

Clickable, Oxidation-Responsive and Enzyme-Degradable Polypeptide: Synthesis, Characterization and Side Chain Modification

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Electronic Supplementary Information

Abstract Modifications at polypeptide side chains have been found to affect conformational and properties, which is a vital strategy to prepare functional polypeptides. This contribution describes the synthesis of homo and co-polypeptides containing unsaturated bonds and thioether moieties, poly(*S*-allylcysteine) (PAC). The pendant vinyl groups provide an active binding site which are able to be further modified with cysteine by thiol-ene click reaction. The sulfoxide from thioether oxidation bestows the polypeptide water solubility and enzymatic catalytic sites. The self-assembly micelles of PEG-*b*-PAC are ruptured along with oxidation to release the Nile red payloads. Moreover, poly(*S*-allyl-cysteine sulfoxide), *aka*. polyalliin, is the oxidation product of PAC which undergoes side chain cleavage catalyzed by alliinase enzyme. The polypeptides are biocompatible as confirmed by cell viability assays and may find applications in drug delivery, biosensing and nanoreactor.

Keywords Polypeptides; Ring-opening polymerization; Stimuli-responsive polymers; *N*-carboxy anhydrides; *N*-phenoxycarbonyl α -amino acids

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INTRODUCTION

Post-translational modification of proteins expands the diversity of the proteome and allows for tailoring the function of proteins. As a vital approach to prepare functional polypeptides, side chain modification facilitates the investigation of naturally modified protein.^[1–7] The chemical structure of side chain has a decisive impact on biological and physicochemical properties of polypeptides such as solubility and secondary structure.^[8–13] Polypeptides bearing functional side chains have been widely used in self-assembly,^[14,15] drug delivery, tissue engineering, and antibiotics.^[16–21]

Side chain functionalized polypeptides possess high reactivity residues such as amines, thiols, alkenes, alkynes, azides and thioethers.^[5,6,22,23] Polypeptides with pendent thioethers, a competent reactive oxygen species (ROS)-responsive drug carriers, have aroused considerable interest of researchers owing to the discovery that various diseases are highly correlated with the accumulation of ROS *in vivo*.^[19,24] Thiol-ene reaction is a powerful strategy to modify the side chain of polypeptides.^[25,26] Unsaturated bonds provide a platform to obtain functional biomaterials by combining molecules containing thiols.^[27,28] Specifically, Schlaad and coworkers

reported thiol-ene clickable polypeptides based on allyl-glycine.^[9,10,29,30]

Advanced synthetic techniques toward polypeptides have paved the way to design polypeptides with desired structure and properties. The ring-opening polymerization (ROP) of α -amino acid *N*-carboxyanhydrides (NCAs) is a controllable and feasible pathway to obtain well-defined polypeptides with high molecular weights (MW) and low dispersities (*D*).^[13,31] The polymerization of *N*-phenoxycarbonyl α -amino acids (NPCs), precursors of NCAs, is a phosgene-free synthetic approach of polypeptides. NPC is more stable than NCA to normal nucleophilic compounds like water and alcohol.^[32–34] These advantages enable NPC to serve as an indispensable monomer to synthesize polypeptides. *S*-allylcysteine (AC), a water-soluble natural amino acid in garlic, is known for inhibiting tumor proliferation and high antioxidant activities.^[35–39] Poly(*S*-allylcysteine) (PAC) was firstly mentioned in 1955.^[40] However, its synthetic method and characterization were very limited^[5,13,23] and no further studies were followed in the past 60 years. Herein, we report the synthesis of PAC in controllable manners from L-AC-NCA and DL-AC-NPC. Vinyl and thioether groups in side chain are proven to be active for functionalization. The polypeptides containing alkene group are able to be conjugated by thiol-ene click reaction. The designed amphiphilic diblock copolymer, composed of hydrophilic poly(ethylene glycol) (PEG) and hydrophobic PAC segments, self-assembles into micelles

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which is readily to rupture during oxidation of hydrophobic thioethers into hydrophilic sulfoxides and to release Nile red (NR) loaded as a model drug. Furthermore, poly(*S*-allylcysteine sulfoxide) (PAll), aka. polyalliin, is prepared for the first time by the oxidation of PAC. The side chains of PAll are decomposed in the presence of alliinase.

EXPERIMENTAL

Materials

All polymerizations were carried out using Schlenk technique under anhydrous conditions, and all polymerization tubes were purged with argon. Neopentylamine (98%) and benzylamine (BnNH_2) were distilled over CaH_2 before use. DL-cysteine HCl (98%, Shuya Medical Technology, Shanghai, China), L-cysteine (99%, Adamas-beta), allyl bromide (98%, Macklin), pyridoxal phosphate (98%, Macklin), phenyl chloroformate (98%, Aladdin Chemical Co., Ltd.), triphosgene (99%, Aladdin Chemical Co., Ltd), *m*-PEG- NH_2 (Aladdin Chemical Co., Ltd., samples with MW of 2000 and 5000), *m*-PEG- NH_2 (J&K samples with MW of 5000), and alliinase (EC 4.4.1.4, enzyme activity ≥ 1000 U/g, Aladdin Chemical Co., Ltd.), NR (97%, Energy Chemical), dimethyl sulfoxide (DMSO, super dry, Energy Chemical), *N,N*-dimethylformamide (DMF, super dry, Energy Chemical), dulbecco's modified Eagle's medium (DMEM, Gibico), penicillin (Gibico), streptomycin (Gibico), fetal bovine serum (FBS, Gibico), Cell Counting Kit-8 (CCK-8, Dojindo) were used as received. All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. and used as received.

Measurements

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance DMX 400 spectrometer (^1H : 400 MHz and ^{13}C : 100 MHz). A size-exclusion chromatography (SEC) equipment composed of Waters 1515 isocratic HPLC pump, Waters 2414 refractive index detector and Hexafluoroisopropanol (HFIP) series columns (HFIP-803 and HFIP-805 from Shodex, MW between 1000 and 6×10^5) was used to determine MW and $\bar{M}_w/\bar{M}_n$ of polymers. HFIP, containing 3 g/L of CF_3COOK , was used as eluent with a flow rate of 0.8 mL/min at 40 °C, and commercial poly(methyl methacrylate) were used as calibration standards. In alternative measurements, SEC equipped with two MZ-Gel SDPlus columns (particle size: 10 μm , porosity: 100 and 1×10^4 Å) and a Wyatt Optilab T-rEX interferometric refractometer were used to determine the MW and $\bar{M}_w/\bar{M}_n$ of polymers. DMF (containing 0.05 mol/L LiBr and 0.2% (V/V) triethylamine) was used as eluent with a flow rate of 0.8 mL/min at 50 °C, and commercial poly(methyl methacrylate) were used as calibration standards. Matrix-assisted laser desorption ionization-time of flight (MALDI-ToF) mass spectra were collected on a Bruker UltraFLEX MALDI-ToF mass spectrometer. 2,5-Dihydroxybenzoic acid (DHB) was used as matrix and potassium trifluoroacetate was used as the cationic agent. Fourier transform infrared (FTIR) spectra were recorded between 400 and 4000 cm^{-1} using a Tensor II FTIR spectrometer (Bruker, Germany). Hydrodynamic diameters (D_h) of nanoparticles were detected at 25 °C by dynamic light scattering (DLS) on Zetasizer Nano Series of Malvern Instruments with the wavelength of 657 nm and fixed angle of 173°. Transmission electron microscopy (TEM) images were taken from HT7700 equipment (Hitachi, Japan). Polymer micelle solution (1

mg/mL, 8 μL) was placed on a carbon-film coated copper grid in ambient condition followed by 5 μL of 1% phosphotungstic acid solution. The fluorescence spectra were obtained using a PTI TimeMaster fluorimeter equipped with a PTI nitrogen laser (GL-3300, $\lambda = 266$ nm, pulse width ~ 1 ns, pulse energy = 1.45 mJ). Circular dichroism (CD) spectra were detected using circular dichroism spectroscopy (JASCO, J-1500-150ST). Electrospray ionization mass spectrometry (ESI-MS) spectra were detected at LCMS-IT-TOF (Shimadzu). Ultraviolet (UV) irradiation experiments were carried out through a Hamamatsu LC-8 light source equipped with a mercury xenon lamp of 2 kW. Gas chromatography mass spectrometry were recorded on GCMS-QP2010 (Shimadzu).

Synthesis of L-AC-NCA and DL-AC-NPC

Synthesis of L/DL-AC

L-cysteine (24.2316 g, 200 mmol) and allyl bromide (36.2931 g, 300 mmol) were added in 2 mol/L of $\text{NH}_3 \cdot \text{H}_2\text{O}$ (300 mL) and stirred at room temperature for 20 h. White product was obtained by precipitation in ethanol for three times and dried under reduced pressure (16.84 g, 52%). $^1\text{H-NMR}$ (400 MHz, D_2O , δ , ppm) 5.82 (ddt, $J = 17.1, 9.9, 7.2$ Hz, 1H), 5.33–5.08 (m, 2H), 3.89 (dd, $J = 7.5, 4.3$ Hz, 1H), 3.11 (dq, $J = 7.4, 1.2$ Hz, 2H), 3.13–2.80 (m, 2H). ESI-MS (+) (m/z): $[\text{M}+\text{H}]^+$ calculated for $\text{C}_6\text{H}_{12}\text{NO}_2\text{S}^+$: 162.0590, found: 162.0608; $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_6\text{H}_{11}\text{NO}_2\text{SNa}^+$: 184.0408, found: 184.0437; $[\text{M}+\text{H}_2\text{O}+\text{Na}]^+$ calculated for $\text{C}_6\text{H}_{13}\text{NO}_3\text{SNa}^+$: 202.0543, found: 202.0927. ESI-MS (–) (m/z): $[\text{M}-\text{H}]^-$ calculated for $\text{C}_6\text{H}_{10}\text{NO}_2\text{S}^-$: 160.0430; found: 160.0473.

DL-AC was synthesized by the same procedure above with yield of 48%. $^1\text{H-NMR}$ (400 MHz, D_2O , δ , ppm) 5.82 (ddt, $J = 17.2, 10.0, 7.2$ Hz, 1H), 5.35–5.03 (m, 2H), 3.89 (dd, $J = 7.5, 4.3$ Hz, 1H), 3.20 (dq, $J = 7.2, 1.2$ Hz, 2H), 3.09–2.85 (m, 2H).

Synthesis of L-AC-NCA

To a pressure vessel with a heavy wall, L-AC (4.8366 g, 30 mmol, 1.0 eq.), tetrahydrofuran (THF, 100 mL) and methyloxirane (9 mL, 120 mmol, 4 eq.) were added under magnetic stirring. Triphosgene (4.4 g, 15 mmol, 0.5 eq.) was then added in one portion and the vessel was sealed immediately. The mixture was stirred at room temperature for 0.5 h and filtered. After the removal of the solvent by rotatory evaporation in a vacuum under 25 °C, the crude product was purified by crystallization in *n*-hexane/THF. The pure product was obtained as a white powder (4.02 g, yield = 72%). The NCA was stored at –20 °C. $^1\text{H-NMR}$ (500 MHz, $\text{DMSO}-d_6$, δ , ppm): 9.15 (s, 1H), 5.74 (ddt, $J = 17.1, 9.9, 7.2$ Hz, 1H), 5.36–4.89 (m, 2H), 4.77 (d, $J = 1.2$ Hz, 1H), 3.50–2.98 (m, 2H), 2.85 (d, $J = 4.4$ Hz, 2H). $^{13}\text{C-NMR}$ (101 MHz, $\text{DMSO}-d_6$, δ , ppm): 170.69, 152.19, 134.21, 118.13, 58.15, 34.94, 31.07. ESI-MS (m/z): $[\text{M}-\text{H}]^-$ calculated for $\text{C}_7\text{H}_8\text{NO}_3\text{S}^-$: 186.0223, found: 186.0271.

Synthesis of DL-AC-NPC

Phenyl chloroformate (36.5 mmol, 5.715 g) was added into a suspension of DL-AC (36.5 mmol, 5.885 g) in ethyl acetate (150 mL). The reaction mixture was stirred at 45 °C for 48 h and filtrated. The filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography from 10:1 to 1:1 PE/EA and then recrystallized from PE/EA. The pure product was obtained as a white solid powder (8.89 g, yield = 45%). $^1\text{H-NMR}$ (400 MHz, $\text{DMSO}-d_6$, δ , ppm) 12.99 (s, 1H), 8.19 (d, $J = 8.4$ Hz, 1H), 7.57–6.82 (m, 5H), 5.78 (ddt, $J = 17.1, 9.9, 7.1$

Hz, 1H), 5.38–4.82 (m, 2H), 4.17 (td, $J=9.1, 4.6$ Hz, 1H), 3.32–3.04 (m, 2H), 3.04–2.57 (m, 2H). ^{13}C -NMR (101 MHz, DMSO- d_6 , δ , ppm) 172.00, 154.33, 150.87, 134.24, 129.31, 125.06, 121.57, 117.45, 53.82, 33.91, 31.39. ESI-MS (+) (m/z): $[\text{M}+\text{H}]^+$ calculated for $\text{C}_{13}\text{H}_{16}\text{NO}_4\text{S}^+$: 282.0802, found: 282.0816; $[\text{M}+\text{Na}]^+$ calculated for $\text{C}_{13}\text{H}_{15}\text{NO}_4\text{SNa}^+$: 304.3158, found: 304.0638. ESI-MS (–) (m/z): $[\text{M}-\text{phenol}-\text{H}]^-$ calculated for $\text{C}_7\text{H}_8\text{NO}_3\text{S}^-$: 186.2050, found: 186.0260.

Polymerization of L-AC-NCA and Postmodification by Thiol-ene Click Reaction

Polymerization of L-AC-NCA

As an example, L-AC-NCA (564 mg, 3.0 mmol, 30 eq.) was dissolved in anhydrous DMF (5 mL), followed by addition of BnNH_2 in DMF (1 mL, 0.1 mol/L, 1 eq.). The polymerization was carried out at 30 °C for 24 h. After precipitation in ether, centrifugation, and vacuum drying, poly(S-allyl-L-cysteine) (PLAC) was obtained as a white powder (362 mg, yield=82%).

Thiol-ene click reaction

Into a PLAC/HFIP solution (10 mg/mL), H_2O_2 (30 wt%, 2 eq. per thioether residue) was added. The solution was vigorously shaken at 25 °C for 20 h. The excess oxidant was then quenched with a few drops of aqueous Na_2SO_3 solution (10 wt%). Subsequently, the reaction mixture was dialyzed against deionized water for 24 h in a dialysis bag with a molecular weight cutoff (MWCO) of 500 g/mol. The retentate was lyophilized to provide PLAll.

PLAll (100 mg, 0.63 mmol All units, 1 eq.) and benzophenone (11.48 mg, 0.1 eq.) were dissolved in 3 mL of HFIP. L-cysteine (152 mg, 2 eq.) was dissolved in 3 mL of deionized water. The two solutions were mixed and reacted under UV light (365 nm, 3000 MW/cm ^{2}) for 70 min. The mixtures were dialyzed against deionized water for 30 h in a dialysis bag with a MWCO of 500 g/mol. The retentate was lyophilized to obtain a white powder (PLAll-g-cys, 80.3 mg, yield=50%).

Copolymerization of DL-AC-NPC and Oxidation-Responsive Behavior of PEG-*b*-PAC Micelles

Copolymerization of DL-AC-NPC

DL-AC-NPC (422 mg, 1.5 mmol) and *m*-PEG-NH $_2$ (250 mg, 0.05 mmol, MW=5000) were dissolved in 3 mL of anhydrous DMSO in a flame-dried Schlenk tube. The tube was placed in an oil bath at 80 °C for 24 h. The reaction mixture was cooled to room temperature and precipitated by diethyl ether. After centrifugation and vacuum drying, PEG-*b*-PAC was obtained with the yield of 71% (330 mg).

Oxidation-responsive behavior of PEG-*b*-PAC micelles

PEG-*b*-PAC (10 mg) was dissolved in 2 mL of DMF. A total 4 mL of deionized water (33 $\mu\text{L}/\text{min}$) was injected slowly in 2 h to the above solution under mild shaking at 25 °C. The mixture was then dialyzed against deionized water for 2 days in a dialysis bag with a MWCO of 500 g/mol to remove DMF. Aqueous solutions of NR-encapsulated micelles were prepared by a similar process. The solution of NR (1 mL, 0.14 mg/mL in THF) was exposed in the air overnight to completely evaporate the solvent before 10 mg of PEG-*b*-PAC was added.

DLS spectra and fluorescence emission spectra were obtained by following process. Micelle solution (1 mg/mL, 5 mL), H_2O_2 (30 wt%, 2.5 mL), and 0.5 mL of acetic acid were mixed under mildly magnetic stirring at room temperature.

1 mL of solution was taken each time for test and the sample was discarded directly without recycling.

Oxidation kinetic curves of PEG-*b*-PAC determined by ^1H -NMR were obtained by the following process. PEG-*b*-PAC (200 mg), H_2O (50 mL), H_2O_2 (30 wt%, 1 mL) and acetic acid (0.5 mL) were mixed under mildly magnetic stirring at room temperature. 6 mL of solution was taken each time and 1 mL of Na_2SO_3 (10 wt%) was added to terminate the reaction. The samples were purified by dialysis and lyophilized.

Homopolymerization of DL-AC-NPC and Enzymatic Decomposition

Homopolymerization of DL-AC-NPC

DL-AC-NPC (2.813 g, 10 mmol) was dissolved in 7.4 mL of anhydrous DMSO in a flame-dried Schlenk tube, followed by the addition of 2.6 mL of neopentylamine in DMSO solution (0.127 mol/L). The tube was placed in an oil bath at 80 °C for 48 h. The reaction mixture was cooled to room temperature and precipitated by diethyl ether. After centrifugation and vacuum drying, PAC was obtained with the yield of 71% (1.038 g).

Enzymatic decomposition of PAll

PAC (500 mg, 3.5 mmol repeat units) was dissolved in 6 mL of deionized water. H_2O_2 (30 wt%, 3 mL), and acetic acid (1 mL) were added under mildly magnetic stirring at room temperature (below 10 °C) for overnight until the system became completely transparent. Na_2SO_3 solution (10 wt%, 3 mL) was added to terminate the reaction. PAll was isolated after dialysis and lyophilization.

PAll (30 mg) and alliinase (60 mg) suspended in 6 mL of phosphate buffered saline (pH=6.5) containing pyridoxal phosphate (0.02 mmol/L) were placed in an incubator at 37 °C for 14 days. The enzymatic hydrolysis process was monitored by SEC. The solvent was removed by rotary evaporator before the products were dissolved in HFIP for SEC measurement.

Cell Viability Test

CCK-8 assay was performed to characterize the cytotoxicity of PAC. NIH 3T3 cells incubated in DMEM medium were seeded in a 96-well plate at 1×10^4 cells per well and cultivated at 37 °C and 5% CO_2 atmosphere for 24 h. Then, the medium was replaced with DMEM medium containing PAll-1, *m*-PEG-*b*-PAC-5 and PEG-*b*-PAll-5, respectively, with different concentrations (250, 500, 1000 and 2500 $\mu\text{g}/\text{mL}$). An equal volume of DMEM medium served as a control. After 24 h incubation, 10 μL of CCK-8 solution was added to each well and cultured for another 2 h. Finally, the absorption value at 450 nm of each well was measured using a microplate reader (iMark, Bio-red, USA). The cell viability (%) was calculate by:

$$\text{Cell viability} = \frac{\text{OD}_{\text{ex}}}{\text{OD}_{\text{control}}} \times 100\% \quad (1)$$

where OD_{ex} and $\text{OD}_{\text{control}}$ were the absorption values of the experimental and control wells, respectively. Three replicates were used for cytotoxicity assay.

RESULTS AND DISCUSSION

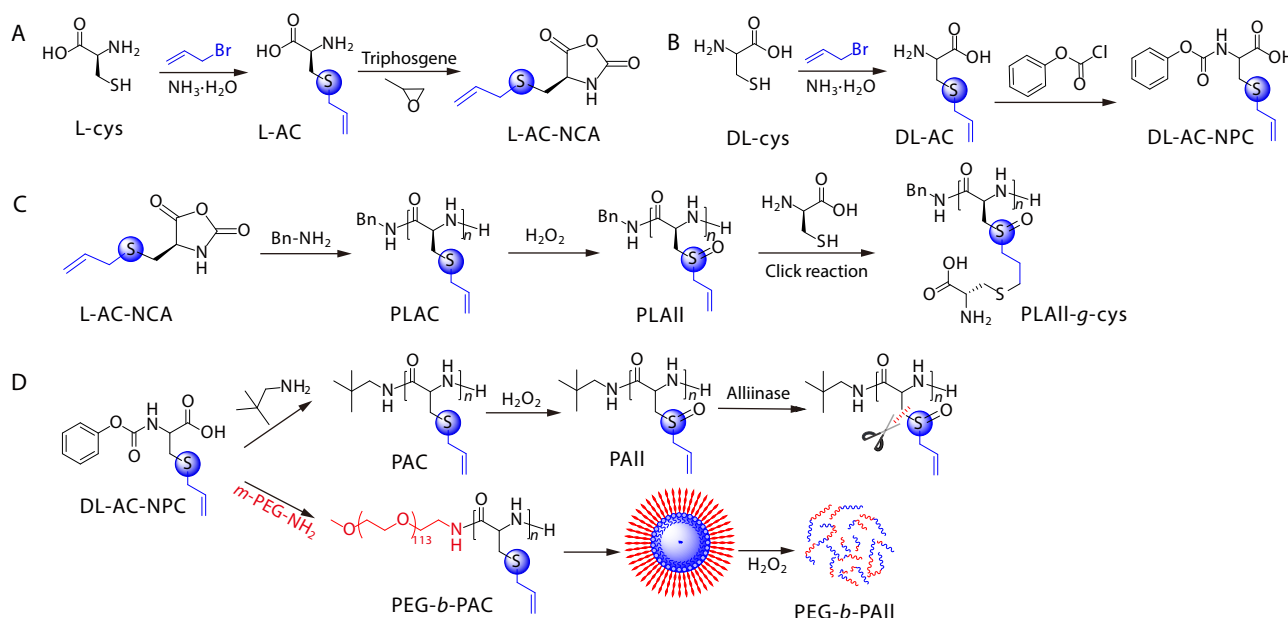
Synthesis of PLAC from L-AC-NCA and Modification by Thiol-ene Click

Amino acid L-AC is prepared by the reaction of L-cysteine with allyl bromide, which is characterized by ^1H -NMR (Fig. S1 in the

electronic supplementary information, ESI) and ESI-MS (Fig. S2 in ESI). L-AC-NCA is synthesized according to Lu's method (Scheme 1A). The signals at 5.74 (H^a), 5.36–4.89 (H^b) and 3.50–2.98 ppm (H^d) are characteristic allyl protons of L-AC-NCA (Fig. 1A). ¹³C-NMR and ESI-MS (Figs. S3 and S4 in ESI) further confirm the structure of L-AC-NCA.

Poly(S-allyl-L-cysteine) (PLAC) are synthesized by ROP of L-AC-NCA with benzylamine as initiator (Scheme 1C, samples

1–7 in Table 1). Typical PLAC proton signals are observed in ¹H-NMR spectrum (Fig. 1B). The methylene protons (H^e and H^f) adjacent to sulfur atoms in PLAC are identified at 3.18 and 2.89 ppm, respectively. The signals of benzylamine residues (H^a) at chain end and unsaturated protons (H^b and H^c) in repeat units are well assigned at 7.33, 5.78 and 5.17 ppm, respectively. The degrees of polymerization (DPs) of PLAC in Table 1 are calculated from the ratios of the intensities of H^a



Scheme 1 Synthetic routes to (A) L-AC-NCA and (B) DL-AC-NPC, and (C and D) their polymerizations.

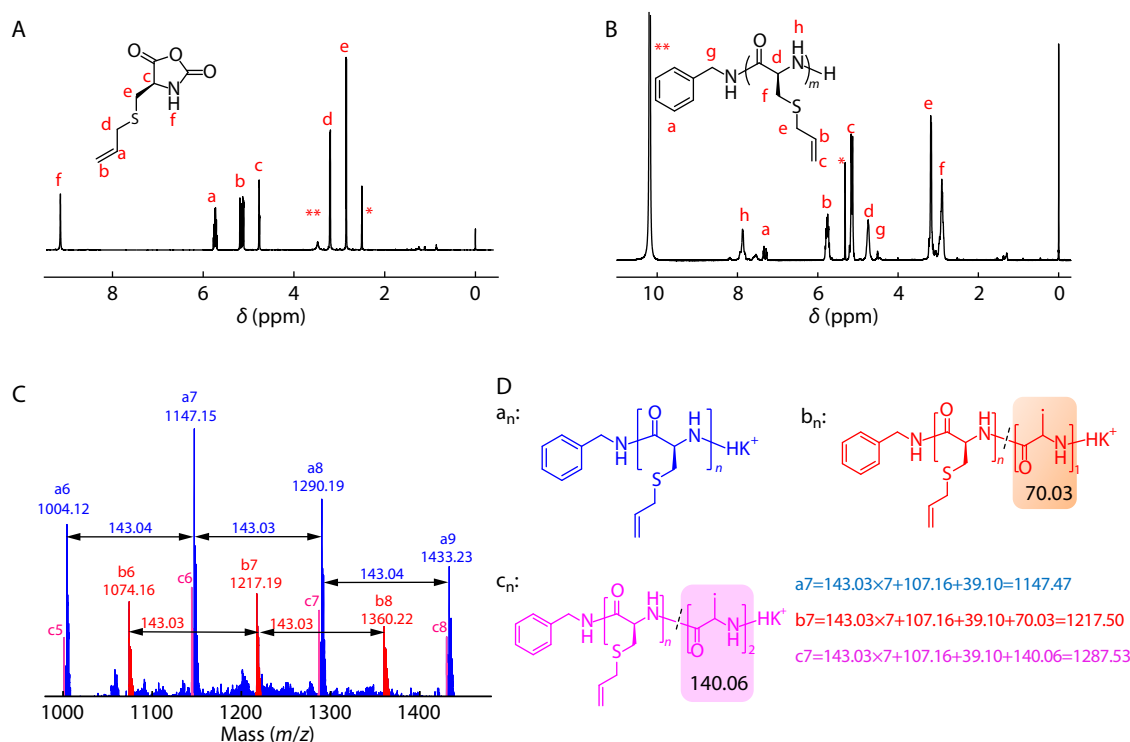


Fig. 1 ¹H-NMR spectra of (A) L-AC-NCA in DMSO-d₆ (* DMSO-d₆, ** H₂O) and (B) PLAC-4 in CD₂Cl₂ with trifluoroacetic acid (TFA) (V:V=4:1, * CD₂Cl₂, ** TFA). (C) MALDI-ToF MS spectrum of PLAC-1 and (D) the corresponding chemical structures.

and H^b signals. The structure of PLAC is further confirmed by MALDI-ToF MS analysis. The spectra (Figs. 1C and S5 in ESI) exhibit prominent population corresponding to the benzylamine initiated PLAC carrying a cation. In addition, PLAC with one or two alanine residues are also observed (populations b_n and c_n in Figs. 1C and 1D) because the side chain is fragile during the MALDI-ToF analysis.

The DP of PLAC reaches 40 with the yield of 47.4% when the initial ratio of [AC]/[BnNH₂] is 100 in feed. The increase of initial molar ratio of [AC]/[BnNH₂] leads to the decrease of yield. PLAC precipitates from the polymerization solution in DMF within 10 min and it exhibits poor solubility in most solvents due to secondary structure. Though HFIP is a well-known solvent for polypeptides with secondary structures, PLAC with DP above 30 cannot dissolve in HFIP either. The poor solubility affects the controllability of polymerization, the characterization of PLAC, and post-modification. We oxidize PLAC into PLAll (samples 1–7 in Table 1) to improve the solubility of the polypeptide for further analyses and modification.^[41]

The structure of PLAll is confirmed by ¹H-NMR (Fig. 2A). Compared to the signals of PLAC, the obvious down-field shift of the methylene protons (H^e and H^f) adjacent to sulfenyl group in PLAll from 2.99 to 3.80 ppm proves the oxidation of thioether groups (Fig. 2A and Fig. S6 in ESI). In FTIR spectra, the sulfoxide groups are responsible for the absorption band at 1027 cm⁻¹ (Fig. S7 in ESI), also indicating the successful ox-

dation. Besides, the bands at 1694 and 1639 cm⁻¹ in the amide-I regions in FTIR disappear after oxidation, which reveals the oxidation-induced conformation transition. As expected, PLAll and low-MW PLAC samples in HFIP do not show characteristic α -helix or β -sheet conformations in CD spectra (Fig. S8 in ESI). Monomodal SEC traces of PLAll (Fig. 2B) are found in the same trends with the DP_{NMR} of PLAC, and narrow $\bar{D}$ of PLAll exhibits good controllability of polymerization.

PLAll has abundant unsaturated bonds in the side chains, which provides a platform for post-polymerization modification. L-cysteine is the only natural amino acid containing thiol group. PLAll-*g*-cys is produced through UV-induced thiol-ene click reaction. In its ¹H-NMR spectra (Fig. 2A), the signals of Hⁱ, H^g and H^h of PLAll-*g*-cys at 3.57–3.39 ppm are attributed to L-cysteine moieties. The decrease in the intensities of vinyl groups (H^b and H^c) also confirms the successful modification, according to which the conversion of the alkene moiety is calculated up to 78%. The H^j signal at 11.60 ppm is characteristic of zwitterionic protons resulted from intermolecular ionic cross-linking. Therefore, compared to alkaline solution, PLAll-*g*-cys exhibits relatively poor solubility in water and DMSO.

Synthesis of PEG-*b*-PAC from DL-AC-NPC and Oxidation-Responsive Behavior

To avoid the formation of secondary structure of PLAC and the usage of highly toxic phosgene derivatives during the preparation of L-AC-NCA, racemic monomer of DL-AC-NPC (a stable

Table 1 Synthesis of PLAC and PLAll from L-AC-NCA. ^a

Sample	Bn-NH ₂ :NCA	Time (h)	DP _{NMR} ^b	Yield (%)	Sample ^c	M _{n, SEC} ^d (kg/mol)	$\bar{D}$ ^d
PLAC-1	1:20	3	12	44.4	PLAll-1	3.3	1.18
PLAC-2	1:15	24	13	88.2	PLAll-2	3.7	1.17
PLAC-3	1:20	24	16	85.3	PLAll-3	5.3	1.21
PLAC-4	1:30	24	23	82.0	PLAll-4	5.0	1.19
PLAC-5	1:50	24	30	61.2	PLAll-5	5.4	1.21
PLAC-6	1:75	24	35	51.9	PLAll-6	5.4	1.21
PLAC-7	1:100	24	40	47.4	PLAll-7	5.5	1.22

^a Polymerizations are carried out at 30 °C in DMF with benzylamine as initiator, [NCA]=0.5 mol/L; ^b Determined by ¹H-NMR; ^c The oxidation of PLAC was conducted in the presence of H₂O₂ using HFIP as solvent; ^d Determined by SEC in HFIP.

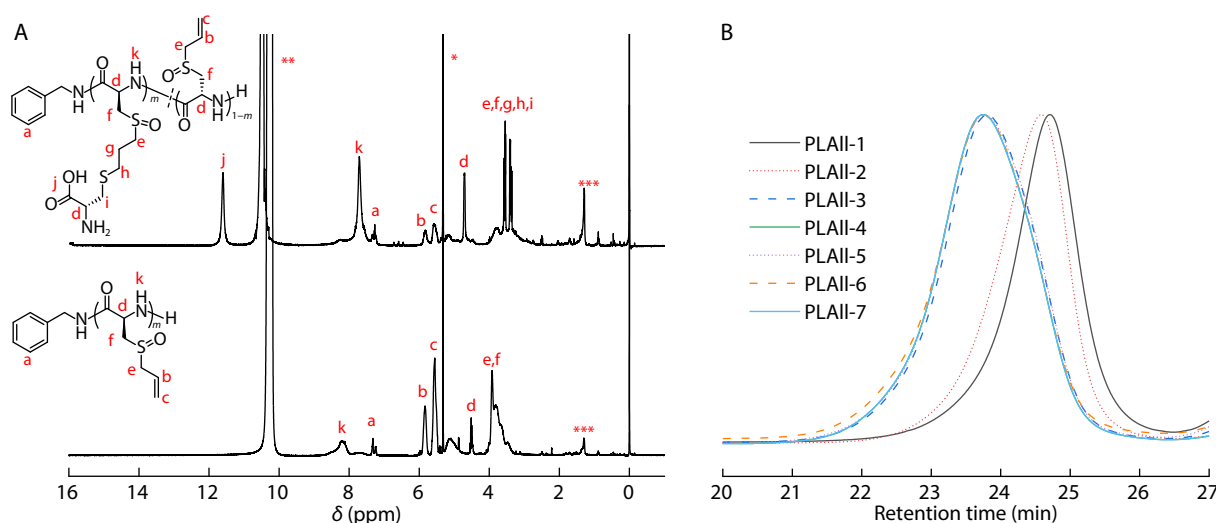


Fig. 2 (A) ¹H-NMR spectra of PLAll-4 and PLAll-*g*-cys in CD₂Cl₂ with TFA (V:V=4:1) (* CD₂Cl₂, ** TFA and *** ethyl ether); (B) SEC traces of PLAll samples 1–7 in Table 1.

precursor of NCA) is synthesized *via* a phosgene-free protocol (Scheme 1B). DL-AC is prepared in the same way of L-AC and confirmed by $^1\text{H-NMR}$ (Fig. S9 in ESI). The structure of DL-AC-NPC is characterized by $^1\text{H-NMR}$ (Fig. 3A), $^{13}\text{C-NMR}$, HSQC and ESI-MS spectra (Figs. S10–S12 in ESI). In $^1\text{H-NMR}$, allyl groups are observed at 5.78 (H^{a}), 5.38–4.82 (H^{b}) and 3.32–3.04 (H^{d}) ppm. It is worth mentioning that an ion signal of DL-AC-NCA is observed in the negative mode of ESI-MS analysis, which demonstrates that the deprotonated DL-AC-NPC ion undergoes ring-closure and produces the corresponding NCA *in situ*.^[33] It is exciting evidence that NPC generates NCA immediately after deprotonation in either ESI-MS analysis or amine-mediated polymerization.

Amphiphilic block copolymers PEG-*b*-PAC are prepared by using *m*-PEG-NH₂ (MW=2000 and 5000 g/mol) as macroinitiators (Table 2 and Table S1 in ESI, Scheme 1D). The methyl (H^{g})

of PEG segments and unsaturated (H^{a}) protons of PAC segments are identified at 3.24 and 5.73 ppm, respectively. DP_{NMR} is calculated according to the ratio of integrals of H^{a} and H^{g} (Fig. 3B). In SEC measurements (Figs. S13–S15 in ESI), PEG-*b*-PAC samples exhibit higher MW than the corresponding *m*-PEG-NH₂ initiators. Hydrophobic thioethers groups can convert to hydrophilic sulfoxide moieties under the treatment of 200 mmol/L H_2O_2 . The oxidation process is monitored by $^1\text{H-NMR}$ (Fig. 3C) and confirmed to be first-order kinetic (Fig. S16 in ESI). The shift of methylene signals (H^{e}) adjacent to sulfur atoms from 2.61 to 3.08 ppm is observed in $^1\text{H NMR}$ after oxidation. Meanwhile, the improved water solubility also supports the successful oxidation of PAC segments (Fig. 3C).

Based on the transformation of solubility caused by oxidation, oxidation-responsibility and drug release behavior of PEG-*b*-PAC micelles are investigated. DLS measurements (Fig.

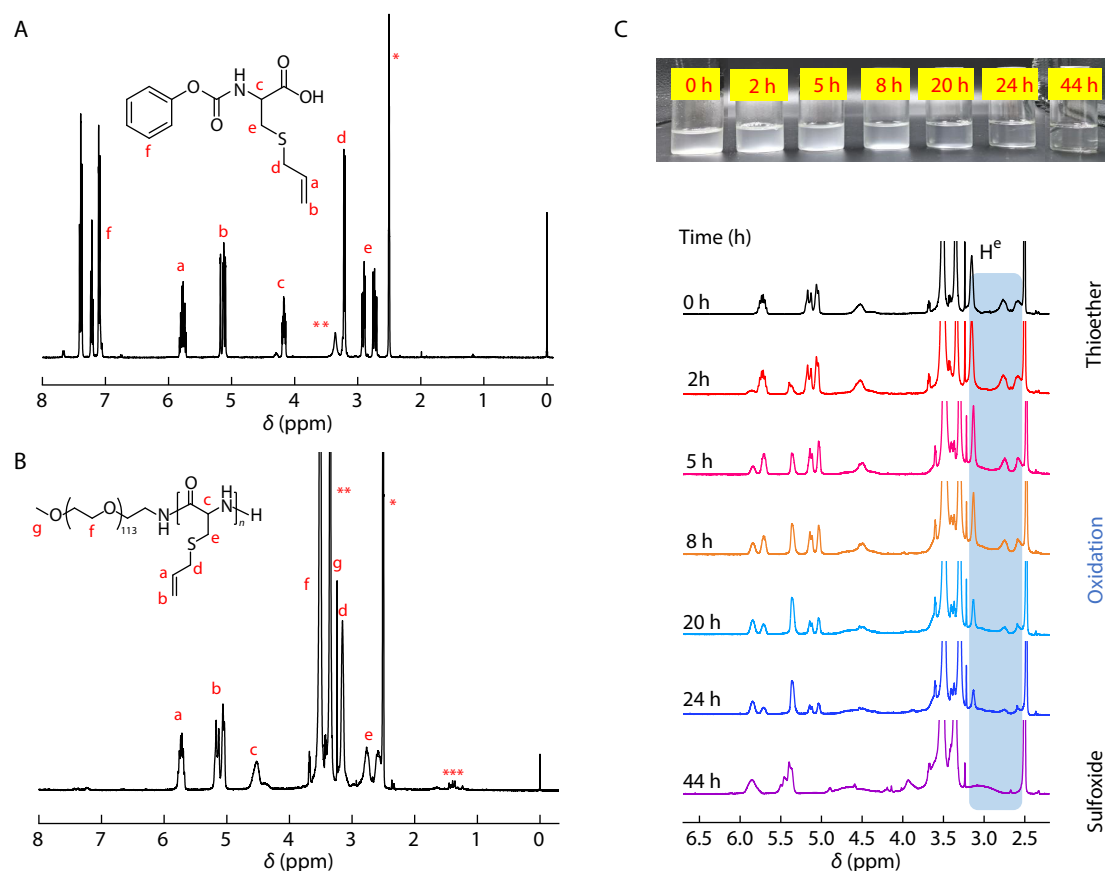


Fig. 3 $^1\text{H-NMR}$ spectra of (A) DL-AC-NPC (* DMSO-d_6), (B) PEG-*b*-PAC-5 (* DMSO-d_6 , ** H_2O , *** ethyl ether), (C) the oxidation process of PEG-*b*-PAC-5 in the presence of 200 mmol/L H_2O_2 and the corresponding optical photos.

Table 2 Synthesis of PEG-*b*-PAC from DL-AC-NPC.^a

Sample	[R-NH ₂]:[NPC]	Time (h)	Yield (%)	$\text{DP}_{\text{NMR}}^{\text{d}}$	$M_{\text{n,SEC}}^{\text{e}}$ (kg/mol)	$\bar{D}^{\text{e}}$	Hydrophilic fraction (wt%)
PEG- <i>b</i> -PAC-1 ^b	1:50	24	45	40	8.5	1.04	26
PEG- <i>b</i> -PAC-2 ^b	1:70	48	52	33	8.7	1.07	30
PEG- <i>b</i> -PAC-3 ^c	1:30	24	71	12	16.4	1.08	74
PEG- <i>b</i> -PAC-4 ^c	1:40	24	61	14	16.7	1.09	71
PEG- <i>b</i> -PAC-5 ^c	1:30	48	73	12	15.3	1.11	74

^a Polymerizations are carried out at 80 °C in DMSO with *m*-PEG-NH₂ as initiator, [NPC]=1 mol/L; ^b R-NH₂=*m*-PEG-NH₂ (MW=2000); ^c R-NH₂=*m*-PEG-NH₂ (MW=5000); ^d Determined by $^1\text{H-NMR}$; ^e Determined by SEC in DMF.

4A) and TEM images (Fig. 4B) demonstrate spherical micellar morphology with a diameter of ca. 63 nm. Oxidation-induced micelle dissociation (Fig. 4C) is monitored by DLS. The size of micelles decreases from 50 nm to 10 nm after the addition of H_2O_2 in 40 min and is less than 5 nm after 60 min, which evidences the complete dissociation of micelles. Hydrophobic NR is employed as a model drug loaded in the core of PEG-*b*-PAC micelles to simulate oxidation-responsive drug delivery. The intensity of the maximum peak at 614 nm declines after the treatment with H_2O_2 within 30 min when the hydrophobic core is destroyed and NR fluorescence is quenched after being released into aqueous solution (Fig. 4D). Thus, PEG-*b*-PAC have great potential as oxidation-responsive nanocarriers for hydrophobic drug.

Synthesis of PAC from DL-AC-NPC and Enzymatic Decomposition

Enzymatic decomposition of the oxidized PAC, i.e., PAII (Scheme 1D) is of interest since the amino acid *S*-allylcysteine sulfoxide,

aka. alliin, can convert to allicin and pyruvic acid by alliinase enzyme. PAC is prepared by DL-AC-NPC using neopentylamine as initiator (Table 3, Scheme 1D). In ^1H -NMR spectra (Fig. 5A), the signals at 0.83 (H^a), 5.73 (H^b) and 5.33–4.86 ppm (H^c) are assigned to neopentylamine residues and unsaturated bonds of AC residues, respectively. The MWs and $\bar{D}$ are detected by SEC in DMF after oxidation of PAC. SEC traces of PAII show unimodal distribution (Fig. 5B). However, the MWs of PAII detected by SEC is not consistent with the DP_{NMR} of PAC, which may be caused by slight breakage of backbone during oxidation. The structure of PAII is further characterized by ^1H -NMR and FTIR (Figs. 5A and 5C). The peaks slowly shift to lower MW region within 14 days (Fig. 5D). Meanwhile, two small peaks (red and purple lines in enzymatic hydrolysis process is monitored by SEC in Fig. 5D) are observed at 25.5 min which are small molecular byproducts generated during enzymatic decomposition. To further confirm the structure after decomposition, the components are extracted by ethanol and detected by GS-MS, where diallyl disulfide, the products of enzymatic decomposition of alliin,

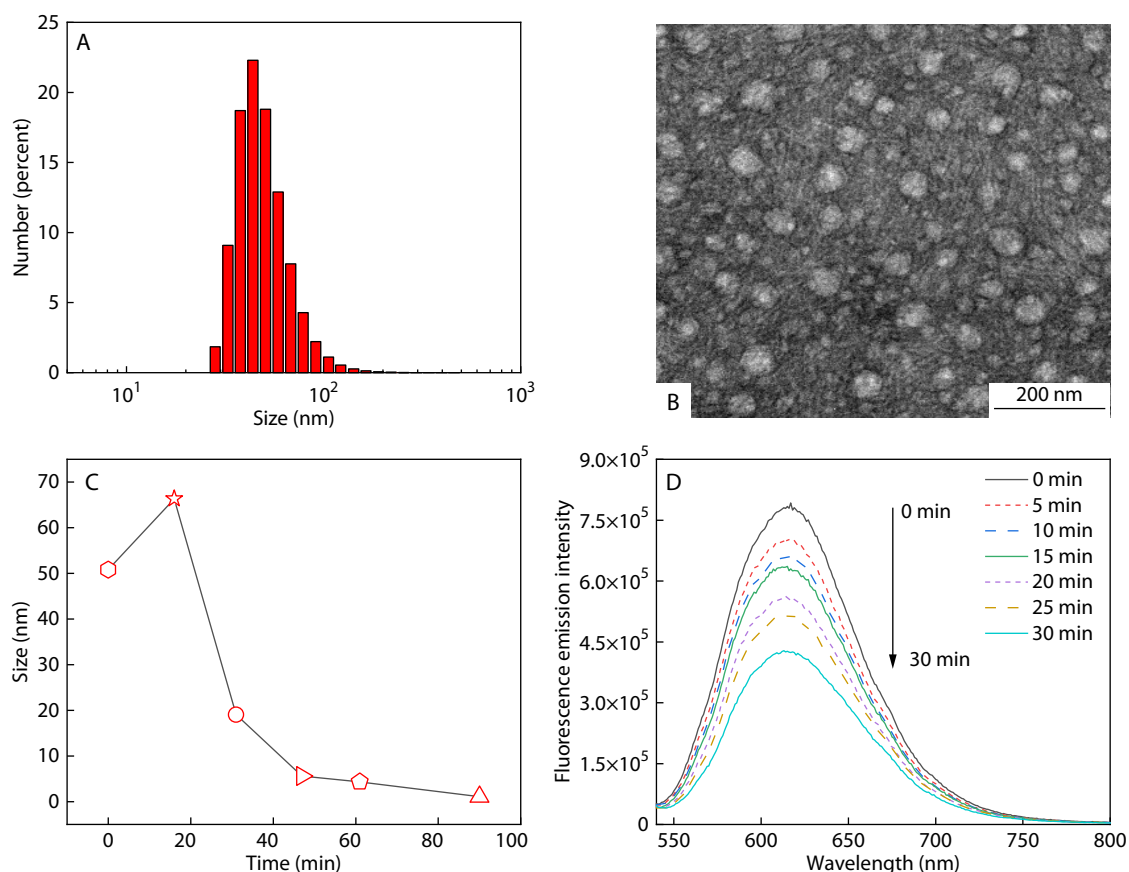


Fig. 4 (A) DLS results and (B) TEM images of micelles (PEG-*b*-PAC-3) in diluted solution (1 mg/mL); (C) DLS spectra of PEG-*b*-PAC-3 upon H_2O_2 oxidation; (D) Fluorescence emission spectra of NR-loaded micelles upon H_2O_2 oxidation.

Table 3 The synthesis of PAC and PAII from DL-AC-NPC. ^a

Sample	R-NH ₂ :NPC	Time (h)	DP _{NMR} ^b	Yield (%)	Sample ^c	M _{n, SEC} ^d (kg/mol)	$\bar{D}$ ^d
PAC-1	1:30	48	25	71	PAII-1	8.4	1.17
PAC-2	1:50	96	33	52	PAII-2	7.1	1.41
PAC-3	1:80	96	49	32	PAII-3	6.6	1.15

^a Polymerizations are carried out at 80 °C in DMSO with neopentylamine as initiator, [NPC]=1 mol/L; ^b Determined by ^1H -NMR; ^c The oxidation of PAC was conducted in the presence of H_2O_2 using acetic acid as catalyst; ^d Determined by SEC in DMF.

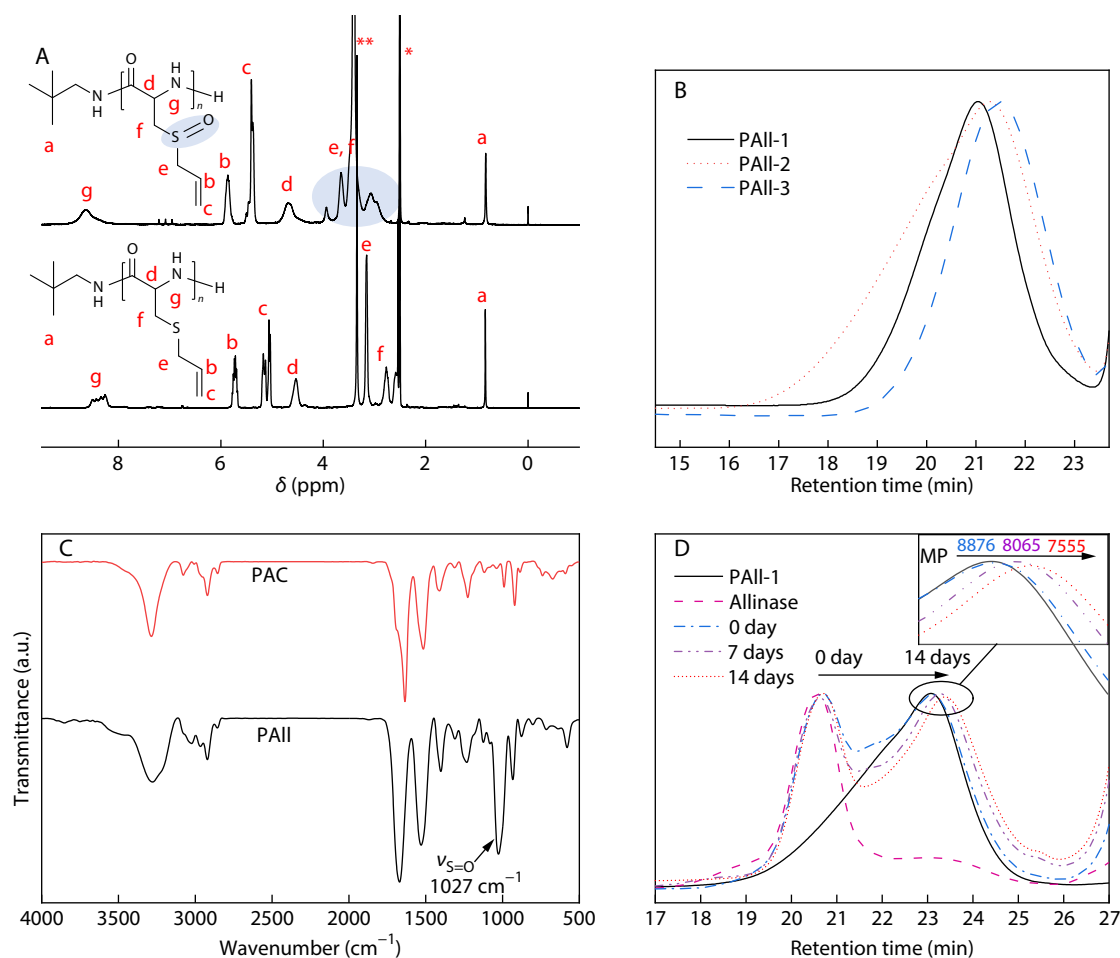


Fig. 5 (A) ¹H-NMR spectra of PAC-1 and PAIL-1 in DMSO-d₆. (* DMSO-d₆, ** H₂O). (B) SEC traces of PAIL (samples 1–3 in Table 3). (C) FTIR spectra of PAC-2 and PAIL-2 in the solid state. (D) SEC traces of enzymatic decomposition of PAIL-1.

can be observed (Fig. S18 in ESI). Low cytotoxicity is a crucial criterion for biomedical materials. The polymer's cytotoxicity is estimated using a CCK-8 assay with NIH 3T3 cells (Fig. S17 in ESI). PEG-*b*-PAC-5 exhibits completely non-toxic even in the concentration of 2500 µg/mL. After oxidation, the cytotoxicity of PEG-*b*-PAIL-5 is slightly increased but still remains non-toxic (>90% cell viability) at the concentrations up to 1000 µg/mL. On the contrary, PAIL-1 is a little toxic because the alliin repeating units contain antioxidant, anti-inflammatory, antibacterial and antifungal properties. The attempts of PAIL-loaded PEG-*b*-PAC micelles for antibacterial application are under investigation.

CONCLUSIONS

Side chain-functionalized PAC is successfully synthesized by two approaches from L-AC-NCA and DL-AC-NPC. Influenced by secondary structure, PLAC shows poor solubility in most common solvents while its oxidized product PAIL is soluble in DMSO and HFIP. PAIL is conjugated with cysteine in high efficiency via thiol-ene click reaction. Amphiphilic diblock PEG-*b*-PAC is prepared by *m*-PEG-NH₂-mediated polymerization of DL-AC-NPC and it forms micelles in aqueous solution. The oxidation of PAC segment by H₂O₂ changes hydrophobic AC residue into hydrophilic one and thus destroys the micelles. Oxidation-

responsive drug release behavior is demonstrated by using NR as a model drug. The side chains of polyalliin are fragile to allinase and are able to break. Together with the oxidation-responsive properties and thiol-ene reaction, PAC is promising for future biomedical applications.

NOTES

The authors declare no competing financial interest.

Electronic Supplementary Information

Electronic supplementary information (ESI) is available free of charge in the online version of this article at <http://doi.org/10.1007/s10118-022-2745-x>.

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