Genetic Engineering of Self-Assembled Protein Hydrogel Based on Elastin-like Sequences with Metal Binding Functionality

U Loi Lao, Minwei Sun, Mark Matsumoto, Ashok Mulchandani, and Wilfred Chen*

Department of Chemical and Environmental Engineering, University of California, Riverside, California 92507

Received June 13, 2007; Revised Manuscript Received September 24, 2007

Recombinant DNA methods have been exploited to enable the creation of protein-based block copolymers with programmable sequences, desired properties, and predictable three-dimensional structures. These advantages over conventional polymer counterparts facilitate the utility of this new class of biomaterials in a wide range of applications. In this project, we exploited the environmental application of protein-based block copolymers based on elastin-like protein (ELP) sequences. Triblock copolymers containing charged and hydrophobic segments were synthesized. Chain lengths of each segment were manipulated in order to maintain a gelation point below room temperature. Polyhistidine sequences were successfully incorporated into the hydrophilic segment without disruption of the self-assembled hydrogel formation. The microscopic structure was further investigated using laser confocal microscopy. The metal binding capability and capacity of resulting hydrogel were studied to demonstrate the functionality of polyhistidine and its environmental application for heavy metal removal. Reversibility of metal binding was demonstrated, indicating the cost-effectiveness of this hydrogel. Significantly, we envision that this versatile strategy of incorporating functional groups within a 3-D protein network provides new possibilities in creation of biomaterials with great control over structure–property relationships.

Introduction

Much effort has been devoted toward the creation of protein block copolymers via genetic engineering.[1,2] By preprogramming the coding information within a DNA template, precise control over block copolymer composition and length enables the preparation of individual blocks with different mechanical, chemical, and biological properties.[3–5] For example, Conticello and colleagues[4] have produced a new class of block copolymers based on elastin-like protein (ELP) sequences, which are composed of Val-Pro-Gly-Val-Gly repeats. By substituting the amino acids in the repeating units, BAB-type block copolymers containing flanking plastic-like end blocks and an elastomeric midblock were generated. Upon a temperature increase, the hydrophobic end blocks, $[(IPAVG)_4(VPAVG)]_n$, undergo a phase separation from the aqueous solution and self-aggregate into micelles, which serve as virtual cross-linkers in the network formation. On the other hand, the elastin midblocks containing periodically placed charged glutamic acid residues remain soluble during the self-assembly process, resulting in formation of a hydrogel. The gelation process is fully reversible due to the stimuli-responsive phase behavior of ELP sequences.[4]

Because of the sequence-directed control over structure and property as well as their intrinsic stimuli-responsive self-assembly capability, protein copolymers based on ELP sequences have been attracting attention as novel biomaterials.[6] A logical extension in expanding the types of application is to introduce additional functionalities into this self-assembled peptide system. Previously, we have demonstrated that ELPs can be engineered to integrate multifunctionalities while preserving the phase-transition property.[7–9] In this report, we detail on incorporating a functional moiety, particularly a hexahistidine metal-binding motif, into the macroscopic gel structure. This biosynthesis strategy was greatly simplified by the use of DNA recombinant technology. The template-driven design highlighted the advantages of the functionalized block copolymers over the conventional synthetic polymers: (a) uniform, monodispersed block copolymers can be produced via the bacteria machinery; (b) functional groups composed of amino acids can be precisely and systematically placed into the sequence to warrant accessibility. In this work, we demonstrate, for the first time, the generation of temperature-responsive, self-assembled block-copolymer gel-like macroscopic structures with embedded metal-binding functionalities.

Experimental Methods

Synthesis of Elastin-Based Triblock Copolymers. The biosysthesis of elastin-based triblock copolymer has been detailed elsewhere.[4] All cloning steps were carried out in *Escherichia coli* SCS110 (Stratagene,CA). The oligonucleotide encoding for hydrophobic domain (B) plastin sequence, gtacctgctgttggtatcccggctgttggtatcccagctgttggcattccggctgtaggtatcccggctgttggtgtacctgctgttggtatc was PCR amplified using primers 5′-tagactaagcttgtacctgctgttggtatc and 5′-tatcgtggatccgataccaacagcaggtac. The PCR product was digested with *Hin*dIII and *Bam*HI and inserted into pBluescriptSK+ (Stratagene, CA). erable amount of DNA for monomer B was obtained from isolated plasmid DNA via restriction digestion with *Bsp*M I. The elongation of monomer B was achieved by self-ligation followed by insertion of the product into the *Bsp*M I site of pEC plasmid, which was modified from pET-24a to accommodate the linker sequence atgggtccgggtgtaggtgtacctggtgttggggtacctgctgttggtattcctgca. The resulting pECP plasmid was digested by *Kpn*I/*Pst*I and the fragment containing the concatemers was cloned into pED plasmid, which was modified from pET-24a to accommodate the linker sequence atggtacctgctgttggtattcctgcagttggcgttccgggtgtaggtgtacctggtgttgggtgatga. Subsequently, plasmids pECP and pEDP were subjected to digestion with *Sex*AI/*Xma*I, and the resultant fragments containing endblocks were ligated to form the plasmid pECDP with a unique restriction site of *Sex*AI.

* Corresponding author. E-mail: wilfred@engr.ucr.edu. Phone: 951-827-2473.

10.1021/bm700662n CCC: $37.00
Published on Web 11/27/2007

Table 1. Coding Sequences of Linker E for Elastin Midblock Construction

*Sex*A I	*Pfl*M I
gta cct ggt gtt ggc ggt ggc ggc agc ggt ggc acc ggc cac cac cac cac cat cac gta cca ggc	
V P G V G G G G S G G T G H H H H H H V P G	
	*Sex*A I
gtt ggc cac cac cat cac cac cac ggc ggt ggc agc ggt ggc acc ggc gta cct ggt gtt ggc	
V G H H H H H H G G G S G G T G V P G V G	

For the construction of midblocks (A) with elastin sequence, two 5′-phosphorylated oligonucleotides, 5′-ttggcgtaccgggcgttggtgttccgggtgttggcgtaccaggcgaaggtgtaccgggtgttggcgtaccaggcg and 5′-ctggtacgccaacacccggtacaccttcgcctggtacgccaacacccggaacaccaacgcccggtacgccaacgc, were annealed to form 75 bp elastin DNA monomers. The concatemers resulting from self-ligation of the monomers were inserted into the adaptor plasmid pBLE *Pfl*M I site.

The adaptor plasmid pBLE for midblock elastin sequence was constructed using pBluescriptSK+ to contain the linker sequence E (Table 1). Linkers of eight amino acids were introduced between hexahistidine sequence and plastin end blocks. The control adaptor without hexahistidine was constructed in a similar fashion.

The midblock (A) with the desirable length of elastin repeats was released from the plasmid by *Sex*AI digestion and subsequently inserted into the unique *Sex*AI site of plasmid pECD. The final plasmid pECDElastin was tranformed into expression strain BLR-Gold(DE3) and confirmed by DNA sequencing.

All cultivations were carried out in terrific broth media supplemented with 30 μg/mL kanamycin at 300 rpm for 36 h at 30 °C. Cells were then harvested by centrifugation, washed in 0.9% NaCl, and resuspended in 50 mM Tris-Cl, pH 8 (TB8). After cell lysis with sonication and removal of cell debris by centrifugation for 30 min at 30 000*g*, the biopolymers were purified from the cell extract by three cycles of inverse temperature transition.[7] For each cycle, the sample was heated to 30 °C and centrifuged at 30 000*g* at 30 °C, and the pellet containing the biopolymer was dissolved in ice-cold TB8. NaCl was added to a final concentration of 0.5 M. Purity of the purified protein was verified by SDS−PAGE electrophoresis followed by silver staining.

Laser Scanning Confocal Microscope Observation. The preparation of dye-stained hydrogel samples was achieved by modifying the method of Nowak et al.[10] Lipophilic dye $DiOC_{18}$ (Invitrogen, Inc.) was dissolved in DMF at 0.001 wt % by sonication. Five microliters of dye solution was added to 1 mL of copolymer solution in TB8 and incubated overnight at 4 °C. The solutions were transferred onto glass slides and immediately imaged using a Zeiss 510 laser scanning confocal microscope (LSCM) with a 488 nm excitation wavelength.

Electron Microscopy. Scanning electron microscopy (SEM) was performed on a Philips XL30 FEG operating at 15 kV. The hydrogel samples (1–10% mg/mL) were prepared according to the procedure described by Xu et al.[5] Briefly, concentrated elastin copolymers were mixed thoroughly with ice-cold deionized water to prepare the samples and left at room temperature for 3 days to ensure the network formation. Then, the samples were subjected to shock-freezing by liquid nitrogen, followed by lyophilization. After drying, copolymer samples were carefully fractured, transferred onto aluminum stubs, and sputtered with gold for 60 s.

Characterization of Metal-Binding Hydrogels. Metal binding experiments to determine the binding capacity of triblock copolymer hydrogel were performed. Thirty microliters of 10% w/v (30 nmol of copolymers) hydrogel samples were incubated overnight with 400 μL of TB8 containing varying amounts of cadmium. Subsequently, the hydrogel samples were washed with TB8 three times and redissolved in 400 μL TB at 4 °C. The metal−copolymer complex was precipitated by the addition of 0.5 M NaCl at 37 °C and centrifuged for 5 min at 14 000*g*. The resulting pellets were redissolved overnight in 100 μL of concentrated HNO_3 to extract the bound/complexed cadmium and analyzed with an atomic absorption spectrometer. Prior to measurements, each sample was diluted by adding water to the appropriate dilutions. In addition, the metal binding capability of free triblock copolymer in aqueous solution as well as $(His)_6(VPGVG)_{78}(His)_6$ constructed previously[11] were examined. Ten nanomoles of biopolymers was used in the experiments, and the same procedures were performed.

For the reversible metal-binding experiment, 50 μL of 10 wt % hydrogel samples was prepared at room temperature. Cadmium (135 nmol) in 400 μL of TB8 was added in each cycle, incubated overnight, and removed by suction, followed by washing with TB8 3 times. "Stripping buffer" composed of 400 μL of TB8 with 25 mM EDTA was used to remove the bound cadmium, followed by washing with TB8 for three times. Samples from each step were subjected to cadmium measurements.

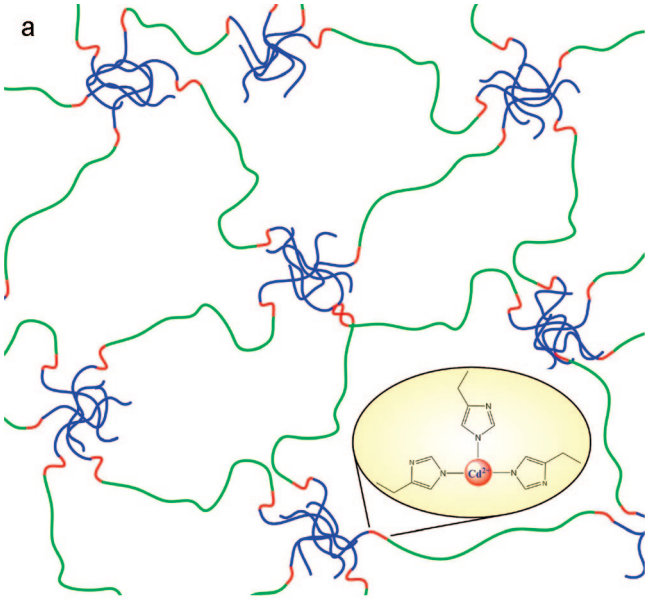
Results and Discussion

Design and Production of Elastin-Based Triblock Copolymer. Wright el al. has developed a modular convergent biosynthetic strategy that facilitated the synthesis of high-molecular weight recombinant protein block copolymers with significant flexibility in the selection and assembly of blocks of diverse sizes and structures.[4] By modifying this genetically directed biosynthesis strategy, protein triblock copolymers based on ELP sequences were biosynthesized with metal-binding capability. Specifically, hydrophobic endblocks (B) that incorporate hydrophobic plastic sequence $[(IPAVG)_4(VPAVG)]_n$ were constructed in a similar fashion to that described by Wright el al.[4] The number of repeating units was selected such that the hydrophobic endblocks aggregate at a desired temperature, preferably lower than 20 °C. To demonstrate that metal-binding functionality can be embedded within the hydrophilic midblock without interfering with hydrogel formation, a hexahistidine domain was inserted to each end of the DNA template coding for the hydrophilic elastomeric block, $[(VPGVG)_2VPGEG(VPGVG)_2]_n$. The precise incorporation of functional groups at designated locations facilitates the analysis of functionality, bypassing the statistical nature of the traditional polymer system. Additionally, linkers of eight amino acids were introduced in the junction area between the endblock and the hexahistidine sequence, ensuring the availability of hexahistidine for metal chelation (Scheme 1a). In this report, a triblock copolymer of 100 kDa molecular weight, named PE1.6, was produced uniformly in *E. coli* BLR-Gold(DE3) and purified by taking advantage of their inherent temperature-responsive properties. SDS−PAGE analysis indicated the expected molecular weight of 100 kDa (Figure 1), corresponding to the composition of this copolymer (Scheme 1b). In addition, DNA sequencing was used to confirm the correct coding sequence for the protein PE1.6.

Temperature-Triggered Self-Assembly and Morphology of the Gels. To investigate whether the modified BAB triblock copolymers retained their self-assembling ability in aqueous solution due to the temperature-triggered hydrophobic interactions between plastin endblocks, the formation of the network (gel) was observed above a critical concentration (6 wt %) at room temperature. The opacity of the gel suggested that microscopic phase separation occurred during gelation.[12] However, the opacity disappeared after the gel was cooled to 4 °C, and the reversible gelation of hydrogels upon cycling the temperature is shown in Figure 2. These results indicate that the presence of the hexahistidine domains within the hydrophilic midblock has no effect on the reversible gelation.

LSCM was applied to further probe the internal structures of self-assembled hydrogel materials.[10,13,14] In this work, a lipo

Scheme 1. (a) Schematic Showing the Metal Binding Structure and (b) Amino Acid Composition of Elastin-Based Triblock Copolymer PE1.6 with Hexahistidine Metal Binding Repeats



philic fluorescent dye was used to identify the aggregated hydrophobic cores within the hydrogel network. Figure 3A shows the morphology of PE1.6 at different concentrations, 1, 5, and 10 wt %. The concentrations were chosen to represent three different phases of the copolymers: (a) the liquid phase, (b) the transition phase between liquid and solid, and (c) the solid gel phase. The bright spots in LCSM images, which started appearing and became more dense in the concentrated samples (5 wt % and above), are indicative of the hydrophobic cores as the lipophilic dye associated only with the hydrophobic domains. The images indicated the change in the microscale morphology from a relatively homogeneous liquid to a heterogeneous gel as the concentration of copolymers increased. The results well correspond to what have been reported in the literature.[13]

Scanning electron microscopy (SEM) was employed to probe the microstructure of the representative hydrogel samples chosen above and validate the observations of LCSM. Samples were treated with liquid nitrogen followed by lyophilization to preserve the hydrogel structure. SEM visualization of the copolymer samples (See Figure 3B) revealed an interconnected network structure only at the higher concentrations (5–10%), and the pore size decreased with increasing copolymer concentrations, as expected. In contrast, only inhomogeneous formation of fibrils and micelles was observed for the 1% copolymer sample, due to the artifacts introduced during the sample preparation.

Metal-Binding Functionality of Self-Assembled Hydrogels. Metal binding experiments were performed to evaluate the functionality of hexahistidine moieties embedded within the copolymer sequence. Previously, ELP biopolymers containing a C-terminal histidine tag have been demonstrated to remove heavy metals from aqueous solutions through stimuli-responsive aggregation.[7] We speculate that embedding the hexahistidine domains into the hydrophilic midblock would afford similar heavy metal removal capability within the macrostructure. The metal uptake capacity of the self-assembled gels was demonstrated by incubating the gels with excess cadmium. After overnight incubation, the gels were redissolved at 4 °C and recovered by precipitation. The amount of Cd^{2+} remaining in the solution and bound was measured (Table 2). A binding ratio of 1.31 ± 0.05 nmol Cd/nmol protein was obtained. In contrast, a similar copolymer gel without the hexahistidine tags removed a negligible amount of Cd^{2+} (data not shown). However, the saturation binding ratio is slightly lower than the ratio of 2.5 for other ELP biopolymers tagged with two tandem repeats of hexahistidine.[15] This reduction in the binding may be the result of limited availability of the hexahistidine repeats within the hydrogel microstructure. To investigate the possibility, similar cadmium binding experiments were performed with an aqueous solution of the copolymer rather than a hydrogel. Nevertheless, no substantial difference in the binding ratio was detected, indicating that this reduced binding capability may be the result of the limited coordination between the hexahistidine repeats and Cd due to the increased rigidity inside the BAB-type block copolymer.

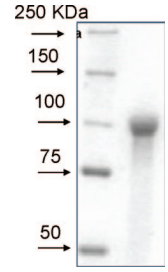


Figure 1. SDS−PAGE gel analysis of the purified PE1.6 block copolymer. Sample after three cycles of reversible temperature transition was loaded on a 10% SDS−PAGE gel.

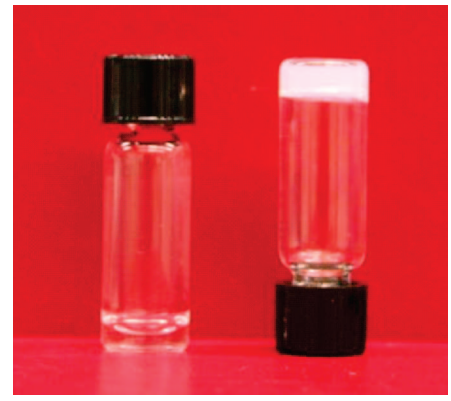


Figure 2. Digital photographic images of (left) an aqueous solution of copolymers at 4 °C and (right) the physical gel formed at 25 °C by the triblock copolymer based on elastin-like sequences.

Regeneration of metal binding sites was enabled by the reported strategy of treating with chelators such as EDTA[7] without disrupting the integrity of the gel structure. After removing traces of EDTA by rigorous washing, the gel structure remained stable and fully functional after three repeating cycles (Figure 4).

Conclusions

Using a tandem hexahistidine repeat as the model functional motif, we have successfully incorporated metal-binding functionality into a self-assembled triblock ELP copolymer hydrogel macroscopic structure. The creation of the functional protein-based hydrogel was enabled by recombinant DNA technology, allowing precise insertion of functional groups

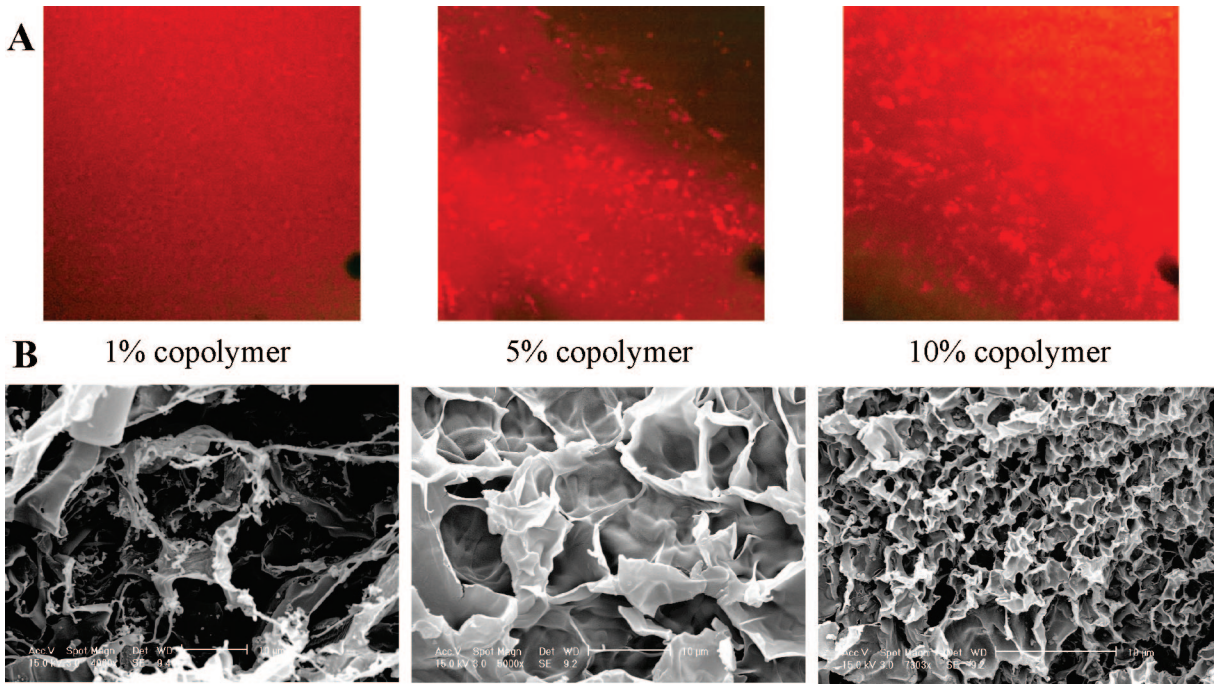


Figure 3. LSCM (A) analysis of micelle formation within the hydrogel network using a lipophilic fluorescent dye, DiO, and SEM (B) images of PE1.6 solutions at 1, 5, and 10 wt % copolymer concentration. Color is arbitrarily chosen and does not depict the actual color.

Table 2. The Maximum (Equilibrium) Cd^{2+} Binding Capacity for 10 wt % PE1.6 Hydrogel, Free PE1.6 Copolymer Solution and $(His)_6(VPGVG)_{78}(His)_6$ Biopolymers in TB8 after Overnight Incubation[a]

protein	hexahistidine per protein	maximum bound Cd^{2+} per protein
PE1.6 hydrogel (10 wt %)	2	1.322 ± 0.008
PE1.6 free copolymer	2	1.833 ± 0.021
H6ELP78H6	2	2.625

[a] The latter two biopolymers bound to Cd^{2+} were recovered through temperature-induced phase separation.

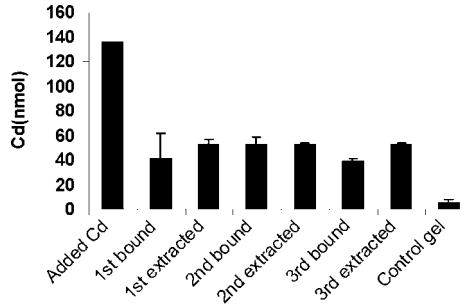


Figure 4. Regeneration of metal binding sites within the hydrogel network. For each cycle, 130 nmol of cadmium was added, and 25 mM EDTA solution was used as the "stripping buffer" for regeneration.

at a designated region within the DNA template. The resulting hydrogel retained both the temperature-responsive self-assembly capability and metal-binding functionality. This strategy of tailoring additional functionalities to self-assembled 3-D network in a precise manner has created possibilities toward new generations of protein-based functional biomaterials. It is easy to envision that different metal-binding moieties can be incorporated into the network, generating selective gel structure for heavy metal removal. Alternatively, we can extend this strategy in an application-oriented fashion to provide protein-based materials with desired properties.

Acknowledgment. This work was supported by a grant from the National Science Foundation (Grant No. BES0329482). We thank Mr. Philip Hehn, Ms. Wong, and Ms. Wang for their help with molecular cloning and protein purification.

References and Notes

(1) Petka, W. A.; Harden, J. L.; McGrath, K. P.; Wirtz, D.; Tirrell, D. A. *Science* **1998**, *281*, 389–392.
(2) Langer, R.; Tirrell, D. A. *Nature* **2004**, *428*, 487–492.
(3) Cappello, J.; Crissman, J. W.; Crissman, M.; Ferrari, F. A.; Textor, G.; Wallis, O.; Whitledge, J. R.; Zhou, X.; Burman, D.; Aukerman, L.; Stedronsky, E. R. *J. Controlled Release* **1998**, *53*, 105–117.
(4) Wright, E. R.; McMillan, R. A.; Cooper, A.; Apkarian, R. P.; Conticello, V. P. *Adv. Funct. Mater.* **2002**, *12*, 149–154.
(5) Xu, C.; Breedveld, V.; Kopeek, J. *Biomacromolecules* **2005**, *6*, 1739–1749.
(6) Kumar, M.; Sanford, K. j.; Cuevas, W. P.; Du, M.; Collier, K. D.; Chow, N. *Biomacromolecules* **2006**, *7*, 2543–2551.
(7) Kostal, J.; Mulchandani, A.; Chen, W. *Macromolecules* **2001**, *34*, 2257–2261.
(8) Kostal, J.; Mulchandani, A.; Gropp, K. E.; Chen, W. *Environ. Sci. Technol.* **2003**, *37*, 4457–4462.
(9) Lao, U. L.; Mulchandani, A.; Chen, W. *J. Am. Chem. Soc.* **2006**, *128*, 14756–14757.
(10) Nowak, A. P.; Breedveld, V.; Pakstis, L.; Ozbas, B.; Pine, D.; Pochan, D.; Deming, T. *Nature* **2002**, *417*, 424–428.
(11) Wang, A. A.; Lee, J.; Jenikova, G.; Mulchandani, A.; Myung, N. V.; Chen, W. *Nanotechnology* **2006**, *17*, 3375–3379.
(12) Wright, E. R.; Conticello, V. P. *Adv. Drug Deliv. Rev.* **2002**, *54*, 1057–1073.
(13) Pakstis, L.; Ozbas, B.; Hales, K.; Nowak, A. P.; Deming, T.; Pochan, D. *Biomacromolecules* **2004**, *5*, 312–318.
(14) Hirokawa, Y.; Jinnai, H.; Nishikawa, Y.; Okamoto, T.; Hashimoto, T. *Macromolecules* **1999**, *32*, 7093–7099.
(15) Prabhukumar, G.; Matsumoto, M.; Mulchandani, A.; Chen, W. *Environ. Sci. Technol.* **2004**, *38*, 3148–3152.

BM700662N