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Disulfide bond as a cleavable linker for molecular self-assembly and hydrogelation†

Chunhua Ren,[ab] Zhijian Song,[c] Wenting Zheng,[a] Xuemei Chen,[c] Ling Wang,[b] Deling Kong[a] and Zhimou Yang*[ad]

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Here we reported on the first example of using the disulfide bond as a cleavable linker to control molecular self-assembly and the formation of small molecular (SM) hydrogels.

Maintaining the homogeneity during the formation of SM hydrogels[1] is crucial for their applications in the fields of tissue engineering,[2] controlled drug release,[3] and biosensing.[4] Up to now, several methodologies have been developed to achieve this requirement including the methods of a heating–cooling cycle,[5] controlled hydrolysis of GdL,[6] light irradiation,[7] and enzymatic triggeration.[8] Of the above methodologies, light irradiation and enzymatic triggeration are more beneficial than the other ones for the applications of SM hydrogels in biomedical research because they caused minimum damage to the encapsulated components in gels. Recently, Rao's group firstly used the strategy of disulfide bond reduction to initiate a condensation reaction for the preparation of hydrogels.[9] Nilsson and Bowerman also applied the strategy of disulfide bond reduction to release the conformational constraint of a cyclic peptide, thus resulting in relaxation to a β-strand linear peptide and the formation of SM hydrogels.[10] Both examples demonstrated that the strategy of disulfide bond reduction might offer great opportunities for the application of hydrogels in biomedical research. Stimulated by both results, we proved herein that the disulfide bond could also be used as a cleavable linker to connect a SM hydrogelator with a hydrophilic molecule. Thereafter, the cleavage of the disulfide bond would lead to the formation of SM hydrogels in a homogeneous way and by a controllable manner.

We designed the molecule of Nap-GFFYE-CS-EERGD (**2** in Fig. 1) as the precursor of the SM hydrogelator. Compound **2** had several features: (1) Nap-GFFY guaranteed the formation of the gels after disulfide bond cleavage because the derivatives of it had been demonstrated as 'super gelators';[11]

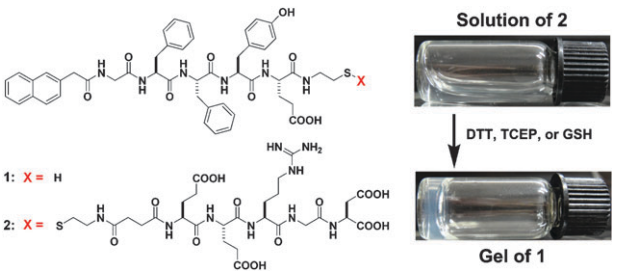

Fig. 1 Chemical structures of the precursor of the gelator (**2**) and the SM gelator (**1**) and optical images to show hydrogelation by the addition of reductants to PBS buffer solutions (pH = 7.4) of **2** (0.4 wt%, 2.42 mM).

(2) the part of CS had a disulfide bond that could be cleaved by reductants such as Tris(2-carboxyethyl)phosphine (TCEP), Dithiothreitol (DTT), and Glutathione (GSH); (3) the part of EERGD ensured the formation of clear solutions of **2** in aqueous solutions. The reasons for using the hydrophilic peptide of EERGD but not other biocompatible and water soluble polymers to increase the solubility of **2** are the easy synthesis of peptides and the fact that polymers have big influences on the property of SM hydrogels.[12] The synthesis of **2** was easy and straightforward. As shown in Scheme S1 (ESI†),[13] the cystamine was firstly reacted with succinic anhydride and then with Fmoc-OSu to produce Fmoc-Cystamine Succinate (Fmoc-CS). Then compound **2** was obtained by standard Fmoc solid-phase peptide synthesis (SPPS) using Fmoc-CS and other Fmoc-amino acids with side chains properly protected.

The self-assembly behavior of **2** was then evaluated in phosphate buffer saline (PBS) buffer solution (Na_2CO_3 was used to adjust the final pH value to 7.4).[13] Compound **2** was highly soluble in PBS buffer solutions with a solubility of >2 wt%. The addition of 1.0 equiv. of TCEP to the solution of **2** (0.4 wt%, 2.42 mM) led to the immediate and nearly total reduction of the disulfide bond and the formation of a SM gelator of **1**, as indicated by the LC-MS result that more than 99% mol of the original **2** had been converted to **1**.[13] However, it took about 10 minutes for the formation of the hydrogel by the invert-tube method. The transmission electron microscopy (TEM) images revealed that **1** firstly self-assembled into short fibrils 10 minutes after the addition of TCEP (Fig. 2A) and then into long ones after the overnight incubation (Fig. 2B). The LC-MS result indicated that **1** gradually converted to its dimer at atmosphere and only small amounts of **2** (3.7% mol) and **1** (0.5% mol) were observed in the gel after 7 days,[13] suggesting the formation of disulfide bond between **1** within

[a] *Key Laboratory of Bioactive Materials, Ministry of Education, College of Life Sciences, Nankai University, Tianjin 300071, P. R. China*
[b] *College of Pharmacy, Nankai University, Tianjin 300071, P. R. China. E-mail: yangzm@nankai.edu.cn; Tel: +86-22-23502875*
[c] *College of Chemical Engineering, School of Chemical and Materials Engineering, Huangshi Institute of Technology, Huangshi 435003, P. R. China*
[d] *Department of Biochemistry and Molecular Biology, College of Life Sciences, Nankai University, Tianjin 300071, P. R. China*

† Electronic supplementary information (ESI) available: Synthesis and characterization of **2**, details in the formation of the hydrogels, emission spectra, percentage of different components in gels at different time scales determined by the HPLC, and rheological measurements of the gels. See DOI: 10.1039/c0cc04135a

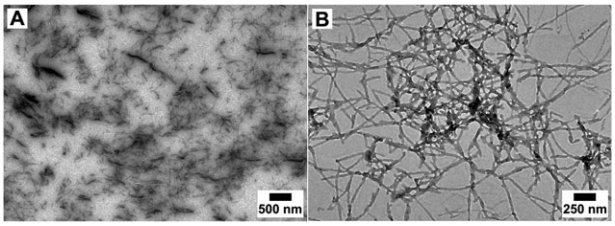

Fig. 2 Transmission electron microscopy (TEM) images of gels formed by the addition of 1 equiv. of TCEP to the PBS solutions (pH = 7.4) containing 0.4 wt% of **2** (2.42 mM): (A) the gel as formed and (B) the gel after overnight incubation.

the nanofibers dominated.[10] The minimum concentration needed for gelation of **2** was 0.2 wt% (1.21 mM), which meant that 1 equiv. of **1** generated from **2** by TCEP could gel 45,900 molecules of H_2O in PBS buffer solutions. Similar results were observed when 1.0 equiv. of DTT was used to trigger the disulfide bond cleavage. However, it took a longer time (about 60 minutes) for the formation of gels when 2 equiv. of GSH was used probably due to the fact that GSH was a less effective reductant than DTT and TCEP.

To investigate the kinetics of the gelation triggered by TCEP, the mode of dynamic time sweep was performed for the PBS buffer solution of **2** with 1 equiv. of TCEP. As shown in Fig. 3A, the hydrogelation happened at about 2 minutes, as indicated by the value of G' (elasticity) dominating that of G'' (viscosity). Both values of G' and G'' kept increasing in the first one hour, which was consistent with the TEM images shown in Fig. 2 that it took time for the self-assembly of **1** into long fibers and the formation of a rigid network for the gel. The gel formed by **1** was a thixotropic one—as shown in the ESI†, the gel would change to a viscous solution by shaking or by vortex, and then the viscous solution could change back to the hydrogel after keeping at room temperature (22–25 °C) within half an hour. The recovery property of the gel was then studied by the mode of dynamic time sweep (Fig. 3B). Both values of G' and G'' dropped rapidly and only about 11% of the original value of G' was observed after 10 minutes under the external large stress (strain = 50%), indicating that the gel possessed a shear-thinning property. After the removal of the external large stress, a rapid recovery of the strength of the gel was observed—58% of the original value of G' was achieved right after the removal of the external stress and more than

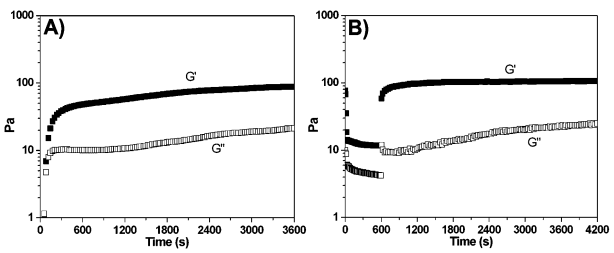

Fig. 3 (A) Dynamic time sweep of a PBS buffer solution containing 0.4 wt% of **2** with 1 equiv. of TCEP at the strain of 2% and the frequency of 2 rad s^{-1} and (B) the recovery property of the resulting hydrogel (the gel was firstly subjected to a large amplitude of strain of 50% at the frequency of 2 rad s^{-1} for 600 seconds and then its recovery was probed at the strain of 2% and the frequency of 2 rad s^{-1} for 3600 seconds).

95% of the original gel strength recovered after 600 seconds. The rapid recovery property would be beneficial for the incorporation of the cells and proteins into the hydrogels for biomedical applications.

The emission spectra provided useful information about the aromatic interaction between naphthalene groups on **1** in the gels. As shown in the ESI†,[13] solution of **2** exhibited two distinctive peaks at 326 and 335 nm, which corresponded to the ^1L_b transition of naphthalene.[14] And there was only one broad peak center at 343 nm being observed in gels formed by TCEP. Though the position of the peak didn't change, the intensity of the peak increased by time in the first 30 minutes after the addition of TCEP, which suggested the formation of more and more efficient aromatic interactions between naphthalene groups. The tiny peaks at 428 nm from the excimer of naphthalene groups suggested that most of naphthalene groups were separated by other groups, which was consistent with the information obtained from the crystal structure of Nap-GFF.[15]

The faster gelation could be achieved by changing the PBS buffer solution to the Dulbecco's Modified Eagle's Medium (DMEM) solution—only 3, 3, and 20 minutes were needed for gelation of DMEM solutions containing 0.4 wt% of **2** treated with 1.0 equiv. of TCEP, 1.0 equiv. of DTT, and 2.0 equiv. of GSH, respectively, probably due to the existence of other reductants such as cysteine in the DMEM solutions and the more complex components in DMEM solutions. For the gel formed by 1 equiv. of TCEP in DMEM solution, compound **1** generated from **2** gradually converted to its dimer and back to its precursor (**2**)—there were 88.6% mol of dimer of **1** and 9.8% mol of **2** in the gel at day 7. The mechanical strength and the recovery property of the resulting gel formed by TCEP in DMEM solutions were similar to those in PBS buffer solutions.[13] These observations suggested that gels of **1** formed by the disulfide bond reduction might be suitable for 3D cell culture and tissue engineering. Increasing the amount of TCEP from 1 equiv. to 2 equiv. led to a gel with more than 99% mol of **1** even after being kept at atmosphere for 7 days probably due to the presence of the excess amount of TCEP.[13] Replacing the 1 equiv. of TCEP with 2 equiv. of GSH in the DMEM solution resulted in the partial reduction of the disulfide bond of **2** and a rapid oxidation of **1**—the gel formed by GSH at 3 minutes consisted of 27.6% mol of **2**, 27.5% mol of **1**, and 44.9% mol of dimer of **1** and then the constituents of the gel gradually changed to 17.1% mol of **2**, 0.3% mol of **1**, and 82.6% mol of dimer of **1** at day 7, which also suggested that GSH was a less efficient reductant than TCEP and DTT. These observations suggested that the percentage of the components in the gels might be manipulated by using different amounts of the reductants and less than 1 equiv. of TCEP/DTT or 2 equiv. of GSH should be used to keep the precursor of the gelator (**2**) bearing the bioactive peptide (RGD) remained in the resulting gels.

In summary, we reported on the first example of using the disulfide bond as a cleavable linker to control molecular self-assembly and the formation of hydrogels, which could be performed smoothly in different kinds of buffer solutions. Unlike Nilsson's design that needs elaborate molecular design and only works in un-buffer solutions,[10] our strategy can be

applied to many reported gelators bearing carboxylic acid groups; bioactive hydrogels can also be obtained by firstly integrating bioactive hydrophilic molecules with the gelators and then partially reducing the disulfide bond. Since the GSH is an endogenous intracellular antioxidant, using GSH to trigger the self-assembly of small molecules could be developed into a biocompatible methodology for the formation of SM hydrogels for biomedical applications. The strategy reported here not only provided a simple and versatile method to form SM hydrogels in a homogeneous way that would be useful for tissue engineering and controlled drug release, but also offered a possible method to control the self-assembly of small molecules within specific cells because the hydrophilic part on **2** could be replaced with a cell-penetration peptide, the nucleus localization peptide, or a peptide that could selectively target to a specific cell line. We believed that the strategy described in this study would offer imaginative opportunities for the exploration of applications of SM self-assembled systems in tissue engineering, regenerative medicine, and cancer therapy.

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