

## A Method to Accelerate the Gelation of Disulfide-crosslinked Hydrogels<sup>\*</sup>

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**Abstract** A glutathione-disulfide-ended poly(ethylene glycol) (GSSG-PEG-GSSG) was designed. It is a much more efficient accelerator than glutathione disulfide (GSSG) for the gelation of an 8arm-PEG-SH polymer solution, and the gelation time can be tuned from hours to minutes at the physiological pH and temperature. A mechanism was proposed to explain the different behaviors of the GSSG and GSSG-PEG-GSSG gelation systems. Due to the ever-going thiol-disulfide exchange reaction, the thiol-disulfide hydrogels also showed interesting swelling behavior.

**Keywords:** Thiol-disulfide exchange; *In situ* gelation; Glutathione; PEG.

### INTRODUCTION

Hydrogels have been widely used for biomedical applications including drug delivery<sup>[1–3]</sup> and tissue engineering<sup>[4–6]</sup>, due to their tissue-like water content and easily tunable properties. Among all types of hydrogels, *in situ* forming hydrogels are injected through a needle in the solution state and gel inside the body *via* physical association and/or chemical reactions<sup>[7]</sup>. The *in situ* gelation avoids the invasive surgical implantation required for the pre-formed hydrogels and is highly desirable for many practical applications<sup>[8]</sup>.

Hydrogels crosslinked by physical interactions possess intrinsic responsiveness to environmental stimuli, because these crosslinking interactions, such as hydrophobic association, ionic bonding and host-guest complexation, are reversible. However, the downside of these physical hydrogels is the lack of stability and prone to premature erosion or dissolution<sup>[9]</sup>. In contrast, hydrogels can also be crosslinked by covalent bonds formed by many *in situ* coupling chemical reactions, such as the Michael addition, hydrazone condensation, oxime formation, alkyne azide 1, 3-dipolar Huisgen cycloaddition and Diels-Alder cycloaddition<sup>[7, 10–13]</sup>. Compared to the physical hydrogels, most of these covalently crosslinked hydrogels are more stable and better controllable, but the network structure is often 'fixed' once the crosslinking reaction is complete. To combine the advantages of both physical and chemical hydrogels, dynamical covalent linkages such as disulfide bonds, which are capable of undergoing the reversible thiol-disulfide exchange with endogenous and/or exogenous thiol compounds, have emerged as promising candidates to crosslink *in situ* hydrogels.

While disulfide-crosslinked hydrogels have unique properties and potential in biomedical applications, problems remain in its slow gelation rate (especially for those with low thiol concentrations), unless strong and cytotoxic oxidants such as hydrogen peroxide are added. One way to form disulfide crosslinks *in situ* is to oxidize free thiol groups of the precursor polymers by the oxygen dissolved in solution, but this reaction is

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usually slow and the gelation time can be longer than 10 h<sup>[14]</sup>. This slow gelation impedes the practical use, as the polymer components may diffuse away from the injection site before gelation ever occurs. In recent studies, gelation was accelerated by adding disulfide compounds to the thiol-polymer precursor solution, such as cystamine, 2-hydroxyethyl disulfide, 3,3'-dithiodipropionic acid and glutathione disulfide. These disulfide compounds can facilitate disulfide crosslink formation through thiol-disulfide exchange<sup>[14, 15]</sup>. However, there is still a limit to the shortest gelation time achievable with these small molecular disulfide compounds, and the gelation even becomes slower with excessive disulfide compounds<sup>[16]</sup>.

To overcome the problems with the small molecular disulfide compounds, we here developed a polymer with disulfide groups at both ends, specifically a poly (ethylene glycol) chain with two glutathione disulfides (GSSG) as its end groups (GSSG-PEG-GSSG). Then, we used 8-arm poly(ethylene glycol) with free thiol end groups (8arm-PEG-SH) as the thiol-containing polymer and demonstrated its gelation can be tuned from hours to minutes with the addition of the GSSG-PEG-GSSG polymers (Fig. 1). In addition to the tunable gelation rate, the system uses two types of biocompatible PEG polymers, results in a disulfide-crosslinked *in situ* PEG hydrogel, and releases the endogenously-abundant glutathione (GSH) as the by-product, all of which make it interesting for biomedical applications.

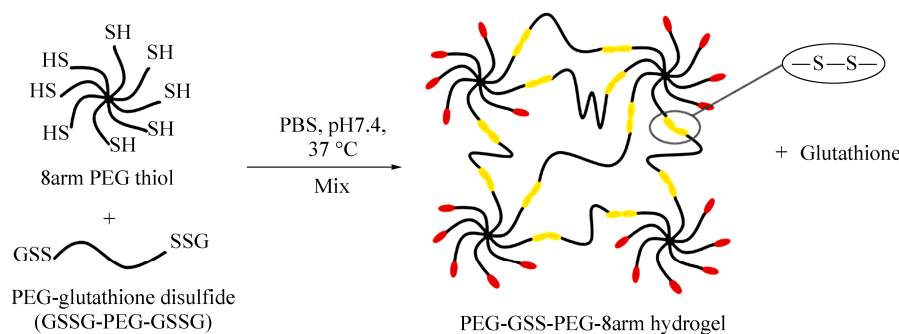


**Fig. 1** Scheme of hydrogels formed by the 8arm-PEG-SH and GSSG-PEG-GSSG polymers

## EXPERIMENTAL

### Materials

Polyethylene glycol (PEG2K, MW 2000 Da), triethylamine (TEA) and calcium hydride ( $\text{CaH}_2$ , 90%–95%) were purchased from Alfa Aesar. *p*-Nitrophenyl chloroformate (PNC) was purchased from J&K Chemical. Glutathione disulfide (GSSG) was purchased from Amresco. Toluene, dichloromethane (DCM), diethyl ether, tetrahydrofuran (THF), sodium hydroxide (NaOH) and hydrochloric acid (HCl, 36.5%) were purchased from Beijing Chemical Company. 8arm PEG thiol (8arm-PEG-SH, MW 20 kDa) was purchased from JenKem Technology. DCM was distilled prior to use and PEG2K was dried by azeotropic distillation with toluene. Other chemicals were used as received.

### Synthesis of PEG-*p*-Nitrophenyl Carbonate (PNC-PEG-PNC)<sup>[17]</sup> (Fig. 2)

PEG2K (1.0 g, 0.5 mmol) was dissolved in 20 mL dichloromethane, and then *p*-nitrophenyl chloroformate (PNC, 0.6 g, 3.0 mmol) was added to the solution with magnetic stirring. Triethylamine (TEA, 0.28 mL, 2.0 mmol) was added dropwise into the solution, and then the mixture was stirred for 36 h at room temperature. After the reaction, most of DCM was removed with rotary evaporator and then THF (5 mL) was added to precipitate the unreacted PNC and TEA salts. After removal of the insoluble solid by filtration, the clear solution was added dropwise into ice-cold ethyl ether to precipitate out the product. White solid powder was obtained and washed three times with 500 mL ice-cold ethyl ether and dried in vacuum (yield about 80%).

### Synthesis of PEG-glutathione Disulfide (GSSG-PEG-GSSG)<sup>[18]</sup> (Fig. 2)

Glutathione disulfide (GSSG, 1.8 g, 2.9 mmol) was dissolved in deionized water (25 mL) and NaOH powder was added to adjust the pH of the solution to around 8.30. PNC-PEG-PNC (1 g, 0.38 mmol) was added into the GSSG solution, and the solution turned yellow. NaOH powder was repeatedly added to the mixture to keep the pH around 8.30 in the next 2 h. Afterwards, the mixture was further stirred for 36 h at room temperature. The mixture was purified in a dialysis tube with cut-off molecular weight of 2000 Da for two days in deionized water to remove unreacted small molecules. After the dialysis, the product was collected by lyophilization as white powder with a yield of 80% and was characterized by <sup>1</sup>H-NMR and <sup>13</sup>C-NMR in D<sub>2</sub>O (300 MHz JOEL spectrometer, Fig. 3). <sup>1</sup>H-NMR (301 MHz, D<sub>2</sub>O)  $\delta$ : 4.79 (overlapped with solvent), 4.23 (m, 3.6H), 3.50–4.00 (m, 212H),  $\delta$  3.30 (m, 3.6H), 2.97 (m, 3.6H), 2.53 (m, 3.6H), 2.44 (m, 3.6H), 2.18 (m, 5.4H), 1.94 (m, 1.8H). <sup>13</sup>C-NMR (76 MHz, D<sub>2</sub>O)  $\delta$ : 178.94, 176.26, 175.82, 174.94, 173.96, 171.89, 157.69, 69.65, 64.20, 55.76, 54.07, 52.64, 52.55, 43.48, 38.67, 32.10, 31.34, 27.75, 26.23.

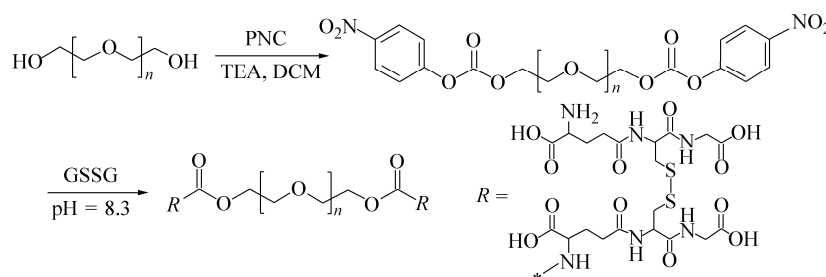


**Fig. 2** Scheme of synthesis of PEG-glutathione disulfide (GSSG-PEG-GSSG)

### Gelation Time Determination

All hydrogels were prepared at pH 7.4 and 37 °C. The concentration of 8arm-PEG-SH was kept constant at 10 wt% unless otherwise specified, and the concentrations of the disulfide components (GSSG and GSSG-PEG-GSSG) were varied. Gelation time was determined by the vial-inversion method, and recorded as the moment when the sample could hold its own weight<sup>[19]</sup>. As an example, formation of a hydrogel (8arm-PEG-SH & GSSG, SS/SH group molar ratio = 0.5) was described as follows. PBS (phosphate buffer solution, 137.0 mmol/L NaCl, 2.7 mmol/L KCl, 10.0 mmol/L phosphate buffer, pH = 7.4) was prepared to simulate the physiological environment. 8Arm-PEG-SH (100.0 mg,  $5.0 \times 10^{-3}$  mmol) was dissolved in PBS (888.0  $\mu$ L) with sonication and NaOH solution (0.1 mol/L) was used to adjust the pH to 7.4. Next, GSSG (12.2 mg,  $2.0 \times 10^{-2}$  mmol) was added into the solution and followed by sonication for 10 s. After pH was again adjusted to 7.4 using NaOH solution (0.1 mol/L), the mixture solution was transferred into a small glass vial and kept in incubator at 37 °C.

### In situ <sup>1</sup>H-NMR Monitoring

*In situ* <sup>1</sup>H-NMR monitoring was carried out to study the mechanism of the gelation. 8arm-PEG-SH was kept at 10 wt% in D<sub>2</sub>O solutions, and pD was adjusted to 7.4 by using sodium deuterium oxide (40 wt%) solution, and reaction was conducted at room temperature. As an example, the detail of a system (8arm-PEG-SH & GSSG, SS/SH ratio = 0.5) was described as follows: 8arm-PEG-SH (100.0 mg,  $5.0 \times 10^{-3}$  mmol) was dissolved in 500.0  $\mu$ L D<sub>2</sub>O, and the pD of solution was adjusted to 7.4 with sodium deuterium oxide (40 wt%). Afterwards, GSSG (12.2 mg,  $2.0 \times 10^{-2}$  mmol) was dissolved in 400.0  $\mu$ L D<sub>2</sub>O and the pD of solution was also adjusted to 7.4 in the same way mentioned above. The two solutions were mixed, adjusted to 1.0 mL with D<sub>2</sub>O, and added into an NMR tube for <sup>1</sup>H-NMR characterization. The initial concentrations of GSSG and 8arm-PEG-SH, designated as [GSSG]<sub>0</sub> and [8arm-PEG-SH]<sub>0</sub>, were 20.0 mmol/L and 5.0 mmol/L, respectively. Therefore, the thiol concentration was  $5.0 \times 8 = 40$  mmol/L, and the SS/SH ratio was 0.5. NMR spectrum was measured in each predetermined time point for two days.

### Swelling Studies

The hydrogels were prepared as described above and incubated for 36 h at 37 °C to allow complete gelation of all samples before swelling experiments. PBS (phosphate buffer solution, 137.0 mmol/L NaCl, 2.7 mmol/L KCl, 10.0 mmol/L phosphate buffer, pH = 7.4) was used as the swelling media. First, the weight of prepared hydrogels was measured as  $W_0$ . Next the individual hydrogel samples were immersed in 6 mL swelling media in the glass vials. Weight of hydrogels ( $W_t$ ) was recorded at predetermined time points (time interval was 1 to 3 h). Swelling ratio was calculated as  $Q = W_t/W_0$ . Experiments were carried out in duplicate and the average value was reported.

## RESULTS AND DISCUSSION

### Characterization of GSSG-PEG-GSSG

PEG-glutathione disulfide (GSSG-PEG-GSSG) was obtained as white powder and the structure was confirmed by  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR in  $\text{D}_2\text{O}$  (Fig. 3). The conversion of PEG end groups was more than 90%, calculated from integrals of peak Hg and the PEG backbone proton peak. In the  $^{13}\text{C}$ -NMR spectrum, the absence of two peaks at  $\delta = 71.78$  and  $60.40$ , which correspond to the two methylene carbons next to the end hydroxyl groups, indicated the complete conversion of PEG end groups.

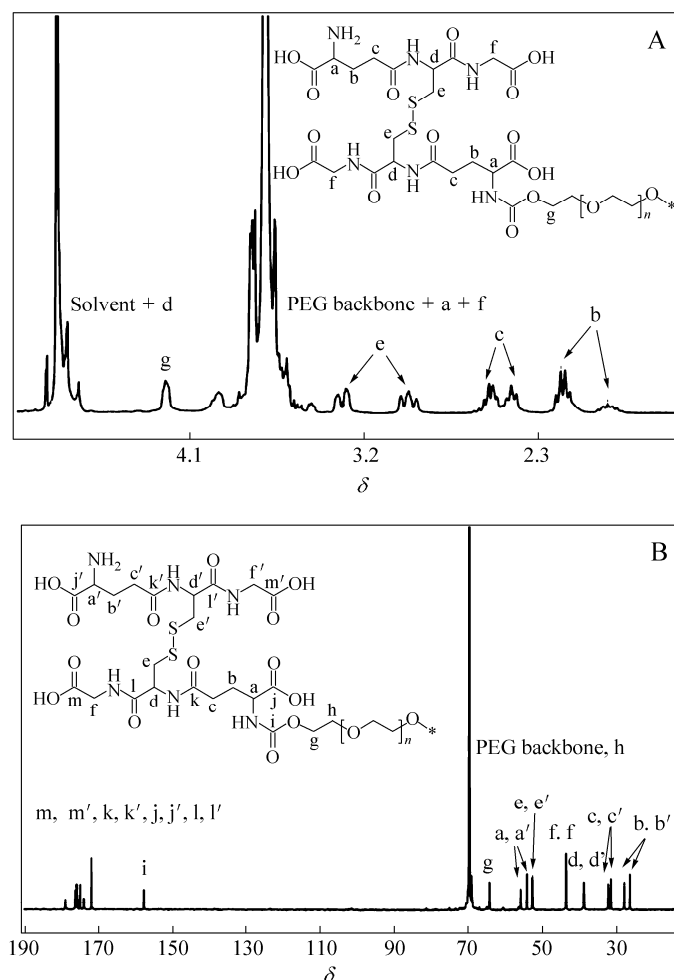


**Fig. 3**  $^1\text{H}$ -NMR (A) and  $^{13}\text{C}$ -NMR (B) spectra of GSSG-PEG-GSSG ( $\text{D}_2\text{O}$ , 300 MHz)

### Gelation Time

Appropriate gelation time is essential for *in situ* hydrogels in clinical use. Usually the time window between a few minutes to an hour is favorable, which allows enough time for handling solution but avoids incomplete gelation *in vivo*<sup>[7, 11, 20]</sup>. We first studied how gelation time of 8arm-PEG-SH was affected by the ratio of disulfide to free thiol groups (SS/SH). We kept the concentration of 8arm-PEG-SH constant but changed the amount of disulfide compounds, including glutathione disulfide (GSSG) and GSSG-ended PEG (GSSG-PEG-GSSG), to change the SS/SH ratio. As a control group, auto oxidation of 8arm-PEG-SH without additional disulfide compounds (SS/SH = 0) was also studied.

At pH 7.4 and 37 °C, gelation of 10 wt% 8arm-PEG-SH solution by auto oxidation took about 16 h (Fig. 4). The addition of GSSG and GSSG-PEG-GSSG speeded up the gelation in a dose dependent manner. When the amount of GSSG was increased, the gelation time decreased initially but reached minimum of 4 h at the SS/SH ratio of 0.25. Further increasing the GSSG amount slowed down the gelation. Similar phenomena were observed in the literature<sup>[16]</sup>. In contrast, increasing the amount of GSSG-PEG-GSSG decreased the gelation time monotonically. The gelation occurred within 20 min when the SS/SH ratio was 0.5, and the gelation time was further reduced to a few minutes when more GSSG-PEG-GSSG was added.

To test whether or not the faster gelation was due to the increase in total polymer concentration in the GSSG-PEG-GSSG group, we did two additional experiments in which the total concentration of polymer, *i.e.* [8arm-PEG-SH + GSSG-PEG-GSSG] was kept as 10 wt% (Fig. 4, filled circles). It was found that the gelation time of GSSG-PEG-GSSG groups was still dramatically shorter than that of GSSG groups at the same SS/SH ratios, indicating that the increase in total polymer concentration was not the major reason for the dramatic decrease in gelation time.

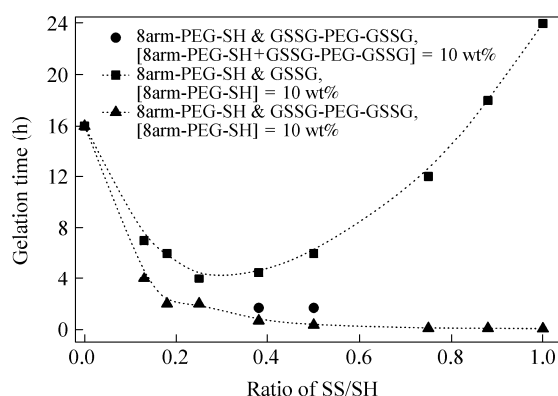


Fig. 4 Dependence of gelation time on SS/SH ratio of three types of hydrogels (pH = 7.4, temperature = 37 °C)

### Gelation Mechanism of Hydrogel Formed by 8arm-PEG-SH and GSSG

GSSG accelerates disulfide crosslinking through a spontaneous thiol-disulfide exchange reaction<sup>[21]</sup>, which is in addition to the oxygen oxidation process. This thiol-disulfide exchange can be described as equations below.



Initially, increasing the amount of GSSG speeded up the formation of disulfide bonds and resulted in shorter gelation time. However, further increasing the amount of GSSG didn't lead to faster gelation, but instead decreased the gelation rate. The slower rate at higher GSSG concentrations is likely due to the fact that most 8arm-PEG-SH was converted into 8arm-PEG-SSG as in reaction A and resulted in insufficient amount of 8arm-PEG-SH to undergo reaction B, which is actually the reaction to create valid crosslinking points for gelation.

To support this hypothesis, we monitored the amount of various species in solution over time by *in situ*

$^1\text{H-NMR}$ . To differentiate glutathione in its reduced form (GSH) from its oxidized form (GSSG), we compared their  $^1\text{H-NMR}$  spectra and identified that peaks with chemical shift at 4.58 (Hd), 3.27 (He') and 2.95 (He) could be used as indicators for the two states of glutathione species (Figs. 5A and 5B). Peak of Hd at  $\delta = 4.58$  and peak clusters of He at  $\delta = 2.95$  only belonged to GSH (Fig. 5A). In contrast, peaks of Hb and Hc did not change between the two states of glutathione, and hence could be used as references of total amount of GSH and GSSG. Similarly, the peak with chemical shift at  $\delta = 2.73$  (Hh, Fig. 5C) represents the methylene proton next to the end free thiol groups of 8arm-PEG-SH, and it shifts to  $\delta = 2.96$  (Hi, Fig. 5D) after disulfide formation, which overlaps with peak of He in glutathione.

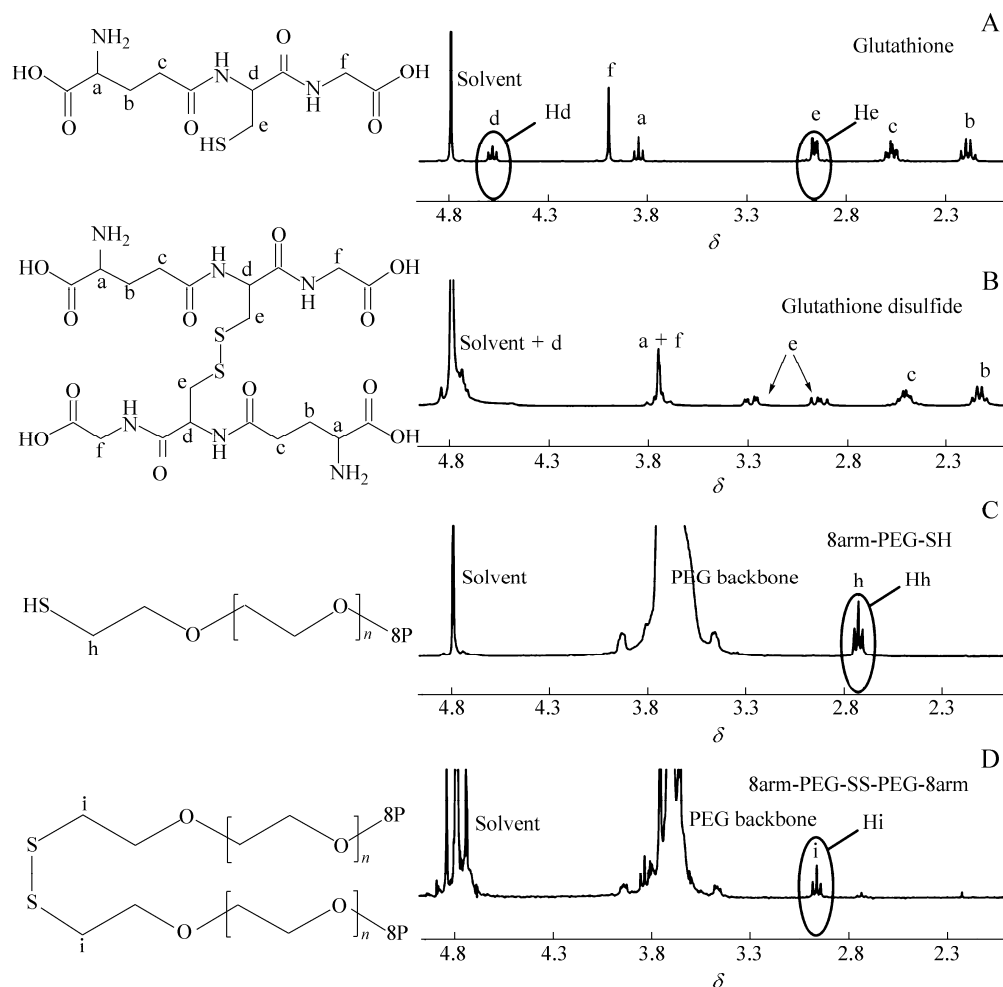


**Fig. 5** Comparison of  $^1\text{H-NMR}$  spectra of glutathione (A), glutathione disulfide (B), 8arm-PEG-SH (C) and 8arm-PEG-SS-PEG-8arm (D)  $\text{D}_2\text{O}$ , 300 MHz  
The sample of 8arm-PEG-SS-PEG-8arm was obtained by auto-oxidation in  $\text{D}_2\text{O}$  for 2 weeks at  $\text{pD} = 7.4$ .

Figure 6 shows the  $^1\text{H-NMR}$  spectrum of the solution right after mixing 8arm-PEG-SH and GSSG ( $\text{SS}/\text{SH} = 0.5$ ). As indicated by the peak at  $\delta = 4.55$ , significant amount of GSH was already generated at the very beginning. In the meantime, the peak at  $\delta = 2.72$  decreased significantly, indicating 8arm-PEG-SH was largely consumed. However, the system is still far away from its gelation, which means that there is little valid crosslink species 8arm-PEG-SS-PEG-8arm existing at this time. Therefore, after mixing the 8arm-PEG-SH and GSSG solutions, reaction A took place very quickly, and new species (GSH, 8arm-PEG-GSSG, and 8arm-PEG-SS-

PEG-8arm in lower concentrations) appeared early in the mixture, which is consistent with the NMR spectra where all species can be identified. It should be noted that, due to peak overlapping, the NMR spectra cannot differentiate between the PEG states of 8arm-PEG-SS-PEG-8arm and 8arm-PEG-SSG, or the glutathione states of GSSG and 8arm-PEG-SSG.

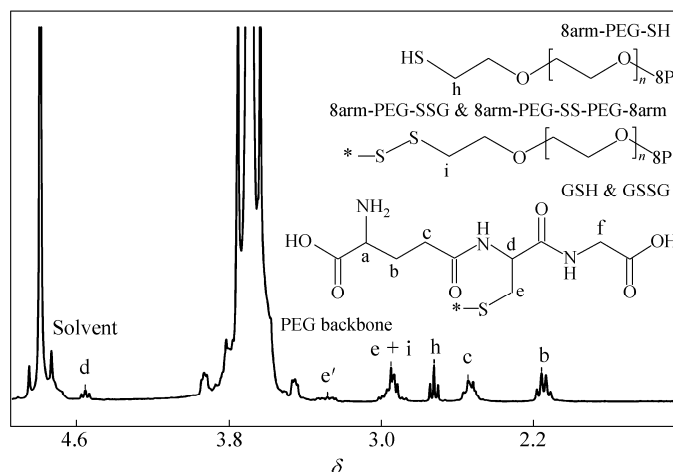


**Fig. 6** *In situ*  $^1\text{H}$ -NMR of the 8arm-PEG-SH and GSSG gelation system in  $\text{D}_2\text{O}$  right after mixing ([8arm-PEG-SH] = 10 wt%, SS/SH ratio = 0.5, 300 MHz)

There was no significant change in the NMR spectra of the mixture in the following two days, within which gelation occurred. This means that the concentrations of 8arm-PEG-SH (indicated by the peak at  $\delta = 2.72$ ) and GSH (indicated by the peak at  $\delta = 4.55$ ) did not change with time, while the concentration changes of 8arm-PEG-SSG and 8arm-PEG-SS-PEG-8arm were not reflected in the spectra due to peak overlapping. The peak at  $\delta = 3.28$  would tell the concentration changes of GSSG, but it was too weak to be reliable. Hisano *et al.* did a detailed kinetic analysis of the multiple thiol-disulfide exchange reactions in a similar system<sup>[21]</sup>. Based on their calculation results and our NMR data, we proposed the concentration-time profiles of all the species in our system as shown in Fig. 7. In the gelation period, the concentrations of the two free thiol species (8arm-PEG-SH and GSH) do not change much, while that of the valid crosslink 8arm-PEG-SS-PEG-8arm increases slowly at the expense of the intermediate species 8arm-PEG-SSG and a slow rebound in the concentration of the GSSG. The total concentrations of thiols and disulfide species remain constant throughout the whole time.

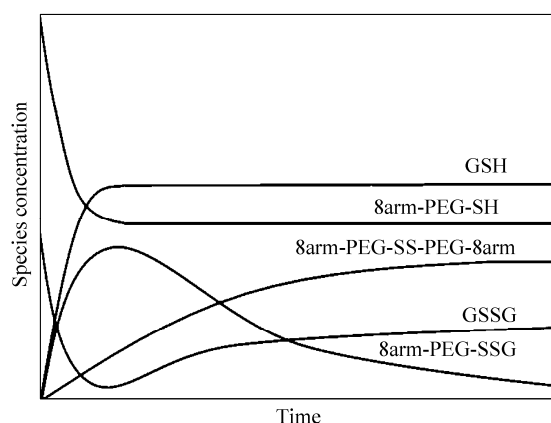


**Fig. 7** The illustration of concentration changes of various species during gelation period in the 8arm-PEG-SH & GSSG systems

As shown in Table 1, increasing the SS/SH ratio from 0.5 to 1.0 decreased the concentration of unreacted 8arm-PEG-SH, which is needed for the crosslinking reaction B, in the mixture from 2.7 mmol/L to 1.5 mmol/L. In the meantime, the concentration of the reducing species GSH was almost doubled, which would act to break down the disulfide crosslinks. These two factors should be the reasons for that the gelation became slower when more GSSG was added in the gelation system.

**Table 1.** Concentration of GSH, unreacted 8arm-PEG-SH and storage modulus of hydrogels in four gelation conditions, calculated by peak areas in  $^1\text{H-NMR}$

|                                                | SS/SH ratio | GSH concentration <sup>a</sup> | Unreacted 8arm-PEG-SH concentration <sup>b</sup> | Storage modulus <sup>c</sup> |
|------------------------------------------------|-------------|--------------------------------|--------------------------------------------------|------------------------------|
| Hydrogel formed by (8arm-PEG-SH & GSSG)        | 0.5         | 28.0 mmol/L                    | 2.7 mmol/L                                       | 2160 Pa                      |
|                                                | 1.0         | 48.0 mmol/L                    | 1.5 mmol/L                                       | 61 Pa                        |
| Hydrogel formed by (8arm-PEG-SH&GSSG-PEG-GSSG) | 0.5         | 28.0 mmol/L                    | 2.5 mmol/L                                       | 4120 Pa                      |
|                                                | 1.0         | 40.8 mmol/L                    | 1.2 mmol/L                                       | 5080 Pa                      |

<sup>a</sup> GSH concentration was defined as  $[\text{GSH}]_t$  and was calculated by equation:  $[\text{GSH}]_t = 2 \times [\text{GSSG}]_0 \times \frac{2 \times \int \text{Hd}}{\int \text{Hb}}$ , the

symbol “ $\int \text{H} \times$ ” means the area integral of peak H  $\times$  in  $^1\text{H-NMR}$  (same below).

<sup>b</sup> Unreacted 8arm-PEG-SH concentration was defined as  $[\text{8arm-PEG-SH}]_t$  and was calculated by equation:

$$[\text{8arm-PEG-SH}]_t = [\text{8arm-PEG-SH}]_0 \times \frac{\int \text{Hh}}{\int \text{Hh} + \int (\text{He} + \text{Hi}) + \int \text{He}' - \int \text{Hb}}$$

<sup>c</sup> Storage modulus of the hydrogels were tested after an incubation of 36 h at 37 °C. Rheology experiments were performed at 37 °C on a rotational rheometer (Anton-Paar MCR 502) using parallel plates (25 mm in diameter). The sample was subjected to an oscillatory strain (amplitude  $\gamma = 1\%$ ) and the storage modulus was monitored at frequency of 1 rad/s.

### **Gelation Mechanism of Hydrogels Formed by 8arm-PEG-SH and GSSG-PEG-GSSG**

With the understanding of the gelation process of the GSSG system, it is easy to understand why the GSSG-PEG-GSSG resulted in much faster gelation when SS/SH ratio is the same. GSSG-PEG-GSSG accelerates disulfide crosslinking through the spontaneous thiol-disulfide exchange reaction as shown in the following equation.



Similar to Eq. (A), the reaction in Eq. (C) took place very quickly after the mixing. However, different from the GSSG system, this reaction alone can form valid crosslinks with 8arm-PEG-SH after both ends of the GSSG-PEG-GSSG participate the reaction. An *in situ*  $^1\text{H-NMR}$  monitoring during hydrogel formation was also performed. Similarly to GSSG systems, much GSH was already generated within seconds accompanied with the decrease of 8arm-PEG-SH, indicating the reaction C took place very rapidly, and the NMR spectra did not change much with time for the next two days either.

### **Swelling and Degradation of Hydrogels Formed by 8arm-PEG-SH and GSSG-PEG-GSSG**

We then performed swelling studies to further characterize the hydrogels formed by 8arm-PEG-SH and GSSG-PEG-GSSG. With  $[\text{8arm-PEG-SH}] = 10 \text{ wt\%}$  and SS/SH ratio = 0.5, the swelling ratio of hydrogel slightly increased in the first hour. Thereafter, this hydrogel was gradually eroded and completely disappeared in 20 h (Fig. 8, filled squares). This is dramatically different from the typical chemically crosslinked hydrogels, although similar phenomena were actually reported in literature with disulfide-crosslinked hydrogels, though without explanation<sup>[22]</sup>. This erosion process might be due to the existence of significant amount of free thiol species after the gelation, which is still undergoing thiol-disulfide exchange reactions during the swelling process. At the lower SS/SH ratio (*i.e.*, 0.5), although the gelation could be complete in a short time, there is still high amount of free thiol groups in 8arm-PEG-SH left in the system (Table 1). These free thiol groups could undergo exchange

with disulfide crosslinks dynamically, and this results in transient polymer chains with no covalent bonds with the network. Before swelling, these free polymers will have the chance to be incorporated back into the network *via* the ever-going thiol-disulfide exchange. However, during the swelling process, these free polymers, especially at the gel surface, may quickly diffuse out and get lost in the swelling media, *i.e.*, behaving like a surface erosion process.

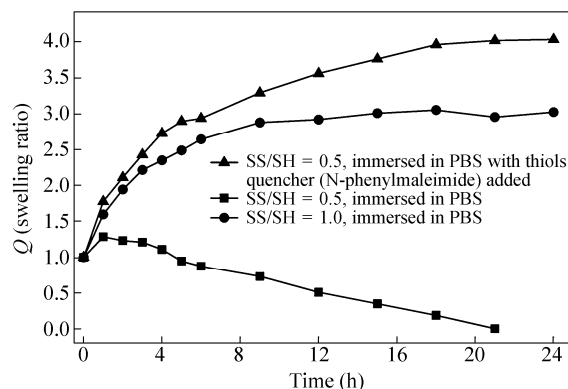


**Fig. 8** Swelling ratio of hydrogels (8arm-PEG-SH & GSSG-PEG-GSSG, [8arm-PEG-SH] = 10 wt%) over time in three conditions

To prove this hypothesis, *N*-phenylmaleimide, a thiol group quencher, was added to the swelling media to block free thiol groups and hence stop the thiol-disulfide exchange reactions. This addition resulted in the changing of swelling behavior from erosion to normal, *i.e.* with swelling ratio increased gradually until reaching equilibrium after 20 h (Fig. 8, filled triangle). This result confirmed our hypothesis and also emphasized the rich and unique properties of disulfide hydrogels.

Besides blocking the free thiol groups, reducing the free thiol concentration in the polymers also can form a more stable hydrogel by increasing the SS/SH ratio to 1.0 (Fig. 8, filled circles). This hydrogel in PBS showed a gradual increase in swelling ratio at the examined time period and reached an equilibrium swelling ratio of 3.0 in 24 h. As shown in Table 1, compared with the hydrogel with SS/SH = 0.5, the system with SS/SH = 1.0 had much lower concentration of the unreacted 8arm-PEG-SH in hydrogel, meaning fewer residual polymer thiols, which makes the hydrogel more stable than the one with SS/SH = 0.5.

## CONCLUSIONS

We have successfully developed a glutathione-disulfide-ended PEG polymer (GSSG-PEG-GSSG) and demonstrated its ability to accelerate the gelation of disulfide-crosslinking hydrogels from thiol containing polymers (8arm-PEG-SH). The gelation time can be tuned from hours to minutes at the physiological conditions. This is in dramatic contrast to those achieved by directly using the small molecule disulfide compound (GSSG), which has a limit to the shortest gelation time achievable. A mechanism was proposed to explain the different gelation behaviors of GSSG and GSSG-PEG-GSSG, based on the *in situ* NMR monitoring results and the kinetic analysis in literature.

For these thiol-consisting polymers and disulfide gelation systems, the multiple and ever-going thiol-disulfide exchange reactions are the origin of their interesting gelation phenomena and rich swelling behavior<sup>[23, 24]</sup>. We showed that, during swelling studies, these hydrogels can go through either an erosion or a normal swelling process, depending on the amount of the initial disulfide compound added to the gelation system. Therefore, in addition to their potential biomedical applications, these thiol-disulfide hydrogels possess rich properties and call for more studies on their structure-property relationship.

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