Engineering tandem modular protein based reversible hydrogels†

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We report the engineering of the first tandem modular protein based hydrogel that exhibits unique properties combining low erosion rate, fast and reversible sol–gel transition and antibody binding ability.

Artificial protein hydrogels are of increasing interest because of their potential biomedical applications as drug delivery carriers, synthetic extracellular matrices and tissue engineering materials.[1–7] Pioneering work has demonstrated that leucine zipper domains are excellent building blocks with which to construct self-assembled protein hydrogels, opening up tremendous opportunities for using genetic engineering to tune the physical and functional properties of hydrogels[8–17] in a precisely controlled manner at the gene level. The use of a flexible random-coil like polypeptide or synthetic polymer as a center block has been the gold standard for the construction of leucine zipper based protein hydrogels.[8,11–17] Although recent progress has allowed for the splicing of a single globular domain into the random-coil like center block,[15] the random-coil nature of the center block remains the key design principle.

However, due to their high flexibility, flexible random-coil like polypeptides are susceptible to forming intramolecular loops, leading to an undesirable fast erosion rate of the hydrogel in open solutions.[13–15] Moreover, many extracellular matrix proteins are tandem modular elastomeric proteins, which are composed of individually folded domains.[18] Such modular extracellular matrix proteins not only have important biological functions, but also display significant mechanical stability. Thus, incorporating tandem modular proteins into hydrogels will not only be important in creating a synthetic extracellular matrix that closely mimics naturally occurring ones, but also may improve the physical properties of the resulting hydrogels. However, direct incorporation of such tandem modular proteins into hydrogels has been challenging. As a proof of principle, here we demonstrate the engineering of the first artificial tandem modular protein based reversible hydrogel and show that the engineered novel hydrogel exhibits unique properties combining much improved erosion properties, fast and reversible sol–gel transition and ability to bind IgG antibodies.

The model tandem modular protein we used here is an artificial polyprotein $(GB1)_8$ (denoted as (G)8 hereafter), which is composed of eight GB1 domains arranged in tandem (Fig. 1A). Previous single molecule atomic force microscopy (AFM) experiments have demonstrated that polyprotein (G)8 exhibits excellent mechanical properties that are either comparable to or superior to those of naturally occurring elastomeric proteins.[19,20] To construct (G)8 based hydrogel, we adopted the standard triblock protein design for hydrogel: we use (G)8 as the center block, in replacing the commonly used flexible random-coil like polypeptide, and use the well-characterized leucine zipper domains A[8] to flank the center block at its N- and C-termini (Fig. 1A). Leucine zipper A, designed by Tirrell and co-workers,[8] can self-associate into oligomers and its use in constructing protein based hydrogels has been studied in detail.[8,13,14]

Using standard molecular biology techniques, we constructed the gene of A(G)8A and expressed the triblock protein in *E. coli* (Supplementary Information†). Fig. 1B shows the denaturing SDS-PAGE picture of the purified protein. The purified A(G)8A appears as a predominant band on SDS-PAGE gel with an apparent molecular weight of ~57 kDa, in close agreement with the theoretic molecular weight of A(G)8A of 63 kDa. Far UV circular dichroism (CD) spectroscopy results provide further supporting information for the structural integrity of A(G)8A (Fig. 2). The CD spectrum of polyprotein (G)8 is characterized by two broad negative minima at 208 nm and 222 nm, consistent with the α/β structure of the constituting GB1 domains (Fig. 2A).[21] In comparison, the CD spectrum of A(G)8A shows a significant increase in the mean residue ellipticity of the bands characterizing α-helix secondary structures, in good agreement with the expectation of appending leucine zipper sequences to the N- and C-termini of (G)8. Furthermore, in dilute solution, A(G)8A exhibits two distinct thermal unfolding transitions (Fig. 2B), as probed by the intensity of the absorbance at 222 nm. The first transition occurs at T_m (temperature of the transition midpoint) of 43 °C and corresponds to the thermal dissociation of the coiled-coil oligomers of A[8]; the second

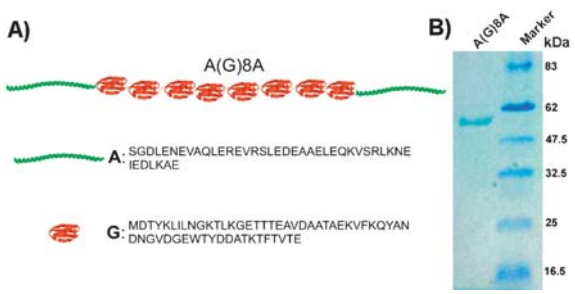

Fig. 1 (A) Schematic of the artificial protein A(G)8A. Green helices represent the leucine zipper domains "A" and red globular proteins represent the GB1 domains. The amino acid sequences of leucine zipper domain A and globular GB1 domains are also shown. (B) 12% denaturing SDS-PAGE picture of A(G)8A protein.

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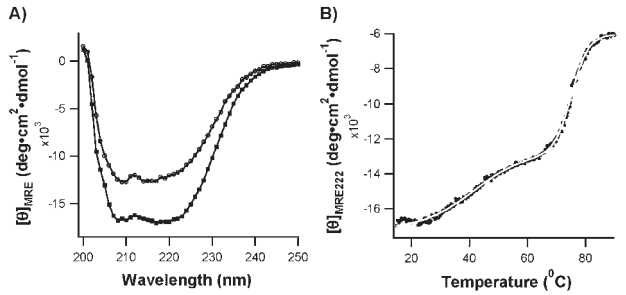

Fig. 2 (A) CD spectra of (G)8 (open circle) and A(G)8A (filled square) in 0.5 × PBS, pH 7.4. (B) Thermal melting of A(G)8A in 0.5 × PBS, pH 7.4. Two melting transitions occur at 43 °C and 75 °C, respectively.

transition occurs at T_m of 75 °C and corresponds to the thermal denaturation of the folded GB1 domains.[21] Moreover, the thermal melting behavior of A(G)8A is fully reversible, providing the possibility of constructing reversible hydrogels.

The coiled-coil domains A can self-associate and dissociate depending on temperature, giving rise to the possibility of A(G)8A forming a thermoreversible hydrogel.[8] Indeed, 7% (w/w) aqueous solution of A(G)8A in PBS buffer (pH 7.6) readily forms an opaque hydrogel (Fig. 3B). The gel can hang at the bottom of the vial without flowing down on a time scale of months, demonstrating its capability of retaining buffer in the protein matrix, the very character of typical hydrogels. For comparison, 7% aqueous solution of (G)8 in PBS (pH 7.6) results in a clear transparent solution, which flows readily (Fig. 3A). This result indicates that the gelation of A(G)8A is not a property intrinsic to polyprotein (G)8, instead it is due to the self-aggregation of coiled-coil domains A flanking the center block of tandem modular protein (G)8. The micro-structure of the hydrogel was investigated using scanning electron microscopy (SEM). As shown in Fig. 3C, the 7% hydrogel shows an interconnected porous network structure, suggesting that the hydrogel is formed *via* physical cross-linking mediated by leucine zipper domains.

Since the self-association and dissociation of coiled-coil domain A are temperature dependent, we expect that A(G)8A hydrogels should undergo sol–gel/gel–sol transitions at similar temperatures. Indeed, increasing the temperature to 60 °C results in gel–sol transition and the hydrogel turning into viscous liquid. Cooling down the solution to room temperature results in the formation of hydrogel again (Fig. S1†). These results confirm that the gel formation is mediated by the formation of physical crosslinking between coiled-coil sequences. In addition, it is also of note that the sol–gel transition for A(G)8A is very fast and can be accomplished typically within 30 s. Such a fast response time is unique for a triblock protein of such high molecular weight. Its origin is yet to be determined.

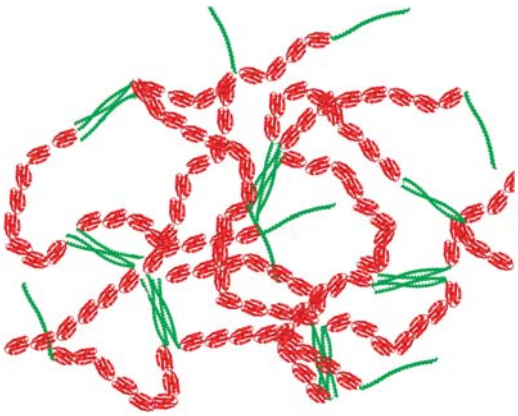

Fig. 4 Schematic drawing of the A(G)8A hydrogel. Hydrogels are formed through the self-association of leucine zipper domains A (green helices).

These results clearly demonstrate the feasibility of engineering tandem modular protein based hydrogels. Fig. 4 shows a schematic of the formation of the A(G)8A hydrogel network at the molecular level, in which the physical crosslinking is formed *via* self-association of leucine zipper domains. The use of a rigid tandem modular protein as the center block to construct leucine zipper based hydrogel is in contrast to the general understanding that flexible random-coil like polypeptide is a necessary requirement for the center block, suggesting that flexible sequences may not be mandatory in constructing leucine zipper based hydrogels.

A previous study on AC10A hydrogel showed that, due to the formation of an intramolecular loop between two terminal leucine zipper domains, leucine zipper domains in the triblock proteins cannot be fully utilized and some remain dangling in the hydrogel, resulting in a high erosion rate and a low storage modulus of the hydrogel. This formation of intramolecular loops mainly resulted

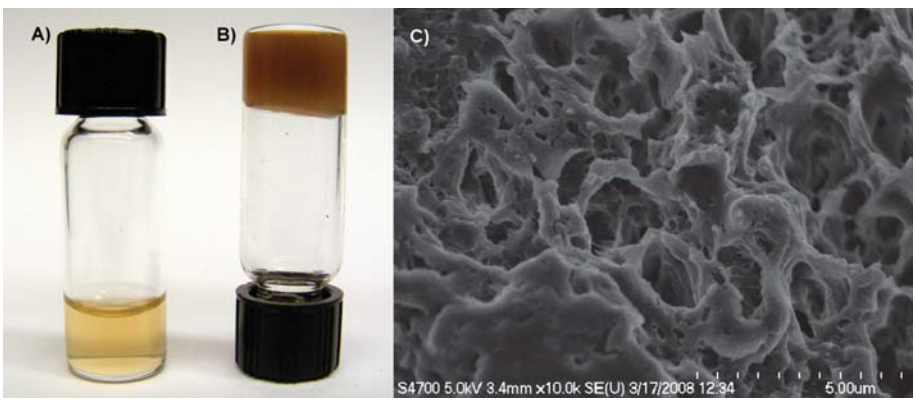

Fig. 3 (A) Aqueous solution of 7% (G)8, (B) hydrogel of 7% A(G)8A in 100 mM phosphate buffer, pH 7.6, and (C) SEM image of 7% A(G)8A hydrogel (the scale bar is 5 μm).

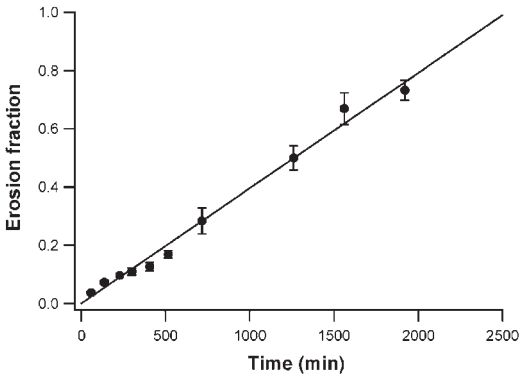

Fig. 5 Erosion profile of 100 mg of 7% A(G)8A hydrogel with a surface area of 0.86 cm^2 at room temperature. A linear regression (solid line) measures an erosion rate of 3.23×10^{-3} mg cm^{-2} min^{-1}.

from the flexibility and short end-to-end distance of the center flexible random-coil like polypeptide sequence. The use of tandem modular proteins in constructing hydrogel not only provides new building blocks for hydrogel construction, but also provides an efficient means to overcome this shortcoming and lead to improved properties. Since tandem modular proteins are more rigid than flexible random-coil like sequences, the end-to-end distance of the tandem modular proteins is much larger than that for flexible random-coil sequences. Therefore, the coiled-coil domains A at both ends of the (G)8 are unlikely to meet each other, thereby effectively preventing the formation of intramolecular loops and increasing the efficiency of intermolecular association. The improvement in intermolecular association mediated by leucine zipper domains has led to some unique properties observed in our tandem modular protein based hydrogel: low gelling point and low erosion rate.

We estimated the gelling point of A(G)8A by preparing A(G)8A aqueous solution at different concentrations (pH 7.6). Photographs of these solutions are shown in Fig. S2.† It is remarkable that A(G)8A can form a gel at a concentration as low as 3.1%. Such a gelling concentration is lower than that for the well-characterized protein AC10A, in which the center block is made of a random-coiled sequence.[8] Considering that the molecular weight of the center block (G)8 is four times that of a C10 sequence, the actual content of coiled-coil domains A in A(G)8A is even lower than that in AC10A.

Similarly, the erosion properties of hydrogel A(G)8A also show significant improvements over the random-coil sequence based hydrogel AC10A. Erosion rate is a measure of the stability of physically crosslinked hydrogel in open solution. The higher the erosion rate is, the less stable the hydrogel is. Improving erosion properties is key for the use of hydrogels in many biomedical applications. As shown in Fig. 5, the erosion profile of A(G)8A in open solution shows a linear mass loss *versus* time profile, indicating that the erosion is occurring at the surface. The measured erosion rate is 3.23×10^{-3} mg cm^{-2} min^{-1}, which is one order of magnitude slower than that for AC10A measured under similar conditions.[13,15] This result is consistent with a previous study on hydrogel A-C10-GFP-A, in which a GFP domain is inserted at the end of a random hydrophilic sequence in AC10A.[15] Such a low erosion rate makes A(G)8A a desirable hydrogel for biomedical application, in that it can retain its shape for a sufficiently long period of time. It is important to point out that the low gelling concentration and slower erosion rate are not likely to result from the solubility of the tandem modular protein center block (G)8, as (G)8 has excellent water solubility and can dissolve in PBS to a concentration of as high as 200 mg ml^{-1}.

Furthermore, the GB1 domain is well known for its ability to bind IgG antibodies.[22] Therefore, our engineered A(G)8A hydrogel also carries IgG binding properties. Detailed studies on the binding behavior of IgG antibody to A(G)8A hydrogel are currently underway.

In summary, here we have demonstrated the engineering of the first tandem modular protein based thermo-reversible hydrogel. This novel type of genetically engineered protein based hydrogel incorporates an artificial tandem modular protein into the hydrogel matrix, thus paving the way for the engineering of intact extracellular matrix protein based hydrogels. Moreover, the use of tandem modular proteins makes it possible to engineer hydrogels with much improved physical properties, allowing them to be used as artificial extracellular matrix and tissue engineering materials.

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