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LETTER

A releasable disulfide carbonate linker for molecular hydrogelations†

Qicai Liu,[a] Caiwen Ou,[a] Chunhua Ren,[b] Ling Wang,[b] Zhimou Yang*[d] and Minsheng Chen*[ac]

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We used a releasable disulfide carbonate linker to construct precursors of gelators and form stable gels.

Molecular hydrogels, formed by the self-assembly of small molecules *via* non-covalent interactions, hold much potential in the fields of 3D cell encapsulation,[1] drug delivery,[2] *etc*. During the formation of molecular hydrogels, molecular gelators are usually in soluble form initially, and then an external stimulus will trigger their self-assembly into 3D networks and hydrogels. These stimuli can decrease the solubility of gelators, trigger their folding and self-assembly, or produce self-assembled molecules from precursors. For example, clear solutions of gelators can be obtained by pH adjustment or heating, and hydrogels will form after changing back the pH values or cooling back to room temperature, respectively.[3] However, for applications involving cells, thermo-sensitive drug molecules and biomacromolecules, biocompatible methods to prepare molecular hydrogels are preferred because of their minimum invasiveness to cells and biopolymers.

Actually, the development of biocompatible methods for hydrogelations keeps attracting research interest and many of them have been reported in the last two decades. The method of ionic strength change can eliminate the charges and decrease the solubility of gelators, which has been widely applied for preparation of peptide-based hydrogels for tissue engineering and the delivery of biomacromolecules;[4] light irradiation can induce *cis–trans* conformation changes or remove capping groups from pre-gelators, resulting in molecular self-assembly and hydrogelations;[5] enzymatic triggers will generate molecular hydrogelators by covalent bond hydrolysis or formation in mild and physiological conditions;[6] self-hydrolysis or enzymatic hydrolysis of some chemicals can alter pH values of solutions and lead to homogeneous hydrogels;[7] specific metal ion–peptide interactions and protein–peptide interactions have also been used to cross-link self-assembled nanofibers, resulting in hydrogelations in mild conditions.[8] These above methods have been demonstrated as powerful methodologies to prepare molecular hydrogels for regenerative medicine.

Recently, the strategy of disulfide bond reduction by reductants such as glutathione (GSH), dithiothreitol (DTT), and *tris*-(2-carboxyethyl)phosphine (TCEP) was reported by the Nilsson group firstly and then our group.[9] The reduction of disulfide bonds can release the conformation strain of a cyclic peptide or produce molecular hydrogelators by cleaving excrescent hydrophilic parts from precursors. This novel method will guarantee the formation of homogeneous gels and the use of biocompatible GSH might cause minimum harm to cells and some biomacromolecules. However, there is at least one thiol group on the molecular gelators formed by disulfide bond reductions. The free thiol groups are reactive and will form disulfide bonds between molecular hydrogelators. Our previous study showed that the gels formed by disulfide bond reduction were unstable and lots of precipitates were observed after being kept at room temperature for 7 days due to the formation of more hydrophobic dimers of gelators. In order to prepare stable molecular hydrogels by disulfide bond reduction, the free thiol group needs to be converted to other groups. In this study, we attempt to use a self-cyclization strategy to convert the thiol group to a hydroxyl group, which leads to the formation of stable molecular hydrogels.

The design of a releasable linker to connect a molecular hydrogelator and a hydrophilic part was based on the results reported by Low and co-workers.[10] They have used the releasable disulfide carbonate linker to connect a camptothecin and the folic acid. Upon endosomal disulfide reduction, the unmodified drug of camptothecin could be released *via* a self-cyclization process. Stimulated by his results, we designed **3** with similar releasable disulfide carbonate linker that could be directly used for solid phase peptide synthesis (SPPS). The synthetic pathway of **3** was described in Scheme S-1 (see ESI†). We firstly used ethanolamine to react with Fmoc-OSu to get **1** in a yield of 60%. We then added triphosgen to the solution of **1** to afford an acid chloride intermediate, which was used directly to react with 2-hydroxyethyl disulfide to get **2** in a yield of 70%. The reaction between **2** and disuccinimidyl carbonate (DSC) afforded **3** that could be directly used for

[a] *Guangzhou Medical University, Guangzhou 510182, P. R. China. E-mail: gzminsheng@vip.163.com*
[b] *College of Pharmacy and Tianjin Key Laboratory of Medicine, Nankai University, Tianjin 300071, P. R. China*
[c] *Southern Medical University, Guangzhou 510515, P. R. China*
[d] *State Key Laboratory of Medicinal Chemical Biology and College of Life Sciences, Nankai University, Tianjin 300071, P. R. China. E-mail: yangzm@nankai.edu.cn*

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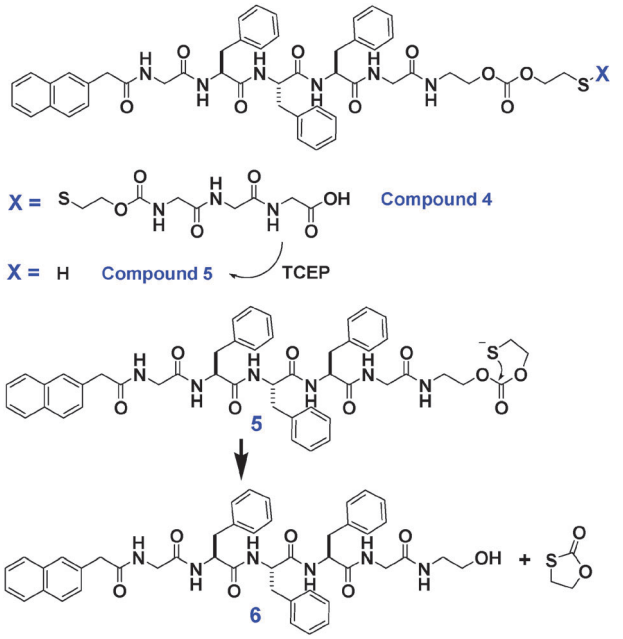

Scheme 1 Disulfide bond reduction leads to the formation of **5** that will undergo cyclization to form **6**.

SPPS to synthesize peptides with the releasable disulfide carbonate linker.

As shown in Scheme 1, we used **3** to synthesize **4** as a precursor of gelators. Compound **4** has several features: (1) Nap-GFFF could assist molecular hydrogelations, which had been demonstrated by us;[11] (2) tripeptide of Gly–Gly–Gly (GGG) was hydrophilic and could increase the solubility of **4**; (3) the cleavable disulfide carbonate linker could be cleaved by reductants to produce **5** following a self-cyclization process to generate **6**. The Xu group has demonstrated that hydrogels formed by a molecular hydrogelator terminating in a hydroxyl group were anistropic and highly stable in aqueous solutions at all pH values.[12] Therefore, we opted to evaluate the possibility of our design in Scheme 1 to produce stable molecular hydrogels of gelators terminating in a hydroxyl group but not the reactive thiol group.

After the synthesis of **4** by SPPS and purification by reverse phase HPLC, we first tested its gelation ability by disulfide bond reduction. Compound **4** could form clear solutions in unbuffered solutions at concentrations lower than 1.2 wt%. Upon the addition of 1 equiv. of TCEP to a clear solution containing 0.4 wt% of **4**, we observed the formation of a gel at about 18 min time point (determined by the invert-tube method, Fig. 1, insert). This phenomenon indicated that **4** could be converted to molecular hydrogelators by disulfide bond reduction. The LC-MS results indicated that the gel at the gelling point contained about 54 mol% of **5** and 46 mol% of **4**. And there were only trace amounts of the dimer of **5** and **6** in the gel. For the gel at the 0.5 h time point, there was nearly total conversion of **4** to **5** and only small amounts of the dimer of **5** and **6**. In atmosphere, compound **5** would gradually change to **6** *via* self-cyclization as we designed and be oxidized to a dimer of **5** through disulfide bond formation. In a balanced gel after 120 h, there was about 76 mol% of **6** and 23 mol% of dimer of **5**. Though we did not observe a total

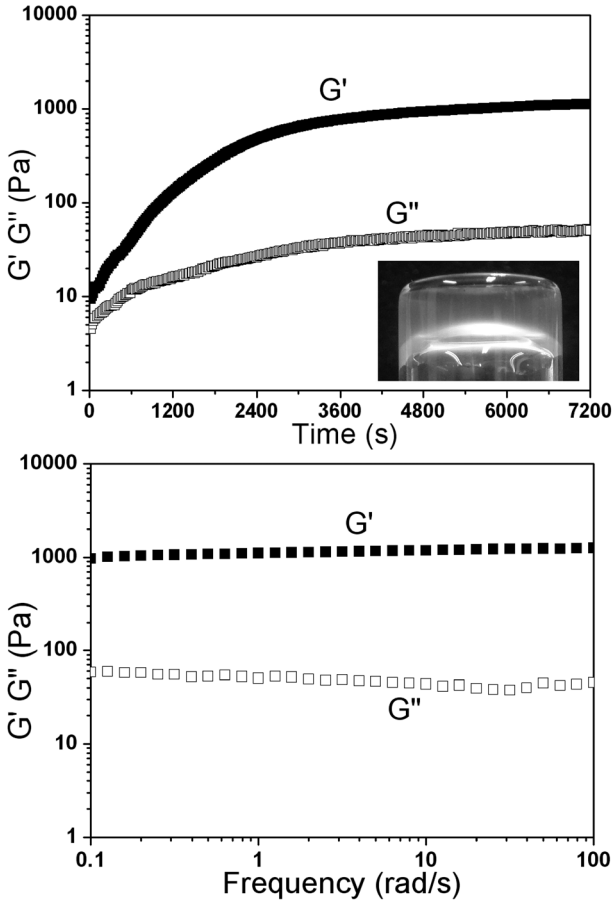

Fig. 1 Rheological measurements in the dynamic time sweep mode (top) for the solution containing 0.4 wt% of **4** with 1 equiv. of TCEP a strain of 1% and a frequency of 1 rad s^{-1} and in the dynamic frequency sweep mode (down) for the gel at 2 h time point at the strain of 1%.

conversion from **5** to **6**, the gel was stable for at least 3 months in the atmosphere, which was much more stable than the gel of the gelator with a free thiol group we reported before (the gelator with a free thiol group would form dimers of it, resulting in unstable gels with large amounts of precipitates after about one week). These results indicated that the use of cleavable disulfide carbonate linker could lead to stable hydrogels. Similar to the results published by the Xu group,[12] compound **6** itself could not form gels by the heating–cooling process due to its limited water solubility, which indicated that the hydrolysis process was crucial for the gel formation.

We then characterized the gelation process and the mechanical properties of the resulting gels by rheology. As shown in Fig. 1, the dynamic time sweep indicated that a gel formed rapidly after the addition of 1 equiv. of TCEP to a solution of **4**. Both values of G′ (storage modulus and elasticity) and G″ (loss modulus and viscosity) increased rapidly in the first hour (3600 s) due to the fast conversion of **4** to **5** (Table 1). The increase of both values of G′ and G″ was relatively slow after one hour compared with that in the first hour probably because of the slow dimerization process of **5**. Both G′ and G″ of the gel at 2 h time point exhibited weak frequency dependence in the range 0.1 to 100 rad s^{-1}. And the value of G′ was more than ten times bigger than that of G″.

Table 1 The ratio of different compounds in the gel formed by 1 equiv. of TCEP at different time points

Time (h)	**5**	**4**	Dimer of **5**	**6**
Gelling point (~18 min)[a]	>53.8%	46.0%	<0.1%	<0.1%
0.5	>98.5%	<0.5%	<0.5%	<0.5%
1	>65.6%	<0.5%	4.4%	30.5%
2	>57.2%	<0.5%	8.2%	34.1%
6	>49.4%	<0.5%	12.9%	38.2%
12	38.3%	<0.5%	15.7%	>45.5%
24	24.4%	<0.5%	17.1%	>58.0%
48	15.9%	<0.5%	20.8%	>62.8%
72	7.7%	<0.5%	22.3%	>70.5%
120	<0.5%	<0.5%	23.0%	>76.0%
168	<0.5%	<0.5%	22.6%	>76.4%

[a] Determined by the inverted tube method.

Both results suggested the formation of a true gel and a highly elastic network in the gel.

Negative-stained transmission electron microscopy (TEM) images of hydrogels were obtained to characterize the self-assembled structures in the hydrogel at the 2 h time point. As shown in Fig. 2A, we observed networks of nanofibers in the hydrogel. The diameter of nanofibers was very uniform and about 20 nm. They were longer than 1 μm and entangled with each other to form the three dimensional network for hydrogels formation. We also collected the emission spectra of the solution of **4** and gels at different time scales. As shown in Fig. 2B, the solution of **4** exhibited a broad peak from 315 to near 400 nm, indicating partial aggregation of **4** in the solution phase. After the addition of TCEP, there were new peaks at about 355 nm and shoulder peaks above 400 nm, indicating efficient aromatic stacking of naphthalene groups in gels.

In summary, we have attempted to use a reseasable disulfide carbonate linker to construct a precursor of a gelator and tried to form stable molecular hydrogels. Though we were unable to totally convert the gelator terminating in a free thiol group to the one terminating in a hydroxyl group, we succeeded in the formation of stable molecular hydrogels mainly formed by the gelator terminating in a hydroxyl group. Our study provided useful information on using a cleavable linker for molecular hydrogelations and might offer a useful and biocompatible method to form hydrogels for tissue engineering, drug delivery, *etc*. One shortcoming of the cleavable disulfide carbonate linker is its sensitivity to acid, which means that the carbonate linker is not very stable when treated with 95% TFA. This property hinders its wide application for solid phase peptide synthesis. Other alternative cleavable linkers should be developed and used for hydrogel formation.

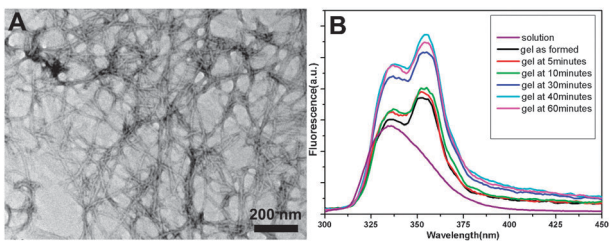

Fig. 2 (A) A negative-stained TEM image of the gel and (B) emission spectra of solution of **4** and gels at different time scales formed by 1 equiv. of TCEP.

Experimental

General methods

The synthesized compounds were characterized using ^{1}H NMR (Bruker ARX 300) using DMSO-d_6 as the solvent and ESI-MS spectrometric analyses were performed at the Thermo Finnigan LCQ AD System. HPLC was conducted on a LUMTECH HPLC (Germany) system using a C_{18} RP column with methanol (1% TFA) and water (1% TFA) as the eluents. TEM samples were prepared as follows: 10 μL of gel was added to a copper grid coated with a thin layer of carbon for 5 s, and then the gel was removed by a filter paper. The sample was washed with 20 μL of PBS twice and then stained with uranium acetate solution (half saturated solution) for 5 s. The dried sample, after being kept in a desiccator for about half an hour, was tested on a Tecnai G2 F20 system, operating at 200 kV. LC-MS was conducted on a LCMS-2020 (Shimadzu) system.

Characterization of compound 4

^{1}H NMR (300 MHz, DMSO-d_6) δ 7.777–7.854 (m, 4H), 7.721 (s, 1H), 7.407–7.481 (m, 4H), 7.226–7.245 (t, 4H), 7.114–7.187 (m, 9H), 4.463–4.595 (m, 5H), 4.151–4.192 (t, 4H), 4.020–4.057 (t, 2H), 3.843–3.865 (m, 2H), 3.717–3.754 (m, 6H), 3.569–3.642 (m, 5H), 3.156–3.240 (m, 3H), 2.840–2.943 (m, 6H) (the signals above 8.0 ppm were from NH groups). HR-MS: calc. M^+ = 1179.4, obsvd. $(M + NH_4)^+$ = 1197.4372.

Preparation of the hydrogel (0.4 wt%)

2 mg of compound 4 was dissolved in 0.47 mL of pure water, and then 17.0 μL of Na_2CO_3 solution (0.1 mmol mL^{-1}) was added to adjust the pH to 7.4. 1.0 equiv. of *tris*(2-carboxyethyl)phosphine (TCEP) in 13 μL of aqueous solution (pH = 7.4, adjusted by Na_2CO_3) was then added to the above solution. A gel would form after the solution being kept at room temperature (22–25 °C) within 20 min. Similar observations could be obtained when using 1 equiv. of dithiothreitol (DTT) or 2 equiv. of glutathione (GSH) instead of 1 equiv. of TCEP.

Rheological measurements

All rheological experiments were performed at 25 °C ± 0.1 °C in different modes (dynamic time sweep and dynamic frequency sweep) using an AR 1500ex rheometer (TA company, America) with 40 mm parallel plates. To minimize evaporation, a solvent trap was employed and a low viscosity mineral oil was applied around the sample. Time sweep experiment data were collected at 1.0 rad s^{-1} frequency and 1.0% strain. Dynamic frequency sweep experiments were performed in the range of 0.1–100 rad s^{-1} at 1.0% strain.

Determination of the ratio of the compounds in gels

At different time points, the gel was dissolved in DMSO and the solution was used to run LC-MS to determine the ratio of compounds in gels. The areas of peaks at 272 nm were used to determine the molar ratio of compounds containing naphthalene groups. The experiment was repeated 3 times.

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