Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information

One-pot Preparation of Polypeptide Nanogels in Aqueous Solution via Ring-Opening Polymerization-Induced Nano-gelation

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Experimental Procedures

Materials and Instrument.

Hexane, *N,N*-dimethylformamide (DMF) and tetrahydrofuran (THF) were purified by first purging with dry nitrogen, followed by passing through columns of activated alumina. L-Cystine (99%)，DL-dithiothreitol (DTT, 99%)，indomethacin (99%), sodium dodecyl sulfate (SDS，98.5%)，ethyl acrylate (99%) and ethyleneglycol monomethyl ether acrylate (99%) were purchased from Aladdin reagent. α-amino-poly(ethylene oxide) macroinitiator ($mPEG_{113}$-NH_2, Mn = 5000 Da) was purchased from Jenkem Technology Co, Ltd (Beijing, China). γ-Benzyl-L-glutamic acid was purchased from GL Biochem (Shanghai) Ltd. All other chemicals were purchased from commercial suppliers and used without further purification unless otherwise noted. ^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a Bruker AVANCE NEO 400 MHz NMR spectrometer. All samples were prepared at a concentration of approximately 5 mg/mL. FTIR spectra from 4000 to 400 cm^{-1} were recorded on a JASCO FT/IR-4700 spectrofluorometer. The solution samples were cast on KBr salt tablets before measurement**.** The solid samples were milled with potassium bromide at mass ratio of 1:99 and pressed into disk before IR measurements. Circular dichroism (CD) spectra were recorded on a JASCO1500 spectrometer in deionized water at a concentration of 1 mg/mL at 25 °C. Transmission electron microscopy (TEM) experiments were conducted on a FEI TECNAI 20 with an accelerating voltage of 200 kV. Atomic Force Microscopy (AFM) studies were conducted using tapping mode AFM under ambient conditions.[1] The mica disk was pretreated in a plasma cleaner. Minimal processing of the images was done using NanoScope Analysis software from Bruker. The intensity-averaged hydrodynamic diameter (D_h) of the assemblies was recorded at 25 °C by dynamic light scattering (DLS) using a Malvern NANO ZS90 instrument. Polymer aqueous solutions at different concentrations were prepared by adding deionized water or DTT aqueous solution (10×10^{-3} M). The UV–Vis spectra of the samples were recorded at room temperature using a Shimadzu UV-2910 spectrometer. Fluorescence spectra of the samples were recorded on a F-2700 fluorescence spectrophotometer using 4J1-0008 model at room temperature.

Tandem gel permeation chromatography (GPC) was performed at 50 °C using an SSI pump connected to Wyatt Optilab DSP with 0.02 M LiBr in DMF as the eluent at flow rate of 1.0 mL/min, using monodispersed polystyrene as the calibration standard. All GPC samples were prepared at concentrations of ~5-10 mg/mL.[2]

Synthesis of CYS-NCA and BLG-NCA

L-Cystine (4.5 g, 18.6mmol, 1.0 equiv) was suspended in dry THF (150 mL), and triphosgene (7.5 g, 24.9 mmol, 1.3 equiv) was then added. The mixture was refluxing and stirred at 65 °C under N_2 for 24 h. The turbid mixture was concentrated to 40 mL by rotary evaporation, and the crude product was purified by flash column chromatography using THF/hexane (1:1/v:v) as eluent. After three times dissolving/precipitating with THF/hexane in a glovebox, CYS-NCA was obtained as the light pink solid (1.15 g, 25% yield).[3]

γ-Benzyl-L-glutamate *N*-carboxyanhydride (BLG-NCA) was synthesized by reacting γ-benzyl-L-glutamate with triphosgene in a THF solution as according to the literature procedures.[4]

Typical Synthesis Procedure of poly(ethylene glycol)-b-poly(cystine) (PEG-b-PCYS) and poly(ethylene glycol)-b-poly(cystine-co-PBLG) (PEG-b-P(CYS-co-BLG)).

The polymerization was conducted according to a reported method.[5] In a glove box, the CYS-NCA (350.4 mg, 1.2 mmol) is weight in a Schlenk tube containing a magnetic stirring bar. The Schlenk is removed from the glove box and cooled on ice. Typically, 8 mL of an ice-cooled solution of $NaHCO_3$ (0.05 M) containing the initiator $mPEG_{113}$-NH_2 (300 mg, 0.06 mmol, [M]/[I] = 20:1) is added to the CYS-NCA powder under a strong agitation. The reaction was first stirred in an ice water bath and then stirred overnight at room temperature. The obtained suspension was transferred to a 3.5 kDa dialysis membrane and dialyzed in deionized water for 3 days. An aliquot is kept for further microscopy imaging and dynamic light scattering and the remaining dispersion is lyophilized. Finally, the dispersion was freeze-dried to obtain white flocculent powder (85% yield).

Poly(ethylene glycol)-*b*-poly(cystine-*co*-PBLG) **(**PEG-*b*-P(CYS-*co*-BLG)**)** was also prepared by polymerization of mixtures of CYS-NCA (M_1) and BLG-NCA (M_2) using similar protocol. In this case, 8 mL of an ice-cooled solution of $NaHCO_3$ (0.05 M) containing the initiator $mPEG_{113}$-NH_2 (300 mg, 0.06 mmol, $[M]_1/[M]_2/[I]$ = 15:5:1) is added to the monomers of CYS-NCA (262.8 mg, 0.9 mmol) and BLG-NCA (79.0 mg, 0.3 mmol) under vigorous stirring.

Synthesis of poly(ethylene glycol)-b-poly(cystine-co-glutamic acid) (PEG-b-P(CYS-co-GA)).

The benzyl groups in PEG-*b*-P(CYS-*co*-BLG) was removed by reacting with 4 mol equiv of HBr (C = 33%, in HAc) with respect to BLG repeat units in CF_3COOH (35 mg/mL) at 0 °C for 2.5 h.[4] The final product poly(ethylene glycol)-*b*-poly(cystine-*co*-glutamic acid) (PEG-*b*-P(CYS-*co*-GA)) was obtained after dialysis and lyophilization (75% yield).

Preparation and Disassembly of the Nanogels

When the polymerization is completed, the nanogel has been formed and the pure nanogel product can be obtained after dialysis and removal of impurities without further manipulation. They were lyophilized and collected for easy preservation. In the

subsequent experiments, the freeze-dried samples were dissolved in in DI H_2O and subjected to ultrasonic treatment to obtain nanogel samples at a certain concentration.

The nanogel (6 mg) was dispersed in 0.6 mL deuterated dimethyl sulfoxide (DMSO), followed by addition of 3.6 mg DTT. The resulting solution was stirred at 37 °C for 30 h, and then analyzed by NMR without further purification. For GPC studies, a 5 mg/mL nanogel solution containing 10×10^{-3} M DTT in DMF was incubated at 37 °C for 30 h, which was directly analyzed by GPC without further purification. Nanogel solution (5 mg/mL) without DTT addition was also subjected to GPC measurement and used as a reference.[6] The nanogels can easily dissolve in DMF before and after DTT treatment.

Transmission electron microscopy (TEM) test sample preparation

Samples were prepared by taking liquid from aqueous nanogel solution (1 mg/mL pH=7.5) at ambient temperature. 6μL of the nanogel solution was transferred to a carbon-coated copper grating that had undergone surface hydrophilization in a plasma cleaner, and after allowing the solution to remain on the copper grid for 45 s, excess liquid on the membrane was absorbed with filter paper. The samples were then stained with 0.1% uranyl acetate as the stain for 40 s. After the staining, the grids were similarly subjected to aspiration to remove excess stain. A sample corresponding to the reduced disulfide bond was prepared, and 10×10^{-3}M DTT was added and reacted in a warm bath at 38°C for 30h to obtain an aqueous solution of the degradation product (1 mg/mL, pH = 6.6). The above operation was repeated without staining treatment. Samples were dried and stored under ambient conditions prior to TEM testing.

Atomic Force Microscopy (AFM) test sample preparation

AFM topography and phase maps of nanoparticles were obtained in Tapping mode. Samples were prepared from aqueous nanogel solutions (3mg/mL) at ambient temperature. One drop (7μL) of the clarified solution was deposited on the newly cut mica. Note that the newly cut mica sheets need to be treated with surface hydrophilic treatment in a plasma cleaner in advance. The solution is dispersed well using a homogenizer and the sample is dried and stored under ambient conditions prior to AFM testing. The nanogel DMF solution (3 mg/mL) was repeated, and the mica flakes did not require hydrophilic treatment. Finally, length and width were measured using the cross-sectional particle analysis tool provided by AFM software (Bruker's Nanoscope Analysis 1.7).

Drug Loading and Release

The core cross-linked nanogel (10 mg) and indomethacin (10 mg) were dissolved in 10 mL of DMF, followed by the slow addition of 10 mL of DI H_2O under stirring. The mixture was stirred at room temperature overnight, the free drug and organic solvent was removed by dialysis against DI H_2O to obtain drug-loaded nanogel. The drug loading content as well as loading efficiency was determined by UV-Vis measurement: (DMF solution): λex=320 nm.[7]

The content of drug loaded (DLC) and the drug loading efficiency (DLE) were calculated by the following equations:

DLC (wt.-%)=(weight of loaded drug/weight of polymer)×100%

DLE (wt.-%)=(weight of loaded drug/weight of drug in feed)×100%

In vitro drug release profiles were performed under both reduction insensitive and reduction-sensitive conditions. For the reduction insensitive experiment, 3 mg of the drug-loaded nanogel was suspended in 3 mL of PBS (0.1 mol/L, pH 7.4) containing 0.5% (w/v) SDS and transferred into a dialysis bag (MWCO = 3500 Da). The end-sealed dialysis bag was placed into 17.0 mL of PBS (pH 7.4) at 37 °C with continuous shaking at 100 rpm. 1.0 mL of external release medium was sampled at certain time intervals and an equal volume of fresh release medium was replaced.[8] All of the release experiments were carried out in triplicate, and the averaged results were reported. For the reduction-sensitive experiment, the same method was also applied except for the replacement of the pure PBS by PBS solution with 10×10^{-3} M DTT. Indomethacin was also as a model drug for the drug-loaded and released experiments using the similar procedures. The amount of drug release was determined by UV-Vis spectroscopy at 320 nm using the standard method.[6]

FTIR and NMR Tracing and Conversion Calculation

FTIR and NMR spectra were used to trace the NCA polymerization and conversion determination. FTIR (Abs@1780 cm^{-1}) was chosen to follow the NCA polymerization initiated by $mPEG_{113}$-NH_2 in $NaHCO_3$ aqueous solution at 4 °C. As an example of monomer to initiator [M]/[I] = 20:1: A parallel set of experiments was designed in which individual samples were prepared as follows: CYS-NCA (13 mg, 44.5 mmol) was placed in a 2 mL centrifuge tube with a magnetic stirring bar in a glove box. The tubes were removed from the glove box and cooled in an ice bath, to which 250 μL of $NaHCO_3$ ice-cold water solution containing $mPEG_{113}$-NH_2 initiator at the desired concentration for the polymerization reaction was added. At various time points, the insoluble unreacted monomers were separated and 250 μL of THF was added to individual samples to dissolve the monomers. Ten microliters of the solution were instantly taken and directly coated on KBr salt tablets for FTIR measurements. The reaction rates for different reactant concentration ratios ([M]/[I] = 10:1, [M]/[I] = 20:1, [M]/[I] = 30:1, [M]/[I] = 40:1) were examined by varying the molar ratio of CYS-NCA monomer to initiator $mPEG_{113}$-NH_2 feeding.

The consumption of NCA was also monitored by ^1H NMR in DMSO-d_6. Samples were taken out at certain time intervals for calculation of the monomer conversion. The same set of parallel experiments was prepared with the same pre-dosing ratios as well

as the same experimental methods compared to the Fourier transform infrared spectroscopy (FTIR) tracking experiments. When different time points were reached, the insoluble monomer was separated from the dissolved polymer by centrifugation, and 0.5 mL of deuterated DMSO solution containing the quencher FITC 2 mg/mL was taken and the monomer was dissolved for NMR hydrogen spectroscopy.

Thiol-initiated Cleavage of Disulfide Linkages and Further Modification by Thiol−ene Addition

The purified, freeze-dried nanogel (200 mg) was dissolved in DMF (5 mL) and mixed with DTT (10×10^{-3} M) under stirring at 37 °C for 38 hrs. The products were precipitated from cold hexane for 4 times, and then dried under vacuum. Aliquots were taken for ^{1}H NMR analysis to confirm the cleavage of pendant disulfide linkages.[9] The pendant thiols were further modified using azobisisobutyronitrile (AIBN) as radical source at elevated temperature. A solution of reduced product in DMF (50 mg/mL) was mixed with ethyleneglycol monomethyl ether acrylate (or ethyl acrylate) and AIBN (molar ratio: [C=C]：[SH]：[AIBN]=5：1：1).[10, 11] Three freeze/thaw/pump cycles were performed for the reaction mixture and the resulting solution was stirred for 24 h at 70 °C under a nitrogen atmosphere. The solution was then precipitated in cold ethyl ether three times, and then dried under vacuum. The functionalization efficiency was determined by ^{1}H NMR analysis.

Supporting Figures and Tables

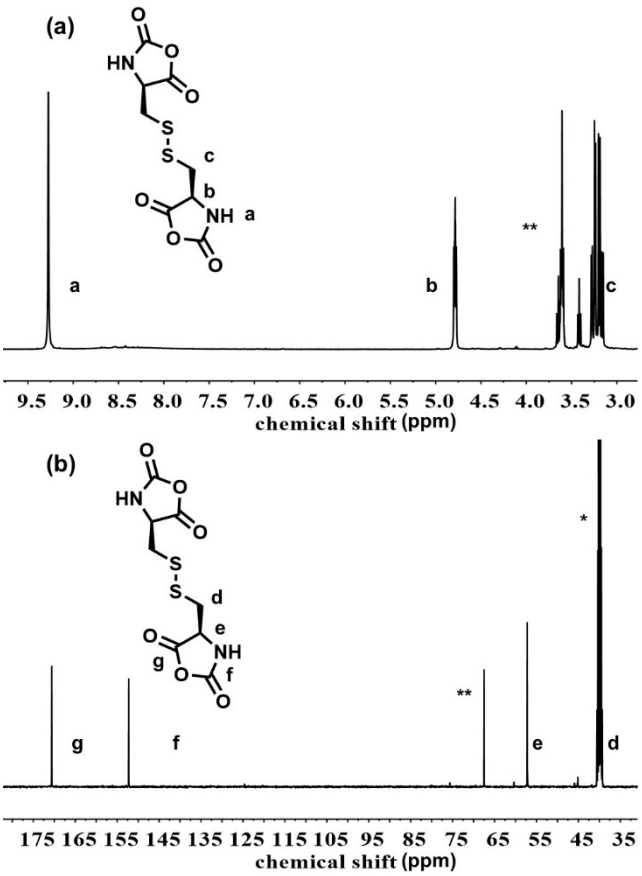

Figure S1. ^{1}H NMR (a) and ^{13}C NMR (b) spectra of CYS-NCA in DMSO-d_6. (* indicates DMSO-d_6. ** indicates residual THF.)

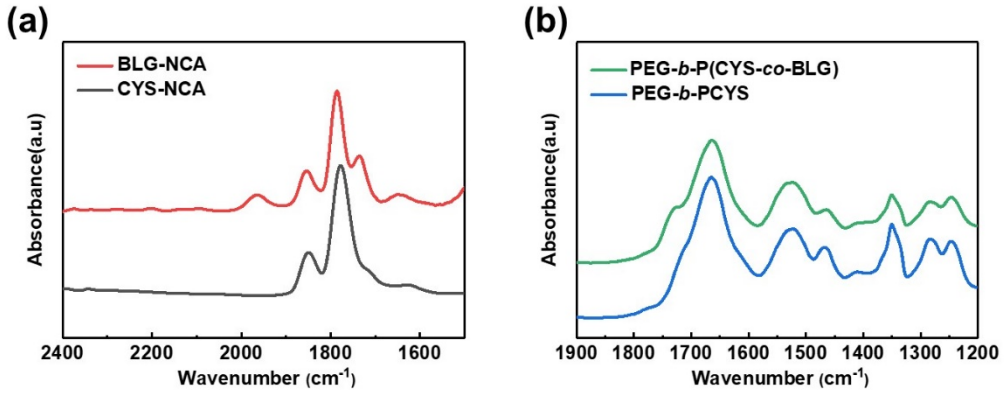

Figure S2. (a) FTIR spectra of BLG-NCA and CYS-NCA. (b) FTIR spectra of PEG-*b*-PCYS and PEG-*b*-P(CYS-*co*-BLG) in solid state.

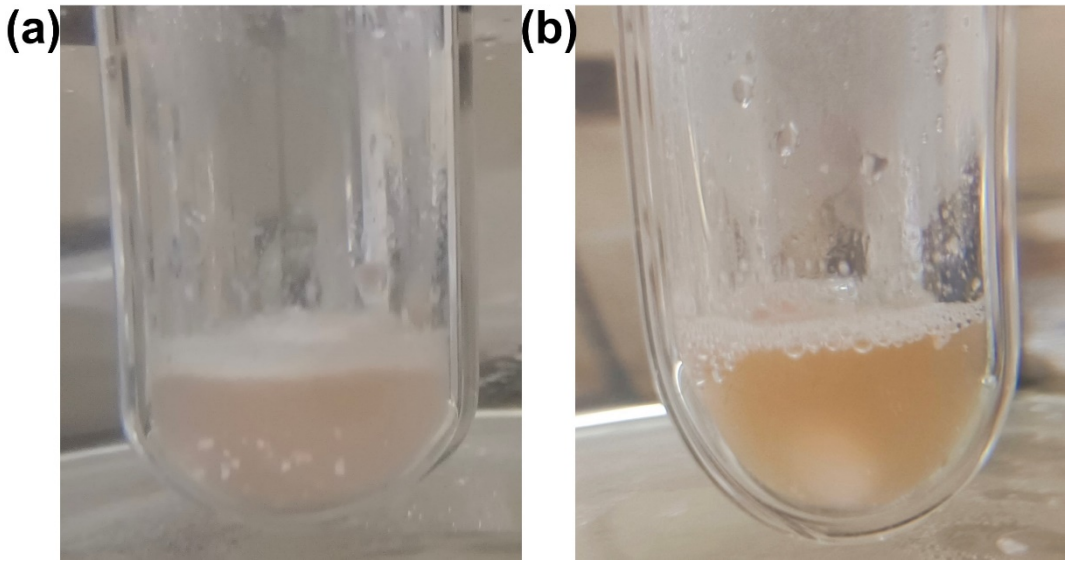

Figure S3. The pictures of (a) PEG-*b*-PCYS and (b) PEG-*b*-P(CYS-*co*-BLG) nanogels at the end of the polymerization.

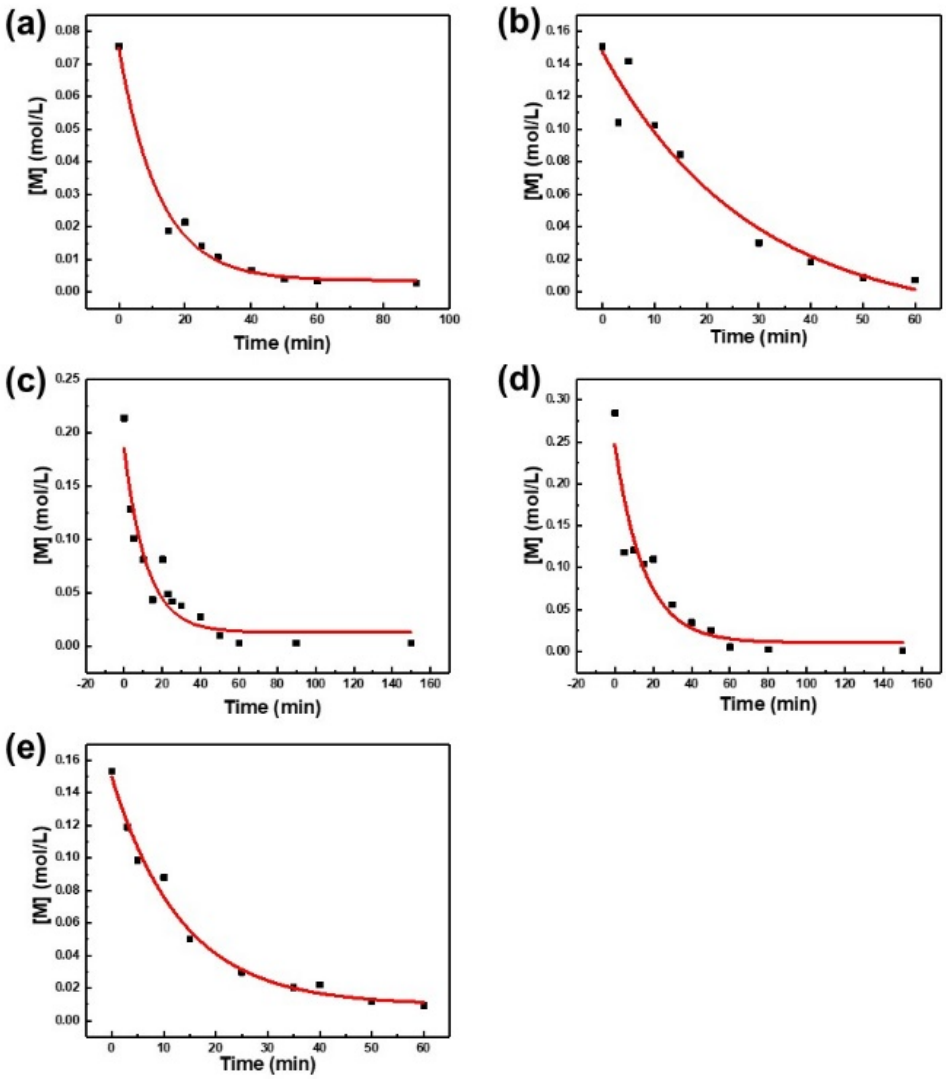

Figure S4. Kinetic study by FTIR (Abs@1780 cm^{-1}) of $mPEG_{113}$-NH_2 initiated (a) CYS-NCA ([CYS-NCA: I] = 10:1) , (b) CYS-NCA ([CYS-NCA: I] = 20:1) , (c) CYS-NCA ([CYS-NCA: I] = 30:1) , (d) CYS-NCA ([CYS-NCA: I] = 40:1) and (e) CYS-NCA and BLG-NCA ([CYS-NCA: BLG-NCA: I] = 15:5:1) polymerization at 4 °C. All of the initiator $mPEG_{113}$-NH_2 concentrations were 0.0075 M. (a, b, c and d) Evolution of the CYS-NCA concentration over time and fit using an exponential decay. (e) Evolution of the CYS-NCA and BLG-NCA concentration over time and fit using an exponential decay.

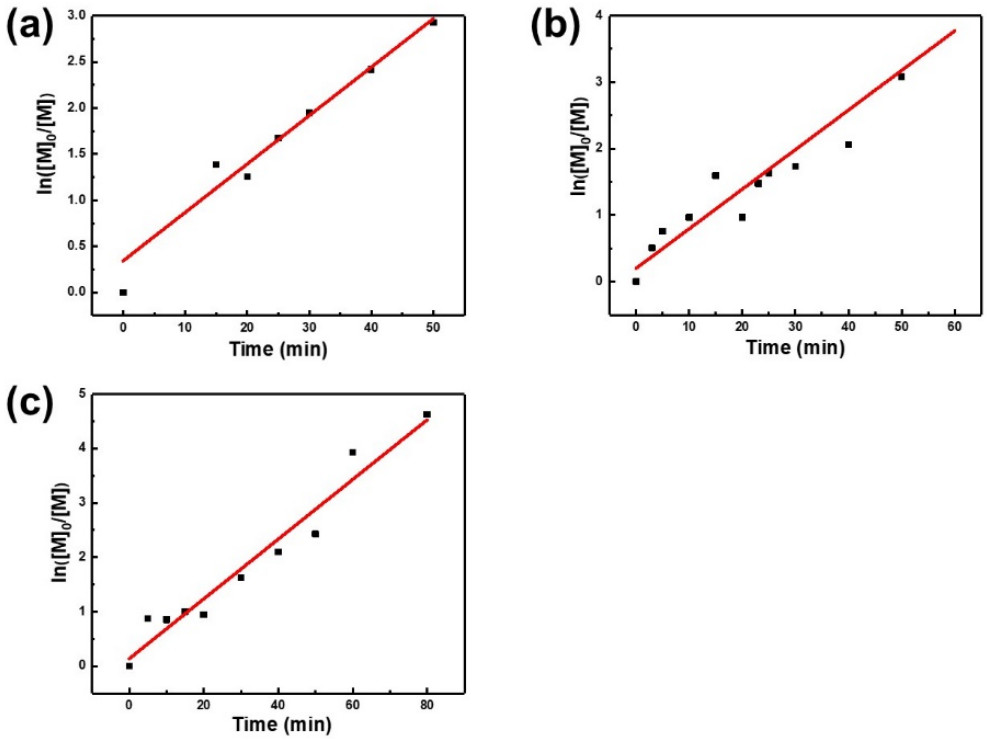

Figure S5. Kinetic study by FTIR (Abs@1780 cm^{-1}) of $mPEG_{113}$-NH_2 initiated (a) CYS-NCA ([CYS-NCA: I] = 10:1), (b) CYS-NCA ([CYS-NCA: I] = 30:1) and (c) CYS-NCA ([CYS-NCA: I] = 40:1) polymerization at 4 °C. All of the initiator $mPEG_{113}$-NH_2 concentrations were 0.0075 M. (a, b and c) Logarithm of initial CYS-NCA concentration normalized by the CYS-NCA concentration over time.

Table S1 Solids content and corresponding polymerization apparent kinetics rate constant. Diblock copolymers obtained by ROPIG.

Monomer	$[M]_0/[I]_0$	Conversion [%]	Solid content (τ_s)%	k_p [L mol^{-1} s^{-1}]^a	k_p [L mol^{-1} s^{-1}]^b
CYS-NCA	10/1	96.44	4.79	0.12	
CYS-NCA	20/1	98.93	5.51	0.11	0.13
CYS-NCA+BLG-NCA	15/5/1 [a]	94.04	5.87	0.10	0.10
CYS-NCA	30/1	93.24	8.12	0.13	
CYS-NCA	40/1	99.51	8.74	0.12	

All polymerizations were carried out using $mPEG_{113}$-NH_2 at a concentration of 0.0075 M as initiator. *a*: Kinetic study by FTIR (Abs@1780 cm^{-1}). *b*: Kinetic study followed by ^1H NMR in DMSO. [a][CYS-NCA: BLG-NCA: I] = 15:5:1.

Solid contentwere determined by the following formula:

solid content (wt.-%)= dry constant weight / material original weight × 100%

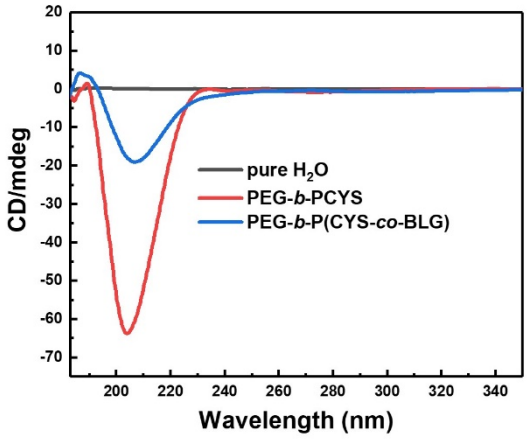

Figure S6. CD (circular dichroism) spectra of PEG-*b*-PCYS and PEG-*b*-P(CYS-*co*-BLG) nanogels in aqueous solution.

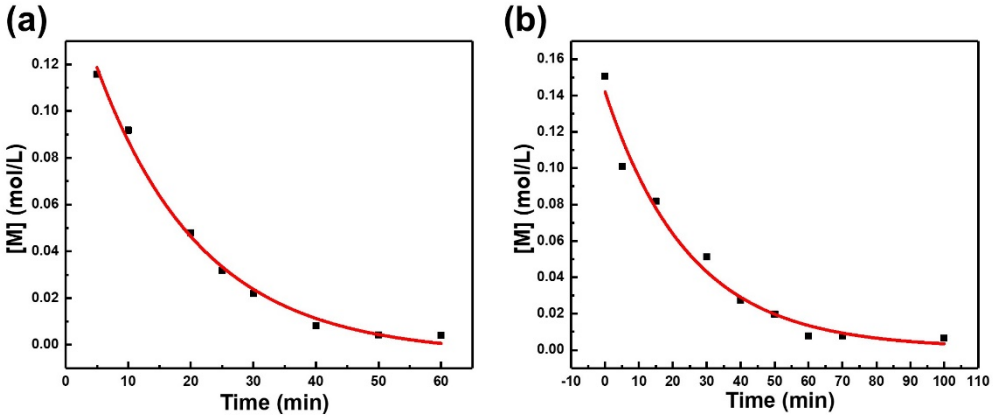

Figure S7. Kinetic study by ^{1}H NMR in DMSO of $mPEG_{113}$-NH_2 initiated (a) CYS-NCA ([CYS-NCA: I] = 20:1) and (b) CYS-NCA and BLG-NCA ([CYS-NCA: BLG-NCA: I] = 15:5:1) polymerization at 4 °C. Both of the initial NCA concentrations were 0.15 M. (a) Evolution of the CYS-NCA concentration over time and fit using an exponential decay. (b) Evolution of both the CYS-NCA and the BLG-NCA concentration over time and fit using an exponential decay. At each point of the kinetic, an aliquot of the reaction media is quenched using fluorescein isothiocyanate (FITC).

Table S2. Molecular parameters of nanogels before and after DTT treatment.

Monomer	$[M]_0/[I]_0$[a]	Mn/kDa[b]	$Đ$[b]	Percentage/%
PEG-*b*-PCYS	20/1	116	1.08	100
PEG-*b*-PCYS-SH	20/1	12, 1111[d]	1.08, 1.21[d]	63, 37[e]
PEG-*b*-P(CYS-*co*-BLG)	15/5/1[c]	133	1.10	100
PEG-*b*-P(CYS-*co*-BLG-SH)	15/5/1[c]	12, 1277	1.26, 1.21	90, 10[e]

[a] Indicates monomer/initiator ratio. [b] Molecular weight and polydispersity index determined by GPC with 0.02 M LiBr in DMF at 50 °C, calibrated by polystyrene standard. [c] [CYS-NCA: BLG-NCA: I] = 15:5:1. [d] The molecular parameters for two peaks in GPC traces. [e] Percentages of the two peak areas determined by GPC.

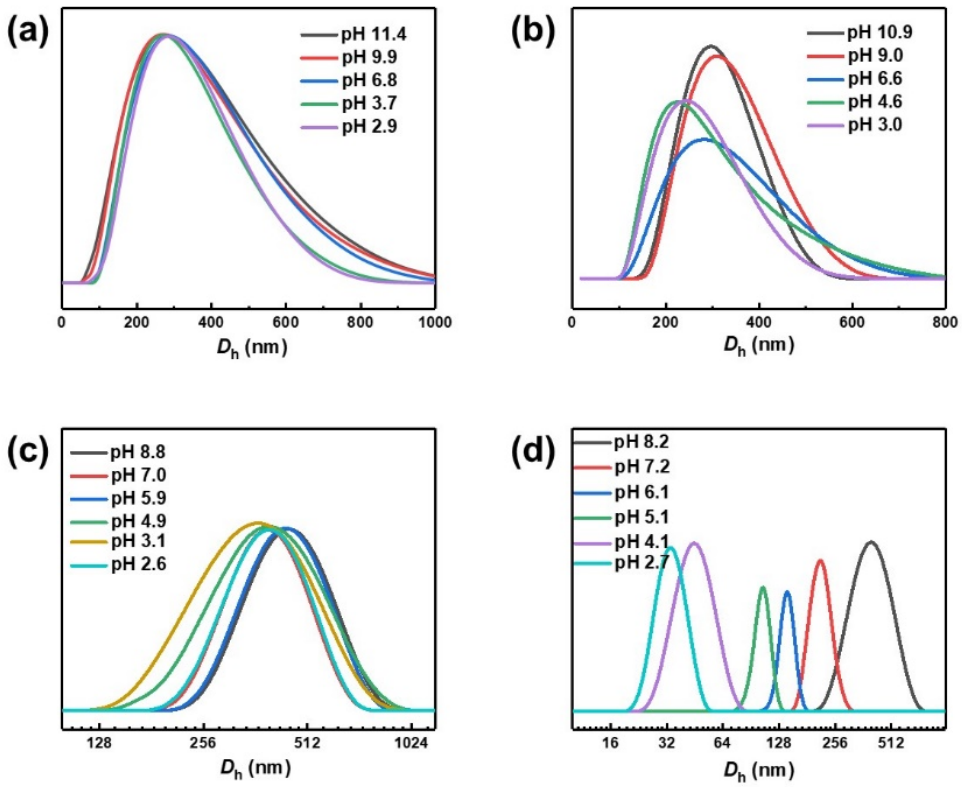

Figure S8. D_h of PEG-*b*-P(CYS-*co*-BLG) (a) before and (b) after DTT treatment. D_h of PEG-*b*-P(CYS-*co*-GA) (c) before and (d) after DTT treatment. The D_h was measured by DLS in MQ water at different pH values.

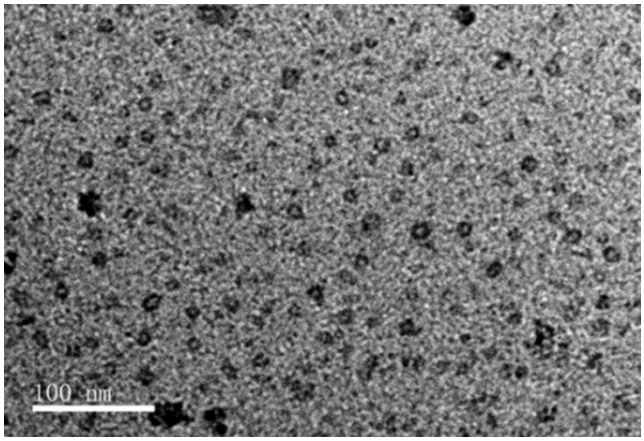

Figure S9. TEM images of PEG-*b*-PCYS-SH. The sample was not stained.

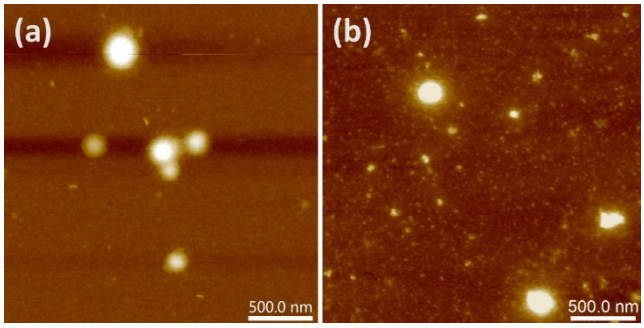

Figure S10. AFM images of PEG-*b*-PCYS nanogel (a) and PEG-*b*-P(CYS-*co*-BLG) nanogel (b) assembled in DMF for 3 hrs.

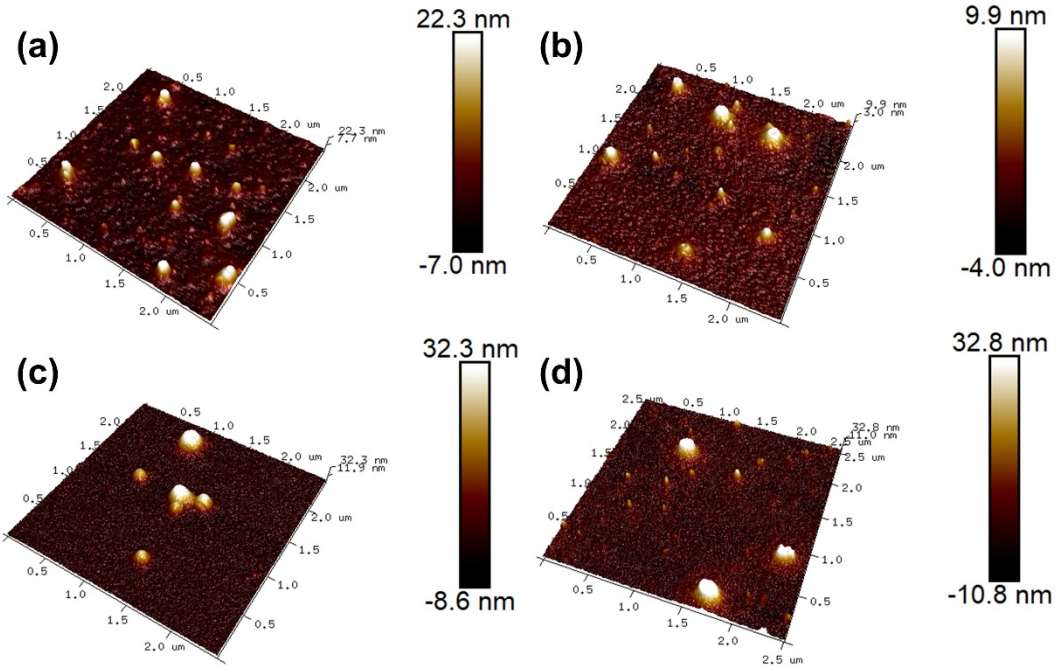

Figure S11. A scale and height profile of AFM images. PEG-*b*-PCYS nanogel assembled in (a) water and (c) DMF for 3 h. PEG-*b*-P(CYS-*co*-BLG) nanogel assembled in (b) water and (d) DMF for 3 h.

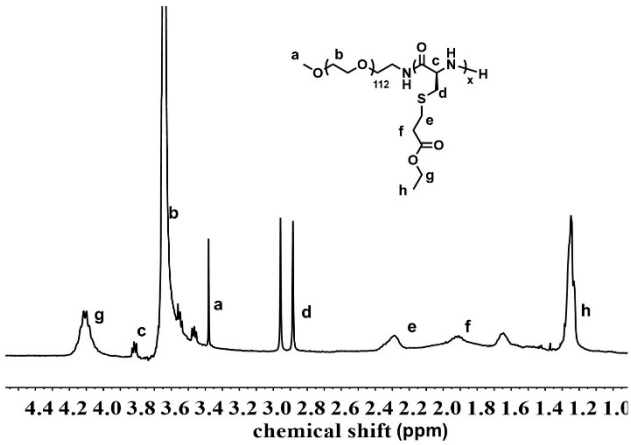

Figure S12. ^{1}H NMR spectra of PEG-*b*-(PCYS-*g*-EA) in $CDCl_3$.

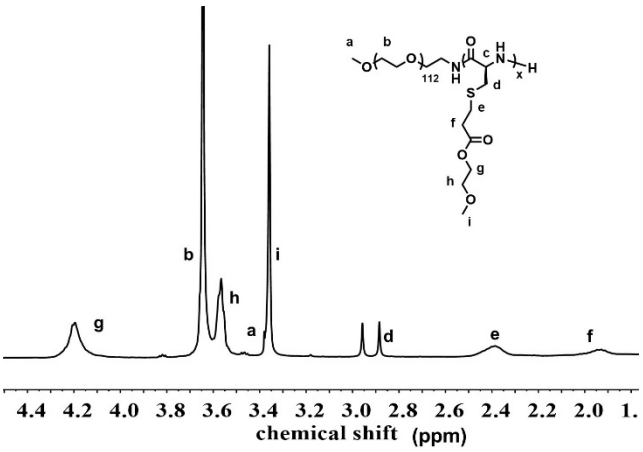

Figure S13. ^{1}H NMR spectra of PEG-*b*-(PCYS-*g*-EGMEA) in $CDCl_3$.

References

1 J. Wei, J. Sun, X. Yang, S. Ji, Y. Wei and Z. Li, *Polym Chem-Uk*, 2020, **11**, 337-343.
2 C. Xing, Z. Shi, J. Tian, J. Sun and Z. Li, *Biomacromolecules*, 2018, **19**, 2109-2116.
3 E. D. Raftery, E. G. Gharkhanian, N. G. Ricapito, J. McNamara and T. J. Deming, *Chem Asian J*, 2018, **13**, 3547-3553.
4 Y. Ni, J. Sun, Y. Wei, X. Fu, C. Zhu and Z. Li, *Biomacromolecules*, 2017, **18**, 3367-3374.
5 C. Grazon, P. Salas-Ambrosio, E. Ibarboure, A. Buol, E. Garanger, M. W. Grinstaff, S. Lecommandoux and C. Bonduelle, *Angew Chem Int Ed Engl*, 2020, **59**, 622-626.
6 T. Xing, B. Lai, X. Ye and L. Yan, *Macromol Biosci*, 2011, **11**, 962-969.
7 G. Mocanu, D. Mihai, D. LeCerf, L. Picton and M. Moscovici, *J Appl Polym Sci*, 2009, **112**, 1175-1183.
8 L. Dong, H. Chen, T. Liu, J. Zhu, M. Yu and Q. Yuan, *Biomacromolecules*, 2021, **22**, 5374-5381.
9 N. Chan, S. Y. An, N. Yee and J. K. Oh, *Macromol Rapid Commun*, 2014, **35**, 752-757.
10 Y. Xiao, C. Tang, Y. Chen and M. Lang, *Biopolymers*, 2019, **110**, 23318-23327.
11 J. Sun and H. Schlaad, *Macromolecules*, 2010, **43**, 4445-4448.