Oligopeptides[a]

peptide	sequence of positively charged modules
*K*VW15	acetyl-*KWKVKVKVKVKVKVK*-amide
*K*VW10	acetyl-W*KVKVKVKVK*-amide
peptide	sequence of a negatively charged module
EVW10	acetyl-**E**W**E**V**E**V**E**V**E**V-amide

[a] The numbers represent the chain length of the peptide. Modular material assembly is achieved by pairing a positive module with a negative module. Positively charged amino acids are in italics, and negatively charged amino acids are in bold. To demonstrate the versatility of modular assembly, peptides with equal chain length and unequal chain length were co-assembled to create blunt- and sticky-end pairs, respectively. K, lysine; V, valine; E, glutamic acid; and W, tryptophan.

For in vivo applications, hydrogels should satisfy two important requirements: first, hydrogel formation should be controllable, that is, unintended spontaneous gelation or aggregation should be prevented, and second, hydrogels should be biocompatible. Previous design of peptide-based hydrogels was based on the principle of self-attraction where material assembly is governed by the presence of complementary chemical groups within each peptide molecule.[13,14] The problem with such a design is that, as a result of self-attraction, hydrogelation can be triggered by small variations in the environment of the peptide solution. For example, an ionic strength of less than 5 mM is sufficient to cause gelation.[8] Such an ionic strength is much lower than the physiological ionic strength. As a result, extra precaution needs to be taken to prevent gelation in such systems. For instance, additives such as sucrose need to be added to the peptide solution to prevent uninitiated gelation.[15] To exert better control over gelation and prevent unintended aggregation at the peptide level, we chose a modular design strategy based on mutual attraction but self-repulsion. In this approach, a peptide carries either multiple negative charges or multiple positive charges, but not both (Table 1). The electrostatic charges carried by each peptide module act as a brake mechanism to inhibit spontaneous gelation or aggregation. Such an electrostatic brake can be relieved by different means, including changing pH (pH-induced gelation), ionic strength (salt-induced gelation), or mixing two oppositely charged modules (mixing-induced gelation). Our previous work focused on mixing-induced gelation.[12] In this manuscript, pH- and salt-induced gelation will also be demonstrated.

Another focus of the present work is to develop a method to verify in situ molecular biocompatibility and use this method to compare the molecular biocompatibility of pH-induced hydrogels with mixing-induced hydrogels. Biocompatibility is a complex issue that has manifestation at multiple levels.[16] At the molecular level, the criterion for material biocompatibility can be defined as no alteration of key molecular characteristics, that is, a material should not change key features of biomolecules in contact with the material. If a material is not biocompatible at the molecule level, then it is unlikely for the material to be compatible at the cellular and systemic levels. Previous studies used enzymatic activities to verify molecular biocompatibility in semi-wet, supramolecular, peptide-based hydrogel arrays.[17,18] However, many therapeutic proteins such as insulin are not enzymes. To test the biocompatibility of a material with nonenzymes, other methods are needed. For proteins, molecular biocompatibility translates into maintenance of nativelike structure because the structure and function of a protein molecule are closely related. Traditionally, the degree of retention of secondary or tertiary structures of proteins released from their encapsulation media [monitored using circular dichroism (CD), Fourier transform infrared spectroscopy, differential scanning calorimetry, fluorescence spectroscopy, etc.] is used as an indirect measure of molecular biocompatibiltiy.[19,20] However, these methods do not address the question of in situ biocompatibility. In situ molecular biocompatibility is important for certain tissue engineering and drug delivery applications. For example, an encapsulated growth hormone should retain its native conformation and, hence, its bioactivity in its encapsulated form to enhance the proliferation and growth of co-encapsulated cells. In this work, a two-dimensional NMR fingerprinting method is used to verify the retention of protein tertiary structure inside a hydrogel. Compared with enzymology-based assays, the NMR fingerprint method has general applicability.

Materials and Methods

Preparation of Oligopeptides. All the peptides were synthesized using standard Fmoc Chemistry on Rink Amide MBHA resin and purified using HPLC as described earlier.[12] The purity and the molecular weight of each purified peptide were verified by analytical HPLC and mass spectrometry, respectively (see Supporting Information for more details). Each purified peptide sample was dissolved in the respective buffer at appropriate pH and dialyzed at room temperature for 2−4 h using a dialysis membrane with a molecular weight cutoff of 100 Da. The concentration of each peptide sample was determined on the basis of the UV absorption of the Trp residue in each peptide, using an extinction coefficient of 5690 $M^{-1}\cdot cm^{-1}$ at 280 nm,[21] with a correction for the light scattering.[22]

Preparation of ^{15}N-Enriched Ubiquitin. The gene encoding for human ubiquitin, a 76 amino acid protein, was subcloned into the commercial plasmid pET11a in the polyclonal region between NdeI and BamHI. In addition to the main coding sequence, a nucleotide segment that specifies six consecutive histidine residues (6 × His) has been engineered at the *C*-terminal of the amino acid sequence that supports highly efficient purification using nickel nitriloacetic affinity chromatography. The resulting plasmid was used to transform *Escherichia coli* BL21(DE3). Overexpression on a minimal medium (M9) prepared using $^{15}NH_4Cl$ provides a means for generating uniformly ^{15}N-enriched ubiquitin. Approximately 20

(13) Zhang, S.; Holmes, T.; Lockshin, C.; Rich, A. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90* (8), 3334−3338.
(14) Yokoi, H.; Kinoshita, T.; Zhang, S. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (24), 8414−8419.
(15) Narmoneva, D. A.; Oni, O.; Sieminski, A. L.; Zhang, S.; Gertler, J. P.; Kamm, R. D.; Lee, R. T. *Biomaterials* **2005**, *26* (23), 4837−4846.
(16) Anderson, J. M. *Annu. Rev. Mater. Res.* **2001**, *31*, 81−110.
(17) Kiyonaka, S.; Sada, K.; Yoshimura, I.; Shinkai, S.; Kato, N.; Hamachi, I. *Nat. Mater.* **2004**, *3* (1), 58−64.
(18) Yoshimura, I.; Miyahara, Y.; Kasagi, N.; Yamane, H.; Ojida, A.; Hamachi, I. *J. Am. Chem. Soc.* **2004**, *126* (39), 12204−12205.
(19) Jorgensen, L.; Van de Weert, M.; Vermehren, C.; Bjerregaard, S.; Frokjaer, S. *J. Pharm. Sci.* **2004**, *93* (7), 1847−1859.
(20) Tatford, O. C.; Gomme, P. T.; Bertolini, J. *Biotechnol. Appl. Biochem.* **2004**, *40*, 67−81 (Part 1).
(21) Gill, S. C.; von Hippel, P. H. *Anal. Biochem.* **1989**, *182* (2), 319−326.
(22) Winder, A. F.; Gent, W. L. *Biopolymers* **1971**, *10* (7), 1243−1251.

mg of purified ubiquitin is obtained per liter of culture (approximately 4 × 600 μL, 1 mM samples).

Modes of Gelation. For pH-induced gelation, dialyzed peptide solutions in vials were exposed to vapors of acetic acid (acid-induced gelation) or ammonium hydroxide (base-induced gelation). To demonstrate the reversibility of pH-induced gelation, acid- and base-induced hydrogels were exposed to vapors of ammonium hydroxide and acetic acid, respectively. For salt-induced gelation, a saturated sodium chloride solution was added into the dialyzed stock peptide solution so that the final peptide concentration in the hydrogel was 1 wt %. For mixing-induced gelation, dialyzed peptide solutions were mixed at a 1:1 weight ratio such that the final total peptide concentration was 1 wt %.

CD Spectroscopy. CD spectra were obtained using an AVIV 62DS spectropolarimeter equipped with a water bath operated at 25 °C. A cylindrical cell of 0.1 mm path length was used for the measurements. The instrument was calibrated using ammonium *d*-10-camphor sulfonate (0.06%, w/v) before use and flushed with nitrogen during operation. Ellipticity measurement was normalized to the mean residue ellipticity (θ), expressed in units of deg·cm^2·dmol^{-1}.

Rheological Characterization. Rheological studies of hydrogels were conducted by loading the freshly mixed peptide pair into a 50 mm cone-and-plate module of a strain-controlled, software-operated rheometer (ARES-100; TA Instruments, Piscataway, NJ), followed immediately by 8 h of a time sweep test, with an applied strain of 0.2% amplitude at a 1 rad/s frequency.

NMR Characterization. To characterize ubiquitin entrapped in mixing-induced hydrogels, solutions of two peptide modules (KVW10 and EVW10) and a solution of ubiquitin were mixed thoroughly and then loaded into a NMR tube to allow gelation. The total peptide concentration and the ubiquitin concentration were both 0.25 wt % in the gelled sample. To characterize ubiquitin entrapped in pH-induced hydrogels, a solution of one peptide module (KVW15) and a solution of ubiquitin were mixed into a NMR tube. The NMR tube was then exposed to ammonium hydroxide vapor to induce gelation. The peptide concentration and ubiquitin concentration were both 1 wt % in the gelled sample. All samples contained 7.5% D_2O to support the ^2H lock. A Varian INOVA 500 MHz NMR spectrometer equipped with a triple-axis pulsed-field gradient (TRIAX) indirect probe was used to acquire $^1H-^{15}N$ heteronuclear single-quantum coherence (HSQC) spectra.[23] The spectral width in the ^1H dimension was 7000 Hz (~14 ppm) with the carrier frequency set at the water resonance. Water suppression was accomplished using a flip-back type scheme.[24] The spectral width in the ^{15}N dimension was 2000 Hz (~39.5 ppm) with the carrier frequency set at 121 ppm, which corresponds approximately to the center of the peptide/protein amide frequency range. The $^1H-^{15}N$ HSQC spectrum of ubiquitin in 50 mM, pH 6 phosphate buffer was also acquired.

Results and Discussion

As designed, the multiple like charges carried by each peptide indeed prevented spontaneous peptide gelation and aggregation. Peptides remained in solution and were visually clear after dialysis in pH 6.0, 30 mM ammonium acetate buffer. These peptides had a random coil conformation in solution, as assessed by their CD spectra (Figure 1). The electrostatic inhibition of gelation was removed by three

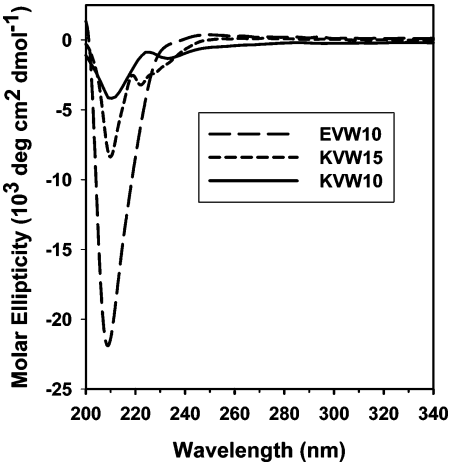

Figure 1. Far UV CD spectra of 1 wt % peptide solutions in 30 mM ammonium acetate aqueous buffer, pH 6.0, 25 °C.

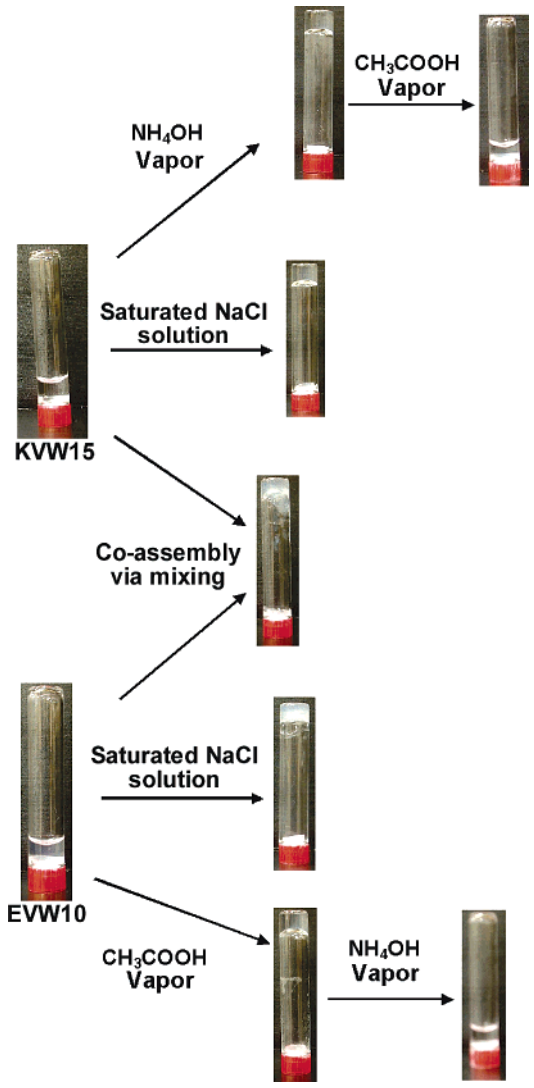

Figure 2. Various means to induce hydrogelation. The vial (with a red cap) was put upside down. All solution and gel samples contained in 30 mM ammonium acetate aqueous buffer, pH 6.0, with a total peptide concentration of 1 wt % except the gel made by adding NaCl to EVW10, which had an EVW10 concentration of 1.25 wt %. For salt-induced gelation, the final NaCl concentration for the KVW15 sample was 1.5 M, and that for the EVW10 sample was 3 M (at 1.5 M salt concentration, EVW10 was still a viscous solution). Note that pH-induced gelation is reversible, forming an on/off switch mechanism.

(23) Kay, L. E.; Keifer, P.; Saarinen, T. *J. Am. Chem. Soc.* **1992**, *114*, 10663−10665.
(24) Grzesiek, S.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 12593−12594.

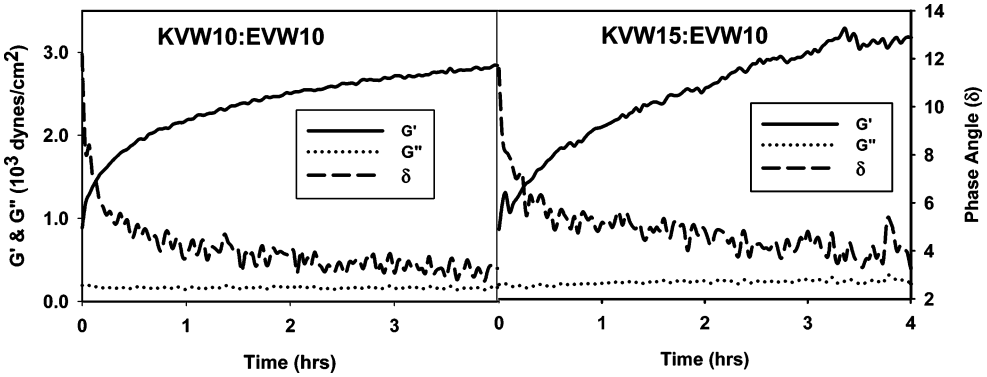

Figure 3. Viscoelastic properties of the co-assembled hydrogels formed by mixing 0.5 wt % of each peptide in 50 mM cacodylate buffer at pH 6 (total peptide concentration in the hydrogel was 0.5 wt % with the concentration of each peptide module being 0.25 wt %). G', elastic modulus; G'', viscous modulus; δ ($= \tan^{-1} G''/G'$), phase angle.

means: changing pH, adding salt, and mixing two oppositely charged peptides (Figure 2). In other words, the electrostatic control is compatible with multiple gelation modes. Another benefit of electrostatic control via self-repulsion is that it significantly elevated the salt concentration needed to induce gelation, which is evident by the observation that each peptide module remains in solution after dialysis in 30 mM buffer, and required a much higher salt concentration to induce gelation (Figure 2). The final NaCl concentration for the KVW15 sample was 1.5 M, and that for the EVW10 sample was 3 M (at 1.5 M salt concentration, EVW10 was still a viscous solution). In contrast, in a self-attractive peptide with the same amino acid composition and sequence pattern, the salt concentration needed to induced gelation was ~5 mM.[8] The elevated salt concentration required to induce gelation also increased the flexibility in peptide purification, handling and preservation. For instance, the EVW10 peptide was purified using 30 mM NH_4HCO_3 buffer, which would have been impossible for its self-attractive counterpart, which gels in the presence of 5 mM salt.[8] The versatility in gelation modes and elevated salt concentration required to trigger gelation clearly demonstrate the advantage of the modular design over the self-complementary design.

Hydrogels are viscoelastic networks.[25] The viscoelasticity of the peptide hydrogels assembled by the oligopeptides was characterized by rheometric measurement. The rheological gelation profiles of the KVW10:EVW10 pair and the KVW15:EVW10 pair are presented in Figure 3. The gelation process is smoother for the KVW10:EVW10 pair than for the KVW15:EVW10 pair (Figure 3). In both pairs, gelation was characterized by two regions: a rapid growth region followed by a slow growth region. The phase angle [$\delta = \tan^{-1}(G''/G')$] is an indicator of the relative elastic and viscous components of a hydrogel. Both pairs had a very low phase angle (~5°), indicating that both hydrogels are highly elastic. Hence, peptide pairs with blunt ends (KVW10:EVW10) and sticky ends (KVW15:EVW10) are both capable of forming hydrogels upon mixing, demonstrating the versatility of the modular design.

The hydrogel matrixes were capable of entrapping small proteins such as lysozyme and ubiquitin during their formation (Figure 4). To verify the retention of the native

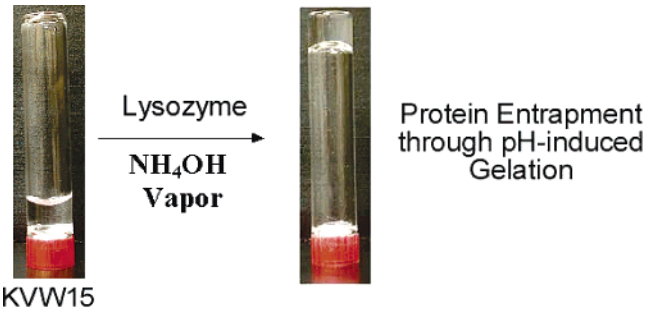

Figure 4. Encapsulation of lysozyme by pH-induced gelation. 1 wt % KVW15 peptide in 30 mM ammonium acetate buffer (pH 6) containing 0.5 wt % lysozyme was exposed to ammonium hydroxide vapor to induce gelation.

conformation of entrapped protein molecules, a $^1H-^{15}N$ HSQC spectrum of uniformly ^{15}N-enriched ubiquitin was used as a model system to study the integrity of entrapped proteins in the hydrogel. Ubiquitin entrapped in the mixing-induced gel retained its native conformation, as evidenced by minimal perturbation of the amide NMR resonances when compared to its native solution conformation (Figure 5). The preservation of ubiquitin native structure is more clearly seen from the overlay of these fingerprints in Figure 6A. Ubiquitin entrapped in the pH-induced gel retained a nativelike overall fold (Figure 6B). However, the higher gel pH accelerated the rate of amide proton exchange, leading to the loss of a number of amide NMR resonances. The resonances of ubiquitin in hydrogel matrixes were comparatively broader, indicating a substantial decrease in the rotational motion of the entrapped protein. Compared to pH- or salt-induced gels, mixing-induced gels cause minimal perturbation to the medium and thereby to the entrapped biomolecule. Hence, mixing-induced gels would be preferred as encapsulation matrixes for applications where preservation of protein conformation is critical, including protein drug delivery, tissue engineering (growth factors), and structural genomics[26] (as alignment media for NMR characterization). Although two-dimensional NMR fingerprinting[27] has been utilized to study the conformation of protein solutions (either alone or entrapped in reverse micelles), to our knowledge, this is the first time it is used to characterize the conformation of

(25) Kopecek, J. *Nature* **2002**, *417* (6887), 388−389, 391.

(26) Prestegard, J. H.; Kishore, A. I. *Curr. Opin. Chem. Biol.* **2001**, *5* (5), 584−590.

(27) Wand, A. J.; Ehrhardt, M. R.; Flynn, P. F. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95* (26), 15299−15302.

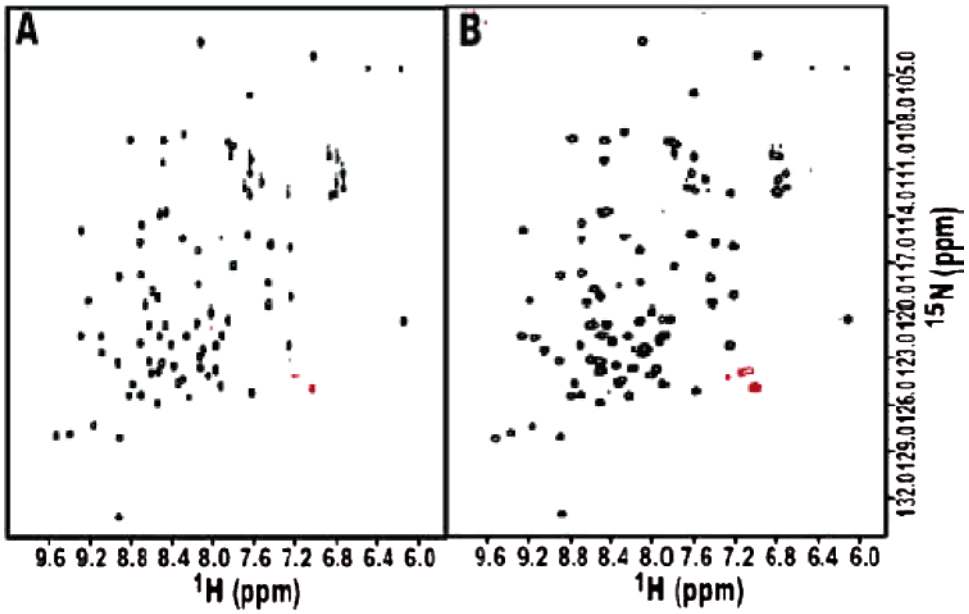

Figure 5. ^{1}H−^{15}N HSQC spectra of ubiquitin at 25 °C in solution (A) and in mixing-induced hydrogel (B). The resonances in red are aliased peaks present outside the spectral window.

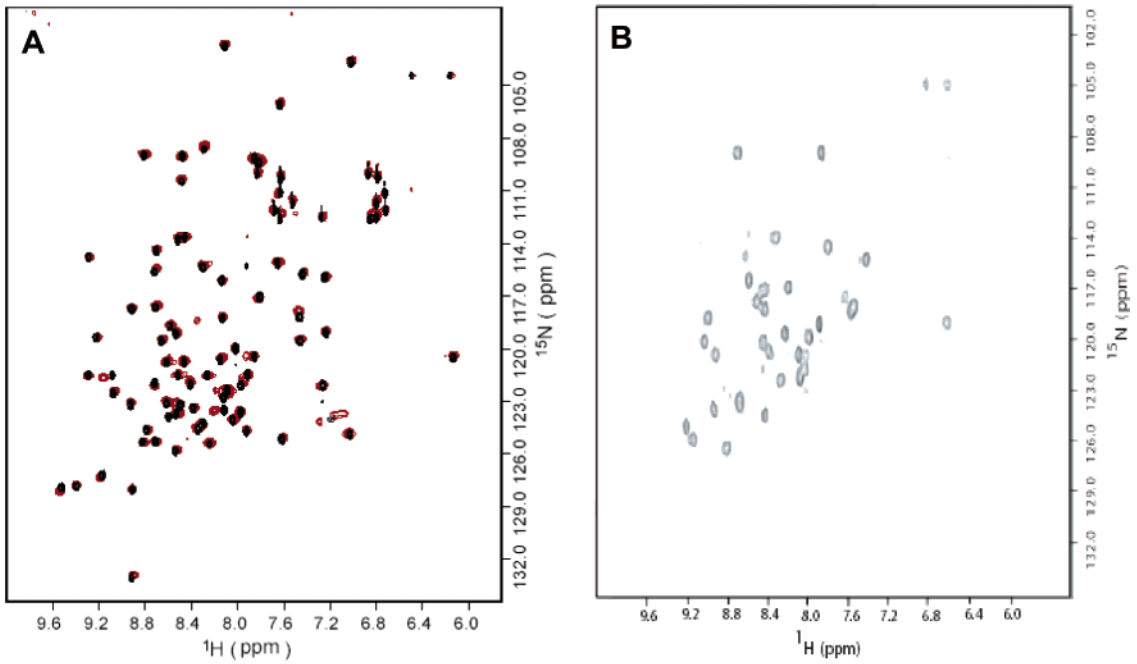

Figure 6. ^{1}H−^{15}N HSQC spectra of backbone amide of ^{15}N-enriched ubiquitin at 25 °C. (A) Overlay of ubiquitin in solution (black) and ubiquitin entrapped in a mixing-induced hydrogel (red). (B) Ubiqutin entrapped in a pH-induced hydrogel. Clearly, the mixing-induced hydrogel better preserves the native conformation of entrapped ubiquitin than the pH-induced hydrogel.

proteins entrapped inside a peptide hydrogel. Our data demonstrate that multidimensional NMR fingerprinting is a suitable technique to monitor protein conformation inside hydrogels.

The modular design along with the electrostatic control provides a basis for constructing dynamic libraries of peptide-based biomaterials. Along with their excellent biodegradability and bioresorbability, the possibility of fine-tuning material properties (e.g., sol−gel transition pH, ionic strength, kinetics of co-assembly) via systematic sequence variation[8,28] is a unique advantage of peptide-based materials. In pH- and salt-induced hydrogels, the material property might be fine-tuned by changing the peptide sequence, length, and concentration. In mixing-induced hydrogels, material properties can be further tuned by varying concentration ratios and length ratios of the pairing peptide modules, offering additional flexibility in adjusting the material properties.

The fibrillar structure[12] and high elasticity of these hydrogels mimic the ECM and might act as a morphogenetic guide during tissue regeneration. Further, cell adhesive motifs[29] (like RGD) could be incorporated into these

(28) Caplan, M. R.; Schwartzfarb, E. M.; Zhang, S.; Kamm, R. D.; Lauffenburger, D. A. *J Biomater. Sci., Polym. Ed.* **2002**, *13* (3), 225−236.

(29) Yamada, K. M. *J. Biol. Chem.* **1991**, *266* (20), 12809−12812.

sequences for specific tissue engineering applications. In short, the protein entrapment in its native state (Figure 5A) and the rapid gelation time (Figure 3) are highly desirable for tissue engineering and drug delivery applications.

Conclusion

Mutually attractive and self-repulsive oligopeptide modules were found to undergo stimuli- and mixing-induced sol−gel phase transitions. Two-dimensional NMR fingerprinting of isotope-enriched protein molecules entrapped in hydrogels revealed that mixing-induced gelation better preserves the native conformation of the entrapped protein, as the gelation process does not involve changes in pH, ionic strength, or temperature. Hence, mixing-induced, co-assembled hydrogels would be preferred for biomedical applications where preservation of protein conformation is crucial.

Acknowledgment. This work is supported by the National Institutes of Health under Grant EB004416. We thank Alana Simorellis and Wade van Horn for providing ^{15}N-enriched ubiquitin.

Supporting Information Available: Analytical HPLC and matrix-assisted laser desorption ionization mass spectra of oligopeptides (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

CM0513076