Supplementary Information

Light-inducible nanodrug-mediated photodynamic and anti-apoptotic synergy for enhanced immunotherapy in triple-negative breast cancer

Jing Huang,[a,b,c] *Xingliang Liu,*[a,b,c] *Minzhao Lin,*[b] *Zecong Xiao,*[a,*] *Xintao Shuai,*[a,b,]

[a]Nanomedicine Research Center, The Third Affiliated Hospital of Sun Yat-sen University, Guangzhou 510630, P. R. China

[b]PCFM Lab of Ministry of Education, School of Materials Science and Engineering, Sun Yat-sen University, Guangzhou 510275, P. R. China

[c]School of Engineering, Westlake University, Hangzhou 310030, P. R. China

Corresponding email: xiaozc5@mail.sysu.edu.cn

Table of Contents

Materials

All commercial reagents including cysteamine hydrochloride, trifluoroacetic acid (TFA), methoxypolyethylene glycol (PEG-OH, M_n = 5000 kDa), succinic anhydride, 4-dimethylaminopridine (DMAP), triethylamine (TEA), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), *N*,*N*-diisopropylethylenediamine (DIP), benzylamine (BA), *N*,*N*-diisopropylethylamine (DIPEA) and *N*,*N*-Di-Boc-1H-pyrazole-1-carboxamidine (PCX(Boc)$_2$ were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) and used as received unless otherwise specified. Amino acids were purchased from Tokyo Chemical Industry (Shanghai, China). Triphosgene and maleic anhydride were purchased from Shanghai Macklin Biochemical Technology Co., Ltd. (Shanghai, China). Solvents including acetone, chloroform, 1,4-dioxane, diethyl ether, ethyl acetate, petroleum ether, dichloromethane (DCM) and tetrahydrofuran (THF) were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). DCM, petroleum ether and THF were dried over CaH_2 prior to use. Monomers including γ-Benzyl-$_L$-aspartic acid *N*-carboxyanhydrides (BLAsp-NCA) and N^{ε}-carboxybenzyl-$_L$-lysine *N*-carboxyanhydride (ZLLys-NCA) were synthesized following literature procedures.[1-3] 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and *N*,*N*-dimethylformamide (DMF, ultra-dry, with molecular sieves) were purchased from J&K Chemical Ltd. (Beijing, China). DMEM culture medium, RPMI-1640 culture medium, PBS, 0.25% trypsin-EDTA and fetal bovine serum (FBS) were purchased from ThermoFisher Scientific (Gibco, USA).

The antibodies for flow cytometry analyses including anti-mouse anti-mouse CD80 (PE conjugated, Cat#104704), anti-mouse CD86 (APC conjugated, Cat#105011), anti-mouse CD11c (FITC conjugated, Cat#117306), anti-mouse CD3 (APC conjugated, Cat#100236), anti-mouse CD8 (PE conjugated, Cat#100707), anti-mouse CD4 (FITC conjugated, Cat#100509) and anti-mouse IFN-γ (APC-Cy7 conjugated, Cat#505849) were purchased from BioLegend (San Diego, CA, USA). Other antibodies for western blot, immunohistochemical staining and immunofluorescence staining including anti-Catalase antibody (ab209721), anti-GAPDH antibody (ab9485), anti-Calreticulin antibody (ab92516), anti-Ki67 antibody (ab15580), anti-CD8 alpha antibody (ab217344) and goat anti-rabbit IgG Alexa Fluor® 488 (ab150077) were purchased from Abcam (Cambridge, UK). Goat anti-Rabbit IgG antibody (HRP, A0208) was purchased from Beyotime Biotechnology (Shanghai, China).

Instrumentation

^{1}H NMR spectra were recorded on a Bruker AVANCE III 400 MHz spectrometer with

tetramethylsilane (TMS) as the internal standard. The hydrodynamic diameter, size distribution of nanoparticles and zeta potentials of nanoparticles were measured using the Zeta-Nanosizer (ZEN3600, Malvern Instruments Ltd, Worcestershire, UK), which was routinely calibrated with a -50mV Zeta-potential standard (Malvern Instruments). Each measurement was performed for 30 runs, and the results were processed with DTS software version 3.32. Transmission electron microscopy (TEM) observations were conducted on a JEOL1400 plus electron microscope at an acceleration voltage of 120 kV. The sample solution was deposited onto a carbon-coated copper grid and the solvent was completely evaporated under infrared lamp before TEM observation.

Synthesis of H_2N-thioketal-NH_2

H_2N-thioketal-NH_2 was synthesized according to a reported method. A mixture of cysteamine hydrochloride (2 g, 17.6 mmol) and anhydrous acetone (2.75 g, 47.3 mmol) were stirred at room temperature for 30 min. After the cysteamine hydrochloride was completely dissolved, trifluoroacetic acid (TFA) (0.5 mL) was added into the reaction mixture and the reaction continued for another 12 h at room temperature. After the reaction, the product was filtered, and the white filtrate was washed with chloroform three times. Then, the product was dried for 3 h and recrystallized from 6 M NaOH aqueous solution (25 mL) three times. Finally, the product was extracted with 3 × 25 mL of DCM to obtain the product.

Synthesis of PEG-thioketal-NH_2

PEG-OH were converted to PEG-COOH following a reported method. Briefly, PEG-OH (5 g, 1 mmol), succinic anhydride (0.5 g, 5 mmol) and DMAP (0.61 g, 5 mmol) were dissolved in 1, 4-Dioxane (32 mL). Triethylamine (TEA) (0.51 g, 5 mmol) was added to reaction mixture and the mixture was stirred at room temperature for 24 h under nitrogen atmosphere. The reaction solution was poured into an excess amount of diethyl ether to obtain PEG-COOH.

PEG-COOH (5 g, 1 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (0.23 g, 1.1 mmol) and *N*-hydroxysuccinimide (NHS) (0.13 g, 1.1 mmol) were dissolved in DCM (10 mL). After reacting for 2 h, the solution of NHS-actived PEG was dropwise added into 10 mL DCM containing NH_2-thioketal-NH_2 (0.2 g, 1.5 mmol) under stirring, and further reacted for 24 h. And then, the mixture was concentrated and precipitated into a cold mixture of diethyl ether/methanol (10/1, v/v) three times to gain the PEG-thioketal-NH_2.

Synthesis of PAsp(DIP/BA)-PLys(Gua)-s-PEG *via* multiple reactions

PZLLys-s-PEG was prepared by ring-opening polymerization of ZLLys-NCA using PEG-thioketal-NH_2 as an initiator. Briefly, PEG-thioketal-NH_2 (0.25 g, 0.05 mmol) and ZLLys-NCA (2 g, 6.54 mmol) was dissolved in anhydrous DMF. The mixture was kept in an oil bath at 40 °C for 48 h. PZLLys-s-PEG was purified by precipitation into diethyl ether for three times. The product was dried under vacuum to obtain white powder.

PZLLys-s-PEG was dissolved in DCM (304.4 mg/mL) for further use as macroinitiators. The stock solution of PZLLys-s-PEG (59.5 μL) was diluted with DCM (340 μL), into which the aqueous buffer (pH = 7.0, 10.6 μL) was added. The resulting mixture was emulsified with a probe sonicator using a pulse sequence (1s pulse on and 1 s pulse off, total pulse on time = 10 s). An aliquot of the emulsion (257 μL) was then mixed with DCM solution of purified BLAsp-NCA (250 μL, 0.1 M) to start the polymerization. FT-IR was used to monitor the reaction process. After the complete reaction of BLAsp-NCA, the reaction mixture was precipitated in a large amount of anhydrous ether. The precipitation was dried in vacuum at room temperature for 24 h, achieving PBLAsp-*b*-PZLys-s-PEG.

PBLAsp-*b*-PZLys-s-PEG (450 mg) was dissolved in DMSO (4.5 mL), to which *N*,*N*-diisopropylethylenediamine (DIP) (33 mg) was added under stirring at 40 °C for 12 h with the protection of dry nitrogen atmosphere. After the reaction, excess benzylamine (BZ) (570 mg) was added directly to the reaction mixture at 40 °C for another 24 h. The mixture was dropped into cold diethyl ether, and the precipitate was filtered and dried under vacuum, achieving PAsp(DIP/BA)-PZLLys-s-PEG.

PAsp(DIP/BA)-PZLLys-s-PEG (1 g) was dissolved in 10 mL trifluoroacetic acid, and added with 1 mL hydrobromic acid. The mixture was stirred at room temperature for 4-5 h. The resulting PAsp(DIP/BA)-PLys-s-PEG was purified by precipitation into diethyl ether to obtain the crude product. The crude product dialyzed against deionized water with dialysis membrane (MWCO, 3500 Da) at room temperature for 48 h. The purified product was lyophilized to obtain white powder.

PAsp(DIP/BA)-PLys-s-PEG (0.28 g) and $PCX(Boc)_2$ (0.524 g) were separately added into *N*-methyl pyrrolidone (1 mL), and then mixed. DIPEA (0.22 g) was dissolved in the above solution and reacted at 25 °C for 24 h. the obtained solution was purified by precipitation into diethyl ether, achieving PAsp(DIP/BA)-PLys(Gua(tBu))-s-PEG. PAsp(DIP/BA)-PLys(Gua(tBu))-s-PEG (50 mg) was added into 2 mL of a solvent mixture containing TFA and DCM (1/1, v/v) and stirred at 25 °C for 12 h. the solution was purified by precipitation into diethyl ether to obtain the crude product. The crude product dialyzed against deionized water with dialysis membrane (MWCO, 3500 Da) at room temperature for 48 h. The purified product was lyophilized to obtain white powder, achieving PAsp(DIP/BA)-

PLys(Gua)-s-PEG.

Preparation of C/A@PN-s-P

PAsp(DIP/BA)-PLys(Gua)-s-PEG was dissolved in DMSO (10 mg mL^{-1}), to which DMSO solution of Ce6 and ABT-737 were added. The above mixture was then added into water dropwise under sonication to achieve C/A@PN-s-P. The micelle suspension was dialyzed to remove free Ce6 and DMSO and then ultrafiltrated for further use.

Assessment of ROS responsibility

In order to prove the ROS responsiveness of C/A@PN-s-P, its suspension was subjected to 670 nm laser irradiation (670 nm, 400 mW/cm^2, 5 min). The particle size and zeta potential of C/A@PN-s-P before and after irradiation were monitored by Zeta-Nanosizer. The morphology of C/A@PN-s-P before and after irradiation were also studies by TEM.

Cellular uptake

Cellular uptake was studied using confocal laser scanning microscope (CLSM, Leica SP8) and Attune NxT flow cytometer (Thermo Scientific, Waltham, USA). Briefly, 4T1 cells were seeded at a density of 1×10^4 cells per well (in a 20 mm confocal dish (Φ =15 mm) for CLSM study) and incubated overnight for cell attachment. Cells were exposed to PBS, C@PN-s-P and C@PN-n-P with/without the pretreatment of H_2O_2 (400 mM). After 1 h incubation, the cells were rinsed with ice bath-cooled PBS and analyzed by flow cytometer. Similarly, the cells were further fixed with fresh 4% paraformaldehyde for 15 min at room temperature. The cells were then counterstained with DAPI for cell nucleus. After the staining, the cells were observed using CLSM (Leica SP8).

MTT assay

The 4T1 cells were seeded in the 96-well plates overnight. C/A@PN-s-P with various Ce6 and ABT-737 concentrations were pretreated with H_2O_2 (400 mM), and then added into 4T1 cell culture medium. After 6 h of incubation, 4T1 cells were washed with PBS and then subjected to irradiation (670 nm, 400 mW/cm^2, 1 min, 50% intensity). The cells were cultured for another 24 h. Finally, 20 μL of MTT was added to each well for another 4 h incubation, and absorbance at 490 nm was measured by microplate reader.

ROS generation

The *in vitro* ROS generation triggered by NIR light in cells was determined using a 2',7'-dichlorofluorescein diacetate (DCFH-DA, 10 mM) ROS detection kit. Briefly, 4T1 cells were seeded at a density of 1×10^4 cells per well in a 20 mm confocal dish (Φ =15 mm) and incubated overnight for cell attachment. PBS, A@PN-s-P, C@PN-s-P and C/A@PN-s-P were pretreated with H_2O_2 (400 mM) and then added into cell culture medium. After 6 h of incubation, the culture medium was replaced with the fresh one without FBS but added with DCFH-DA. After irradiation (670 nm, 400 mW/cm^2, 1 min, 50% intensity), cells were rinsed with PBS cooled in ice bath and further counterstained with DAPI for cell nucleus. After staining, cells were observed using CLSM (Leica SP8).

Apoptosis assay

4T1 cells were incubated in 24-well plates. PBS, A@PN-s-P, C@PN-s-P and C/A@PN-s-P were pretreated with H_2O_2 (400 mM) and then added into cell culture medium. After 6 h of incubation, cells were washed with PBS and irradiated (670 nm, 400 mW/cm^2, 1 min, 50% intensity). After another incubation of 6 h, Annexin V and PI staining were performed and the apoptotic ratio was detected by flow cytometer (Thermo Scientific, Waltham, USA).

***In vitro* immune activation**

CRT exposure: 4T1 cells were seeded in confocal cell dishes and 24-well plates. PBS, A@PN-s-P, C@PN-s-P and C/A@PN-s-P were pretreated with H_2O_2 (400 mM) and then added into cell culture medium. After 6 h of incubation, the cells were washed with PBS, followed by laser irradiation (670 nm, 400 mW/cm^2, 1 min, 50% intensity). Then, cells were incubated with anti-CRT antibody and secondary antibody before detection with flow cytometry and confocal assay.

DC maturation: The BMDCs of mice were extracted from bone marrow and induced to differentiate into DCs by 20 ng/ml IL-4 and 20 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF). On day 5, DCs were cultured with the supernatant of 4T1 cells which were treated with different formulations (PBS, A@PN-s-P, C@PN-s-P and C/A@PN-s-P pretreated with H_2O_2 (400 mM)). On day 7, DCs were collected and CD11c, CD80 and CD86 were detected by flow cytometer (Thermo Scientific, Waltham, USA).

Animal tumor model

Female BALB/c mice aging 4 weeks were purchased from Guangdong Medical Laboratory Animal Center. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Sun Yat-sen University (SYXK 2016-0112) and approved by the Animal Ethics Committee of Sun Yat-sen University. To develop tumors in BALB/c mice, 100 μL of 4T1 cells suspension (1×10^6 4T1 cells suspended in 10 mL of PBS) were subcutaneously implanted in the right lateral hind legs. For flow cytometry assay, immunofluorescent staining, immunohistochemical staining, H&E and Ki67 staining experiments, the experimental endpoint was at 15th day after first drug administration. For tumor growth and cumulative survival experiments, the endpoint was the time when tumor volume was over 2000 cm^3.

***In vivo* tumor accumulation and imaging**

200 μL of DiR@PN-s-P and DiR@PN-n-P were injected *via* tail vein into tumor-bearing mice. Mice were exposed to irradiation 4 h post injection to conduct PEG cleavage. Then, the *in vivo* and *ex vivo* fluorescence imaging experiments were performed on an ima1ging system (In vivo FX, Carestream, America) at different time points.

***In vivo* tumor growth inhibition**

Tumor size was measured with a caliper during *in vivo* PDT experiment. When tumor size reached ~80 mm^3, the mice were tail vein injected with 200 μL of neat PBS or PBS containing one of the following nanomedicines A@PN-s-P, C@PN-s-P and C/A@PN-s-P every 2 days. Ce6 was administrated at 5 mg/kg and ABT-737 was administrated at 10 mg/kg. Tumor sites of animals were exposed to first-wave NIR laser (670 nm, 400 mW/cm^2, 5 min) at 4 h after the injection to conduct PEG cleavage. The second-wave NIR laser (670 nm, 400 mW/cm^2, 5 min irradiation followed by 5 min interval and another 5 min irradiation) was applied at 8 h after the injection. Tumor volumes were calculated using the following equation: $V = AB^2/2$, where A and B represent the maximum diameter and minimum diameter of tumor, respectively. For tumor growth and cumulative survival experiments, the endpoint was the time when tumor volume was over 2000 cm^3.

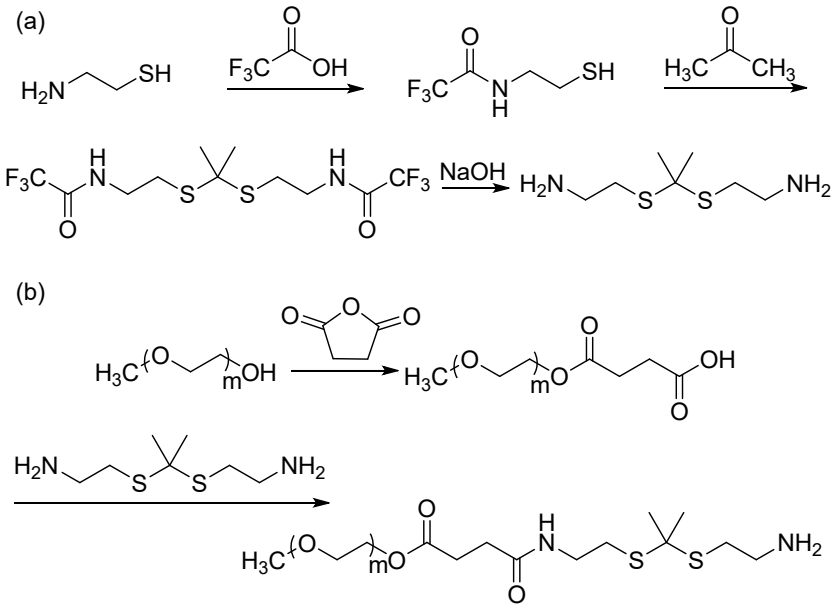
***In vivo* immune activation**

To explore the anti-tumor mechanism of nanomedicines *in vivo*, 4T1 subcutaneous tumor mouse model was established. For flow cytometry assay, Immunofluorescent staining and Ki67 staining

experiments, the experimental endpoint was at 15th day after first drug administration. Mice were treated as described above. On day 15, tumors and lymph nodes were harvested, digested and homogenized. For detection of DC cells, cells collected from lymph nodes were incubated with PE-CD86, APC-CD80 and FITC-CD11c. For T cells detection, the infiltrating lymphocytes were incubated with FITC-CD4, PE-CD8 and APC-CD3. The intracellular IFN-γ were stained after fixing and perforation of T cells. The above cells were detected using NocoCyte 2060R and the data were analyzed with FlowJo 10.0 (Treestar, USA).

Statistical analysis

The statistical significance was analyzed by one-way ANOVA with a Tukey post hoc test. *p*-value: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The statistical significance was analyzed by one-way ANOVA with a Tukey post hoc test. Data were presented as the mean ± standard deviation (SD), and $P < 0.05$ was considered statistically significant.



Supporting Figures

Figure S1. Synthetic illustration of (a) H_2N-thioketal-NH_2 and (b) PEG-thioketal-NH_2.

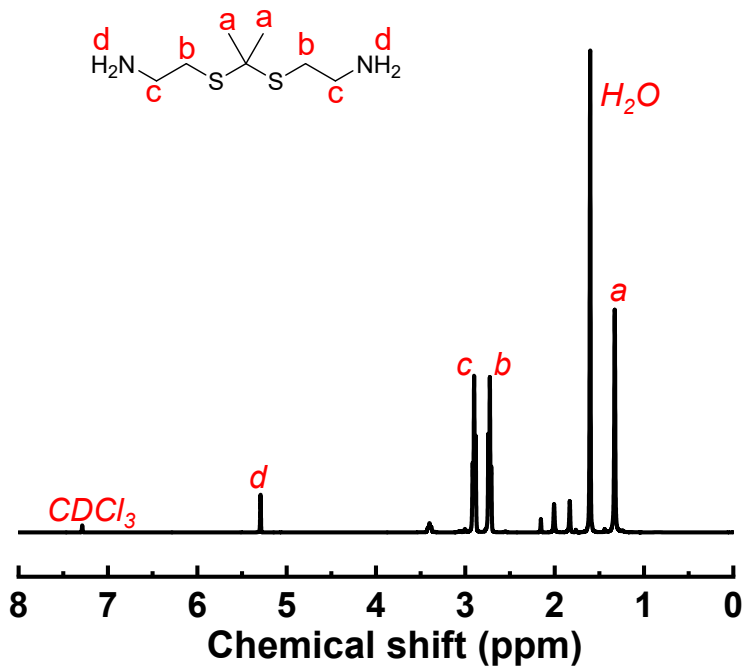


Figure S2. ^{1}H NMR spectrum of H_2N-thioketal-NH_2.

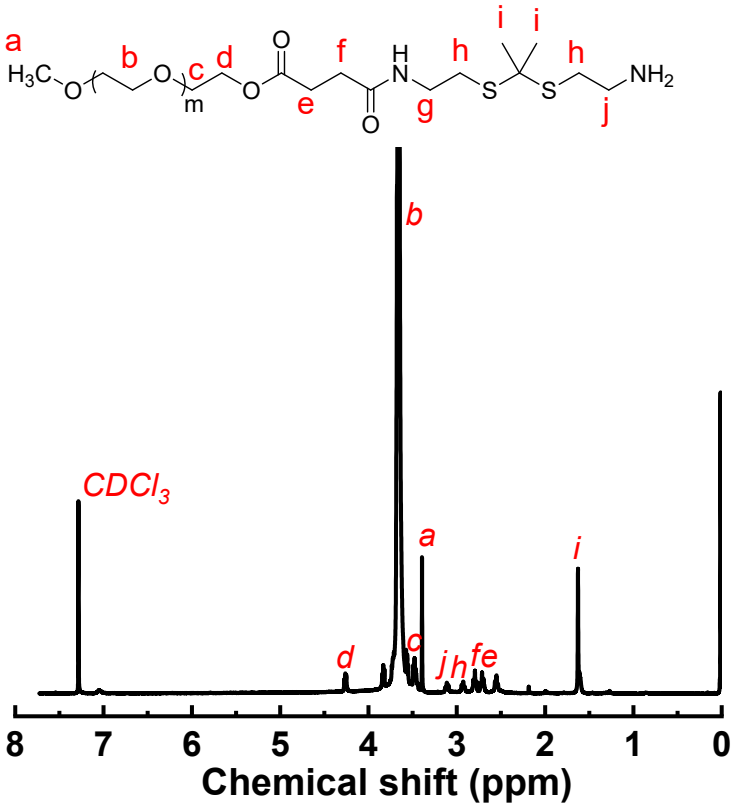


Figure S3. ^{1}H NMR spectrum of PEG-thioketal-NH_2.

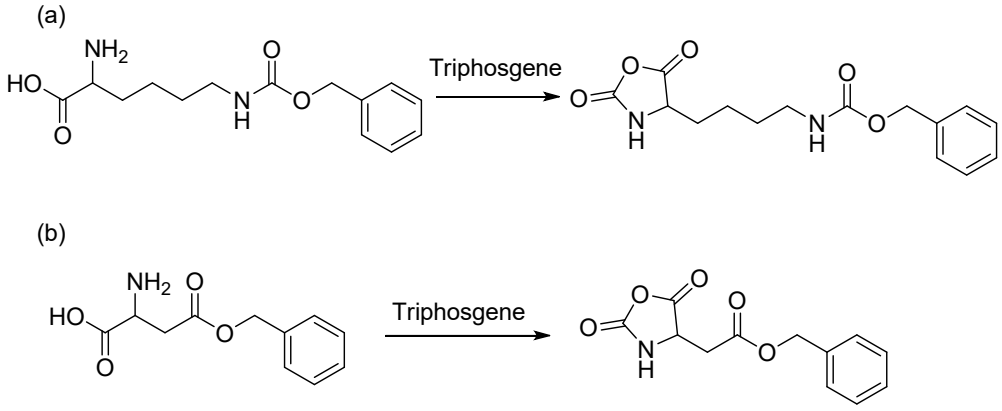


Figure S4. Synthetic illustration of (a) γ-Benzyl-$_{L}$-aspartic acid *N*-carboxyanhydrides (BLAsp-NCA) and (b) N^{ε}-carboxybenzyl-$_{L}$-lysine *N*-carboxyanhydride (ZLL-NCA).

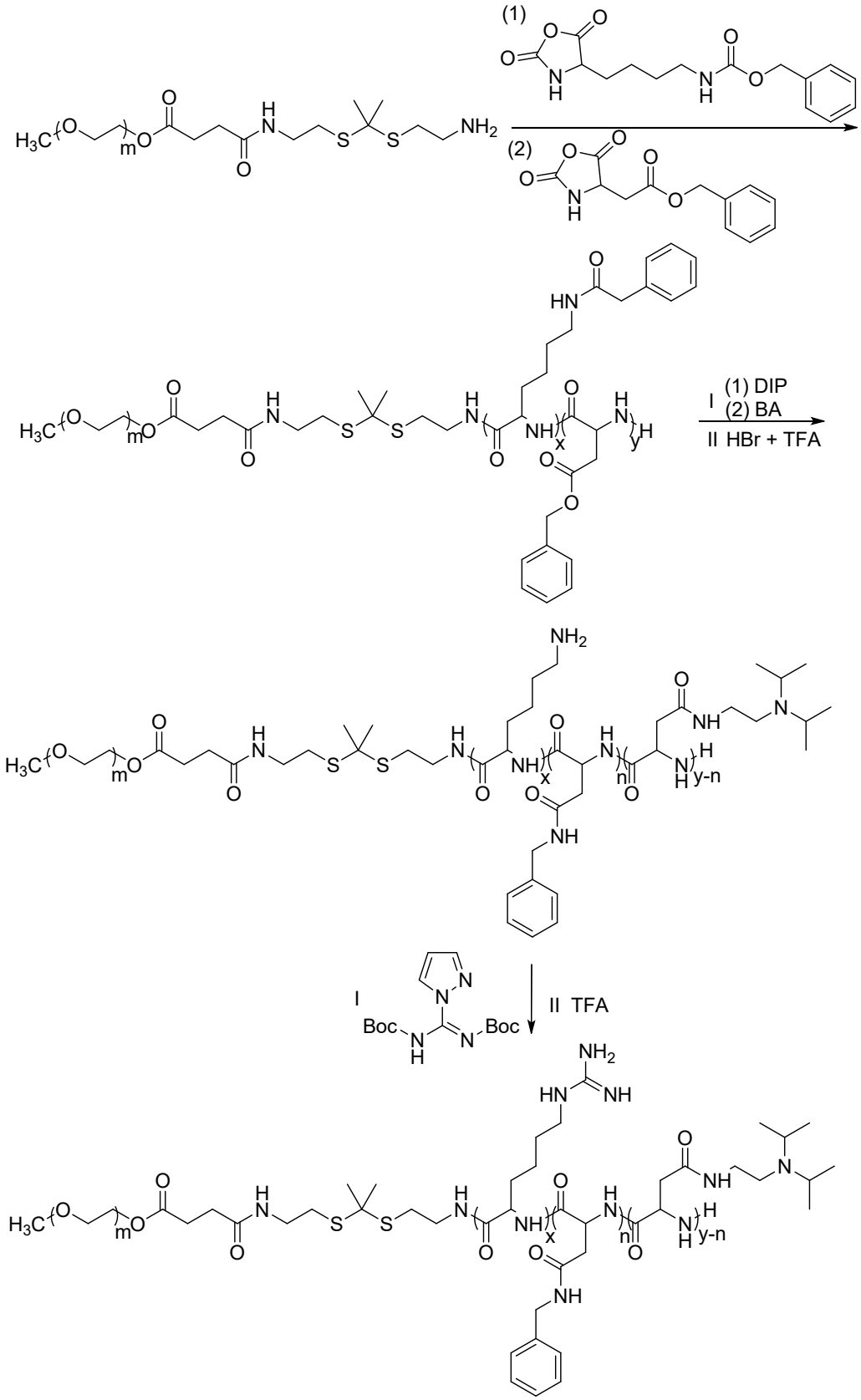


Figure S5. Synthetic illustration of PAsp(DIP/BA)-PLys(Gua)-s-PEG *via* multiple reactions.

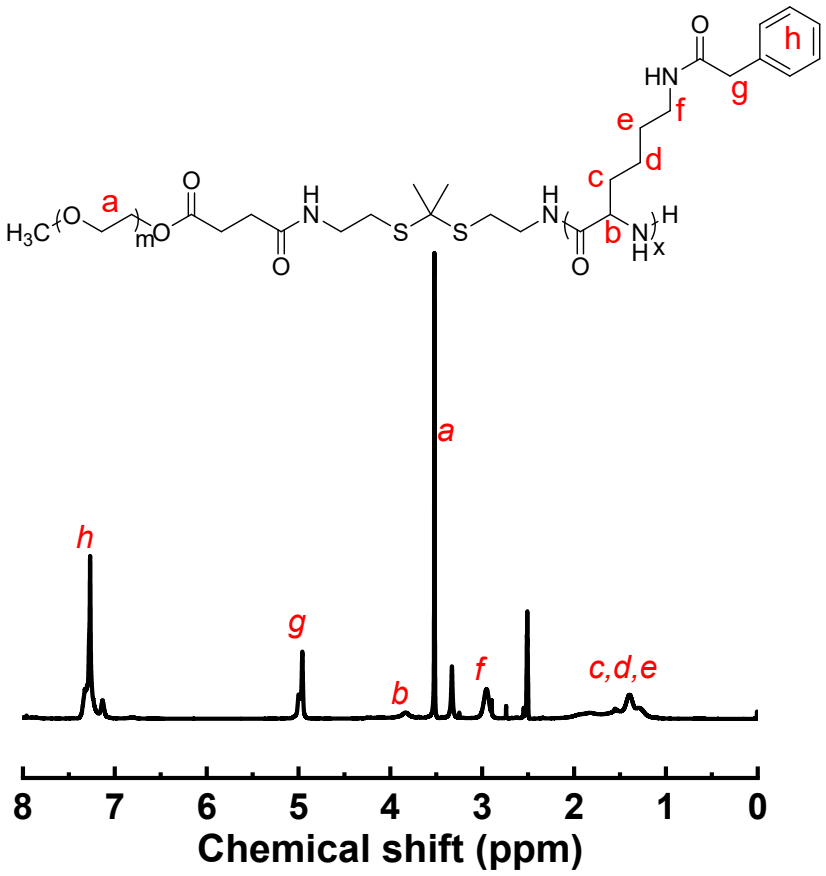


Figure S6. ^{1}H NMR spectrum of poly(N^{ε}-carboxybenzyl-$_{L}$-lysine)-*b*-^{sensitive}PEG (PZLLys-s-PEG).

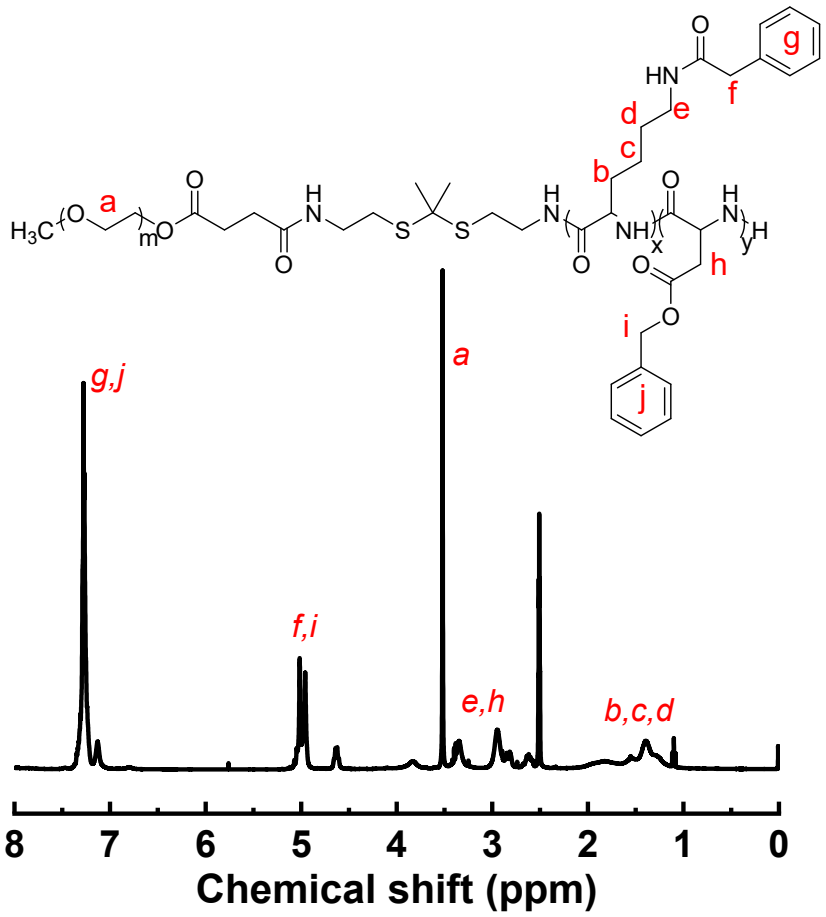


Figure S7. ^{1}H NMR spectrum of poly(γ-Benzyl-$_{L}$-aspartic acid)-*b*-poly(N^{ε}-carboxybenzyl-$_{L}$-lysine)-*b*-^{sensitive}PEG (PBLAsp-PZLLys-s-PEG).

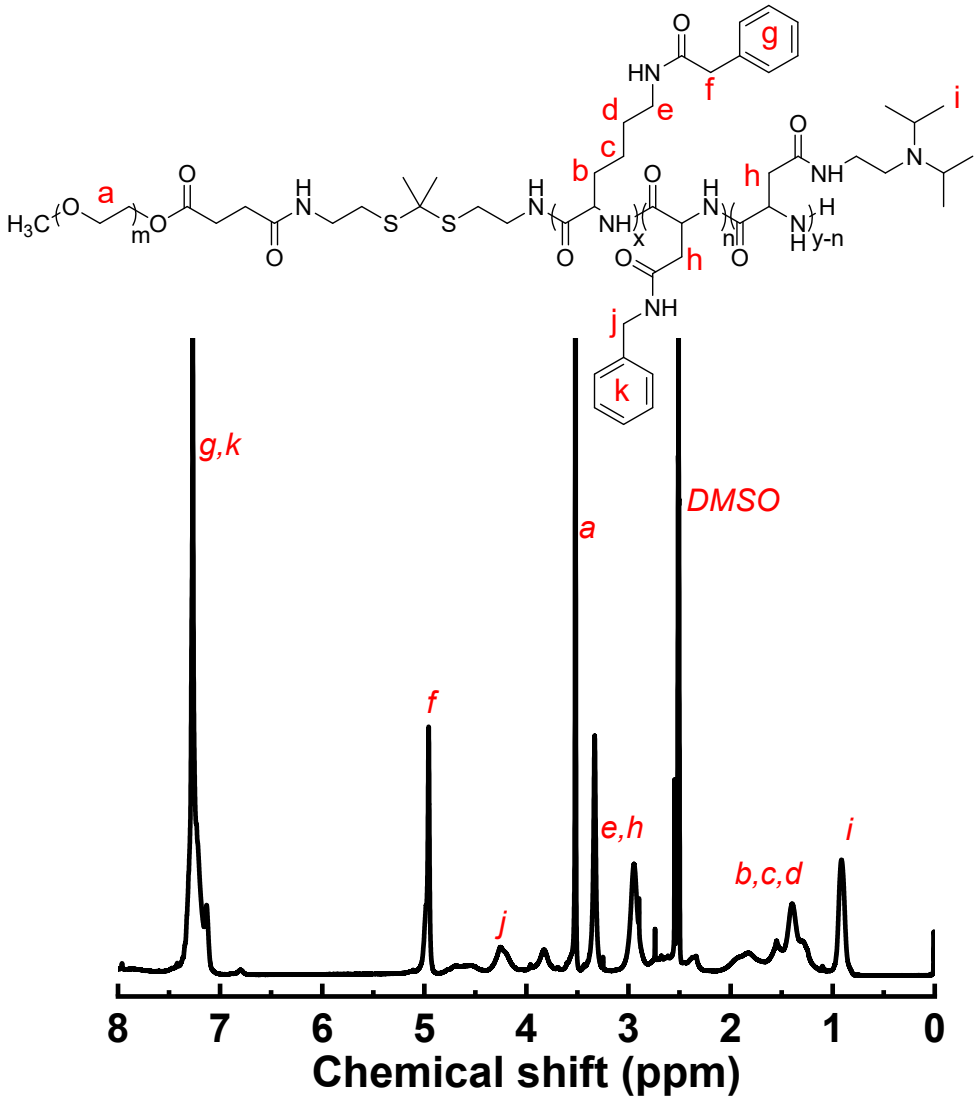


Figure S8. ^{1}H NMR spectrum of poly(aspartic acid (*N,N*-diisopropylethylenediamine-*co*-benzylamine))-*b*-poly(*N*$^{\varepsilon}$-carboxybenzyl-$_{L}$-lysine)-*b*-^{sensitive}PEG (PAsp(DIP/BA)-PZLLys-s-PEG).

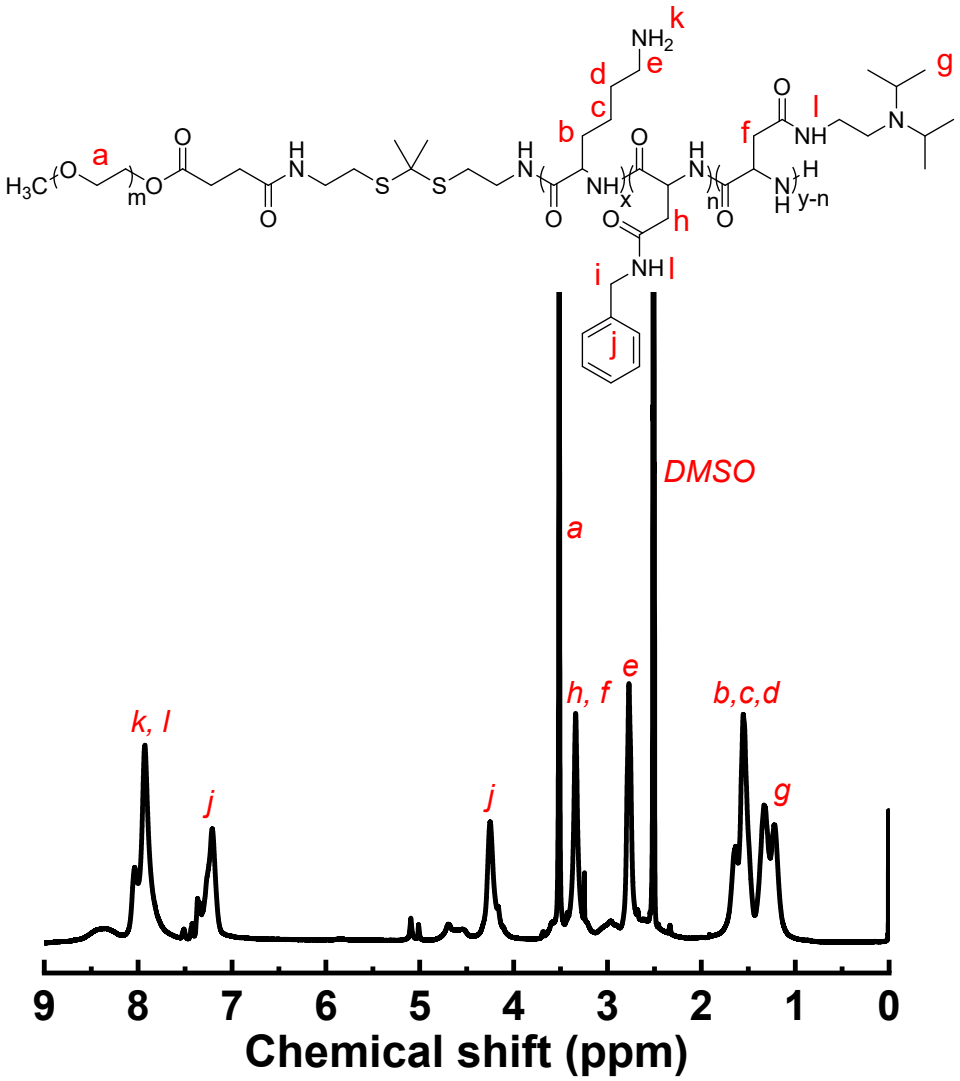


Figure S9. ^{1}H NMR spectrum of poly(aspartic acid (*N,N*-diisopropylethylenediamine-*co*-benzylamine))-*b*-poly(lysine)-*b*-^{sensitive}PEG (PAsp(DIP/BA)-PLys-s-PEG).

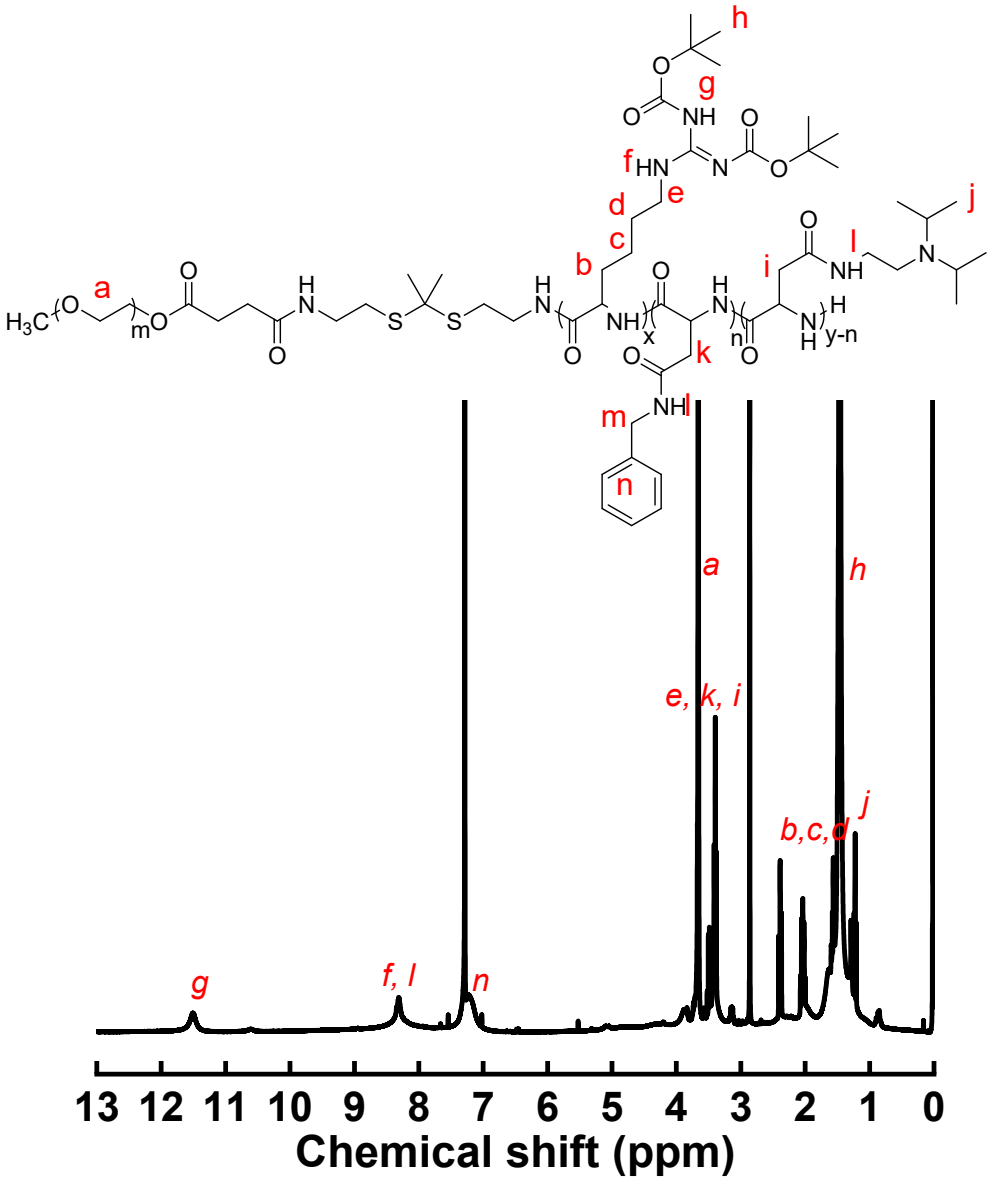


Figure S10. ^{1}H NMR spectrum of poly(aspartic acid (*N,N*-diisopropylethylenediamine-*co*-benzylamine))-*b*-poly(lysine((tertiary butyl)-guanidine))-*b*-^{sensitive}PEG (PAsp(DIP/BA)-PLys(Gua(tBu))-s-PEG).

Figure S11. Schematic illustration of the 4T1 tumor inhibition studies.

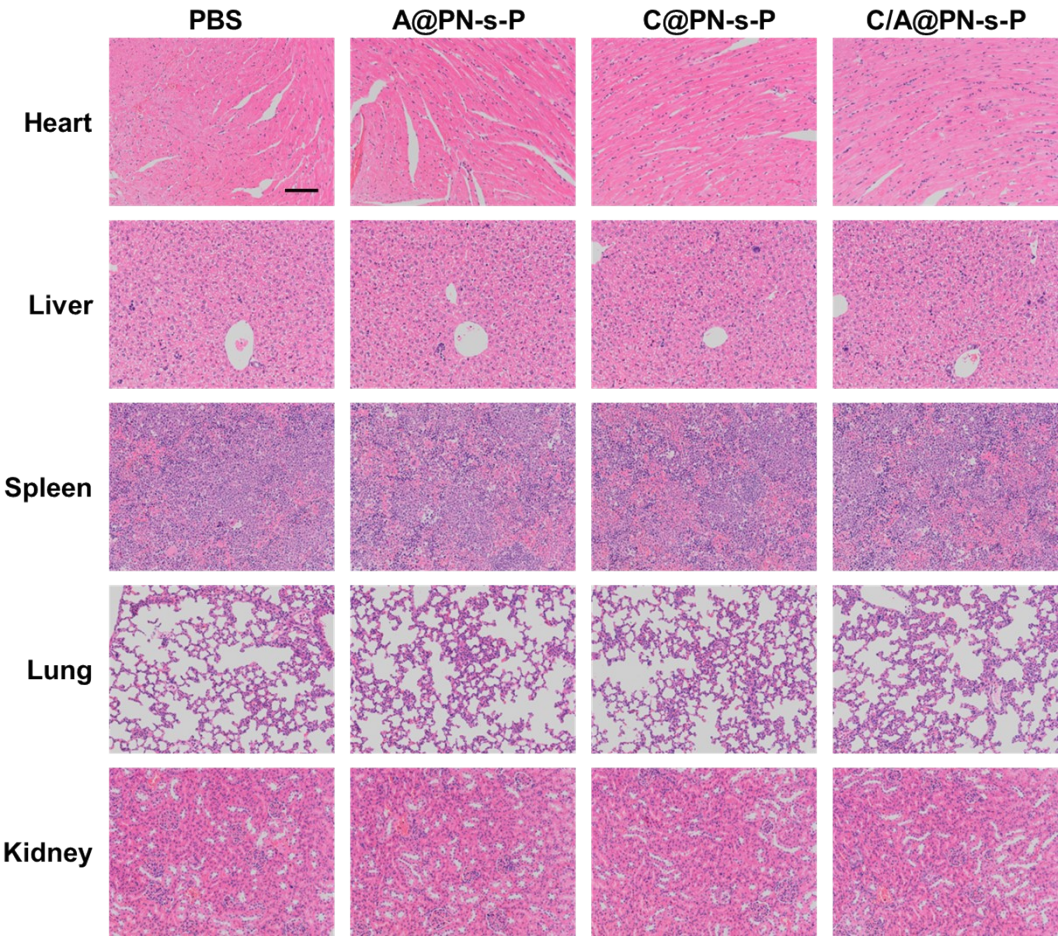


Figure S12. H&E staining of major organs collected from mice receiving different treatments of PBS, A@PN-s-P, C@PN-s-P and C/A@PN-s-P. Scale bar 100 μm.

Supporting References

(1) Tian, Z.-Y.; Zhang, Z.; Wang, S.; Lu, H. A moisture-tolerant route to unprotected α/β-amino acid *N*-carboxyanhydrides and facile synthesis of hyperbranched polypeptides. *Nat. Commun.* **2021,** 12, 5810.
(2) Zhang, R.; Zheng, N.; Song, Z.; Yin, L.; Cheng, J. The effect of side-chain functionality and hydrophobicity on the gene delivery capabilities of cationic helical polypeptides. *Biomaterials* **2014,** 35, 3443-3454.
(3) Xia, Y.; Song, Z.; Tan, Z.; Xue, T.; Wei, S.; Zhu, L.; Yang, Y.; Fu, H.; Jiang, Y.; Lin, Y.; Lu, Y.; Ferguson, A. L.; Cheng, J. Accelerated polymerization of *N*-carboxyanhydrides catalyzed by crown ether. *Nat. Commun.* **2021,** 12, 732.