Supporting Information

Physiologically relevant pH- and temperature-responsive polypeptide hydrogels with adhesive properties

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Experimental

Materials Poly(ethylene glycol) monomethyl ether (mPEG, M_n = 2000) and poly(ethylene glycol) (PEG, M_n = 2000) were obtained from Sigma-Aldrich. L-Glutamic acid, CuBr and 1,1,4,7,7-pentamethyl-diethylenetriamine (PMDETA) were purchased from Aladdin. 1-(2-Hydroxyethyl)-4-methylpiperazine (HMP) was purchased from TCI. Amino-terminated mPEG (mPEG-NH_2), amino-terminated PEG (NH_2-PEG-NH_2), γ-ethyl-L-glutamates (ELG), γ-ethyl-L-glutamate N-carboxyanhydride (ELG-NCA), γ-propargyl-L-glutamates (PLG) and γ-propargyl-L-glutamate N-carboxyanhydride (PLG-NCA) were synthesized according to previously reported methods.[1, 2] N,N-Dimethylformamide (DMF) and tetrahydrofuran (THF) were purchased from CINC High Purity Solvents Co., Ltd. (Shanghai, China) and purified by the solvent purification system (MB SPS-800, MBRAUN, Germany). Chloroform was distilled over CaH_2 before use and stored under the nitrogen atmosphere. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, USA). Newborn bovine serum (NBS) was purchased from TianHang Biotechnology, (Hangzhou, China). Sterile penicillin/streptomycin was purchased from Meilun Biotechnology Co., Ltd. (Dalian, China). Cell Counting Kit-8 was purchased from Beyotime Biotechnology (Shanghai, China). B16F10 cells were purchased from BeNa Culture Collection (Beijing, China). SpragueeDawley (SD) rats (6 ~ 8w, ~200 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China). All other reagents were purchased from Sinopharm Chemical Reagent Co. Ltd., China, and used as obtained.

Characterization

NMR spectra were recorded using Bruker AV 500 NMR spectrometer except for special instructions. ^{13}C NMR analysis of HMP, 1-(2-chloroethyl)-4-methylpiperazine (CMP) and 1-(2-azidoethyl)-4-methylpiperazine (AMP) was conducted in deuterated chloroform ($CDCl_3$) at 25 ^oC. ^1H NMR analysis of ELG and PLG was performed in deuterium oxide (D_2O), ELG NCA and PLG NCA in deuterated chloroform ($CDCl_3$, Bruker AV 300 NMR spectrometer), poly(ethylene glycol)-block-poly((γ-ethyl-L-glutamate)-co-(γ-propargyl-L-glutamate)) (mPEG-b-P(ELG-co-PLG)) and poly((γ-ethyl-L-glutamate)-co-(γ-propargyl-L-glutamate))-block-poly (ethylene glycol)-block-poly((γ-ethyl-L-glutamate)-co-(γ-propargyl-L-glutamate)) (P(ELG-co-PLG)-b-PEG-b-P(ELG-co-PLG)) in deuterated trifluoroacetic acid (CF_3COOD), and poly (ethylene glycol)-block-poly((γ-ethyl-L-glutamate)-co-(γ-propargyl-L-glutamate/AMP)) (mPEG-b-P(ELG-co-PLG/AMP)) and poly((γ-ethyl-L-glutamate)-co-(γ-propargyl-L-glutamate/AMP))-block-poly (ethylene glycol)-block-poly((γ-ethyl-L-glutamate)-co-(γ-propargyl-L-glutamate/AMP)) (P(ELG-co-PLG/AMP)-b-PEG-b-P(ELG-co-PLG/AMP)) in deuterated trifluoroacetic acid (CF_3COOD) at 25 ^oC. ^1H NMR spectra were calibrated by using tetramethyl silane (TMS) as the internal reference.

Electrospray ionization mass spectrometry (ESI-MS) of AMP was measured by a Thermo LTQ XL ion trap mass spectrometer (Thermo Finnigan, CA, United States) in a positive ion mode (ESI+).

Microstructure of micelles and hydrogel was observed using scanning electron microscope (Gemini 2 SEM, Carl Zeiss AG, Germany). PPCAs aqueous solution (0.125 mg/mL, at 25 ^{o}C and 55 ^{o}C) was dropped onto clean silicon and dried naturally in the air. During the shooting of SEM, skip the images about the crystal of salt. The cross-section microstructure of hydrogel was ingested after lyophilization of hydrogel.

The ellipticity of polymer aqueous solution (0.02% (w/v)) was conducted using Chirascan spectrometer (Applied photophysics, Leatherhead, Surrey, UK) as a function of temperature within the range of 10 to 70 °C or pH 6 to 8.

pH was monitored using HI2211 pH/ORP meter equipped with HI1131 glass electrode (Hanna Instruments Inc., Italy).

Synthesis of AMP AMP was synthesized via a two-step process as described in the literature.[3-5] Firstly, thionyl chloride (2 eq.) was dropped into a solution of HMP (1 eq.) in dry chloroform in an ice bath. Then, the reaction mixture was refluxed at 70 ^{o}C for 4 h. After evaporation and precipitation, CMP was obtained as a light brown powder in 95% yield. Next, sodium azide (1.5 eq.) was added to the aqueous solution of CMP (1 eq.). After 24 h, the mixture was adjusted to neutral pH using potassium hydroxide (KOH) and extracted several times with diethyl ether. After thorough removing diethyl ether by vacuum rotary evaporation, AMP was obtained as a yellow liquid in 40% yield. ^{13}C NMR and ESI-MS (ESI+) spectra as shown in Fig. S5 and Fig. S6. ESI-MS (ESI+) m/z: calculated for $C_7H_{15}N_5$, 169.2; found, 170.1.

Synthesis of mPEG-*b*-P(ELG-*co*-PLG) and P(ELG-*co*-PLG)-*b*-PEG-b-P(ELG-*co*-PLG) mPEG-*b*-P(ELG-*co*-PLG) ($EG_{45}(E_xP_k)_m$) and P(ELG-*co*-PLG)s-*b*-PEG-b-

P(ELG-*co*-PLG) ($(E_xP_k)_mEG_{45}(E_xP_k)_m$) were synthesized via ring-open-polymerization (ROP) of ELG-NCA and PLG-NCA with mPEG-NH_2 or H_2N-PEG-NH_2 as macroinitiator. After eliminating residual water by azeotropic distillation with toluene, the macroinitiator was dissolved in anhydrous DMF. After DMF was completely dissolved, ELG NCA and PLG NCA were added to the solution. The reaction mixture was kept at 25 ^oC for 3 days with gentle stirring under dry nitrogen atmosphere, followed by precipitation in cold diethyl ether three times. The final product was obtained as white or pale-yellow powder in above 80% yield. ^1H NMR of $EG_{45}(E_xP_k)_m$ and $(E_xP_k)_mEG_{45}(E_xP_k)_m$ (CF_3COOD, δ): 3.64-3.92 ppm (-*CH_2-CH_2-O*-, PEG), 1.18 ppm (-*CH_3*, ELG), 2.36 ppm (-*C≡CH*, PLG).

Synthesis of mPEG-*b*-P(ELG-*co*-PLG/AMP) ($EG_{45}(E_xPA_y)_m$) and P(ELG-*co*-PLG/AMP)-*b*-PEG-*b*-P(ELG-*co*-PLG/AMP) ($(E_xPA_y)_mEG_{45}(E_xPA_y)_m$) AMP was introduced into the polypeptide chain via "click" chemistry to prepare polypeptide-contained amphiphiles (PPCAs). $EG_{45}(E_xP_k)_m$ and $(E_xP_k)_mEG_{45}(E_xP_k)_m$ were dissolved in DMF. After three freeze-pump-thaw cycle of the mixture, AMP, PMDETA and $CuSO_4·5H_2O$ were added to the system quickly under N_2 atmosphere. The reaction was kept at r.t. for 3 days with stirring. The solution mixture was purified by dialysis (regenerated cellulose membrane; MWCO 500) against distilled water for 72 h, changing the external water once every 2 h in the first day and six hours in the next. Subsequently, the solvent was lyophilized for 72 h generating a white or pale-yellow product in above 80% yield. ^1H NMR of PPCAs (CF_3COOD, δ): 3.64-3.92 ppm (-

CH_2-CH_2-O-, PEG), 1.18 ppm (-*CH_3*, ELG), 5.13-5.32 ppm (two -*CH_2*- adjacent the triazole ring).

Acid-base titration PPCAs of aqueous solution (1 mg/ml) was first prepared. Then, the PPCAs aqueous solution was adjusted to pH 3 via 0.5M HCl. Next, 20 μl 0.1M NaOH was dropped into the PPCAs aqueous solution. After stirring, the pH was recorded. Once pH reached 11, the titration ended. The curves based on the pH value and the volume of NaOH were prepared as Fig. S9.

Critical micelle concentration (CMC) study CMC values of PPCAs were measured by the fluorescence method using pyrene as the probe. Firstly, 20 μL pyrene acetone solution (6.0×10^{-5} mol/L) was transferred into 15 brown vials, followed by evaporation of acetone under vacuum. Meanwhile, PPCAs solution (1 mg/mL) was prepared, which was half-diluted 14 times with distilled water. Then, pH of the series of dual responsive polymer solution with different concentrations was adjusted to 7.4. Next, 2 mL dual responsive polymer solution at different concentrations was added to 15 brown vials, kept at constant temperature and shaken for 24 h to allow for self-assembly. The fluorescence excitation spectra were recorded on a fluorescence spectrophotometer (Photon Technology International Inc., USA) at λ_{em} of 390 nm with bandwidths of 5 nm. The intensity ratio of the peak at 336 nm to that at 333 nm (I_{336} / I_{333}) from the excitation spectra were plotted against the log of PPCAs concentrations. CMC values were obtained from the fitting curves shown in Fig. S12.

Phase Diagram The sol-gel transition behavior of PPCAs in phosphate buffered saline (PBS, pH 7.4 and 6.5) was observed using the inversion tube method. Firstly,

PPCAs were dissolved in PBS (pH 7.4 or 6.5) and stirred vigorously in an ice bath to form a series of solutions with different concentrations about 300 μL in little vials (Φ=8 mm). Details regarding the concentration of each sample is listed in Table S1. The process obeyed programmed increase of temperature at 2 °C per 10 min from 0 °C to 70 °C. The sol-gel and syneresis temperature were recorded as no flow was observed within 30 s after tilting the test tube and phase separation leading to accumulation of free water, respectively. The critical gelation concentration (CGC) was defined as the lowest PPCAs concentration for gelation in the process of phase transition.

Rheological experiments Rheology experiments were performed using MCR 301 rheometer (Anton Paar GmbH., Austria), and diameter of the parallel plates was 25 mm and gap fixed at 0.5 mm. As the higher CGC of $EG_{45}(E_{1/2}PA_{1/2})_{12}$, $EG_{45}(E_{1/2}PA_{1/2})_{16}$, $EG_{45}(E_{1/2}PA_{1/2})_{20}$ and $(E_{1/2}PA_{1/2})_{10}EG_{45}(E_{1/2}PA_{1/2})_{10}$, we chose the concentration of 9% (w/v) to test the rheological property as the function of temperature from 4 °C to 60 °C. 3% (w/v) was employed for $EG_{45}(E_{2/3}PA_{1/3})_{12}$, $EG_{45}(E_{3/4}PA_{1/4})_{16}$ and $EG_{45}(E_{3/4}PA_{1/4})_{20}$. When the sample board was below 4 °C, it was easy to provoke moisture in the atmosphere to condense to water and dilute the sample. Considering the tolerant temperature of the instrument and the fact most sample achieved max storage strength, we set 60 °C as the end.

Adhesion Experiments PPCAs were dissolved by PBS and stirred vigorously in an ice bath until a uniform solution was formed. 10 μL PPCAs solution was placed onto PMMA platelet, followed by different substrates. After maintaining at ambient

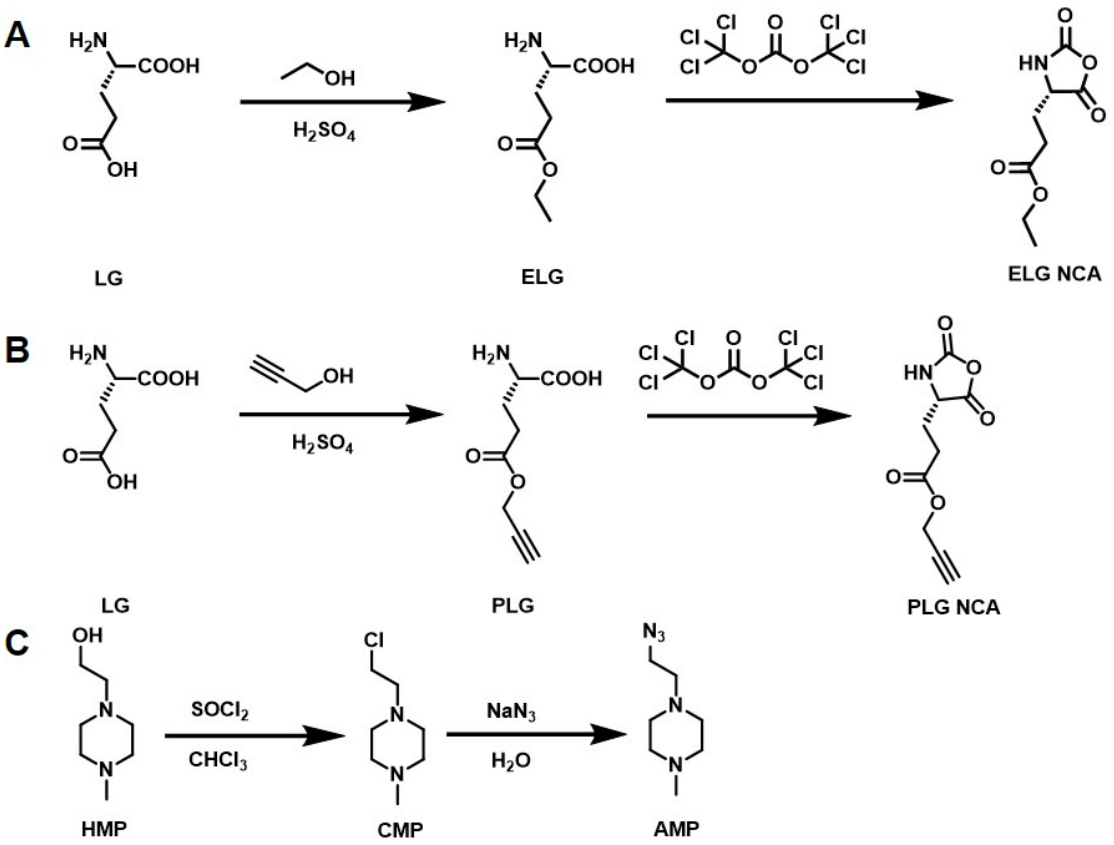
temperature for 10 min, images were recorded. 20 μL PPCAs solution was added onto PMMA platelet, and another PMMA platelet was placed on the polymer solution to form a sandwich structure. The area of overlap between two PMMA platelet was 25*20 mm. The platelets were kept at 37 °C for 24 h, and dumbbells of different weights ad 10 μL PPCAs solution was added onto PMMA platelet, and another PMMA platelet was placed on the polymer solution to form a sandwich structure. The area of overlap between two PMMA platelet was 26*10 mm. The platelets were kept at 37 °C for 24 h, and adhesion strength tested on universal testing machine (AGS-X 1kN, SHIMADZU, Japan) with 0.5 mm/min of tensile rate at 25 ^{o}C. All tests were repeated at least three times.

In vitro **cell cytotoxicit**y In order to investigate the cytotoxicity of PPCAs, B16F10 cells were added into a 96-well plate (1×10^4 cells/well) and cultured for 24 h containing 180 μL DMEM and 20 μL NBS per well. Then, the PPCAs solution in pH 7.4 PBS with different concentrations (20 μL) were added into 96-well plate. Meanwhile, 20 μL PBS per well were added into the control group. 24 h later, cell viabilities were detected by CCK-8 assay.

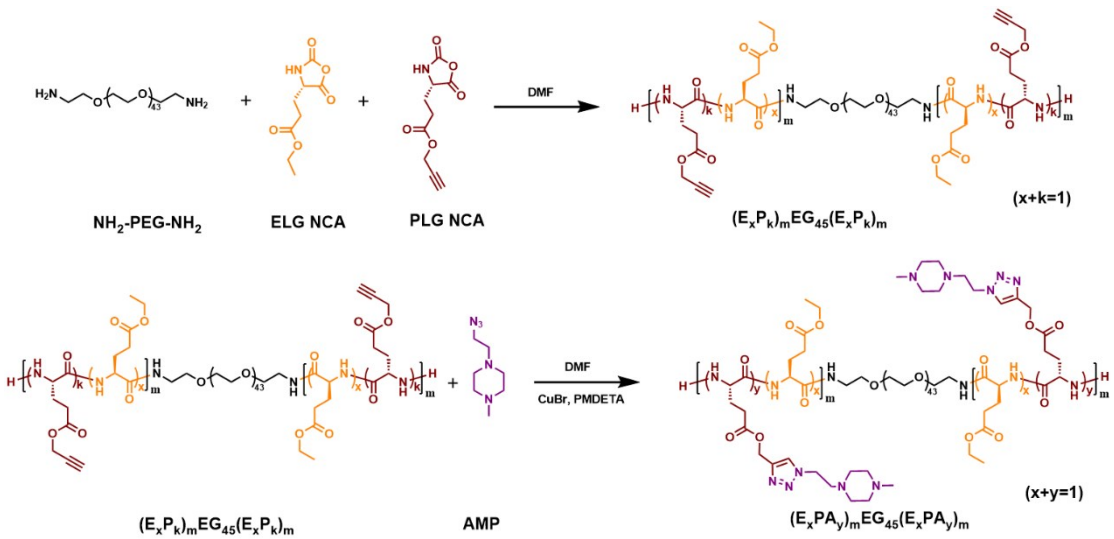
In vitro **hydrogel degradation** $EG_{45}(E_{2/3}PA_{1/3})_{12}$**,** $EG_{45}(E_{1/2}PA_{1/2})_{12}$ and $EG_{45}(E_{3/4}PA_{1/4})_{16}$ were dissolved by PBS to form a solution with certain concentration. Then, pH of polymer solution was adjusted to 7.4 or 6.5, respectively. Three vials (10 mm diameter) with 0.3 mL polymer were defined as a group. After gelation and keeping at 37 °C for 10 min, the weight of the vials was recorded. 1.6 mL degradation medium (10 mM PBS, pH = 6.5 or 7.4) was added to each of the

vials. At the predetermined time (12 h, 24 h, 48 h and other points interval 24 h), the degradation medium was removed and the remaining weight was measured. The detailed about hydrogel concentration and pH, and degradation medium are listed in **Table S3**.

***In vivo* hydrogel degradation** All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jilin University and approved by the Animal Ethics Committee of Changchun Institute of Applied Chemistry, CAS. The degradation experiments *in vivo* employed the same hydrogel to that used *in vitro*. 200 μL $EG_{45}(E_{3/4}PA_{1/4})_{16}$ solution with concentration of 3.0% (w/v) in PBS (pH 7.4) was injected into the left flank of SD rats. At the determined time points (15 min, 1 week, 2 weeks and 4 weeks), four rats were sacrificed. Among them 3 pieces of hydrogel were peeled from the skin and the weight measured, and one hydrogel with skins was collected and stained by H&E to evaluate the biocompatibility *in vivo*.



Scheme S1. Synthetic route of ELG NCA (A), PLG NCA (B) and AMP (C).



Scheme S2. Synthetic route of $(E_xPA_y)_mEG_{45}(E_xPA_y)_m$.

Table S1. ^{1}H NMR and GPC results of $EG_{45}(E_xP_k)_m$ and $(E_xP_k)_mEG_{45}(E_xP_k)_m$.

Polymers	Molar feed ratio[a)] PEG/ELG NCA/PLG NCA	DP[b)]	M_n[b)] (x 10^3)	M_n[c)] (x 10^3)	Đ[c)]
$EG_{45}P_{12}$	1/0/12	13	4.20	7.01	1.32
$EG_{45}(E_{1/3}P_{2/3})_{12}$	1/4/8	12	3.99	6.35	1.23
$EG_{45}(E_{1/2}P_{1/2})_{12}$	1/6/6	12	3.97	5.94	1.24
$EG_{45}(E_{2/3}P_{1/3})_{12}$	1/8/4	12	3.95	5.5	1.19
$EG_{45}E_{12}$	1/12/0	13	3.91	5.98	1.18
$EG_{45}(E_{1/4}P_{3/4})_{16}$	1/4/12	16	4.68	7.24	1.28
$EG_{45}(E_{1/2}P_{1/2})_{16}$	1/8/8	16	4.58	5.27	1.11
$EG_{45}(E_{3/4}P_{1/4})_{16}$	1/12/4	16	4.52	4.59	1.28
$EG_{45}(E_{1/4}P_{3/4})_{20}$	1/5/15	20	5.33	4.66	1.11
$EG_{45}(E_{1/2}P_{1/2})_{20}$	1/10/10	20	5.28	4.3	1.11
$EG_{45}(E_{3/4}P_{1/4})_{20}$	1/15/5	20	4.94	5.29	1.30
$(E_{1/2}P_{1/2})_{10}EG_{45}(E_{1/2}P_{1/2})_{10}$	1/10/10	20	5.28	7.47	1.34

a) $mPEG_{2000}$ refers to EG_{45}. b) DP represents the polymerization degree of the polypeptide calculated by ^{1}H NMR spectra. c) Determined via DMF phase GPC.

Table S2. ^{1}H NMR and GPC results of $EG_{45}(E_xPA_y)_m$ and $(E_xPA_y)_mEG_{45}(E_xPA_y)_m$.

Polymers	M_n[a)] (x 10^3)	M_n[b)] (x 10^3)	Đ[b)]	Grafting ratio of AMP (%)[a)]
$EG_{45}PA_{12}$	4.00	3.44	2.33	92
$EG_{45}(E_{1/3}PA_{2/3})_{12}$	5.17	4.30	2.01	88
$EG_{45}(E_{1/2}PA_{1/2})_{12}$	4.95	4.40	2.12	97
$EG_{45}(E_{2/3}PA_{1/3})_{12}$	4.46	3.93	1.90	75
$EG_{45}(E_{1/4}PA_{3/4})_{16}$	6.71	5.48	1.25	100
$EG_{45}(E_{1/2}PA_{1/2})_{16}$	5.93	3.67	1.21	100
$EG_{45}(E_{3/4}PA_{1/4})_{16}$	5.05	3.38	1.97	80
$EG_{45}(E_{1/4}PA_{3/4})_{20}$	7.7	5.83	1.13	93
$EG_{45}(E_{1/2}PA_{1/2})_{20}$	6.97	4.04	2.17	98
$EG_{45}(E_{3/4}PA_{1/4})_{20}$	5.79	5.58	1.20	100
$(E_{1/2}PA_{1/2})_{10}EG_{45}(E_{1/2}PA_{1/2})_{10}$	6.13	5.44	1.74	76

a) M_n and grafting ratio of AMP related to PLG residues, calculated by ^{1}H NMR spectra. b) M_n and Đ, determined via DMF phase GPC.

Table S3. The pH of the hydrogels and degradation media used for hydrogel degradation tests.

Hydrogel	pH of hydrogel	Degradation medium
3% (w/v) $EG_{45}(E_{2/3}PA_{1/3})_{12}$	7.4	10 mM PBS (pH 7.4)
9% (w/v) $EG_{45}(E_{1/2}PA_{1/2})_{12}$	7.4	10 mM PBS (pH 7.4)
3% (w/v) $EG_{45}(E_{3/4}PA_{1/4})_{16}$	7.4	10 mM PBS (pH 7.4)
3% (w/v) $EG_{45}(E_{3/4}PA_{1/4})_{16}$	7.4	10 mM PBS (pH 6.8)
3% (w/v) $EG_{45}(E_{3/4}PA_{1/4})_{16}$	6.8	10 mM PBS (pH 6.8)

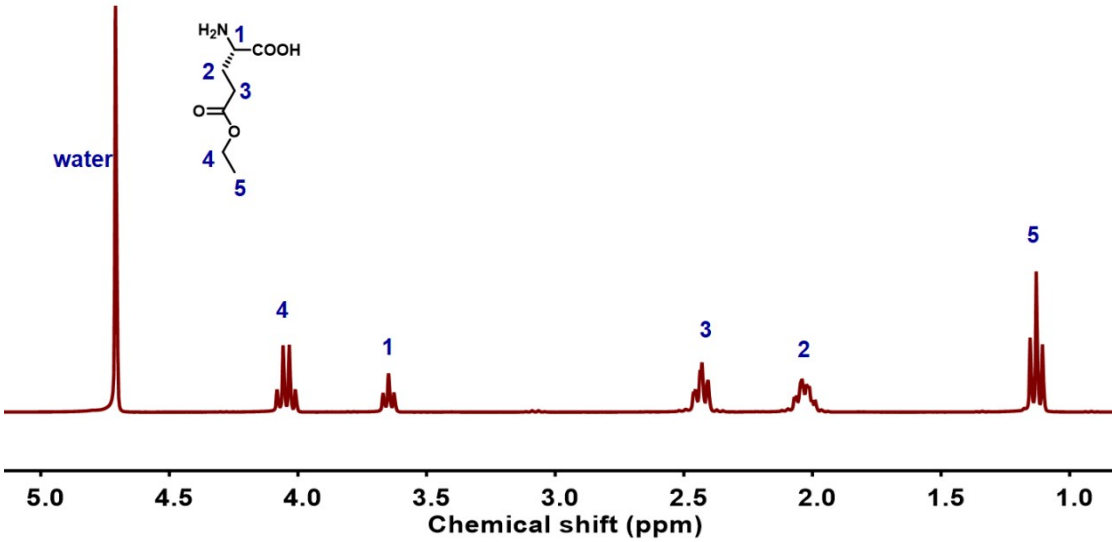


Fig. S1 ^{1}H NMR of ELG (D_2O).

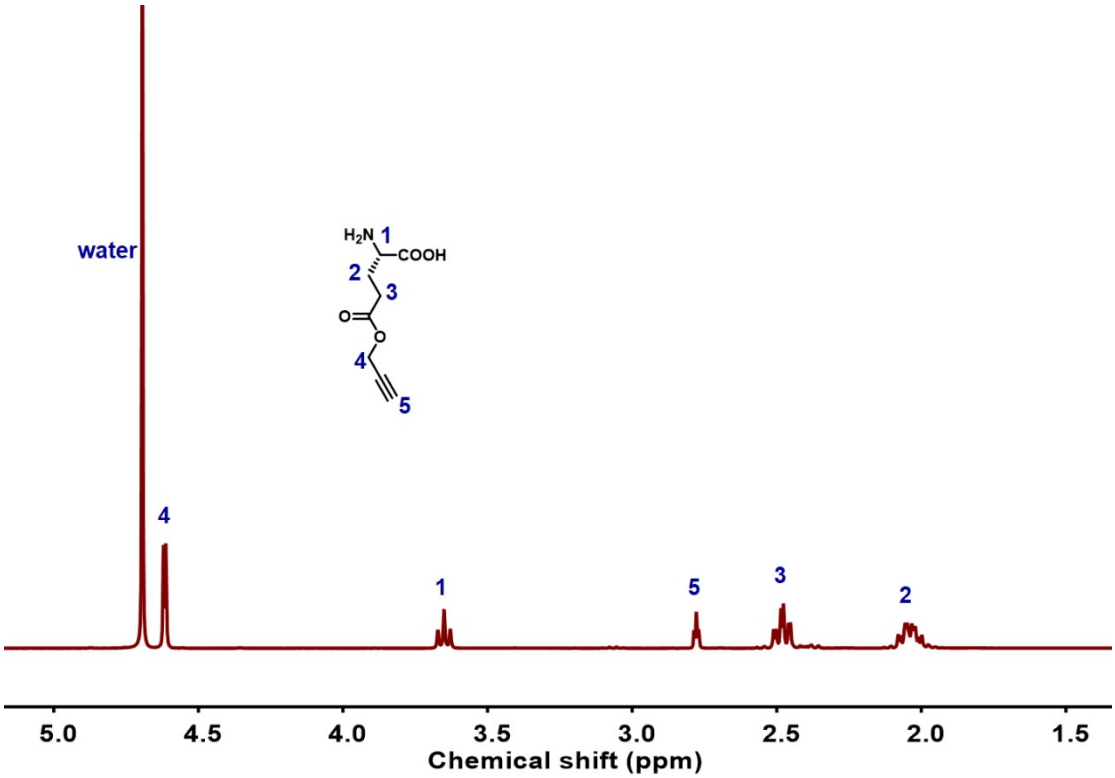


Fig. S2 ^{1}H NMR of PLG (D_2O).

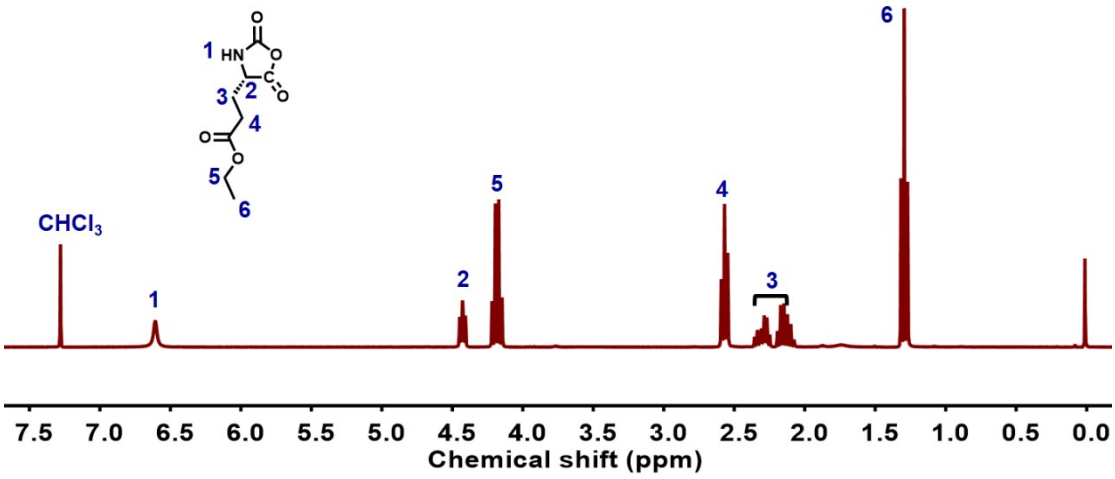


Fig. S3 ^{1}H NMR of ELG NCA ($CDCl_3$).

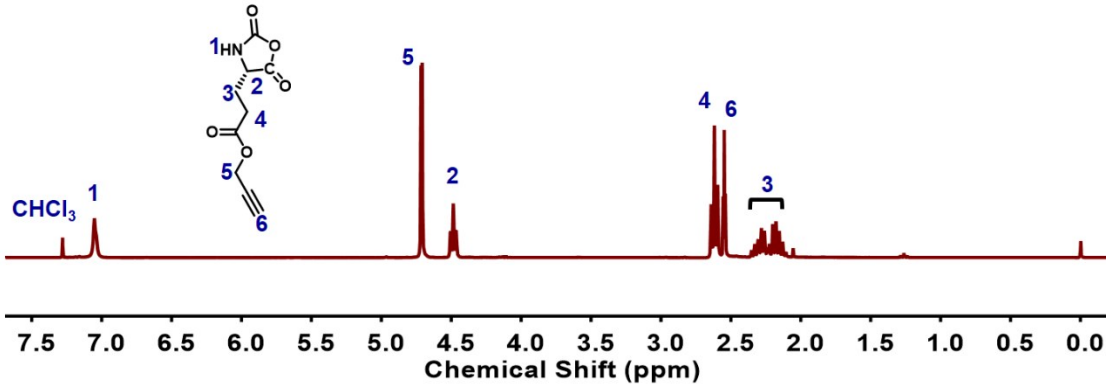


Fig. S4 ^{1}H NMR of PLG NCA ($CDCl_3$).

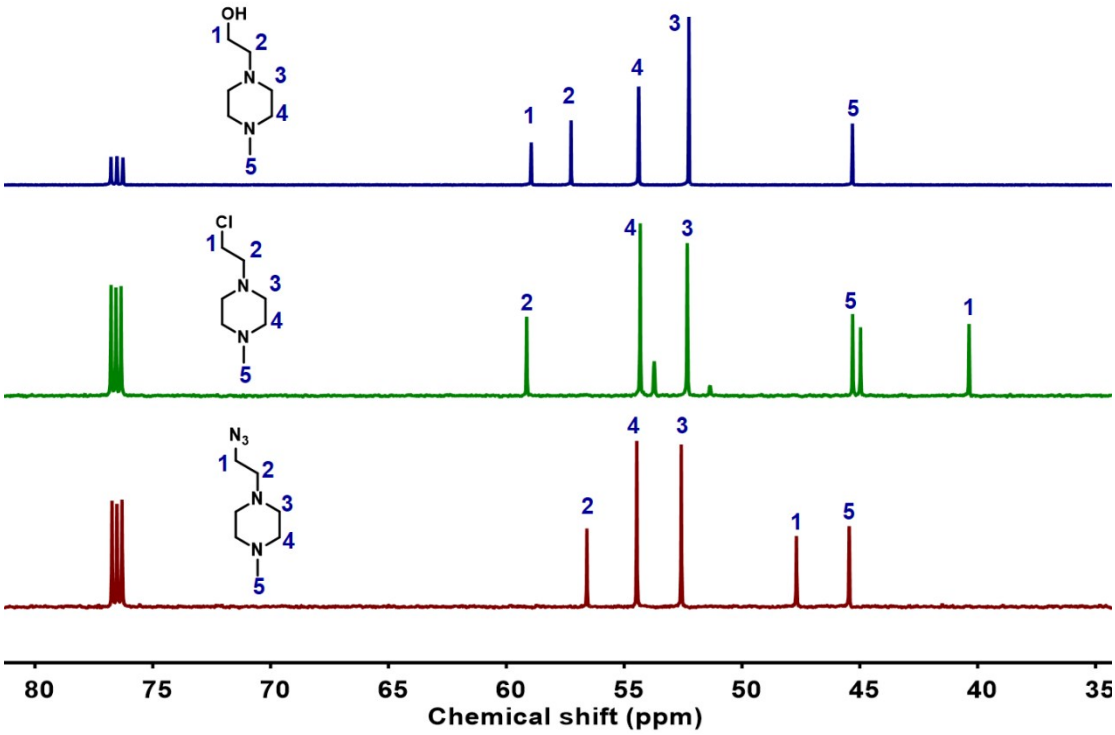


Fig. S5 ^{13}C NMR of HMP ($CDCl_3$), CMP ($CDCl_3$) and AMP ($CDCl_3$), respectively.

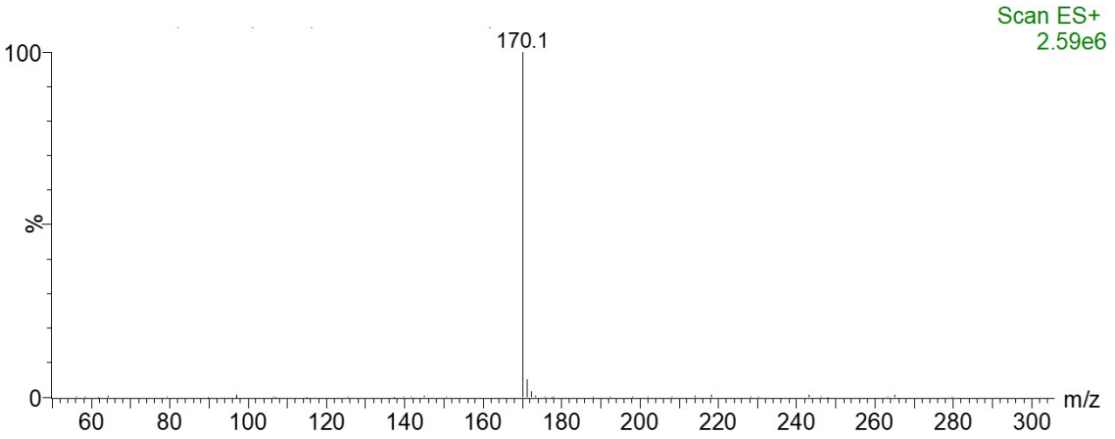


Fig. S6 ESI-MS (ESI+) characterization of AMP.

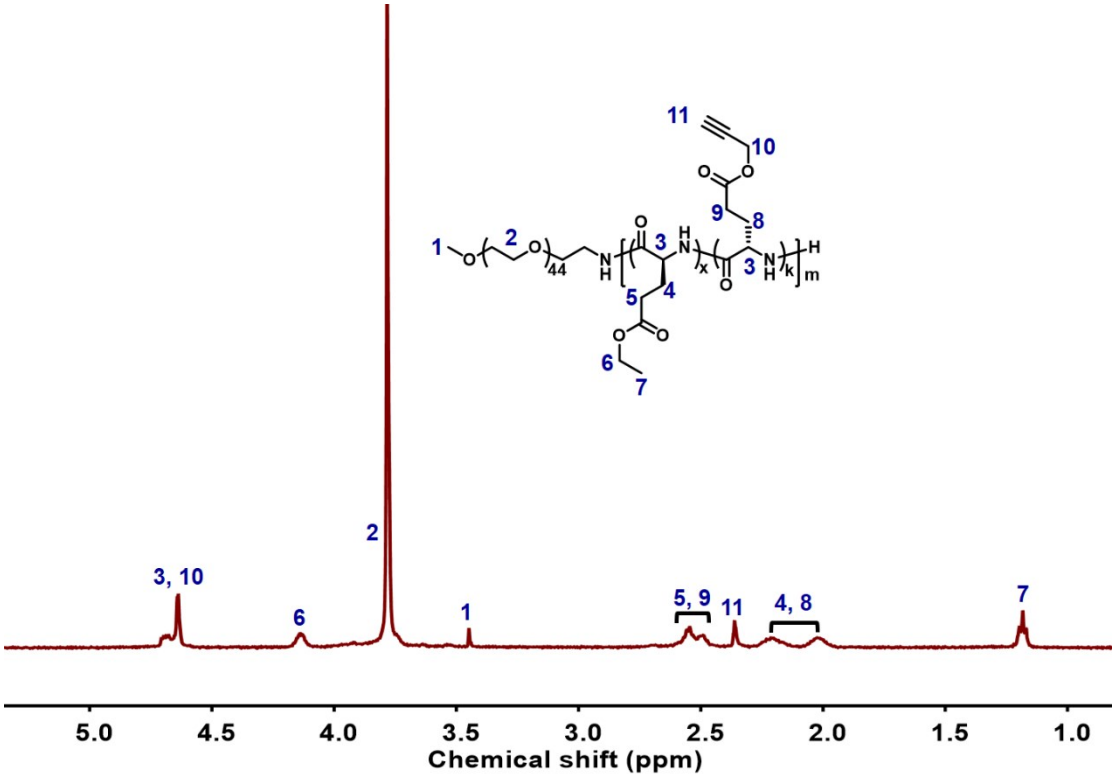


Fig. S7 ^{1}H NMR spectrum of $EG_{45}(E_{1/2}P_{1/2})_{12}$ in CF_3COOD.

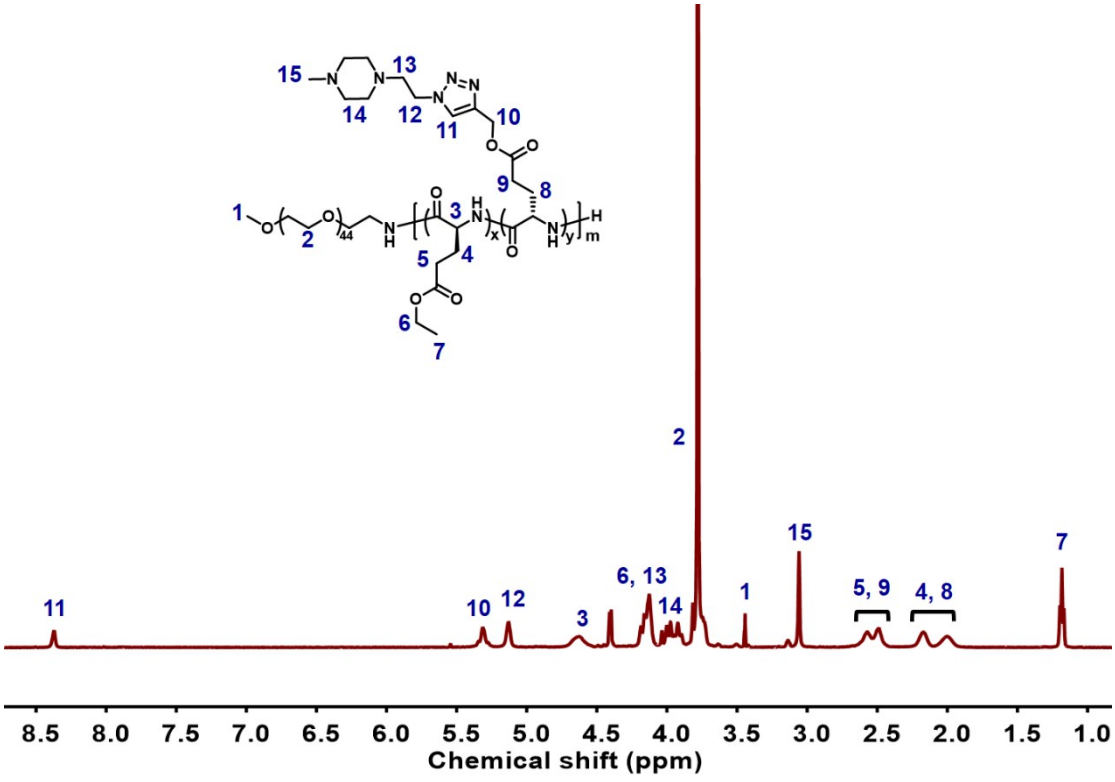


Fig. S8 ^{1}H NMR spectrum of $EG_{45}(E_{1/2}PA_{1/2})_{12}$ in CF_3COOD.

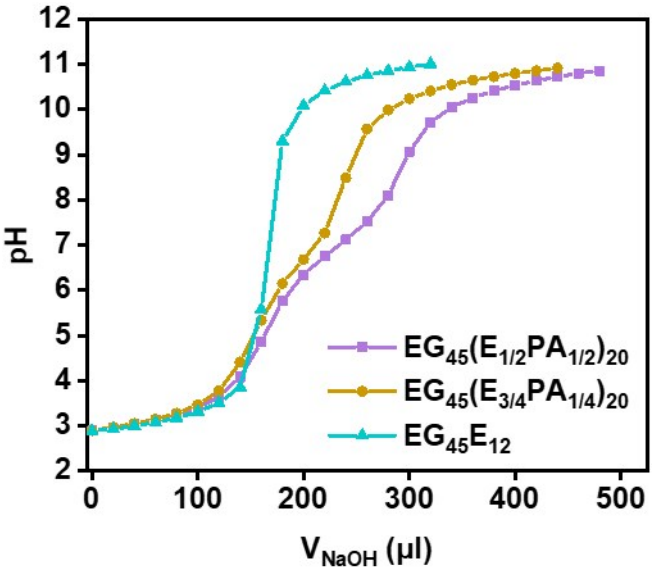


Fig. S9 Acid-base titration curves.

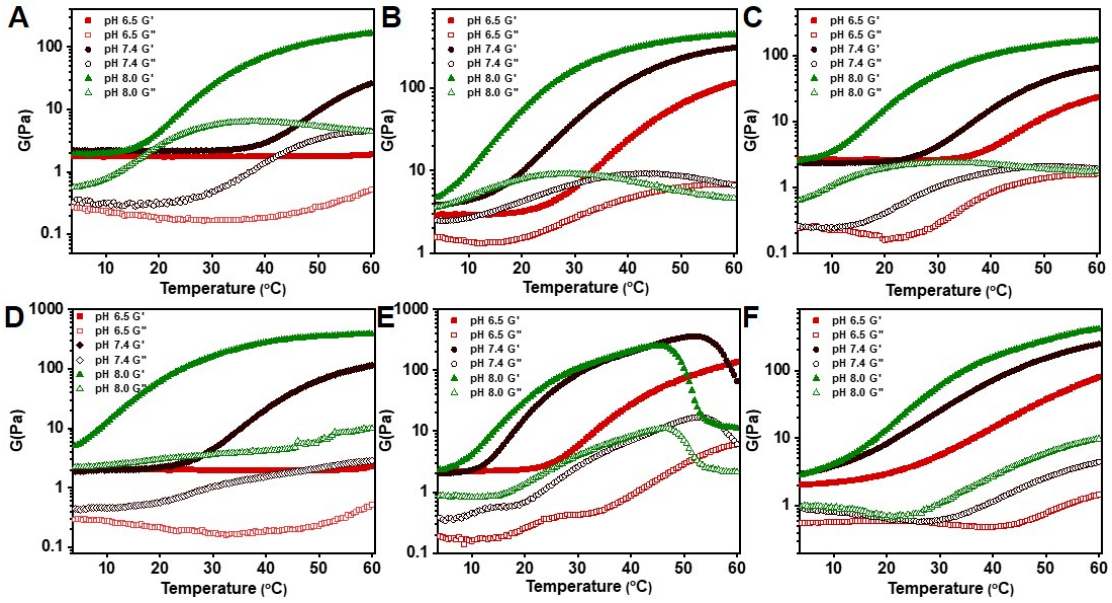


Fig. S10 Storage modulus (G′) and loss modulus (G″) of $EG_{45}(E_{1/2}PA_{1/2})_{20}$ (A), $EG_{45}(E_{1/2}PA_{1/2})_{16}$ (B), $EG_{45}(E_{1/2}PA_{1/2})_{12}$ (C), $(E_{1/2}PA_{1/2})_{10}EG_{45}(E_{1/2}PA_{1/2})_{10}$ (D), $EG_{45}(E_{3/4}PA_{1/4})_{16}$ (E) and $EG_{45}(E_{3/4}PA_{1/4})_{12}$ (F).

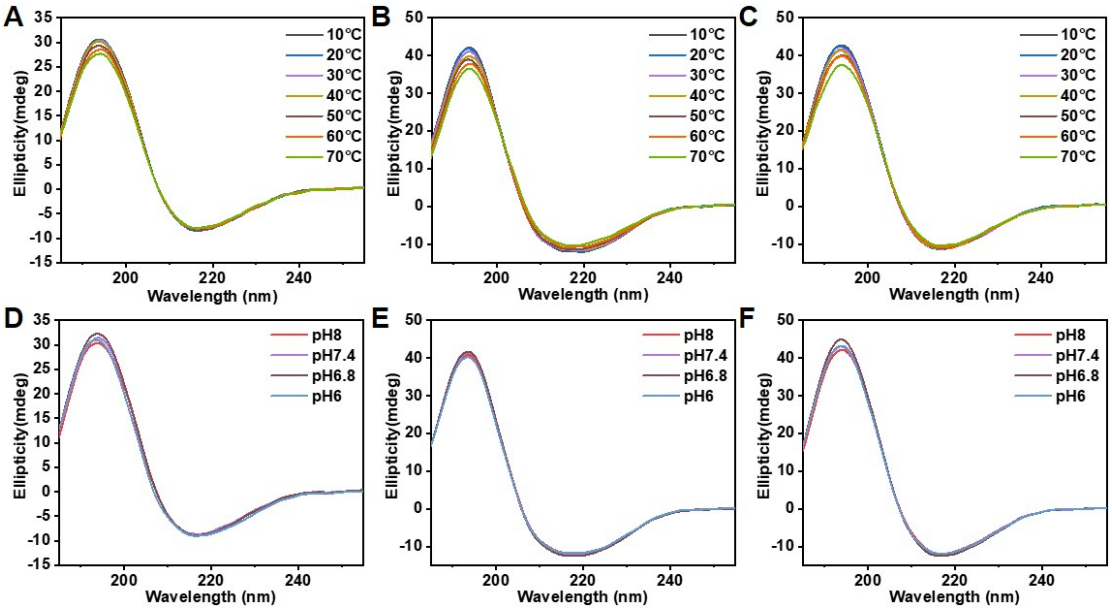


Fig. S11 Circle dichroism spectra as a function of temperature (A-C) and pH (D-F); (A) and (D), $EG_{45}(E_{2/3}PA_{1/3})_{12}$; (B) and (E), $EG_{45}(E_{3/4}PA_{1/2})_{16}$; (C) and (F), $EG_{45}(E_{3/4}PA_{1/4})_{20}$.

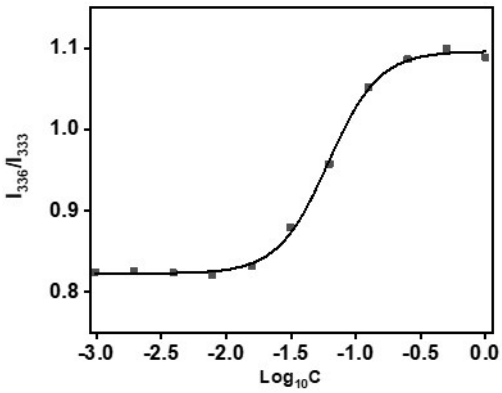


Fig. S12 Plot of I_{336}/I_{333} as a function of $EG_{45}(E_{1/2}PA_{1/2})_{12}$ concentration.

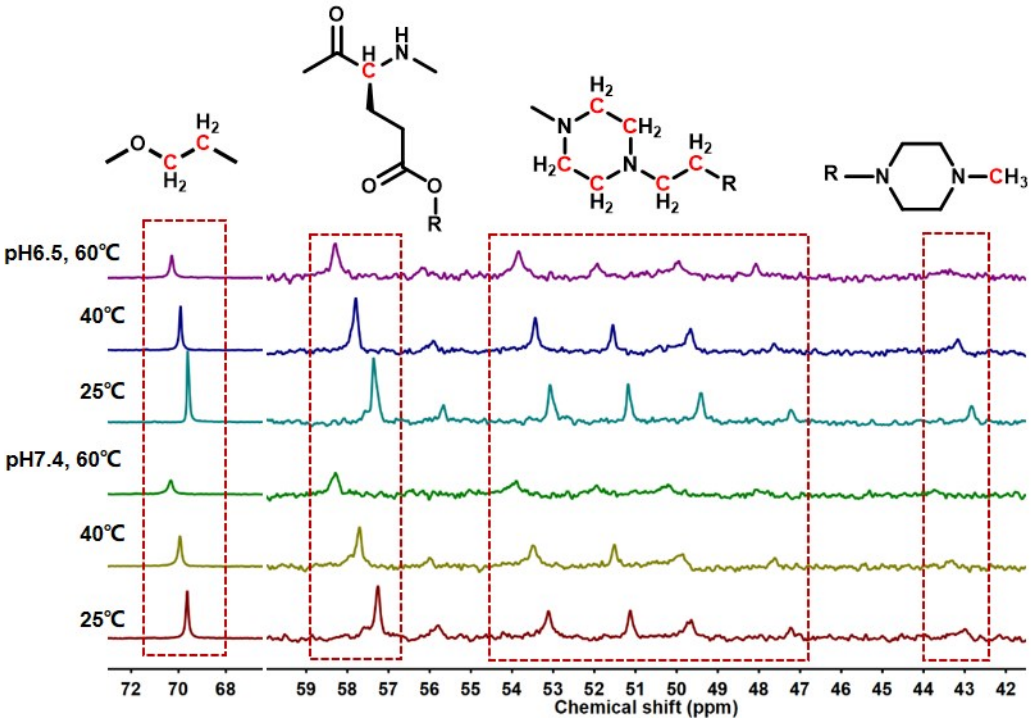


Fig. S13 ^{13}C NMR spectra of $EG_{45}(E_{1/2}PA_{1/2})_{12}$ (14%, (w/v)) in D_2O at indicated pH and temperatures, pH was adjusted using deuterium chloride and sodium deuteroxide.

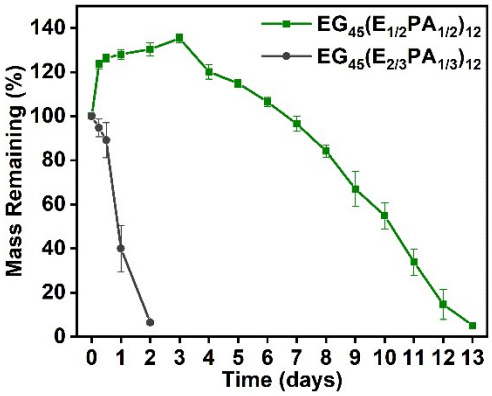


Fig. S14 Degradation of pH 7.4 $EG_{45}(E_{1/2}PA_{1/2})_{12}$ and $EG_{45}(E_{2/3}PA_{1/3})_{12}$ hydrogel used pH 7.4 PBS as degradation medium (n=3).

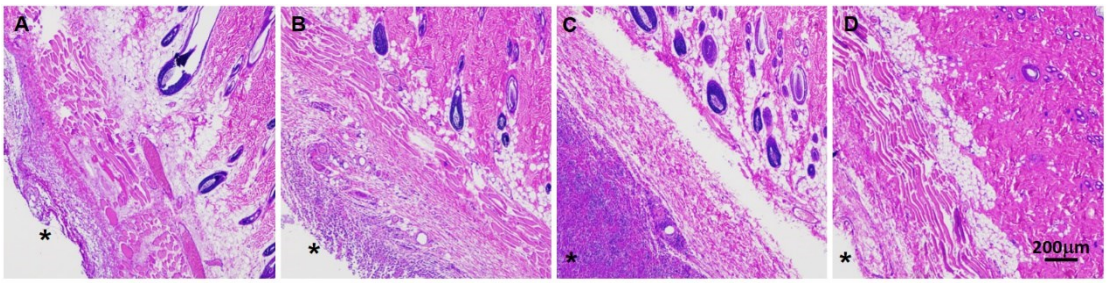


Fig. S15 H&E staining of the skin with $EG_{45}(E_{3/4}P_{1/8}PA_{1/8})_{16}$ hydrogel at 15 min (A), 1 week (B), 2 weeks (C) and 4 weeks (D). *represents the skin contacted with hydrogel.

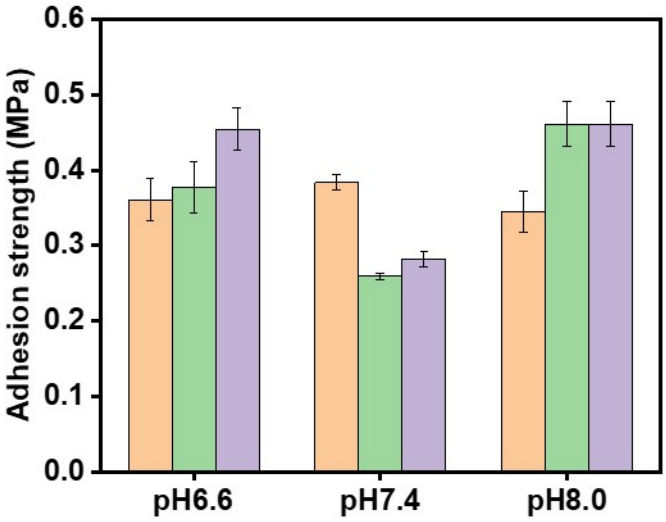


Fig. S16 Adhesion strength of PPCAs (9%, (w/v)). Orange, $EG_{45}(E_{1/2}PA_{1/2})_{12}$; green, $EG_{45}(E_{1/2}PA_{1/2})_{16}$; purple, $EG_{45}(E_{1/2}PA_{1/2})_{20}$.

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