Tandem Modular Protein-Based Hydrogels Constructed Using a Novel Two-Component Approach

Shanshan Lv, Yi Cao,† and Hongbin Li*

Department of Chemistry, University of British Columbia, Vancouver, BC V6T 1Z1 Canada

ABSTRACT: Leucine zipper sequences have been widely used to engineer protein-based hydrogels for biomedical applications. Previously, we have used this method to engineer tandem modular protein-based hydrogels as a step toward developing extracellular matrix-mimetic hydrogels. However, the spontaneous self-association of leucine zipper sequences in solution has made it challenging to express and purify tandem modular proteins carrying leucine zipper under native conditions. To obviate this problem, here we report a novel two-component approach to engineer tandem modular protein-based hydrogels. This methodology makes use of two complementary leucine zipper sequences (CCE and CCK), which do not self-associate but self-assemble into heterodimeric coiled-coils at neutral pH, as functional groups to drive the self-assembly of protein hydrogels. The two protein components are bifunctional and trifunctional tandem modular proteins carrying the leucine zipper functional groups. We found that the two proteins carrying CCE or CCK can be expressed and purified under native conditions with high yield. Upon mixing, the aqueous solution of the two proteins readily forms a transparent hydrogel. The resultant hydrogel can undergo reversible sol–gel transitions as a function of temperature, and shows much improved erosion properties. This method provides a new approach to tune the topology and physical properties of the protein hydrogels via genetic engineering, and opens the possibility to systematically explore the use of large native extracellular proteins to engineer extracellular matrix-mimetic hydrogels.

INTRODUCTION

Hydrogels based on engineered proteins have attracted great interest over the last two decades due to their great potential in a wide range of biomedical applications, ranging from drug delivery carriers, synthetic extracellular matrices, tissue engineering scaffolds, as well as biocatalysts.1–9 Although elastin-mimetic protein and silk-elastin-like proteins have been widely used to engineer protein-based hydrogels,10–15 proteins containing coiled-coil motifs, as pioneered by Tirrell and co-workers,8 have proven a versatile building block for constructing protein-based hydrogels.3,5 Such proteins typically adopt an ABA/ABC triblock architecture with leucine zipper based coiled-coil motifs at both ends and a random coil sequence at the center.8,14–18 Coiled-coil is one of the basic folding motifs found in native proteins and consists of two or more α-helix winding together to form a superhelix.19–21 The gelation of the aqueous solution of block protein ABA/ABC is mediated by the self-assembly/aggregation of the terminal leucine zipper sequences, which form oligomers to serve as the physical cross-linking points.8 Using recombinant DNA technology, it has become possible to precisely tailor coiled-coil-containing triblock proteins at the gene level (i.e., defined amino acid sequence, composition and molecular weight) in order to tune the physical and functional properties of the resultant hydrogels. Since the center block is largely responsible for retaining the water in the hydrogel, various functional motifs have been readily incorporated into the center domain to confer new feats and functionalities to the resultant hydrogels.8,15–18

Inspired by the tandem modular structure of many extracellular matrix proteins,7,22 recently we have successfully used tandem modular protein (GB1)₁₀, which is made of eight identical tandem repeats of a small protein GB1,23 as the center block in a triblock protein ABA to construct a novel protein hydrogel.24 These efforts are not only important for creating a synthetic extracellular matrix that closely mimics naturally occurring ones, but also demonstrate the unique physical properties brought up by the incorporation of tandem modular proteins. However, two limitations of the triblock protein approach have made it difficult to further our efforts in engineering protein hydrogels containing tandem modular proteins for mimicking synthetic extracellular matrix. One is the relatively fast erosion rate due to the formation of intramolecular loops from the same protein chain. The other limitation is the high viscosity of the protein solution, which makes the purification of the protein under native conditions very difficult. Although using a triblock protein ABC containing two dissimilar coiled-coil sequences in the same gelator protein has surpassed the first limitation,19 this approach makes the purification of proteins under native conditions more difficult (sometimes even impossible), as tandem modular proteins carrying two dissimilar terminal coiled-coil sequences could more easily form intermolecular aggregates inside bacterial cells and the solubility of the protein is very poor. On the other hand, because it is difficult to refold tandem modular proteins after denaturation,
purifications of these gelator proteins from denaturing conditions generally gives rise to proteins with compromised functionalities. Therefore, new design strategies must be conceived to overcome these two hurdles to engineer protein hydrogels containing tandem modular proteins. Here we report a novel two-component approach as an alternative method to engineer tandem modular protein-based hydrogels that show improvements in various aspects of the hydrogel properties over the first-generation tandem modular protein-based hydrogels.24

**MATERIALS AND METHODS**

**Protein Engineering.** The gene encoding protein GB1 was a generous gift from David Baker of the University of Washington. The gene that encodes (GB1)4 and (GB1)5 was constructed as previously reported.24 The DNA sequences of coiled-coil domain CCE and CCK,25 flanked with a 5’ BamHI restriction site and 3’ BglII and KpnI restriction sites, were synthesized by PCR (polymerase chain reaction)-based oligonucleotides assembly. The resulting sequences of the CCE and CCK peptides are shown in Figure 1A. The expression vectors of pQE80L-CCE-(GB1)4-CCE was constructed by iterative cloning CCE, (GB1)4, and CCE genes into an empty pQE80L vector, on the basis of the identity of the sticky ends generated by BamHI and BglII restriction enzymes. The expression vectors of pQE80L-CCK-(GB1)5-CCK-(GB1)5-CCK was constructed in the same way. The expression vector was transformed into *Escherichia coli* (E. coli) strain DH5α. Cultures were grown at 37°C in 2.5% lysogeny broth (LB) containing 100 mg/L ampicillin, and induced with 1 mM isopropyl-1-β-D-thiogalactoside (IPTG) when its optical density reached ~1. Protein expression continued for 5 h. The cells were harvested by centrifugation at 4000 rpm for 12 min and lysed using lysozyme. The soluble fraction was purified using Co⁵⁰ affinity chromatography. The yield of the proteins CCE-(GB1)4-CCE (designated as AG₄₄₄) and CCK-(GB1)₅-CCK-(GB1)₅-CCK (designated as CG₅₅₅₅₅C) was around 60 mg and 30 mg per liter of culture, respectively. The purity of the purified protein is above 90%, as estimated from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using AlphaEaseFC software (Version 4.0.0, Alpha Innotech Corporation, San Leandro, CA).

**Circular Dichroism (CD) Spectroscopy.** CD spectra were recorded on a Jasco-J815 spectropolarimeter flushed with nitrogen gas. The spectra were recorded in a 0.1 cm path length cuvette at a scan rate of 50 nm/min. The protein samples were measured in 0.2× phosphate-buffered saline (PBS) at pH 7.2. Data have been corrected for buffer contributions. For each protein sample, the CD signal was converted into mean residue ellipticity (MRE) using the following equation: \( \theta_{\text{MRE}} = \left( \frac{100}{dC(n - 1)} \right) \theta_{\text{obs}}, \) where \( \theta_{\text{obs}} \) is the observed ellipticity (in deg), \( d \) is the path length (in cm), \( C \) is the concentration of protein samples (in M), and \( n \) is total number of amino acids in the protein. For thermal melting measurements, the MRE at 222 nm was monitored when the temperature was increased from 15 to 90 °C with a rate of 3 °C/min.

**Hydrogel Preparation.** The purified protein was then dialyzed against deionized water for 2 days to remove all the salt from elution buffer. During dialysis, the water was changed every 5 h. The protein was then lyophilized after dialysis. The hydrogel was made by redissolving the protein sample into phosphate buffer (100 mM, pH 7.2). Vigorously mixing helps dissolution of proteins. The trapped air bubbles can be removed by fast spinning at 2500 rpm for 30 min. The fast spinning also helps flatten the hydrogel surface.

**Atomic Force Microscopy (AFM).** About 5 μL of the protein hydrogel was placed on the surface of a newly cleaved mica surface and allowed to adsorb for 10 min. Then the mica plate with the hydrogel sample was freeze-dried using liquid nitrogen. Then, the surface of the mica plate was flushed with nitrogen gas to remove unattached proteins before analysis. The images were taken at room temperature by a NanoWizard II AFM (JPK, Germany) operating in intermittent contact mode (conditions: scan rate, 1 Hz; cantilever, AC160 from Olympus, Japan; number of pixels, 512 × 512).

**Erosion Rate Measurement.** The erosion rate of hydrogel was measured using a method similar to that previously reported.24 AG₄₄₄ and CG₅₅₅₅₅C proteins were dissolved in PBS buffer to a concentration of 7%. The two protein solutions are mixed so that the molar ratio of CCE and CCK is 1:1. One hundred milligrams of hydrogel was transferred into a cylindrical glass tube with a flat bottom (1.05 cm diameter). The glass tube with the hydrogel was then centrifuged at 2500 rpm for 30 min to completely flatten the hydrogel sample to the bottom and smooth the surface of the hydrogel. The hydrogel was allowed to stand overnight. Then the thin gel film together with the glass tube was soaked in 5 mL of 100 mM phosphate buffer, pH 7.2, in a scintillation vial. The whole setup was placed on a compact rocker (FINEPCR) tilting at 50 rpm with an amplitude of ±9° at room temperature. The erosion profiles were determined by measuring the protein absorbance at 280 nm of the supernatant at successive time points using a Nanodrop ultraviolet—visible spectrophotometer. Two different samples were measured, and the average value was reported.

**Measurement of the Degree of Hydration.** The hydrogel (weight ranging from 60 mg to 200 mg) was soaked in 1.0 mL of PBS (pH = 7.2) for 30 min at room temperature, and was allowed to sit on a filter paper to drain the excess PBS. The hydrogel was weighed to obtain the swollen weight \( W_s \). Then the hydrogel was lyophilized and weighed again to obtain the dry weight \( W_d \). The degree of hydration of the hydrogel was calculated as \( (W_s - W_d)/W_s \times 100\% \).

**RESULTS**

The Two-Component Methodology to Construct Protein Hydrogels. The new method we developed is a two-component approach, which uses two different triblock proteins to form the hydrogel. The two triblock proteins carry two different and complementary leucine zipper coiled-coil sequences CCE and CCK to drive the gelation process. CCE and CCK are engineered coiled-coil motifs, which were designed by Kopecek and co-workers,25 and have been used to engineer polymer/protein hybrid hydrogels.25–28 The sequences of CCE and CCK are shown in Figure 1A. CCE and CCK have opposite charges at neutral pH: CCE is negatively charged, while CCK is positively charged under the same condition. Due to electrostatic repulsion, CCE or CCK cannot form homo coiled-coils, instead, they can only form antiparallel heterodimers between CCE and CCK (Figure 1A). The two components we engineered to form the hydrogel are CCE-(GB1)₄-CCE (designated as AG₄₄₄) and CCK-(GB1)₅-CCK-(GB1)₅-CCK (designated as CG₅₅₅₅₅C), where G₄ and G₅ are tandem modular proteins containing four and five tandem repeats of GB1 and are to mimic tandem modular extracellular matrix proteins; A represents CCE, and C represents CCK (Figure 1B). Both CCE and CCK are designed to provide intermolecular cross-linking through the
formation of antiparallel heterodimers between CCE and CCK. Tandem modular proteins made of GB1 have superior solubility in aqueous solution and are used to prevent precipitation of the tandem modular protein chain and retain water in the resultant hydrogel. AG₄A is a bifunctional protein, while CG₃CG₃C is a trifunctional one. Thus, a mixture of AG₄A and CG₃CG₃C with equal-molar functional groups A and C should be able to cross-link both proteins into a physical network through the self-association of coiled-coil sequences A and C.

There are a few considerations in the design to improve the properties of the resultant hydrogels against the first-generation gelator protein A-(GB1)₈-A we constructed before. First, since CCK and CCE are positively (or negatively) charged, they cannot aggregate on their own, making it possible to express and purify AG₄A and CG₃CG₃C with high yield under native conditions. Second, the two proteins are bifunctional and trifunctional, respectively, which facilitate the formation of physically cross-linked intermolecular networks. Third, the number of repeats of GB1 between coiled coil sequences in the two proteins is different, preventing the formation of protein dimers between AG₄A and CG₃CG₃C so that the coiled-coil sequences can be fully utilized for cross-linking.

Using standard molecular biology techniques, we expressed and purified the bifunctional AG₄A and trifunctional protein CG₃CG₃C in E. coli under native conditions. The yield for AG₄A and CG₃CG₃C are ∼60 mg and 30 mg per liter of LB culture, respectively. The viscosity of both protein aqueous solutions was low and did not pose any adverse effect on the purification of both proteins under native conditions. Figure 1C shows the SDS-PAGE picture of both purified proteins. The apparent molecular weight is ∼34 kDa for AG₄A and ∼78 kDa for CG₃CG₃C, in close agreement with the theoretical molecular weights.

**Far-UV CD Spectroscopy.** To confirm that CCE and CCK can indeed form a coiled-coil in the presence of GB1 domains, we carried out far-UV CD spectroscopy experiments. Since the CD signal of AG₄A and CG₃CG₃C is dominated by that of GB1, we constructed GB1-CCE and GB1-CCK to confirm that CCE and CCK can form a coiled-coil in the presence of folded GB1 domains. The CD spectra of GB1-CCE and GB1-CCK show two broad negative minima at 208 and 222 nm, which resulted from the α+β structure of the folded GB1 domains (Figure 2). The CD spectrum of 1:1 mixture of GB1-CCK and GB1-CCE shows a clear increase in the MRE of the two bands at 208 nm and 222 nm, suggesting that GB1-CCE and GB1-CCK indeed form a coiled-coil structure, leading to the increase in CD bands (208 and 222 nm) characterized by α-helix structures.

Furthermore, a dilute aqueous solution of GB1-CCE/GB1-CCK mixture (1:1 molar ratio) exhibits two distinct
thermal unfolding transitions (Figure 3A), as probed by the ellipticity at 222 nm. The first transition occurs at $T_m$ (temperature of the transition midpoint) of 38 °C and corresponds to the thermal dissociation of the coiled-coil heterodimer formed by CCE and CCK; the second transition occurs at $T_m$ of 76 °C and corresponds to the thermal denaturation of the folded GB1 domains.\textsuperscript{24,29}

Having established that CCE and CCK can indeed associate to form heterocoiled-coils in the presence of folded GB1 domains, we then characterized the dilute solution of AG\textsubscript{4A}/CG\textsubscript{5CG5C} (1:1 molar ratio of the leucine zipper sequences A and C) using temperature-dependent CD spectroscopy (Figure 3). Two thermal unfolding transitions were observed: a sharp one at 76 °C, which corresponds to the unfolding of folded GB1 domains, and a broad transition at $\sim$38 °C, which can be attributed to the thermal unfolding of CCE/CCK coiled-coils. This broad melting transition of CCE/CCK is consistent with the behavior observed for polymers cross-linked by CCE/CCK sequences,\textsuperscript{25} suggesting that the cooperativity of the thermal melting of CCE/CCK is reduced upon formation of cross-links between AG\textsubscript{4A} and CG\textsubscript{5CG5C}.

**Protein Hydrogels Self-Assembled from CCE/CCK Coiled-Coils.** The CD results suggest that the CCE/CCK sequence can self-associate to form coiled-coils at neutral pH, giving rise to the possibility of constructing protein hydrogels using AG\textsubscript{4A} and CG\textsubscript{5CG5C}. Indeed, a 7% (w/w) aqueous solution of AG\textsubscript{4A} and CG\textsubscript{5CG5C} mixture in PBS buffer (pH 7.2) readily forms a transparent hydrogel (Figure 4). The resultant gel can hang at the bottom of the vial without flowing down for days. In comparison, a 7% aqueous solution of pure AG\textsubscript{4A} or CG\textsubscript{5CG5C} in PBS (pH 7.2) is a clear transparent solution (Figure 4). This result indicates that the AG\textsubscript{4A} or CG\textsubscript{5CG5C} does not self-associate, and the gelation of the AG\textsubscript{4A} and CG\textsubscript{5CG5C} mixture is due to the formation of intermolecular coiled-coils by the CCE and CCK sequences.

In addition, since the association and dissociation of CCE/CCK coiled-coils are temperature dependent, we anticipate that the two-component hydrogel formation is also dependent upon temperature. Indeed, the formation of such two-component hydrogels is fully reversible as a function of temperature. When the temperature is increased to 40 °C, the hydrogel readily dissolves into viscous solution (Figure 4B). These results clearly confirm that this two-component hydrogel formation is mediated by the formation of physical cross-links between CCE/CCK coiled-coil sequences.

By varying the concentration of proteins at pH7.2, we also estimated the gelation point (Figure 4C). We found that the
two-component protein solution can form a hydrogel at a concentration as low as 3.5%. Given the high molecular weight of the two proteins, such a gelation concentration is quite low, indicating the efficiency of the two proteins to form hydrogels.

These results clearly demonstrate the feasibility of engineering protein-based hydrogels using this novel two-component approach. A schematic of the formation of a coiled-coil from CCE and CCK sequences (green and blue helices).

Morphology of Freeze-Dried Two-Component Protein Hydrogels. The morphology of the freeze-dried two-component protein hydrogel self-assembled from 7% AG₄A and CG₃CG₃C solution was characterized using AFM. As shown in Figure 6, the hydrogel shows an interconnected porous network structure, a sponge-like morphology. The pore size shows a big variation, ranging from tens of nanometers to a few hundred nanometers. These results suggest that the physical cross-links mediated by CCE/CCK coiled-coils lead to the formation interconnected network structure. Due to the fast association/dissociation rate of CCE/CCK, the physical cross-linking in the gel is not permanent but in fast equilibrium. Although this feature is beneficial for the prompt formation of hydrogel upon mixing two components, this may also make the resultant hydrogels prone to erosion.

Swelling and Erosion Properties. The degree of hydration for the 7% two-component hydrogel is 93.0% ± 0.2% (n = 6) after being equilibrated in PBS for 30 min. To measure the erosion profile of the two-component hydrogel formed by AG₄A and CG₃CG₃C, we monitored the mass loss as a function of time in an open aqueous environment (Figure 7). The erosion of this two-component hydrogel shows a linear mass loss versus time profile (Figure 7), indicating that the erosion occurs at the surface of the hydrogel. The erosion of this hydrogel is very slow: it takes about 100 h for the hydrogel to completely dissolve in PBS buffer, giving rise to an erosion rate of $1.34 \times 10^{-3}$ mg cm⁻² min⁻¹, which is about a 3-fold improvement over that for AG₈A hydrogel we reported before.²⁴

DISCUSSIONS

Designing protein hydrogels with new functions and improved properties is of imperative importance for realizing the great potential of protein hydrogels in the field of biomaterials and biomedical engineering. The use of coiled-coil sequences to drive the assembly of block protein-based hydrogels has become an important avenue in this area and has enabled the construction of protein hydrogels with various functions and physical properties. Triblock protein, ABA or ABC (where A and C are coiled-coil sequences and can self-associate to drive the gelation process), has been the standard approach.⁸,¹⁴–¹⁸ In this approach, the use of triblock proteins to form hydrogels relies on the efficient oligomerization of the coiled-coils. In our previous work, we have incorporated tandem modular proteins (GB₁)ₙ into the triblock protein as the center block to construct tandem modular protein-based hydrogels.²⁴ However, efficient oligomerization and low gelation point of our triblock tandem modular proteins resulted in significant increase of the viscosity of the

Figure 6. Surface morphology of the freeze-dried hydrogel made of 7% AG₄A and CG₃CG₃C aqueous solution (the molar ratio of A:C is 1:1).

Figure 7. Erosion profile of 100 mg 7% AG₄A and CG₃CG₃C hydrogel with a surface area of 0.865 cm² at room temperature in 100 mM phosphate buffer, pH 7.2. A linear regression (solid line) measures an erosion rate of $1.34 \times 10^{-3}$ mg cm⁻² min⁻¹.
protein solution, making the purification of proteins under native conditions very difficult.

In this work, we have developed a novel two-component methodology to engineer tandem modular protein-based hydrogels. This methodology utilizes two dissimilar yet complementary coiled-coil sequences as the gelation motif, and the gelation is accomplished by the mixing of a bifunctional protein AG₄A and a trifunctional block protein CG₅CG₅C. This new method effectively obviates the problem we encountered in expression and purification of high molecular weight tandem modular proteins carrying coiled-coil sequences for constructing hydrogels. Since CCE and CCK sequences do not self-associate, two proteins AG₄A and CG₅CG₅C were readily expressed and purified at high yield under native conditions. The resultant protein hydrogel shows much improved erosion property than previously engineered tandem modular protein-based-hydrogels. Therefore, this new method will open the possibility to systematically explore large fragments of native extracellular matrix proteins, such as fibronectin and tenasin, for the construction of extracellular matrix-mimicking hydrogels. Furthermore, this method allows for the use of abundant heterodimeric coiled-coil sequences, both naturally occurring and de novo designed ones, for the engineering of protein-based hydrogels, thus significantly expanding the toolbox of protein building blocks for hydrogel formation. Therefore, this novel approach reported here complements the standard triblock approach and will offer some new possibilities to fine-tune the topology and physical properties of the protein hydrogels via genetic engineering.

**AUTHOR INFORMATION**

**Corresponding Author**
*E-mail: Hongbin@chem.ubc.ca.*

**Present Addresses**
Department of Physics, Nanjing University Nanjing, Jiangsu Province P. R. China.

**ACKNOWLEDGMENT**

We thank Dawei Zou for his generous help in AFM imaging of the hydrogel. This work is supported by the Natural Sciences and Engineering Research Council of Canada, Canadian Institutes for Health Research, the Canada Research Chairs Program, Michael Smith Foundation for Health Research, and Canada Foundation for Innovation.

**REFERENCES**

(2) Kopecek, J. *Biomaterials* 2007, 28, 5185.
(18) Xu, C.; Breedveld, V.; Kopecek, J. *Biomacromolecules* 2005, 6, 1739.