Hydrogels

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Rational Design of a Tetrameric Protein to Enhance Interactions between Self-Assembled Fibers Gives Molecular Hydrogels**

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Molecular hydrogels have attracted extensive research interest in recent years because of their inherent properties (e.g., formation by the self-assembly of small molecules and their gel–sol/sol–gel phase transitions can be easily manipulated by external stimulus).[1] They have shown great potential in fields such as three-dimensional (3D) cell culture[2] and controlled drug delivery.[3] During the formation of a molecular hydrogel, a small molecule (molecular hydrogelator) needs to self-assemble into a 3D matrix of nanofibers, nanorods, or nanospheres that can hold water molecules within the cavities of the 3D matrix. To form the 3D matrix, there should be strong or at least medium interactions between self-assembled nanostructures. Otherwise, nanostructures with weak interactions between them will only form dispersions or solutions in the aqueous phase. Actually, there are many examples of this kind of self-assembled system that lack strong interactions between the self-assembled structures.[4] This type of solution/dispersion containing self-assembled nanostructures could change to a hydrogel if the interaction between the nanostructures could be enhanced. For example, several groups have demonstrated that zinc and calcium ions can be used to cross-link self-assembled nanofibers to form molecular hydrogels.[5] In this study, we rationally designed a fusion protein with four binding sites and used the protein–peptide interaction to enhance interactions between self-assembled nanofibers, thus leading to the formation of molecular hydrogels (Figure 1).

There are only a few examples of polymeric hydrogels formed by specific protein–peptide interactions.[6] Specific protein–peptide interaction has also been used to direct self-assembly of peptide nanowires into micrometer-sized crystalline cubes.[7] However, there are no reports about the formation of molecular hydrogels through protein–peptide interactions up to now. As mentioned above, the formation of a 3D matrix is crucial to the formation of molecular hydrogels. To use protein–peptide interactions to enhance interfiber interactions to support 3D structures, fusion proteins with multiple binding sites are needed. These kinds of fusion proteins usually contain two parts, one for multimer formation and the other for peptide binding. However, these fusion proteins are usually in the balance between multimers with multiple binding sites and monomer with only one binding site. The dissociation constants of protein–peptide interactions are also usually in the micro- to millimolar range. It remains a challenge to develop a protein that can predominantly (> 95 %) form multimers with multiple binding sites. It would also be highly interesting for researchers in the field of biomaterials to develop a protein–peptide interaction with

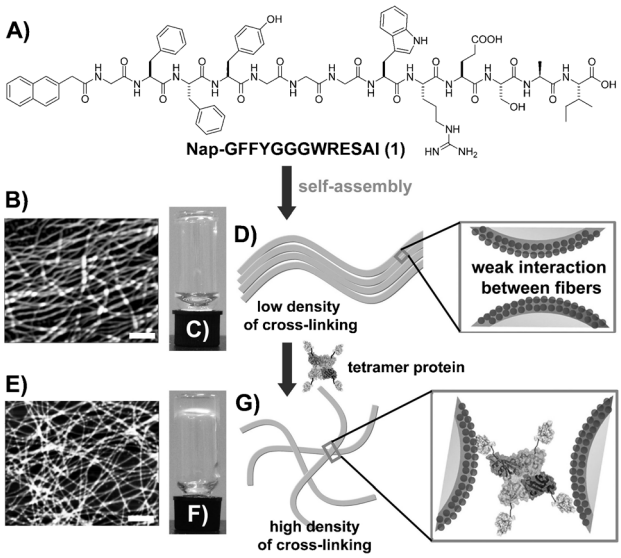

Figure 1. Protein–peptide interaction can be used to enhance interactions between self-assembled fibers, thus leading to molecular hydrogelation. A) Chemical structure of Nap-GFFYGGGWRESAI (**1**: nongelator) with a possible self-assembly ability. B–D) Compound **1** self-assembles into nanofibers that lack strong interactions between fibers in aqueous solution, thus resulting in fiber networks with low density of cross-linking points. E–G) The addition of our rationally designed fusion protein (ULD-TIP-1) enhances the interactions between fibers, thus leading to a 3D fiber network with high density of cross-linking points and hydrogelation. The scale bars in (B) and (E) represent 500 nm; the small balls in the insets of (D) and (G) represent the hydrophilic part of **1** (GGGWRESAI); the images of (D) and (G) do not represent the exact length scale and structure of the components.

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adjustable dissociation constants within a broader range from nano- to millimolar.

Recently, we determined the crystal structures of two important proteins, an ubiquitin-like domain (ULD) from the special AT-rich sequence binding protein 1 (SATB1)[8] and the complex of Tax-interacting protein-1 (TIP-1) with its peptide ligand.[9] The ULD protein spontaneously and predominantly (>99%) formed a tetramer.[8] From the crystal structure of TIP-1 with its natural peptide ligand NISYRRESAI (dissociation constant (K_d) = 6.4 μM) and the hexapeptide RRESAI (especially the amino acids R(−5), S(−2), and I(0)) that was important for binding, we realized that the replacement of R(−5) with W might increase the binding affinity to TIP-1. Therefore, we designed the peptide NISYWRESAI that could specifically and very tightly bind to TIP-1 with a K_d of 8.5 nM.[9] Based on these observations, we designed a fusion protein ULD-TIP-1 that might be able to form a tight tetramer with four binding sites (ULD assisted the formation of tetramer and TIP-1 provided a binding site to peptides). This fusion protein might be used to enhance interactions between self-assembled nanofibers for the formation of molecular hydrogels.

We designed a construct that could express the fusion protein ULD-TIP-1, in which ULD and TIP-1 were joined as a single polypeptide by a hexaglycine (GGGGGG) segment (Figure 2A). As shown in Figure 2B (inset), ULD-TIP-1 could be expressed in *Escherichia coli* and purified to homogeneity (25.44 kDa, single band in SDS-PAGE gel). The purified ULD-TIP-1 proteins were eluted as a single peak from a size-exclusion column with a molecular mass corresponding to a tetramer (Figure 2B). Analytical ultracentrifugation further confirmed that ULD-TIP-1 assembled into a tetramer with a molecular mass of approximately 104.07 kDa (Figure 2C). These data demonstrated that ULD-TIP-1 formed a very stable tetramer in solution. All together, the results clearly indicated the successful design of the fusion protein ULD-TIP-1 with four ligand-binding sites (Figure S-2 in the Supporting Information).

After the successful synthesis and purification of the fusion protein, we designed and synthesized a small molecule with a possible self-assembly ability (Nap-GFFYGGGWRESAI (**1**) in Figure 1A). Nap-GFFY has been demonstrated as an efficient short peptide for the construction of self-assembled peptides and it might assist the self-assembly of **1**,[10] with GGG as a linker, and WRESAI could tightly bind to TIP-1.[9] Compound **1** did not form a hydrogel but a clear solution, even at a high concentration of 5 wt% in phosphate-buffered saline (PBS, pH 7.4) at room temperature (22–25°C). However, the atomic force microscopy (AFM) images (Figure 1B and Figure S-28 in the Supporting Information) indicated the presence of large amounts of nanofibers with diameters of 40–60 nm in a solution of **1** at the concentration of 0.5 wt%. Rheological measurements indicated that the solution of **1** was a viscous one (Figure S-22 in the Supporting Information). The reasons why **1** could not form a hydrogel but only a solution were probably the weak interactions between these self-assembled nanofibers and the lack of adequate cross-linking points to support hydrogel formation. Therefore, compound **1** was an ideal candidate to demonstrate our proposed novel strategy for molecular hydrogelation by enhancing interactions between self-assembled fibers through protein–peptide interactions.

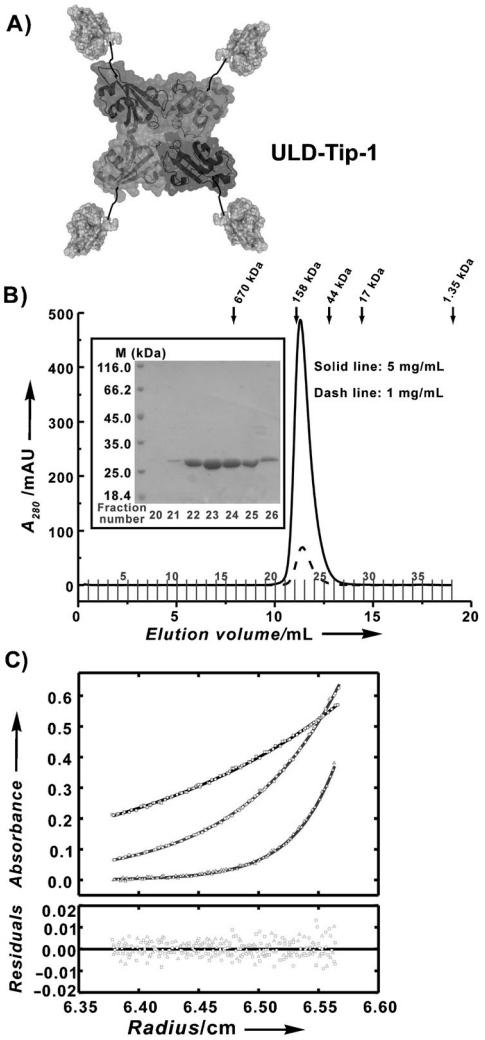

Figure 2. ULD-TIP-1 assembles into a tight tetramer in solution. A) Proposed structure of the tetrameric ULD-TIP-1 protein (modified from the crystal structures of ULD and TIP-1). The linkers of hexaglycine segments are shown as black lines. B) Size-exclusion column chromatography of ULD-TIP-1 was carried out in a Superose 12 column at two concentrations. Inset: 15% SDS-PAGE gel of each fraction. The theoretical molecular weight (MW) of ULD-TIP-1 is 25.44 kDa. The molecular mass standards used to calibrate the column are indicated. C) Representative sedimentation equilibrium profiles of ULD-TIP-1 derived from a global fitted MW ≈ 104.07 kDa (1σ confidence interval 103.67–104.52).

We then tested whether the addition of ULD-TIP-1 to a solution of **1** resulted in hydrogelation or not (final concentration of **1** = 0.5 wt%). As shown in Figure 1F, hydrogelation happened within minutes after the addition of ULD-TIP-1. The minimum amount of ULD-TIP-1 needed for gelation was about 0.2% equiv relative to **1** (about 0.0158 wt% in the hydrogel). Hydrogels could also be obtained within 30 min by the same procedure for Nap-GFFYGGG*G*RESAI (W mutated to *G*, compound **2** in Scheme S-2 in the Supporting Information) due to the

presence of the peptide GGG*G*RESAI that could bind to ULD-TIP-1 with a K_d of 39.21 μM (determined by the ITC assay).[11] However, the sequences of Nap-GFFYGGG*GE*ESAI (W mutated to *G* and R mutated to *E*, compound **3** in Scheme S-3 in the Supporting Information) and Nap-GFFYGGG*G*RESA*G* (W mutated to *G* and I mutated to *G*, compound **4** in Scheme S-4 in the Supporting Information) did not form hydrogels within 6 h in the presence of ULD-TIP-1 because of the very weak interactions between GGG*GE*ESAI/GGG*G*RESA*G* and ULD-TIP-1 (K_d = 211.42 μM/not detectable, determined by ITC assay).[11] All of the critical micelle concentrations (CMCs) of compounds **1**–**4** were lower than 0.8 mg mL^{-1} (determined by dynamic light scattering (DLS), Figure S-19 in the Supporting Information), which suggested the formation of self-assembled structures in solutions containing 5 mg mL^{-1} of compounds **1**–**4**. The cryo-TEM images of these solutions also clearly indicated the formation of uniform nanofibers with diameter of about 7.5 nm in solution (Figure S-20 in the Supporting Information). These results suggested that ULD-TIP-1 enhanced the interactions between self-assembled fibers by a specific protein–peptide interaction. They also suggested that the gelation kinetics and mechanical properties of the resulting gels could be manipulated by using different peptides with different binding affinities to TIP-1. To the best of our knowledge, this is a novel method to trigger molecular hydrogelation. We then characterized the hydrogels under different conditions by several techniques.

The mechanical properties of hydrogels could be adjusted by adding different amounts of ULD-TIP-1 to solutions of **1**. As shown in Figure 3, the results obtained by dynamic time sweep indicated that the values of the storage modulus (G') of the resulting gels were bigger when higher amounts of ULD-TIP-1 were used—the G' value of balanced gels was about 50, 80, 240, and 320 Pa for gels of **1** with 0.25, 0.50, 1.0, and 1.5% equiv ULD-TIP-1 (relative to compound **1**), respectively, at a strain of 0.8% and frequency of 2 rad s^{-1}. The mechanical strength of the resulting hydrogels could also be manipulated by using different peptides with different dissociation constants (K_d) from that of ULD-TIP-1. As shown in Figures S-24 and S-25 in the Supporting Information, a gel containing 0.5 wt% **1** exhibited a higher G' value (320 Pa) than the gel containing 0.5 wt% **2** (85 Pa) in the presence of 1.5% equiv ULD-TIP-1, thus indicating that a stronger protein–peptide interaction would lead to a mechanically stronger hydrogel. These observations clearly indicated that the mechanical properties of hydrogels formed by our method could be controlled by several means, which would be beneficial to their future applications in fields such as stem cell controlled differentiation and controlled drug release.

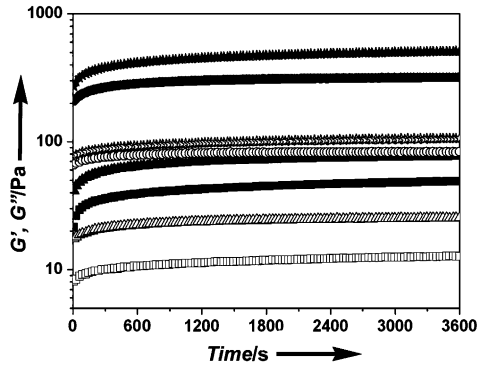

Figure 3. Rheological measurements with the mode of dynamic time sweep at a frequency of 2 rad s^{-1} and strain of 0.8% for PBS solutions containing 0.5 wt% **1** and different amounts of ULD-TIP-1 (squares: 0.25, triangles: 0.50, circles: 1.0, and stars: 1.5% equiv relative to **1**; closed symbols: G' and open symbols: G'').

Hydrogels formed by our method possessed a rapid recovery property. For example, the gel containing 0.5 wt% **1** and 0.25% equiv ULD-TIP-1 exhibited a shear-shining property (Figure 4): both G' and G'' decreased gradually when subjected to a large external stress (50% strain) and the value of G'' changed to being bigger than that of G' after about 5 min, thus indicating a gel–sol phase transition. After the removal of the large external stress, both G' and G'' increased rapidly—about 42% of the original G' value was achieved within seconds and a full recovery of mechanical strength was observed within 600 s. These observations were consistent with the fact that gel containing 0.5 wt% **1** and 0.25% equiv ULD-TIP-1 was thixotropic—the gel could be converted to a viscous solution by gently shaking, vortexing, or pipetting and the resulting viscous solution changed back to a gel within 5 min upon keeping without disturbance (Figure S-27 in the Supporting Information). A similar recovery property was observed for gels of **1** with different amounts of ULD-TIP-1. For example, the result in Figure S-26 (Supporting Information) indicated that a gel of **1** with 0.5% equiv ULD-TIP-1 also showed thixotropic and fast recovery properties. The thixotropic property of our gels would facilitate their application to cell encapsulation in 3D cell culture.[12] The gel changed to a clear solution if firstly mixed with three volumes of PBS buffer solution and then shaken, which would be beneficial to separate encapsulated cells post culture for further analysis.

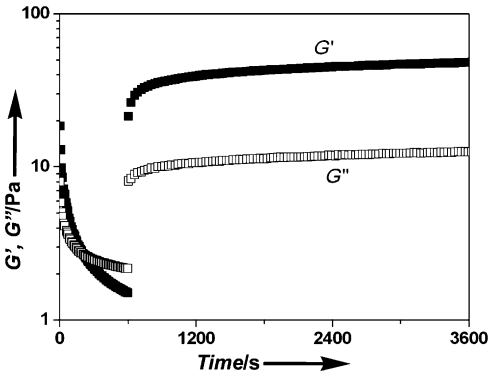

Figure 4. Recovery property of a gel in 100 mM PBS buffer (pH 7.4) containing 0.5 wt% **1** and 0.25% equiv ULD-TIP-1. The gel was first subjected to a large external stress of 50% strain for 600 s, and then the recovery of the gel was measured at a strain of 1% and a frequency of 1 rad s^{-1} for 3000 s.

We then used AFM to characterize the nanostructures in solutions of **1** and the gels. As shown in Figure 1B and Figure S-28 in the Supporting Information, nanofibers with a size of about 40–60 nm are observed in a PBS solution containing 0.5 wt% **1**. These nanofibers did not form stable 3D networks that supported hydrogel formation, probably because of the lack of strong interactions between fibers. This hypothesis was partially proved by the relatively parallel arrangement of these nanofibers. After the addition of ULD-TIP-1 to solutions of **1**, the solutions changed to gels rapidly.

Dense fiber networks were also observed in gels with different amounts of ULD-TIP-1 (Figure 5A–D). These nanofibers formed extensive cross-linking points, thus leading

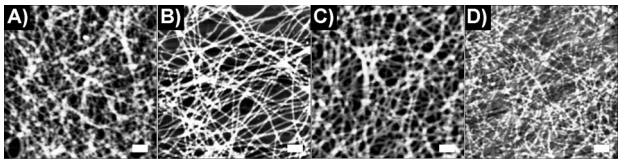

Figure 5. AFM images of gels containing 0.5 wt% **1** and different amounts of ULD-TIP-1: A) 0.25, B) 0.50, C) 1.0, and D) 1.5% equiv relative to **1** (scale bar = 300 nm).

to the formation of 3D networks for hydrogel formation. The sizes of the nanofibers in gels were similar: all were about 40–60 nm in width and longer than 3 μm. Based on the cryo-TEM observations, the fibers observed by AFM were bundles of nanofibers (about 7.5 nm), which formed during the drying process or due to the surface effect. The cross-linking points in solutions of **1** and gels were different—there were about 6, 27, 51, 46, and 91 cross-linking points per μm^2 in solutions of **1**, and gels with 0.25, 0.50, 1.0, and 1.5 equiv ULD-TIP-1, respectively. The phenomenon of more cross-linking points in gels with higher amounts of ULD-TIP-1 was consistent with the results shown in Figure 3, which suggested that gels with higher amounts of ULD-TIP-1 were mechanically stronger. These observations also indicated that ULD-TIP-1 could enhance interfiber interactions and help to form more cross-linking points of nanofibers in gel samples to support hydrogelation.

In summary, we have designed and obtained a fusion protein with four binding sites to hexapeptides. The specific protein–peptide interaction between TIP-1 and peptide GGGWRESAI with $K_d = 36.76$ nM was a very rare and strong interaction. Therefore, we used the specific protein–peptide interaction to enhance the interactions between self-assembled nanofibers, thus resulting in hydrogel formation. This is a novel method to prepare molecular hydrogels, and the mechanical properties of the resulting hydrogels could be managed by using different kinds of peptides with different binding affinities to TIP-1 protein, by changing the concentration of self-assembled peptides and by altering the concentration of the cross-linker (protein of ULD-TIP-1). The thixotropic and fast recovery properties of these gels might allow the homogeneous encapsulation of drug molecules and cells within gels. Although we only used the fusion protein of ULD-TIP-1 to prepare molecular hydrogels, the fusion protein with multiple binding sites can also be applied to polymeric hydrogelation.[13] The four binding sites in the fusion protein allow other bioactive molecules to be incorporated, which would lead to multifunctional hydrogels for tissue engineering and regenerative medicine.

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