Supporting Information

Self-healing Injectable Polymer Hydrogel via Dynamic Thiol-alkynone Double Addition Crosslinks

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1. Materials and Measurements

NMR spectra were recorded on an Agilent-400 MR DD2 (399.7 MHz for ^{1}H and 100.5 MHz for ^{13}C) at 298 K. The rheological measurements were performed using a rheometer (AR G2, TA instruments) equipped with a steel plate-and-plate geometry of 40 mm in diameter and equipped with hexadecane trap. Cell viability were obtained by Andor Inverted Microscope (Zeiss, ×20 air objective). Thiol terminated 4-arm poly(ethylene glycol) (PEG10k-4-SH, Mw = 10 000, PDI ≤ 1.05) was purchased from JenKem Technology (USA). 3-Butyn-2-one was purchased from Fluorochem ltd. 2-Mercaptoethanol, sodium 2-mercaptoethanesulfonate, phosphate buffered saline, Dulbecco's phosphate-buffered saline (DPBS), Calcein AM, Fluorescein，Rhodamine B and Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (Pen-Strep) were purchased from Sigma Aldrich. Propidium iodide (PI) was purchased from Thermo Fisher Scientific Inc. NIH/3T3 cell (mouse fibroblast cells) culture line was obtained from American Type Culture Collection. Deuterium Oxide (D_2O) was purchased from Euriso-top. Hexadecane was purchased from VWR International BV. All chemicals were analytical grade and used without further purification.

2. Experimental Methods

2.1 *Dynamic exchange of thiol-alkynone double addition products*

First, the double addition between 2-mercaptoethanol (thiol#1) and 3-butyn-2-one was followed by ^{1}H NMR. 2-Mercaptoethanol (6.2 μL, 0.088 mmol) and 3-butyn-2-one (3.4 μL, 0.044 mmol)

were dissolved in a 1 mL solution of phosphate buffer solution (100 mM, pH=8.2; 'PB8.2') including 4 drops of D_2O. Sodium trimethylsilylpropanesulfonate (1 mg) was added as the internal standard. The ^1H NMR spectrum was obtained after 1-hour reaction time. Then, the double addition between sodium 2-mercaptoethanesulfonate (thiol#2) and 3-butyn-2-one was followed by ^1H NMR using the same procedure as described above. The ^1H NMR spectrum was obtained after 4 hours reaction time. Next, the thiol#1-alkynone double adduct was prepared as describe above. Sodium 2-mercaptoethanesulfonate (7.23 mg, 0.044 mmol) was added into the thiol#1-alkynone double adduct in PB8.2 at room temperature. The dynamic exchange was monitored by ^1H NMR.

2.2 *Preparation of Thiol-alkynone double addition hydrogel*

The thiol-alkynone double addition hydrogel was prepared by a mixing procedure. 4-arm PEG thiol (25 mg) was dissolved in 150 µL PB8.2 as PEG-thiol solution. 3-butyn-2-one (3.9 µL, 'alkynone') was dissolved in 1 mL PB8.2 as alkynone solution. A transparent colorless hydrogel was obtained by mixing 150 µL PEG-thiol solution (25 mg polymer in 150 µL, 16.7 wt%) and 100 µL alkynone solution (0.39 µL in 100 µL, 50 mM) in a glass vial at room temperature. Starting from these amounts, the total solid concentration is 10 wt % and the ratio of alkynone to thiol groups is 1:2.

2.3 *Monitoring of single addition and double addition products during hydrogel formation*

4-arm PEG thiol (60 mg) was dissolved in 140 µL PB8.2 and 10 µL D_2O ('PEG-thiol solution'). 3-butyn-2-one (9 µL) was dissolved in 950 mL PB8.2 and 50 µL D_2O ('alkynone solution'). 150 µL PEG-thiol solution and 100 µL alkynone solution were mixed in an NMR tube at room temperature. Sodium trimethylsilylpropanesulfonate (1 mg) was added as the internal standard. The reaction during hydrogel formation was monitored by ^1H NMR at room temperature. The hydrogel formation was tested by the tube-inversion method after each NMR measurement.

2.4 *Gelation test: variation of alkynone/thiol ratio*

The same amount of 4-arm PEG thiol PB8.2 solutions (25 mg in 150 μL, 16.7 wt%) was mixed with 100 μL alkynone PB8.2 solutions containing varying amount of alkynone (1.57 μL, 0.78 μL, 0.20 μL and 0.01 μL) to prepare alkynone-thiol pre-gel solutions (alkynone: thiol group= 2:1; 1:1; 1:4; 1:8). Gelation and gelation time were tested by vial-inversion method rheological experiments. The vial is a 12 mm diameter, 32 mm high glass vial. The hydrogel formation was checked by reverting vial every half hour in first 2 hour, then every 2 hours in next 10 hours, then every half day in next 1.5 days. If no hydrogel formed after 2 days, we concluded there is no hydrogel formation under this ratio condition. Once the hydrogels under certain ratios were able to form, the rheological time sweeps of those hydrogels were conducted using the procedures described in 2.6. The total solid content was set as 10 wt %.

2.5 *Preparation of Thiol-alkynone double addition hydrogel with varying solid concentration*

Similar to hydrogels containing 10 wt% solid concentration, we also prepared hydrogels with 2 wt%, 4 wt%, 6 wt%, 8 wt% solid concentration using the same procedure. The ratio of alkynone and thiol group is 1:2 in all cases. Gelation and gelation time were tested by vial-inversion method and rheological experiments.

2.6 *Preparation of Thiol-alkynone double addition hydrogel in PBS7.4*

Gels were prepared using the same preparation procedure as before, except using phosphate buffered saline solution (100 mM, pH=7.4, prepared by dissolving a commercial PBS tablet in 200 mL distilled water, 'PBS7.4') instead of PB8.2.

2.7 *Model Reactions*

The aim of small molecular model reactions is to study the gelation mechanism in two different condition of PB8.2 and PBS7.4. The rate of double additions between 2-mercaptoethanol and 3-butyn-2-one were followed by ^{1}H NMR. 2-Mercaptoethanol (6.2 μL, 0.088 mmol) and 3-butyn-2-one (3.4 μL, 0.044 mmol) were dissolved in a 1 mL solution of PB8.2 including 4 drops of D_2O. Sodium trimethylsilylpropanesulfonate (1 mg) was added as the internal standard. The

rate of formation of double adduct in PB8.2 between 2-mercaptoethanol and 3-butyn-2-one was monitored by ^{1}H-NMR. The small molecular model reaction in PBS7.4 was carried out as described above.

2.8 *Rheological measurements of hydrogels*

Thiol-alkynone double addition hydrogels were prepared as described above, at the different solid concentrations (4 wt %, 6 wt %, 8 wt %, 10 wt%) in PB8.2 and 10 wt% in PBS7.4. After mixing PEG thiol solution and alkynone solution, 0.7 mL of the sample was positioned on the rheometer plate. Time sweep measurements were performed at fix strain (γ = 1%) and frequency (ω =6.28 rad/s = 1 Hz). Frequency sweep measurements were performed from 0.1 to 100 rad/s at fix strain (γ = 1%). All frequency sweeps were measured after storage modulus (G') reached equilibrium state. All measurements were performed in the linear viscoelastic region. The modulus of hydrogels was measured under strain sweep from 1 to 600% at a fixed frequency (ω = 6.28 rad/s). Continuous step strain measurements were measured at fixed frequency (ω = 6.28 rad/s). Oscillatory strains were switched from 1% strain to subsequent 300% strain with 1 minute for every strain period. The viscosity of hydrogels was measured under flow step as a function of shear rate from 0.1 /s to 80 /s to study the shear thinning behavior of the hydrogel.

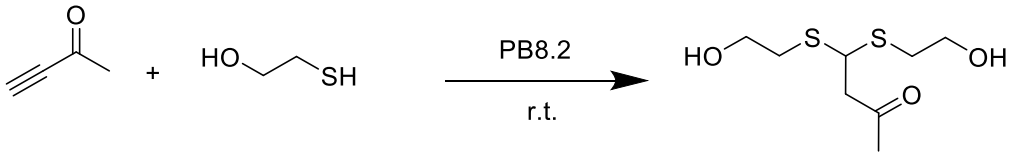
2.9 *Macroscopic Self-healing and Injectable Test of Hydrogels*

Two pieces of disk-shaped hydrogel (thickness: 4 mm; diameter: 9 mm) were prepared as described above stained by rhodamine B (red dye) and fluorescein (yellow dye). Both hydrogels were cut into two equal pieces. Then two piece of different color hydrogels were brought together and kept in a moist environment for 15 mins. Healing of the hydrogel was checked by lifting the combined gel using tweezers. Afterwards, this healed hydrogel was cut to 4 equal pieces using a scalpel. Then a piece of hydrogels was put in a syringe (1 mL volume; 0.5 inner

diameter) using a tweezer and syringe plunger, and subsequently injected through a 20G needle using manual force.

2.10 *Cell Cytotoxicity*

Thiol-alkynone double addition hydrogels were prepared as described above at 10 wt% in PB8.2 and PBS7.4. The hydrogel sample was cut to a ~1 mm layer of square shape (4 x 3 mm) and transferred into a sterile 8-well cell culture plate. The hydrogels of Gel-PB8.2 and Gel-PBS7.4 were sterilized with 70% ethanol (2 times), DPBS (5 times) and cell culture media (3 times). The cell culture media was made with DMEM supplemented 10% (v/v) FBS and 0.5% (v/v) Pen-Strep. Cells were cultured in 25 cm^2 tissue culture flasks and immersed in 5 mL cell culture media. Cells were washed with 3 mL DPBS, trypsinized and centrifuged. Afterwards the cells were resuspended in 3 mL cell culture media. Then the cells were added in the 8-well cell culture plate containing the hydrogel samples and incubated for 48 h. Cell viability was checked by the Live/Dead Cell Double Staining Kit with Calcein AM and propidium iodide (PI). After removing the cell culture media, the integrate pieced of hydrogel cannot be found under microscope, indicating that the hydrogel had dissolved in the medium over the period of incubation. Then 200 µL calcein AM solution (2 µM, DPBS) was added into the culture plate and incubated for 10 minutes, followed by adding 200 µL PI solution (12 µM, DPBS). After 5 minutes, the samples were placed under the fluorescence microscope and imaged by double two excitation wavelengths of 490 and 535 nm channels. In alive cells the nonfluorescent calcein AM is converted to a green-fluorescent calcein ($\lambda_{ex}/\lambda_{em}$: 490/515 nm) after acetoxymethyl ester hydrolysis by intracellular esterases. PI only permeates dead cells leading to red fluorescence ($\lambda_{ex}/\lambda_{em}$: 535/617 nm).



Scheme S1. Small molecule test of thiol-alkynone double reaction between 3-butyn-2-one and 2-mercaptoethanol in sodium phosphate buffer (100 mM, pH=8.2) at room temperature.

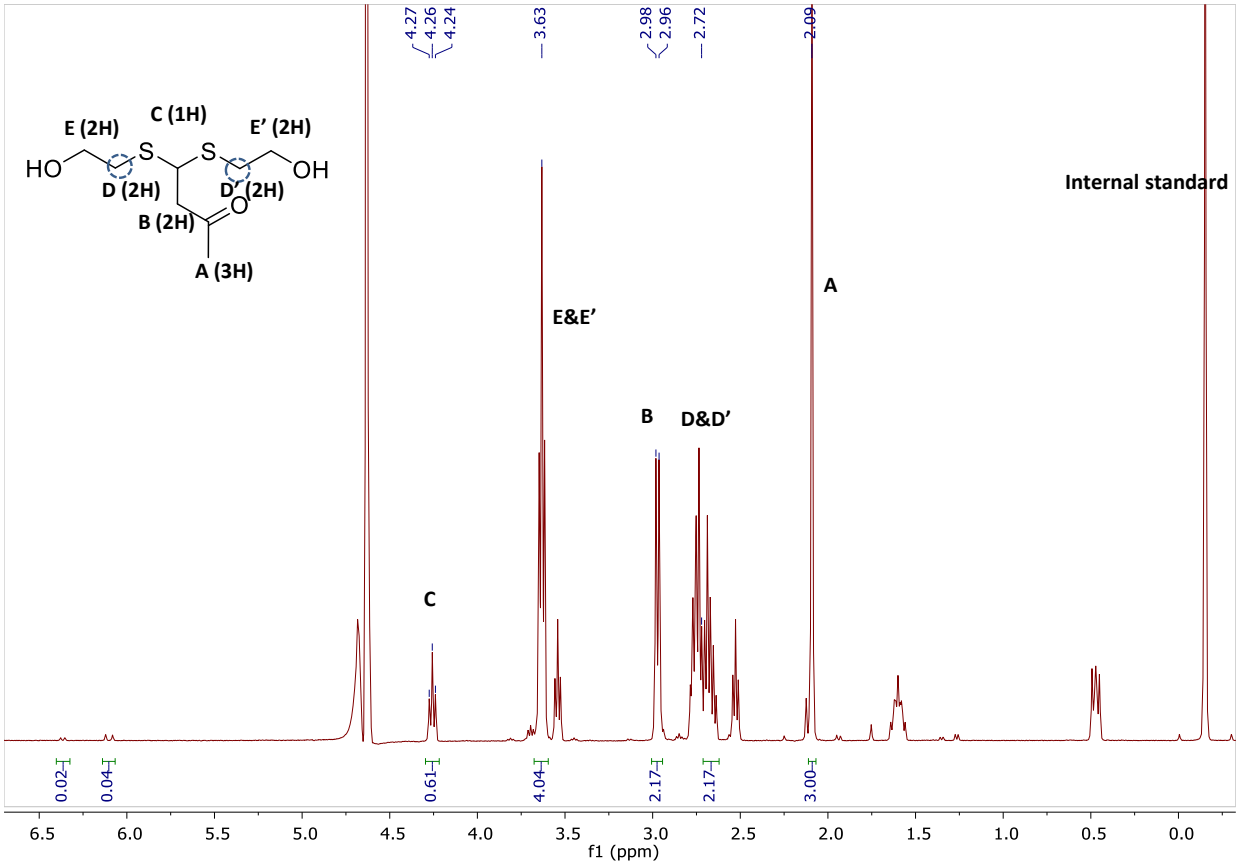
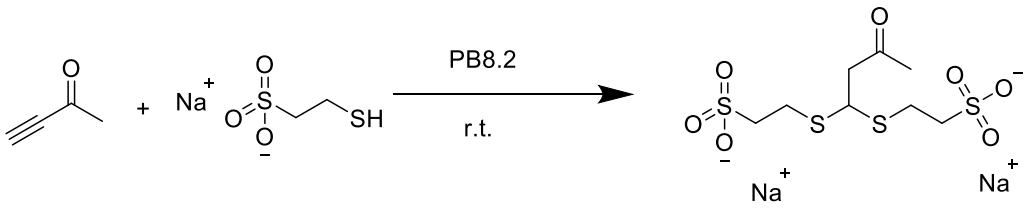


Figure S1. ^{1}H-NMR (399.7 MHz, 298 K in D_2O/ PB8.2, 4 drops of D_2O in 1 mL PB8.2) spectrum of thiol-alkynone double reaction between 3-butyn-2-one and 2-mercaptoethanol. 2-Mercaptoethanol (3 mg, 0.044 mmol) and 3-butyn-2-one (6.2 µL, 0.088 mmol) were dissolved in a 1 mL solution of PB8.2 including 4 drops of D_2O. The spectrum was obtained after 1hour reaction time.



Scheme S2. Small molecule test of thiol-alkynone double reaction between 3-butyn-2-one and sodium 2-mercaptoethanesulfonate in sodium phosphate buffer (100 mM, pH=8.2) at room temperature.

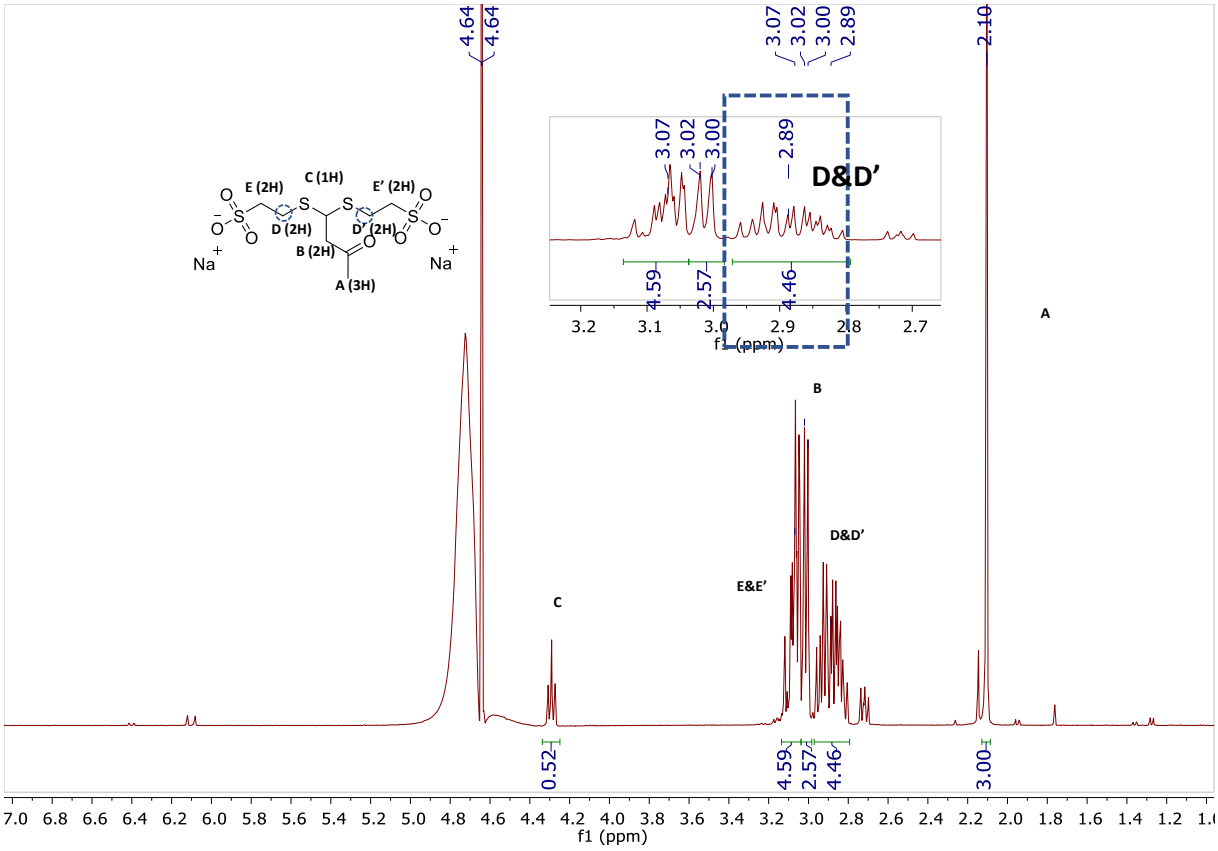
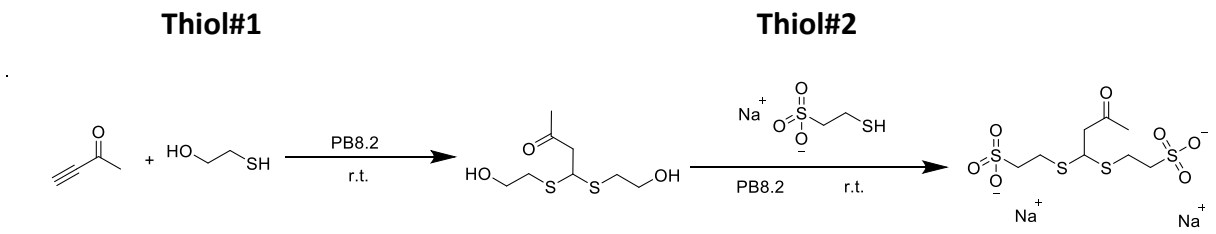


Figure S2. ^{1}H-NMR (399.7 MHz, 298 K in D_2O/ PB8.2, 4 drops of D_2O in 1 mL PB8.2) spectrum of thiol-alkynone double reaction between 3-butyn-2-one and sodium 2-mercaptoethanesulfonate. Sodium 2-mercaptoethanesulfonate (14.46 mg, 0.088 mmol) and 3-butyn-2-one (3 mg, 0.044 mmol) were dissolved in a 1 mL solution of PB8.2 including 4 drops of D_2O. The spectrum was obtained after 4 hours reaction time.



Scheme S3. Small molecule test of dynamic exchange between thiol#1-alkynone double adduct sodium 2-mercaptoethanesulfonate in sodium phosphate buffer (100 mM, pH=8.2) at room temperature. Besides the double 2-mercaptoethanesulfonate addition product, there is likely also formation of a mixed thiol double addition product.

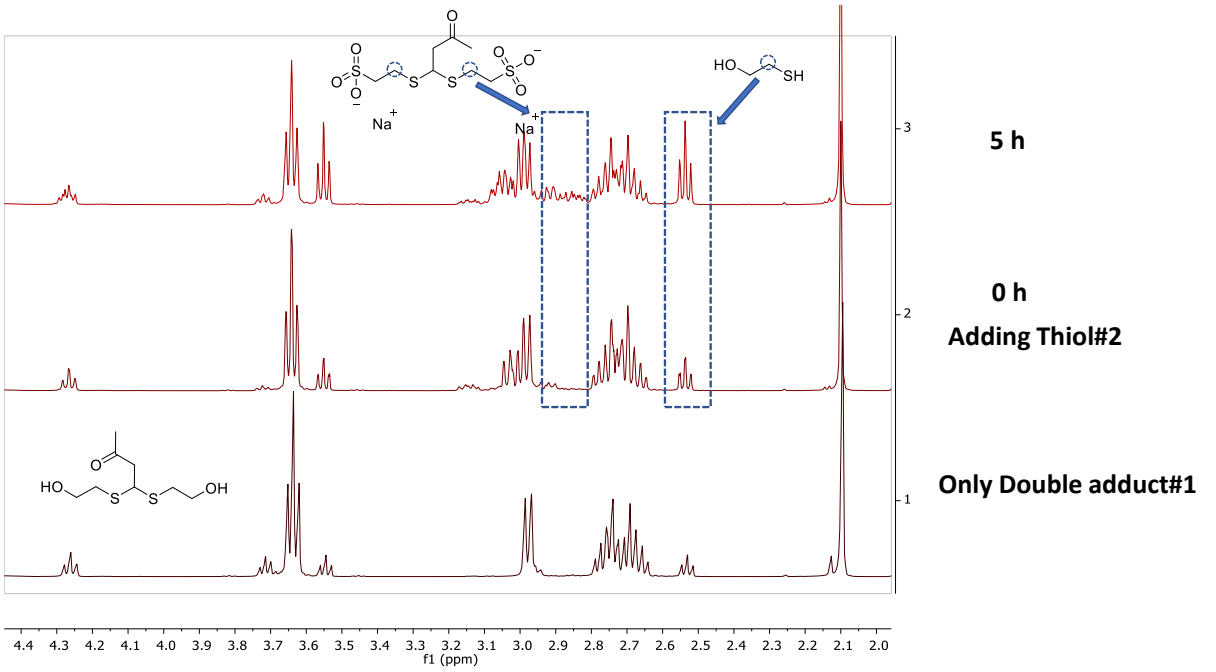


Figure S3. ^{1}H-NMR (399.7 MHz, 298 K in D_2O / PB8.2, 4 drops of D_2O in 1 mL PB8.2) monitoring of dynamic exchange between thiol#1-alkynone double adduct and sodium 2-mercaptoethanesulfonate (thiol#2) in sodium phosphate buffer (100 mM, pH=8.2) at room temperature.

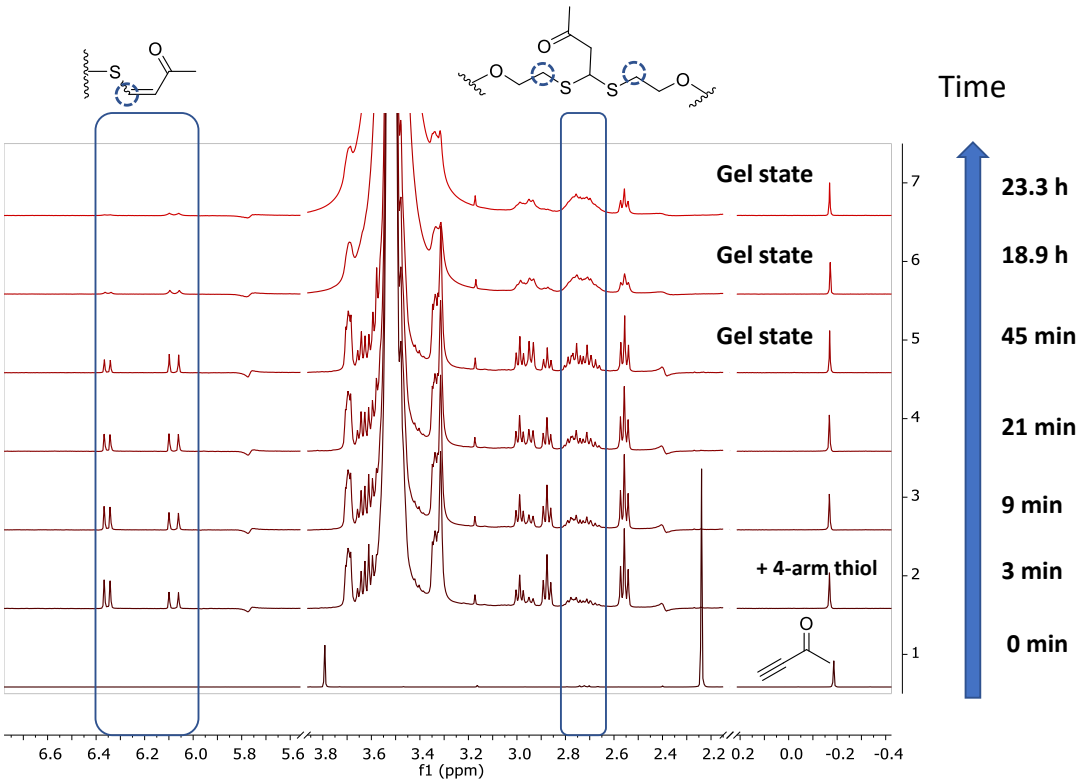


Figure S4. ^{1}H-NMR (399.7 MHz, 298 K in D_2O / PB8.2, 15 µL D_2O in 585 µL PB8.2) monitoring of single addition and double addition during thiol-alkynone double addition hydrogel formation between 3-butyn-2-one and 4-arm PEG thiol.

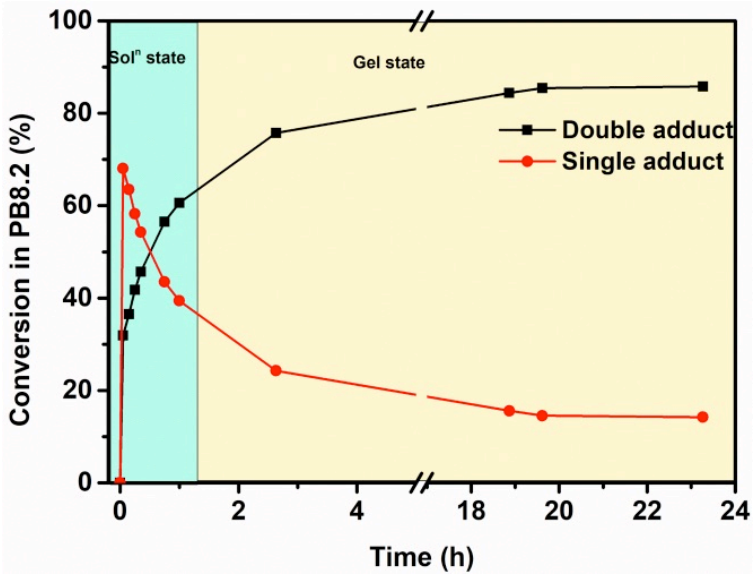


Figure S5. The conversion of double adduct and single adduct during thiol-alkynone double addition hydrogel formation between 3-butyn-2-one and 4-arm PEG thiol. The conversion of single adduct is calculated based on the integration of the alkene protons of the single adducts by ^{1}H NMR. The conversion of double adduct is calculated based on the integration of the methylene group of the double adduct by ^{1}H NMR. The methyl signal of sodium trimethylsilylpropanesulfonate was set as the internal standard. The cyan area in the graph indicates that the sample is in solution state. The yellow area indicates that the sample is in the hydrogel state.

Table S1. Gelation test with varying molar ratio of alkynone and thiol group (2:1; 1:1; 1:2; 1:4; 1:8). All experiments are run at 10% solid content and in PB8.2.

	Ratio of alkynone and thiol group				
Alkynone	1	1	1	1	2
Thiol group	8	4	2	1	1
Gelation Time*	~90 min	~50 min	~30 min	ng**	ng**

* Gelation time is defined as the time it takes to reach the gel point (G'=G") as measured by rheology.

**: no gel, i.e. no hydrogel is observed after two days reaction time, checked by vial-inversion method.

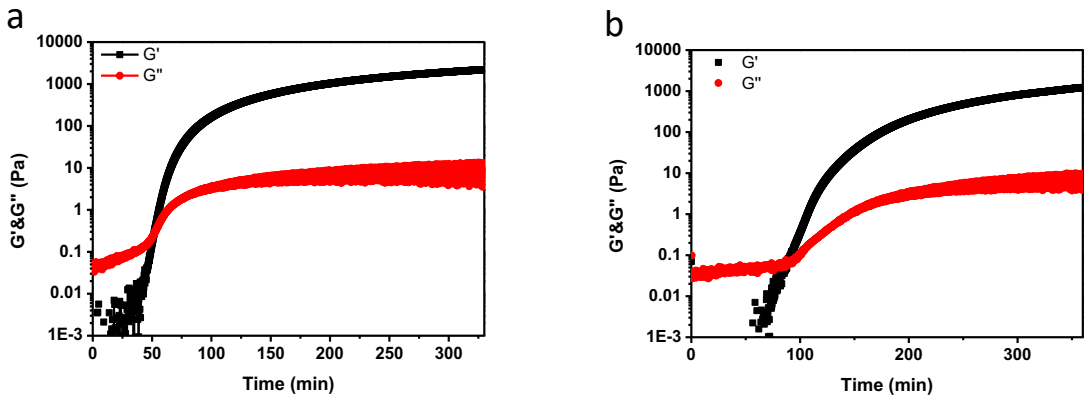
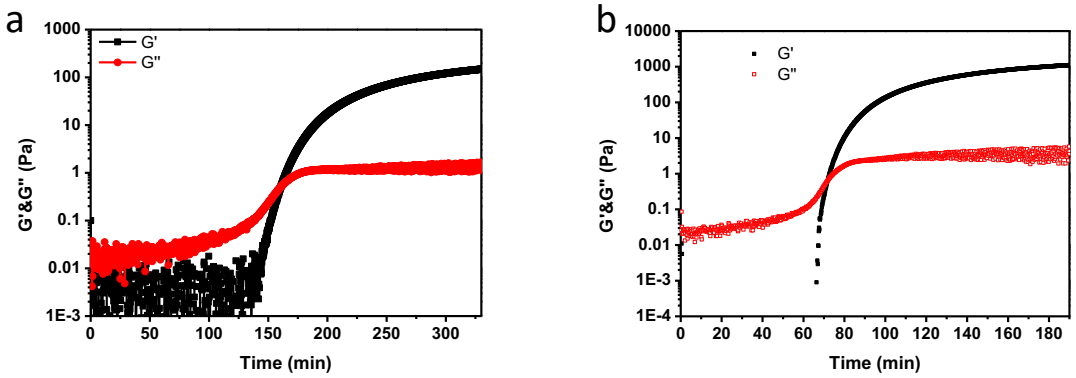


Figure S6. Time sweep measurements of the gelation process of a) hydrogel at 1:4 alkynone: thiol ratio; b) hydrogel at 1:8 alkynone: thiol ratio (γ = 1%, ω = 1 Hz, 25 °C)



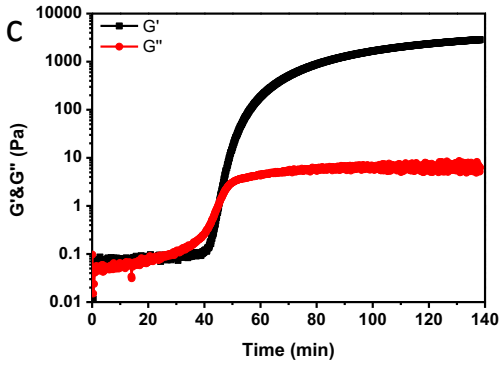


Figure S7. Time sweep measurements of the gelation process of a) 4 wt% hydrogel; b) 6 wt% hydrogel c) 8 wt% hydrogel (γ = 1%, ω = 1 Hz, 25 °C)

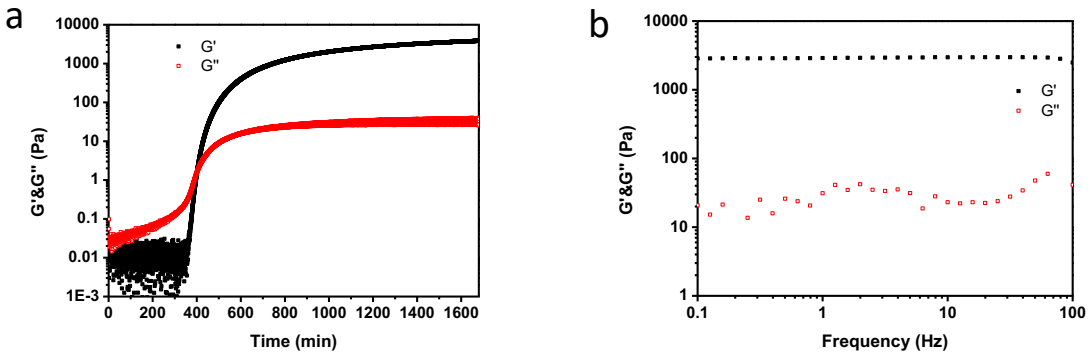
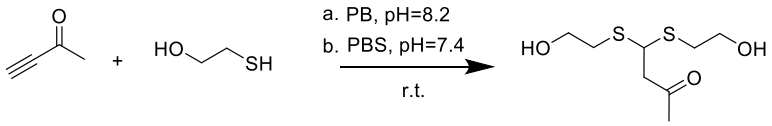


Figure S8. a) Time sweep measurement of the gelation process of 10 wt% hydrogel formed in PBS7.4 (γ = 1%, ω = 1 Hz, 25 °C). b) Frequency sweep measurement of 10 wt% hydrogel formed in PBS7.4 (γ = 1%, ω = 0.1-100 Hz, 25 °C).



Scheme S4. Small molecular test of thiol-alkynone double reaction between 3-butyn-2-one and 2-mercaptoethanol in PB8.2 or PBS7.4 at room temperature.

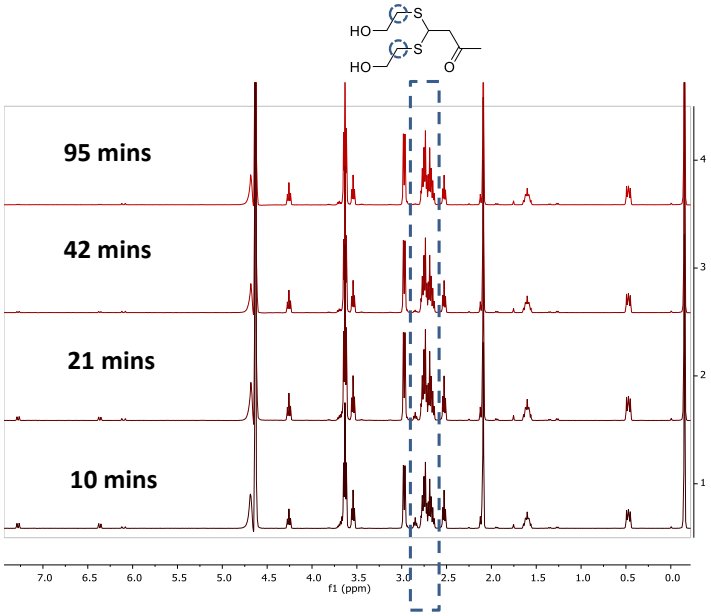


Figure S9. ^{1}H-NMR (399.7 MHz, 298 K in D_2O / PB8.2, 4 drops of D_2O in 1 mL PB8.2) monitoring of thiol-alkynone double reaction between 3-butyn-2-one and 2-mercaptoethanol in PB8.2.

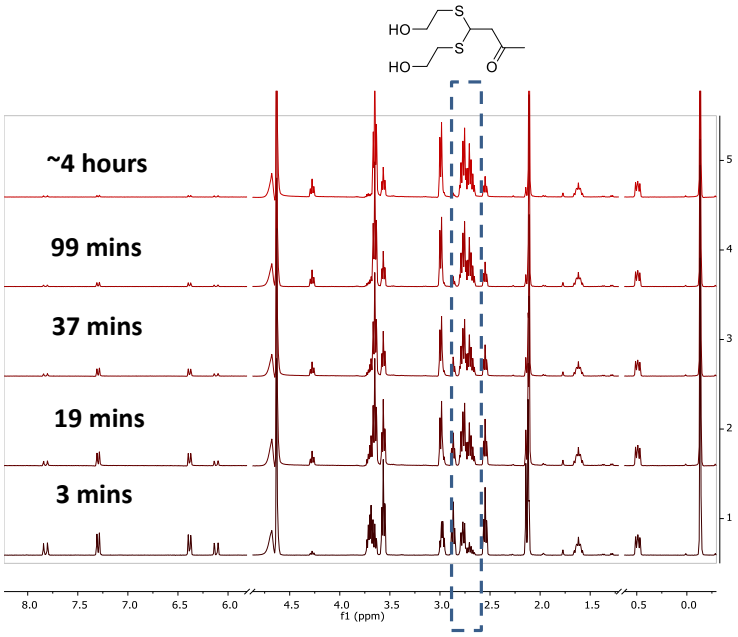


Figure S10. ^{1}H-NMR (399.7 MHz, 298 K in D_2O/PBS, 4 drops of D_2O in 1 mL PBS7.4) monitoring of thiol-alkynone double reaction between 3-butyn-2-one and 2-mercaptoethanol in PBS7.4.

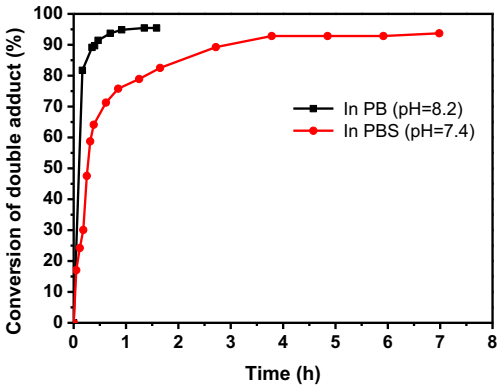


Figure S11. The formation of double adduct product in the thiol-alkynone double addition reaction between 3-butyn-2-one and 2-mercaptoethanol in PB8.2 and PBS7.4 over time.

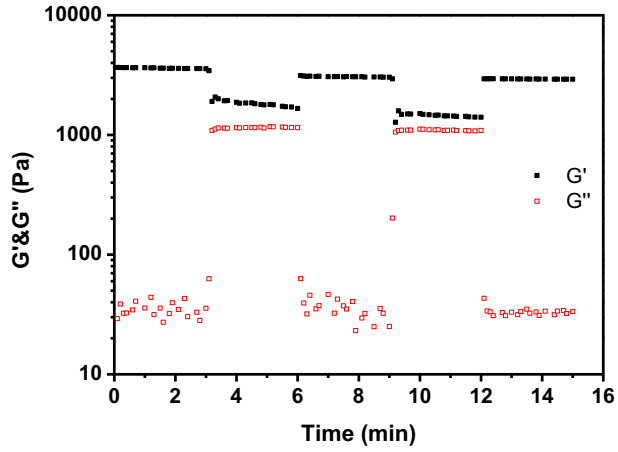


Figure S12. Step-strain sweep of 10 wt% Gel-PBS7.4, alternative strain switched from 1% to 300% twice then back to 1% (ω = 1 Hz, 25 °C).

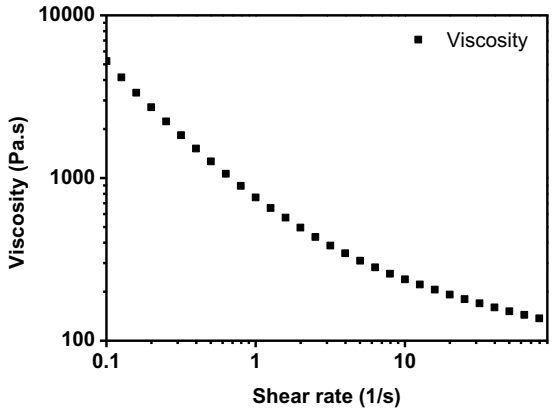


Figure S13. Viscosity of 10 wt% Gel-PB8.2 measured as a function of the shear rate.

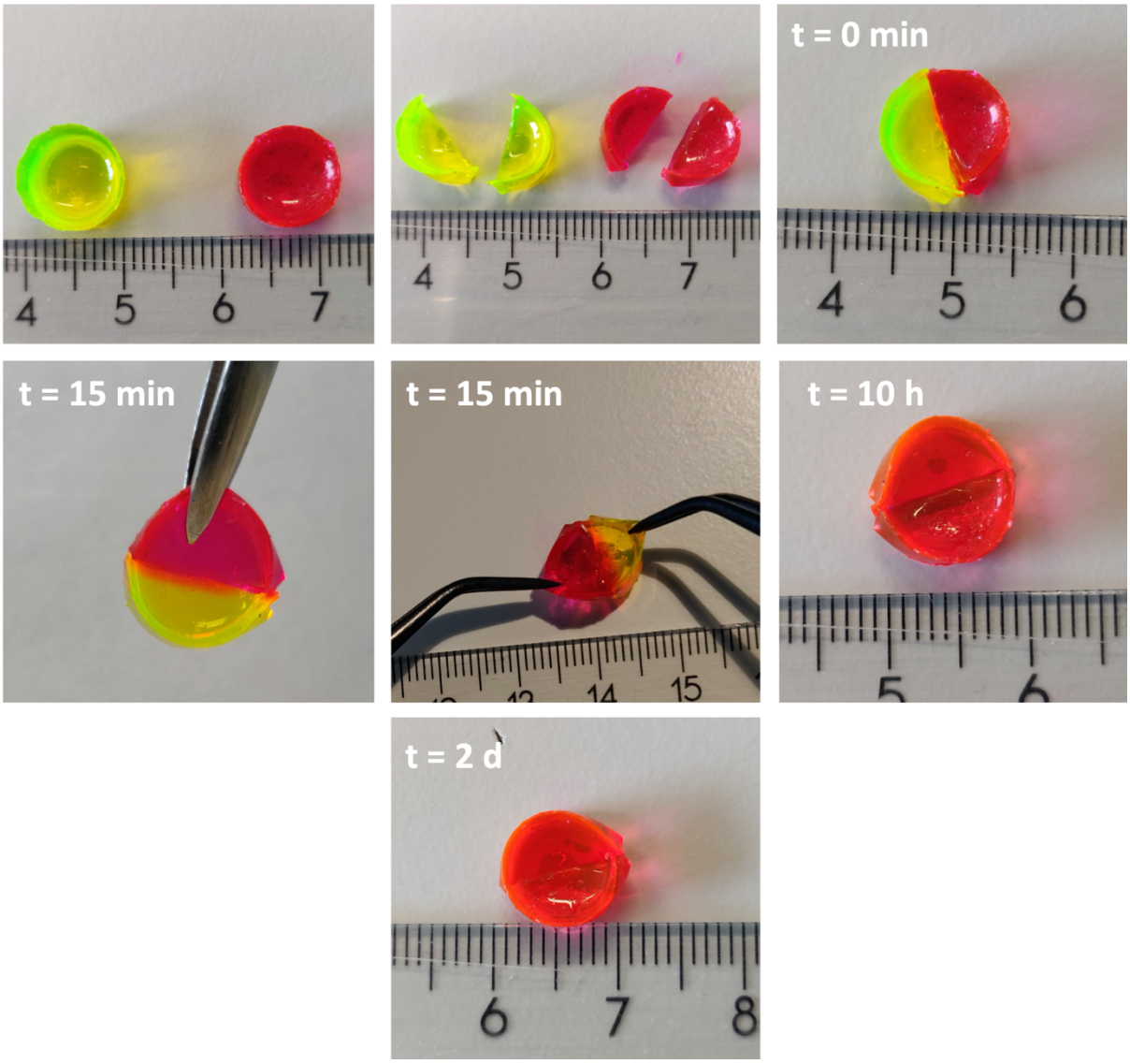


Figure S14. Photographs of self-healing process of 10 wt% Gel-PB8.2.

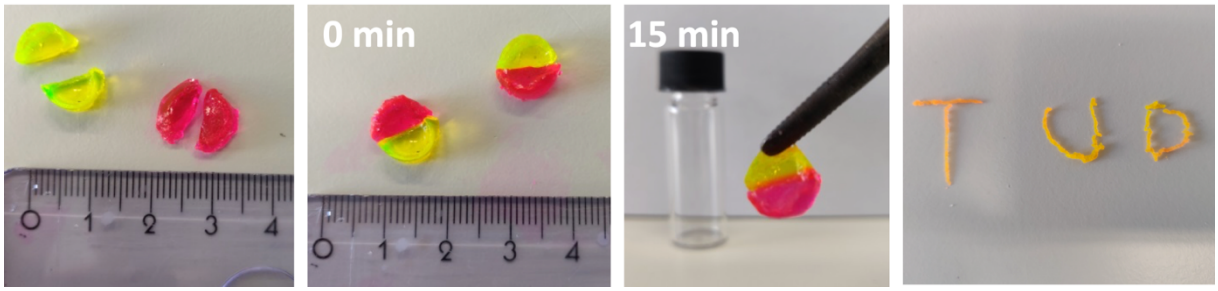


Figure S15. Photographs of self-healing process and injection of 10 wt% Gel-PBS7.4.