Electronic Supporting Information for:

Improved method for synthesis of cysteine modified hyaluronic acid for *in-situ* hydrogel formation

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Experimental Section

Materials.

HA sodium salt (Mη 100k) was purchased from Jiangyin Runhe Bioengineering Inc. (Jiangyin, CN). L-Cysteine hydrochloride, di-*tert*-butyl dicarbonate ($(Boc)_2O$), *N*-hydroxysuccinimide (HOSu) and N,N-dicyclohexylcarbodiimide (DCC) were purchased from GL Biochem (Shanghai, CN). Cystamine dihydrochloride, ninhydrin and Ellman reagent (DTNB) were purchased from Sigma (Shanghai, CN). *N,N*-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), ethyl 3-mercaptopropionate (EMP), sodium borohydride ($NaBH_4$) and 1,4-butanediol diglycidyl ether (BDDE) were purchased from Aladdin (Shanghai, CN). Tetrabutylammonium hydroxide (TBAH) was purchased from Kente Chem (Zhejiang, CN). 4-armed poly(ethylene glycol)-amine (MW 10k) and 4-armed PEG-ACLT were purchased from Jenkem (Beijing, CN). Acetone, acetonitrile, tetrahydrofuran (THF), dichloromethane (DCM), methanol (MeOH), isopropyl alcohol (IPA), ethanol, hydrochloride acid (HCl), sodium bicarbonate (NaHCO3), anhydrous sodium carbonate (Na2CO3) and sodium chloride (NaCl) were purchased from Tianzheng (Tianjing, CN). Dextran standards for GPC (1400000, 668000, 410000, 273000,148000, 48600, 23800, 11600, 5200 Da) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Synthesis of Cysteine-HA conjugate.

Synthesis of *L*-(-)-2, 2-dimethylthiazolidine-4-carboxylic acid hydrochloride (Dmt, 2). L-Cysteine hydrochloride **1** (10 g, 63.7 mmol) was refluxed in dry acetone (1500 ml) for 6 h and concentrated to a small volume under reduced pressure. A large amount of white solid was collected by filtration storing at 4°C overnight to afford **2** (11 g, 88%). ^{1}H NMR (600 MHz, MeOD), δ_H 4.78 (1H, t, J = 8.4 Hz, -C<u>H</u>-COOH), δ_H 3.46 (1H, dd, J = 12.0 Hz, 8.4 Hz, -C<u>H</u>$_2$-SH), δ_H 3.32 (1H, dd, J = 12.0 Hz, 7.8 Hz, -C<u>H</u>$_2$-SH), δ_H 1.61 (3H, s, -C-C<u>H</u>$_3$), δ_H 1.60 (3H, s, -C-C<u>H</u>$_3$).

Synthesis of *L*-(-)-(*tert*-butyloxycarbonyl)-2, 2-dimethylthiazolidine-4-carboxylic acid (Boc-Dmt, 3) To a suspension of **2** (10.9 g, 55 mmol) and di-*tert*-butyl dicarbonate (16.0 g, 73 mmol) in dry MeCN was added DIEA (12.8 g, 99mmol) dropwise, followed by stirring for two days. After evaporation under reduced pressure, the residue was dissolved in Et_2O followed by filtration through Celite, so as to remove the amino salt. The ether filtrate was then washed by 0.1N HCl (x2), water (x2) and brine (x2), dried and continually evaporated in a fume hood to get a white solid. The crude product was then subjected to silica gel column chromatography to afford the pure compound **3** (6g, 40%). ^{1}H NMR (600 MHz, DMSO), δ_H 12.74 (1H, br s, -COOH), δ_H 4.74 (1H, dd, J = 8.4 Hz, 5.4 Hz, -CH-COOH), δ_H 3.36 (1H, d, J = 12.0 Hz, -CH_2-SH), δ_H 3.04 (1H, d, J = 12.0 Hz, -CH_2-SH), δ_H 1.75 (3H, s, -C-CH_3), δ_H 1.73 (3H, s, -C-CH_3), 1.42 (3H, s, *ter*-butyl), δ_H 1.35 (6H, s, *ter*-butyl).

Synthesis of *N*-hydroxysuccinimide easter of *L*-(-)-(*tert*-butyloxycarbonyl)-2, 2-bimethylthiazolidine-4-carboxylic acid (Boc-Dmt-OSu, 4). Compound **3** (3g, 11.5mmol) and *N*-hydroxysuccinimide (1.45g, 12.6mmol) were dissolved together in 20ml THF. The solution was cooled, and *N,N*-dicyclohexylcarbodiimide (2.6g, 12.6mmol) in THF was added. The mixture was stirred for 5h. The reaction was monitored by silica gel TLC plate (solvent system: DCM-MeOH-HOAc = 100:6:1). The spots on the silica gel plate were visualized by ninhydrin reagent. The reaction mixture was kept at 4°C for 30min, and then filtered and wished with THF. The filtrate was concentrated under reduced pressure and the residue was re-crystallized from isopropyl alcohol to yield the purified product 3.7g (90.42%). ^{1}H NMR (600 MHz, $CDCl_3$), δ_H 5.11 (1H, d，J=6.0， -CH-CH_2-),δ_H 3.43 (1H, d, J = 12.0 Hz, -CH_2-S-), δ_H 3.30 (1H, d, J = 12.0 Hz, -CH_2-S-)，δ_H 2.82 (4H, t, J = 6.6 Hz, -CO-CH_2-CH_2-CO-), δ_H 1.87 (3H, s, -C-CH_3), δ_H 1.79 (3H, s, -C-CH_3), 1.48 (3H, s, *ter*-butyl), δ_H 1.45 (6H, s, *ter*-butyl).

Synthesis of Boc-Dmt-Cysta (5). To a solution of cystamine dihydrochloride (2.24 g, 9.94 mmol) in H_2O was added 2.1 equiv Na_2CO_3, followed by the addition of **4** (6.78g, 18.93 mmol) dissolved by THF dropwise, and stirred continuously overnight. The reactant was acidified to pH~5 and then washed by 10mM HCl (x3), 5% $NaHCO_3$ (x3) and brine (x1), respectively, evaporated and dried

in vacuum to give the crude product, which was subjected to silica gel column chromatography to afford the pure compound **5** (5.7g, 94.4%). ^{1}H NMR (600 MHz, DMSO), δ_H 7.97 (2H, br s, -CONH-), δ_H 4.57 (2H, br s, -CH-CONH-), δ_H 3.42 (2H, m, -CONH-CH$_2$-), δ_H 3.34 (2H, d, J = 10.8 Hz, -CH$_2$-SH), δ_H 3.28 (2H, m, -CONH-CH$_2$-), δ_H 2.94 (2H, d, J = 10.8 Hz, -CH$_2$-SH), δ_H 2.79 (4H, br s, -CH$_2$-S-S-), δ_H 1.77 (6H, s, -C-CH$_3$), δ_H 1.70 (6H, s, -C-CH$_3$), δ_H 1.34 (18H, br s, *ter*-butyl).

Synthesis of Boc-Dmt-Cystea (6) Compound **5** (6.37 g, 10.015 mmol) was dissolved and stirred in ethanol/H_2O with cooling, and $NaBH_4$ (10eq) was added to the solution. The mixture was reacted at room temperature for 5h. TLC monitoring of the reaction mixture showed light yellow by spraying of Ellman reagent. Water (2.5 times of the volume of reaction solution) was added to dilute the reaction mixture, followed by acidifying the mixture to below pH 3 with 1M HCl. The product was then isolated with dichloromethane. The dichloromethane solution was washed with water three times and brine (x1) and dried over anhydrous $MgSO_4$. After filtration, the solution was evaporated to afford **6** (6.38g, 99.8%). As the monomer of **5**, compound **6** showed the same chemical shifts as those of **5** in ^{1}HNMR data.

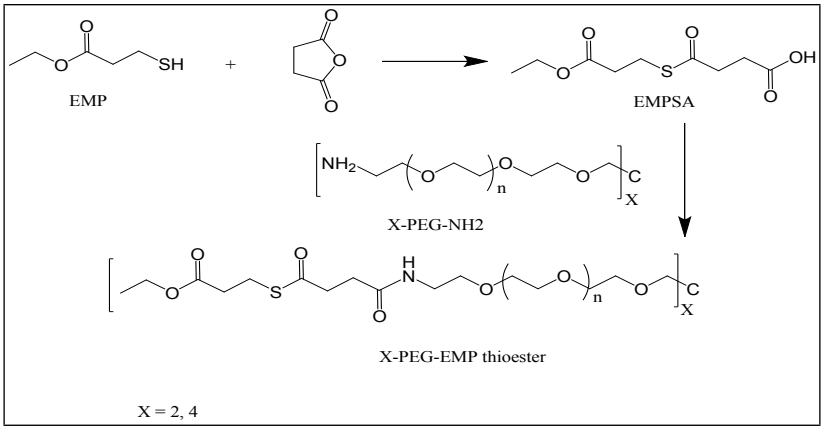
Synthesis of Boc-Dmt-BDDE (7). 1,4-Butanediol diglycidyl ether (BDDE) (8.8g, 25mmol) was dissolved in acetonitrile. To this solution, 1 equivalent of sodium bicarbonate in water was added while stirring at room temperature under argon. Compound **6** (4.6ml, 27.5mmol) in acetonitrile was added into above solution dropwise. The reaction mixture was allowed to stir overnight until the spot corresponding to **6** disappeared on the TLC plate using Ellman test. The mixture was concentrated to dryness under reduced pressure, followed by extraction with dichloromethane, and then dichloromethane solution was washed with water three times, brine (x1), and dried over anhydrous $MgSO_4$. After filtration, the solution was evaporated to afford the crude product (13.08g). The analysis of the crude product by silica gel TLC (solvent system: DCM-MeOH-HOAc = 100:6:1) gave three spots with ninhydrin reagent. The UPLC-MS analysis showed that the three spots were **7**(44.7%), Boc-Dmt-BDDE-Dmt-Boc (the product that both of epoxide were involved in the reaction) and Boc-Dmt-S-S-Dmt-Boc (the oxidation product of **6**). Since both impurities won't participate in the next reaction step, the crude product was used

directly without further purification.

Synthesis of Boc-Dmt-BDDE-HA (8). Sodium hyaluronate (1g, 2.611mmol) was dissolved in H_2O at a concentration of 20% (w/v) with TBAH (1 eq.). To this solution was added compound **7 (**2 eq.), which was added in two batches. The reaction solution (pH 13.0) was rocked for 36h at 25°C. The pH of the mixture was adjusted to 7.0 with 0.1M HCl, followed by dialysis against of 50% (v/v) MeCN with 0.9% (w/v) NaCl. The HA conjugate was precipitated by saturated sodium chloride ethanol solution and then the purified product was dissolved in a small volume of H_2O and lyophilized. ^{1}HNMR (D_2O) indicated that the Boc signal at δ_H 1.34 ppm, and the ratio of the signals of Boc (δ_H 1.34 ppm) and acetyl group (δ_H 1.94 ppm, -NH-COC$\underline{H}_3$) in HA was about 1:10, which suggested that the degree of modification (DS) of disaccharide repeats was 3.3%.

Synthesis of Cys-HA conjugate (9)

To a concentrated solution of **8** (1 g) in H_2O was added TFA (50 mL) and stirred for 4 h, followed by removing the solvent under reduced pressure. The residue was dissolved in water/alcohol (1:1) and evaporated under reduced pressure again, and the process was repeated four times, to free the thiol group of the cysteine from the acetone protection. Finally, the product was subjected to alcohol precipitation, centrifugation, and dried to give **9** as white fluffy solid. The solution of **9** gave a positive reaction with Ellman reagent. ^{1}HNMR (D_2O) indicated that the signals of protecting groups disappeared, which suggested that the protecting groups were fully removed. Due to the interference of other proton signals of HA, ^{1}HNMR analysis of **9** may result in a less accurate estimation of the rate of the modification, thus we used Ellman test. The Ellman test showed that the degree of modification (DS) was 3.5%.



Scheme S1. The synthetic approach of thioester macromonomer **10.**

Synthesis of thioester macromonomer (4-armed PEG-EMP thioester, 10). The synthetic approach is shown in scheme S1. To a stirring solution of succinic anhydride (2.0g, 20mmol) and DMAP (122mg, 1mmol) in 25ml of acetonitrile-pyridine (9:1), EMP (2.95, 22mmol) was added under argon. The mixture was allowed to stir at room temperature overnight before concentrating to dryness under reduced pressure, followed by dissolving the residue in EtOAc, and then washing with 0.1M HCl (×3) and H_2O (×3) before drying over anhydrous $MgSO_4$. The solution was concentrated under reduced pressure and dried *in vacuo* after filtration, to yield the product EMPSA (3.87g, 82.59%). ^{1}H NMR (600 MHz，$CDCl_3$)，δH 9.91 (s, *br*), 4.13 (2H，q，J = 7.2 Hz, -C$\underline{H_2}$-CH_3)，3.12 (2H，t，J = 7.2 Hz, -S-C$\underline{H_2}$-)，2.87 (2H，t，J = 7.2 Hz，-S-CO-C$\underline{H_2}$-)，2.68 (2H, t, J = 7.2 Hz, -S-CH_2-C$\underline{H_2}$-), 2.60 (2H, t, J = 7.2 Hz,-C$\underline{H_2}$-COOH)，1.24 (3H，t，J = 7.2 Hz，-CH_2-C$\underline{H_3}$).

A vial containing 4-armed PEG-NH_2 (1g, 0.4mmol) and BOP (0.221g, 1mmol) was added to a solution of EMPSA (0.234g, 1mmol) in 2ml dichloromethane, and then DIEA (0.696ml, 4mmol) was added. The mixture was vortexed for 5 min and rocked overnight, monitored by silica gel TLC (solvent system: DCM-MeOH-HOAc = 100:6:1), and visualized by ninhydrin reagent. Subsequently, the product was purified by adding 50 ml MeOH and shaking thoroughly, and the solution was cooled at -20°C. The precipitate was collected by centrifugation (-9°C, 7500 rpm, 15min) and the supernatant was decanted. The purified product **10** was precipitated with diethyl ether, dried *in vacuo*, and monitored by the ninhydrin test which gave a yellowish solution after repeating the purification cycle four times. ^{1}H NMR (600 MHz, $CDCl_3$), δ_H 4.15 (2H, q, J=7.0 Hz, -C$\underline{H_2}$-CH_3-), δ_H 3.79-3.41 (m,, -O-C$\underline{H_2}$-CH_2-O-), δ_H 3.12 (2H, t, J=7.0 Hz, -S-C$\underline{H_2}$-), δ_H 2.92 (2H,

t, J = 7.0 Hz, -S-CO-C$\underline{H_2}$-), δ_H 2.61 (2H, t, J = 7.0 Hz, -S- C$\underline{H_2}$ -C$\underline{H_2}$-), δ_H 2.52 (2H, t, J = 7.0 Hz, -C$\underline{H_2}$-COOH), δ_H 1.26 (3H, t, J = 7.0 Hz, -C$\underline{H_2}$-CH_3-).

Hydrogel formation. The gel was formed by mixing Cys-HA conjugates (9) (5% w/v) in buffer solution (5% NaHCO3, pH = 8.0, or phosphate buffer, pH 7.5, or pH 7.2) and 4-armed PEG-EMP thioester (10) (0.5% w/v), linear-PEG-EMP thioester (1% w/v), or 4-armed-PEG-ACLT (0.5% w/v) in buffer solution (5% NaHCO3, pH = 8.0 or phosphate buffer, pH 7.5, or pH 7.2) at 20~37°C. The molar ratio between the cross-linkable groups was 2:1 or 1:1. The results showed that the gel formation time ranged from 3 min to 45 minutes. The pictures of the formed gel are shown in figures S10-S11.

Rheological characterization of gel formation. The measurement of gel formation time and rheological characterization were performed with a Anton Paar MCR302 rheometer at 25°C with a stainless steel cone/plate fixture (CP50). The measurements of the storage and loss moduli were made in the oscillatory mode at 1 Hz frequency and 1% strain during cross-linking. After the storage modulus reached a plateau value during the gelation experiment, a frequency sweep experiment was performed from 0.01 to 10 Hz with 19 data points at 1% strain. Finally, a strain sweep experiment was performed with strain from 1 to 100% at 1 Hz frequency. The data are shown in figures S12-S13 and table S1.

Gel permeation chromatography (GPC) of Cys-HA conjugate 9 and HA starting material. GPC was performed with a Waters 1525 GPC system, a Waters 2414 differential refractive index (DRI) detector, and tandem columns (TSKgel G5000PXL column 7.8 x 300 mm and TSKgel G3000PXL column 7.8 x 300 mm, TOSOH, Japan) at an oven temperature of 35°C, eluted with KH_2PO_4 buffer (20 mmol/L, pH 5) at a flow rate of 0.6ml/min.

Calibration curve. 9 dextran standards for GPC (1400000, 668000, 410000, 273000, 148000, 48600, 23800, 11600, 5200 Da) (Sigma-Aldrich Co.) were dissolved in KH_2PO_4 buffer (20 mmol/L, pH 5) at a concentration of 2mg/ml, subjected to GPC with an injection volume of 20 μl, eluted for 45min, respectively. Calibration curve and calibration equation were obtained by

LogMw vs elution volume plot and curve fitting using Waters Breeze GPC software (R^2 = 0.998946). Results see Fig. S14, Table S2 and equation S1.

$$\text{LogMw} = 5.94e + 9.63eV + 5.77e^{-1}V^2 - 1.18e^{-2}V^3 \qquad \text{(equation S1)}$$

Sample measurements. Samples (Cys-HA conjugate 9 and HA starting material) were dissolved in KH_2PO_4 buffer (20 mmol/L, pH 5) at a concentration of 2mg/ml, subjected to GPC with an injection volume of 10 μl, eluted for 45min, respectively. Each sample ran twice. Molecular weight data were obtained by using Waters Breeze GPC software and calibration equation S1. GPC traces of samples (Cys-HA conjugate 9 and HA starting material) were shown in Fig. S15 and Fig. S16. The MW results see Table S3.

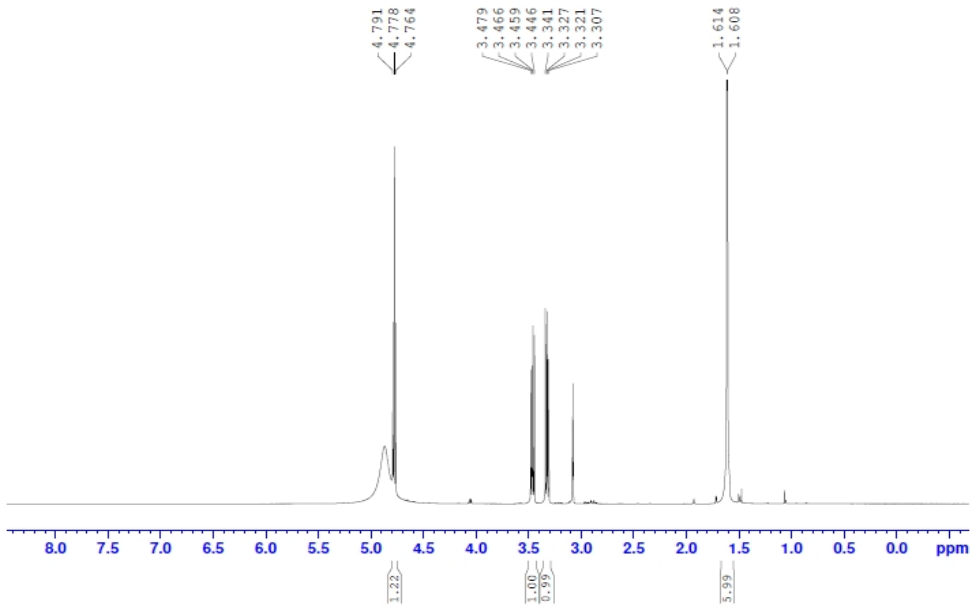


Fig. S1 ^{1}H NMR spectrum of compound **2.**

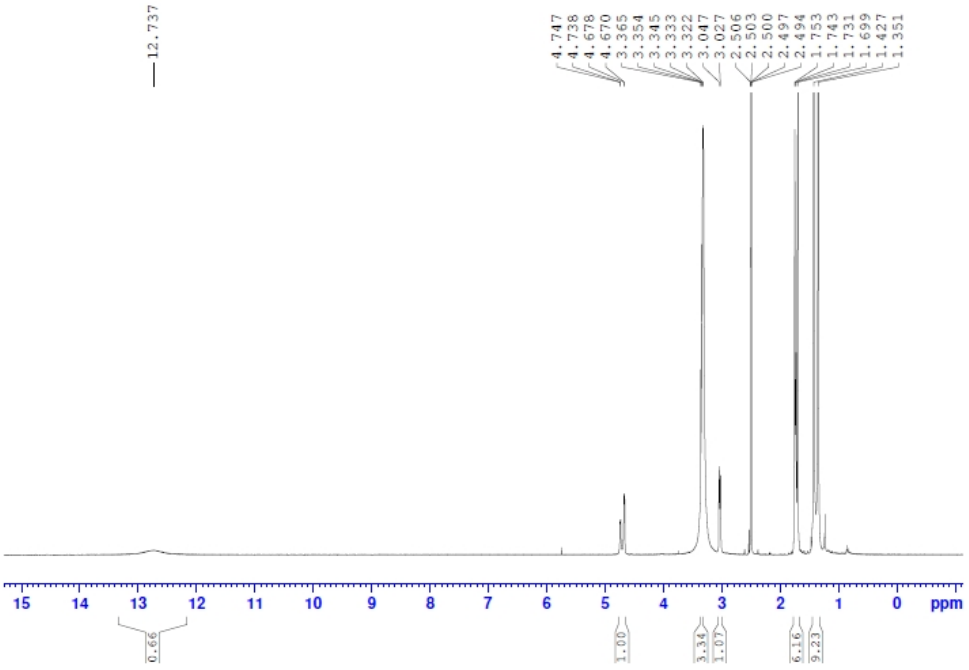


Fig. S2 ^{1}H NMR spectrum of compound **3.**

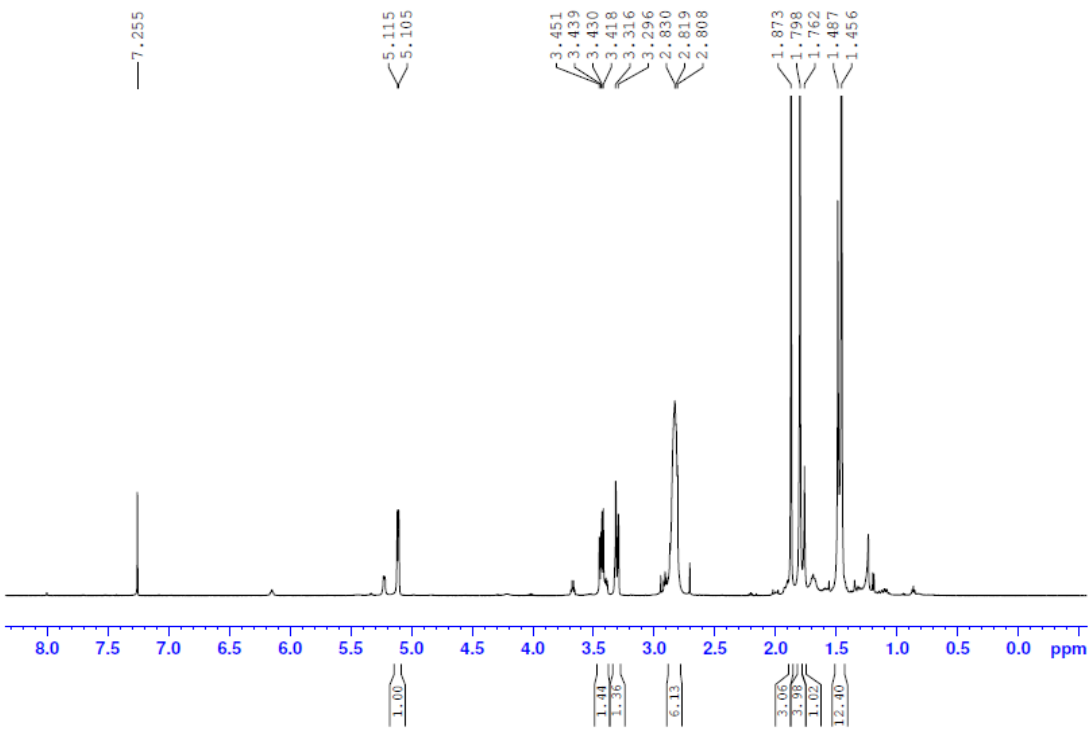


Fig. S3 ^{1}H NMR spectrum of compound **4.**

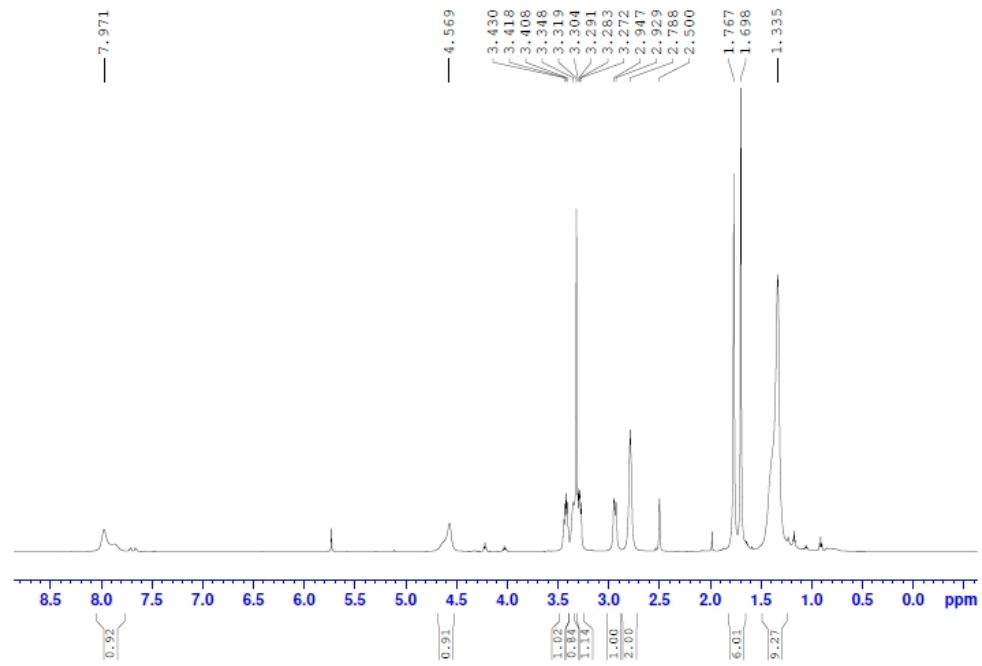


Fig. S4 ^{1}H NMR spectrum of compound **5.**

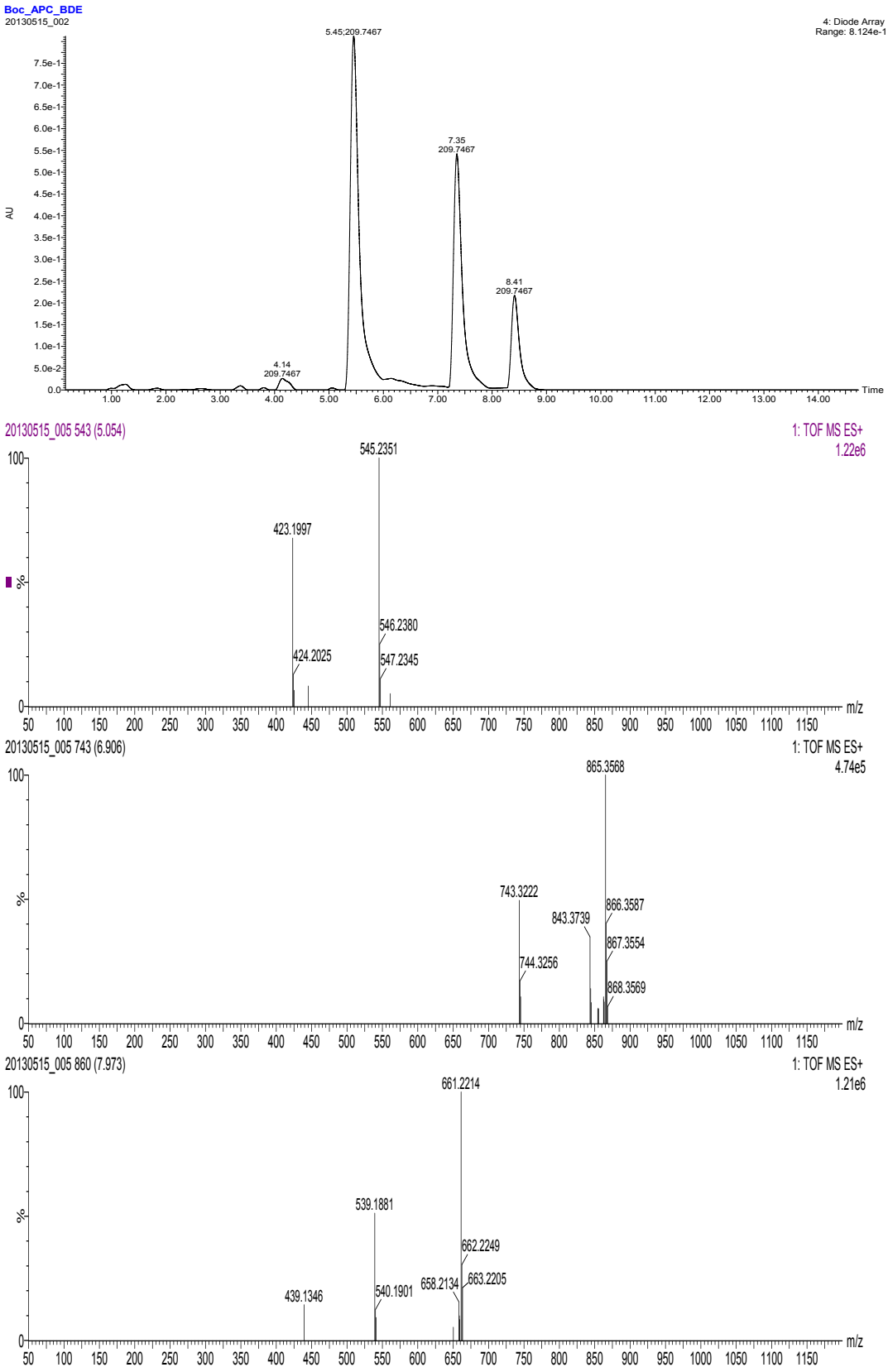


Fig. S5 UPLC-MS spectrum of compound **7.**

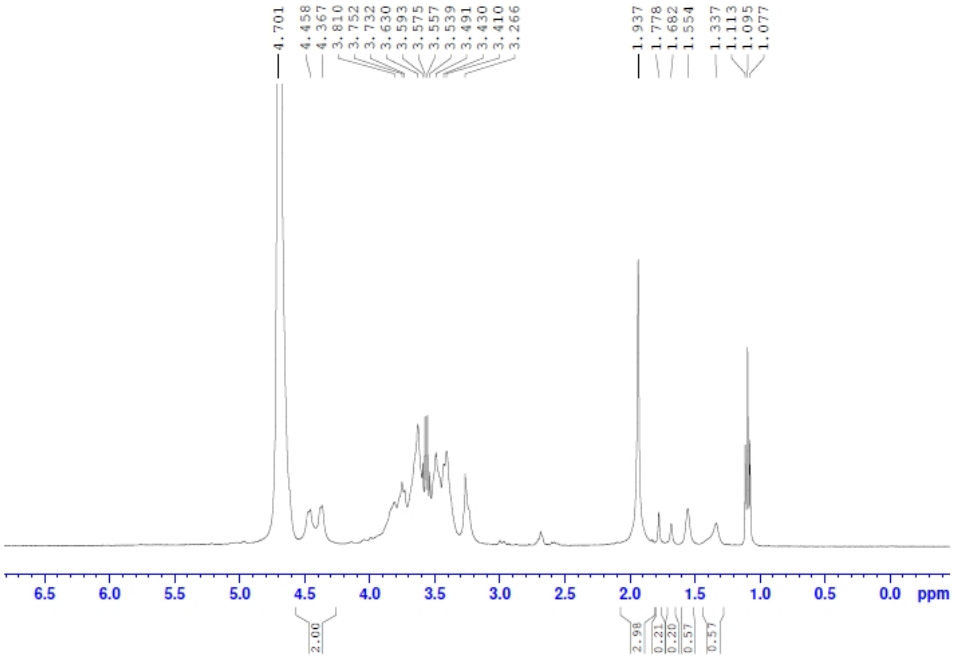


Fig. S6 ^{1}H NMR spectrum of compound **8.**

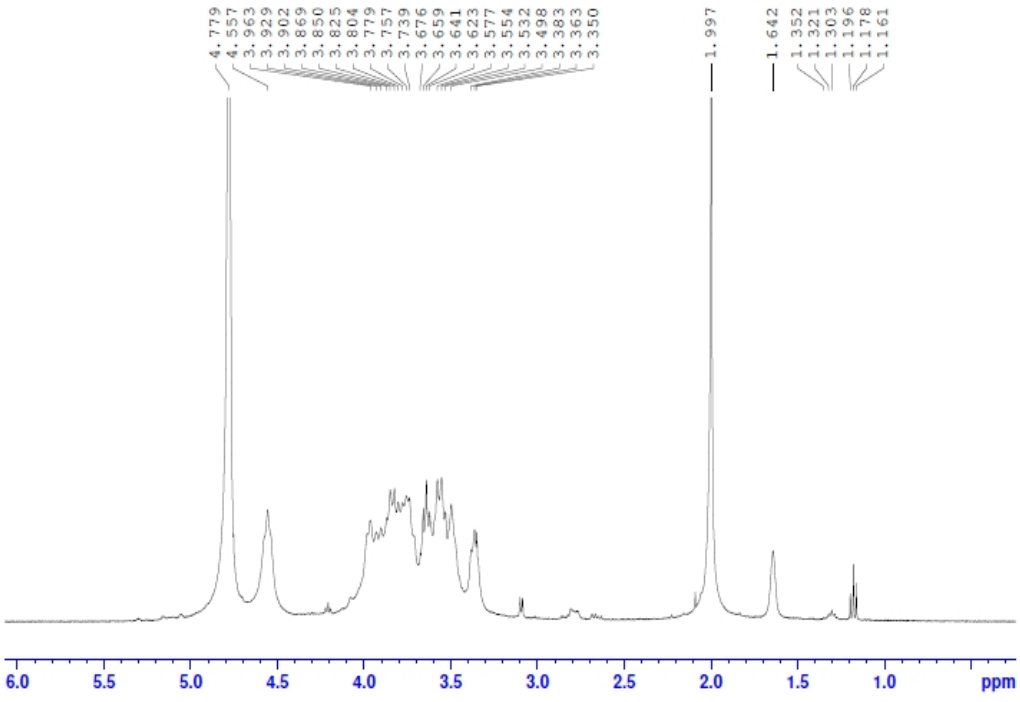


Fig. S7 ^{1}H NMR spectrum of compound **9.**

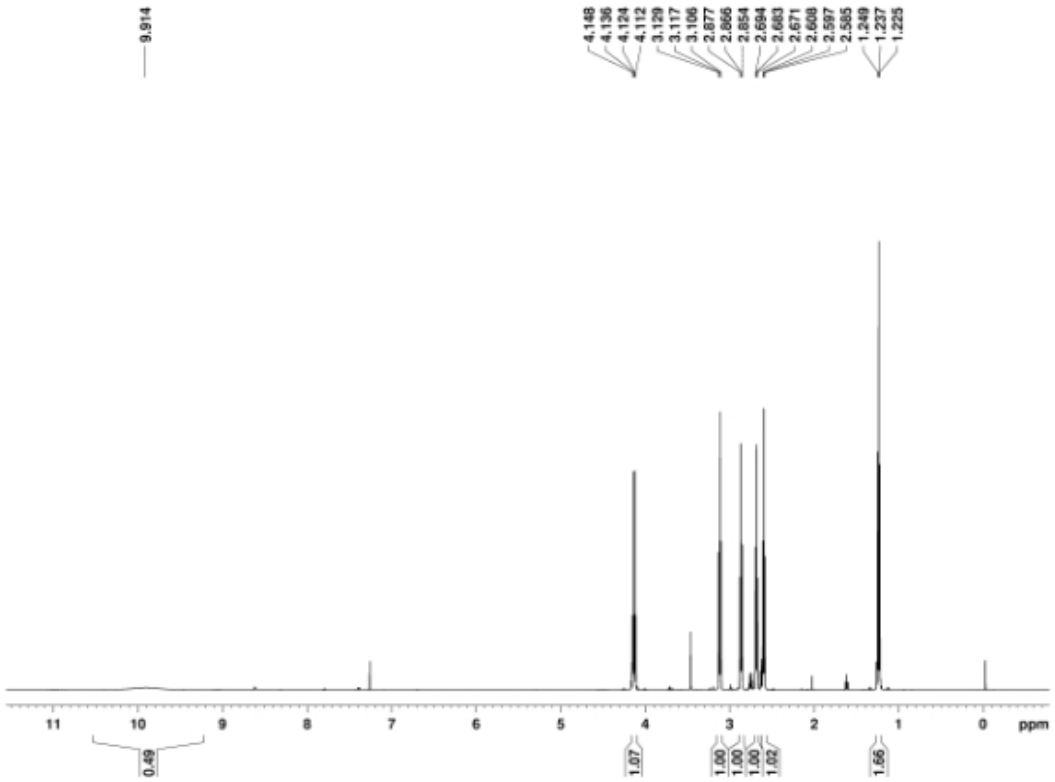


Fig. S8 [1]H NMR spectrum of compound EMPSA.

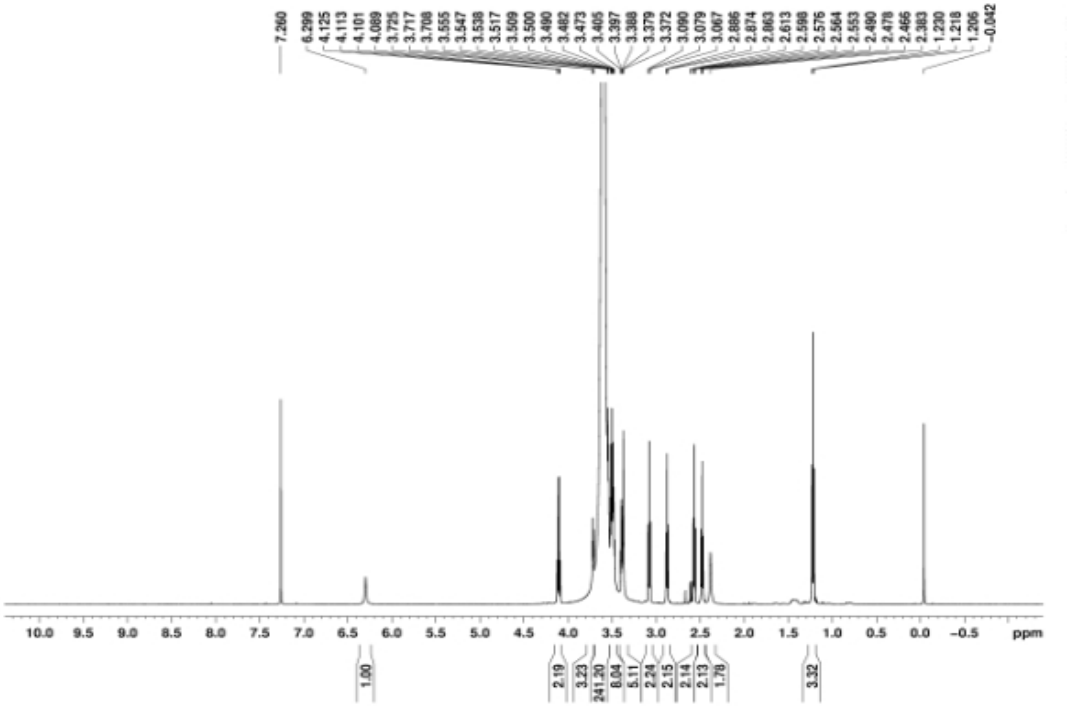


Fig. S9 [1]H NMR spectrum of compound **10.**

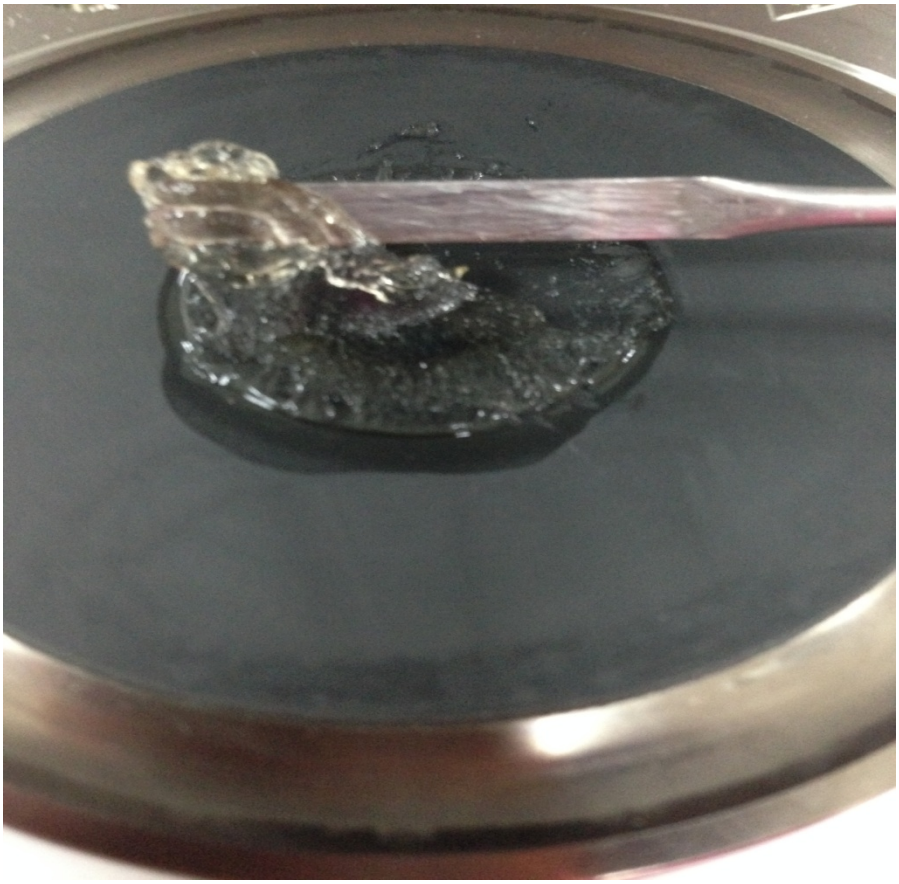


Fig. S10 The gel formed from Cys-HA conjugate **9 (**5%) and 4-armed-PEG-ACLT (0.5%) in phosphate buffer with the molar ratio of cysteine and acrylate 2:1, pH 7.5.

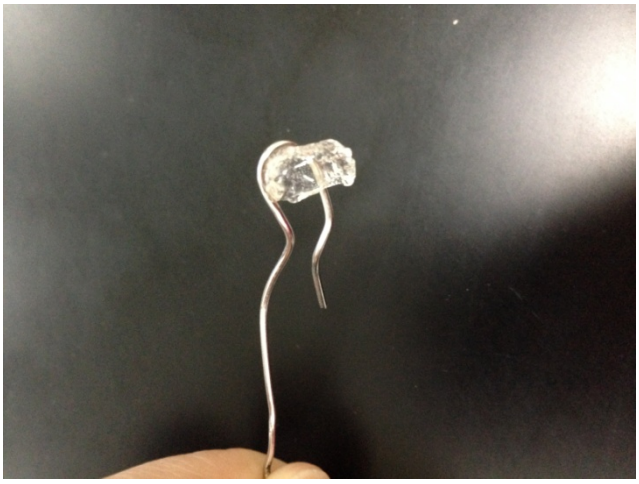


Fig. S11 The gel formed from Cys-HA conjugate **9 (**5%) and 4-armed PEG-EMP thioester **10,** (0.5%) in 5% $NaHCO_3$ aqueous solution with the molar ratio of cysteine and thioester 2:1.

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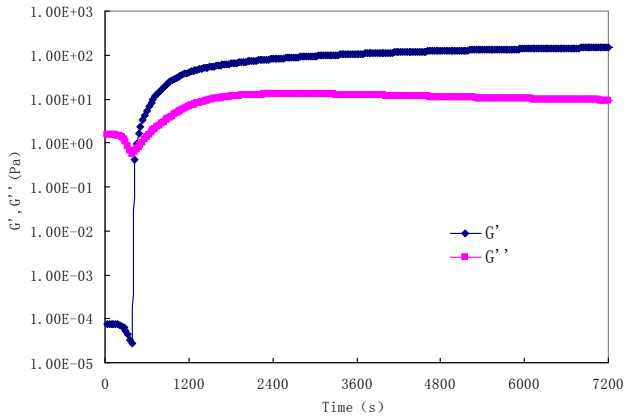
a.



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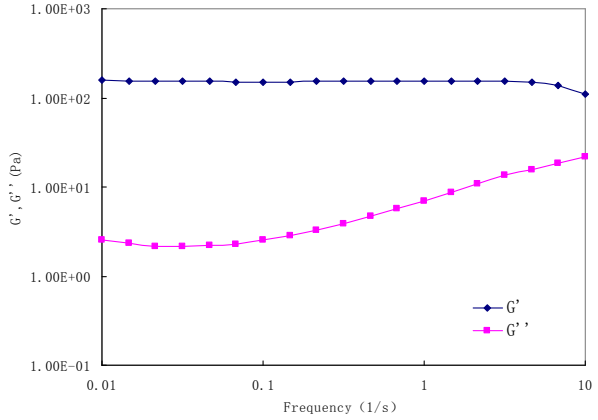

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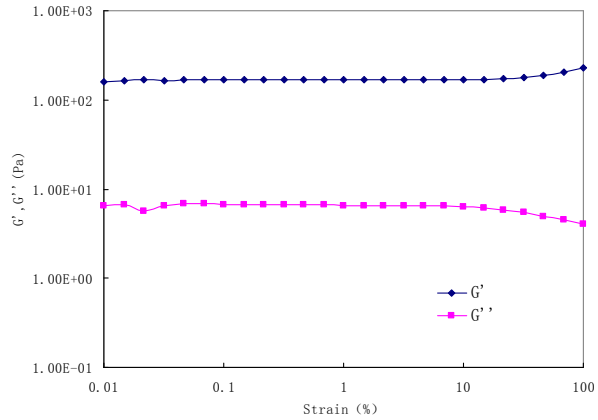
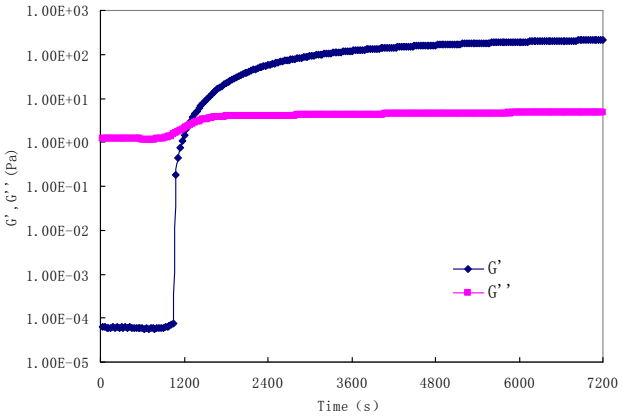


Fig. S12. Oscillatory rheology of hydrogel formed by mixing Cys-HA conjugate **9** (5%) and 4-armed PEG-ACLT (0.5%) in phosphate buffer with the molar ratio of cysteine and acrylate 2:1, pH 7.5.

a. Gel formation vs time at 25°C; b. Frequency sweep at 1% strain at 25°C; c. Strain sweep at 1 Hz frequency. G' = storage modulus; G'' = loss modulus.

a.



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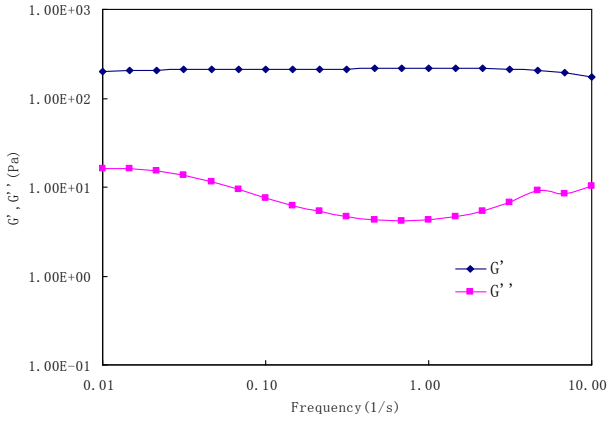

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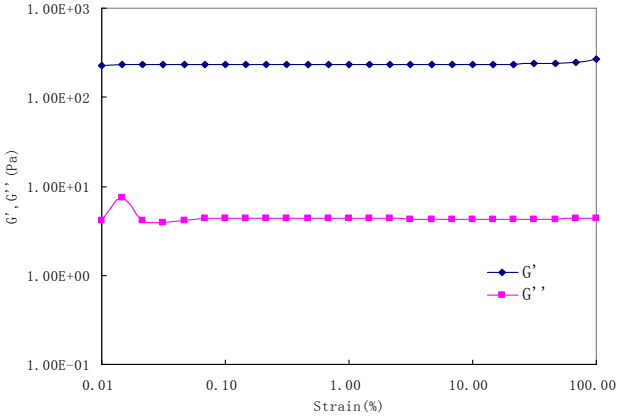

Fig. S13. Oscillatory rheology of hydrogel formed by Cys-HA conjugate **9 (**5%) and 4-armed PEG-EMP thioester **10** (0.5%) in phosphate buffer with the molar ratio of cysteine and thioester 2:1, pH 7.5.

a. Gel formation vs time at 25°C; b. Frequency sweep at 1% strain at 25°C; c. Strain sweep at 1 Hz frequency. G' = storage modulus; G'' = loss modulus.

Table S1. The gelation time of Cys-HA conjugate reacting with different PEG conjugates and buffer solutions measured by the modulus cross-over point using a rheometer at 25°C.

PEG conjugates	**Buffer solutions. pH**	**Gelation time**
4-armed-PEG-ACLT	5% $NaHCO_3$ pH 8.0	instant
4-armed-PEG-EMP thioester	5% $NaHCO_3$ pH 8.0	2 min
linear-PEG-EMP thioester	5% $NaHCO_3$ pH 8.0	30 min
4-armed-PEG-ACLT	Phosphate buffer pH 7.5	8 min
4-armed-PEG-EMP thioester	Phosphate buffer pH 7.5	20 min
4-armed-PEG-EMP thioester	Phosphate buffer pH 7.2	45 min

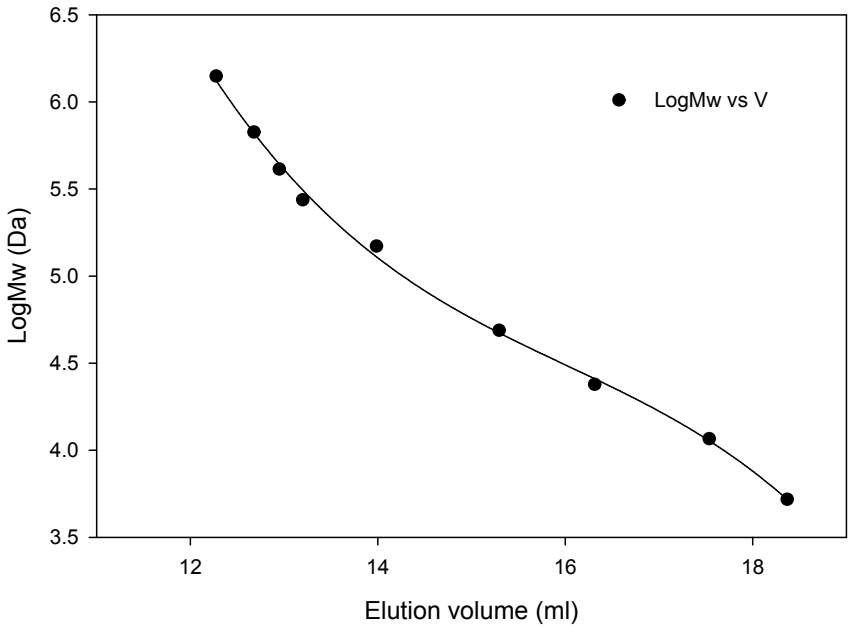

Fig. S14. GPC Calibration curve of dextran standards.

Table S2. GPS data of dextran standards.

Standard	Mw (Dalton)	Log Mw	Elution Volume (ml)
1	1400000	6.146128	12.280
2	668000	5.824776	12.682
3	410000	5.612784	12.952
4	273000	5.436163	13.202
5	148000	5.170262	13.990
6	48600	4.686636	15.298
7	23800	4.376577	16.318
8	11600	4.064458	17.540

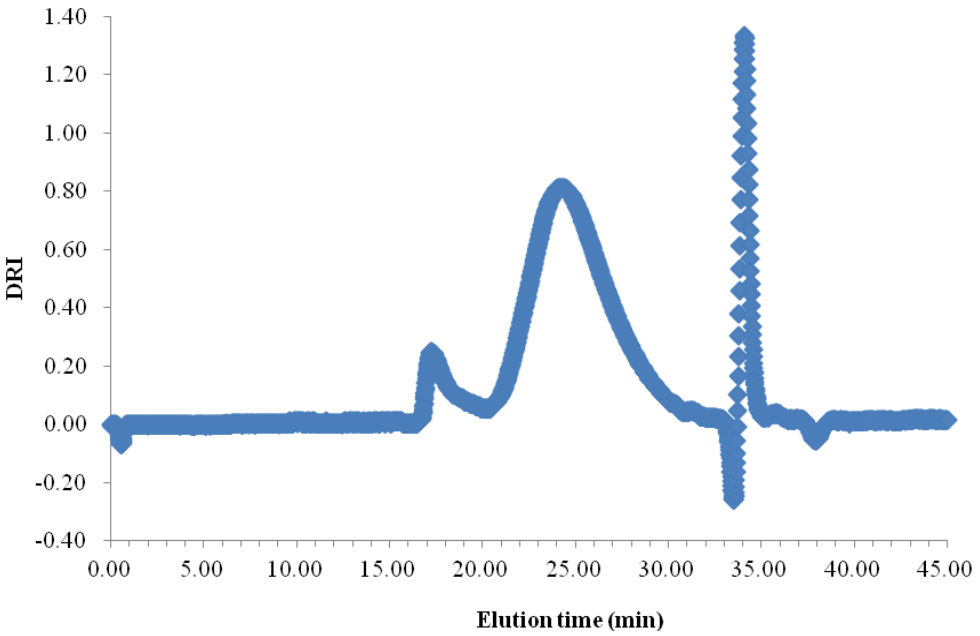

Fig. S15. GPC trace of HA starting material.

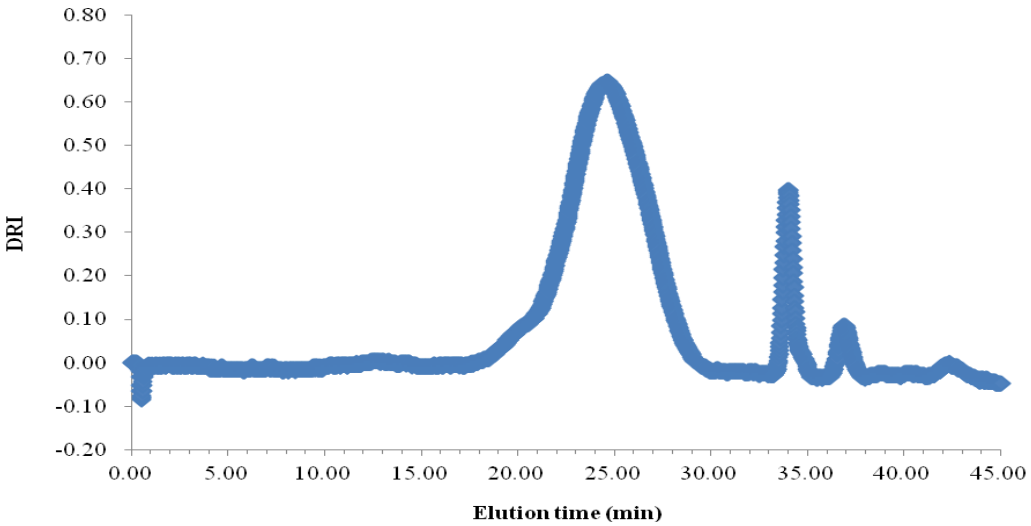

Fig. S16. GPC trace of Cys-HA conjugate 9.

Table S3. GPC results of Cys-HA and HA.

Sample	GPC run	Retention Time (min)	Mn (Da)	Mw (Da)
HA	1	24.050	52780	133051
	2	24.083	51651	131112
	Average		52216	**132082**
Cys-HA 9	1	24.667	65270	142460
	2	24.667	63720	143519
	Average		64495	**142990**