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Supporting Information

Bacteria-Assisted Activation of Antimicrobial Polypeptides by a Random-Coil to Helix Transition

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Experimental procedures:

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified. Anhydrous dimethylformamide (DMF) was dried by a column packed with 4Å molecular sieves and stored in a glove box. Anhydrous tetrahydrofuran (THF), ethyl acetate (EtOAc) and hexane were dried by a column packed with alumina. Hexamethyldisilazane (HMDS) was dissolved in DMF in a glovebox. Spectra/Por® dialysis tubing with a molecular weight cut-off (MWCO) of 1 kDa was purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA). γ-(6-chlorohexyl)-L-glutamate (CH-L-glu), γ-(6-chlorohexyl)-L-glutamic acid based *N*-carboxyanhydride (CH-L-glu-NCA) were synthesized according to previous study.[1] Tyrosine-NCA was obtained from Sigma-Aldrich. ATCC11778 (*Bacillus toyonensis*), ATCC12608 (*Staphylococcus aureus*), and NRS384 (Methicillin-resistant *S. aureus*) were grown in luria broth (LB) medium at 37 °C. Raw 264.7 cells, HEK-293 were incubated in Dulbecco's modified eagle's medium (DMEM) with the supplement of 10% fetal bovine serum (FBS). MCF-10A cells were incubated in Mammary epithelial basal medium (MEBM) along with the additives (from Lonza/Clonetics Corporation as a kit). IMR-90 cells were incubated Eagle's minimum essential medium (EMEM) with the supplement of 10% FBS. Bacterial phosphatase, alkaline from *Escherichia coli*, was purchased from Sigma-Aldrich (Cat No: P4069-25UN).

Characterization. ^{1}H NMR spectra were recorded on a Varian U500 MHz or a VXR-500 MHz spectrometer. Chemical shifts were reported in ppm and referenced to the solvent proton impurities. Gel permeation chromatography (GPC) experiments

were performed on a system equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS multi-angle laser light scattering detector (MALLS) detector (Wyatt Technology, Santa Barbara, CA, USA), and an OptilabrEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The detection wavelength of HELEOS was set at 658 nm. Separations were performed using serially connected size exclusion columns (100 Å, 500 Å, 10^3Å and 10^4 Å Phenogel columns, 5 μm, 300 × 7.8 mm, Phenomenex, Torrance, CA, USA) at 60 °C using DMF containing 0.1 mol/L LiBr as the mobile phase. The MALLS detector was calibrated using pure toluene and can be used for the determination of the absolute molecular weights (MWs). The MWs of polymers were determined based on the dn/dc value of each polymer sample calculated offline by using the internal calibration system processed by the ASTRA V software (version 5.1.7.3, Wyatt Technology, Santa Barbara, CA, USA). Circular dichroism (CD) measurements were carried out on a JASCO J-815 CD spectrometer. The polypeptide samples were prepared at concentrations of 0.20 mg/mL. The solution was placed in a quartz cell with a path length of 0.10 cm. Infrared spectra were recorded on a Perkin Elmer 100 serial FTIR spectrophotometer calibrated with polystyrene film. Lyophilization was performed on a FreeZonelyophilizer (Labconco, Kansas City, MO, USA). High performance liquid chromatography (HPLC) analyses were performed on a Shimadzu CBM-20A system (Shimadzu, Kyoto, Japan) equipped with a SPD-20A PDA detector (190–800 nm), a RF-20A fluorescence detector, and an analytical C18 column (Shimadzu, 3 μm, 50*4.6 mm, Kyoto, Japan) or size exclusion column (SEC,

Phenomenex, PolySep-GFC-P Linear, 300 * 7.8 mm,).

Synthesis of random copolypeptides Poly(γ-6-chlorohexyl-L-glutamate)-*r*-Poly(tyrosine) (PCHT). Inside the glovebox, CH-L-glu-NCA and tyrosine-NCA at various molar ratios (24:6, or 27:3) were dissolved in DMF (1.5 mL), followed by adding the HMDS-DMF solution (0.1 M, M/I=30). The mixture was stirred at room temperature for 48 h. The polymer was then precipitated in cold ether/hexane solution (v/v=1/1) and dried under vacuum at 40 ˚C for 8 h. We synthesized another two batches of PCHT-2, named as PCHT-2-2 and PCHT-2-3, with the same molar ratio of CH-L-glu-NCA and tyrosine-NCA (27:3) and the same procedure. (Yield: PCHT-1: 66 %, PCHT-2: 65%, PCHT-2-2: 58%, PCHT-2-3: 55%). DL-PCHT-2 was synthesized similarly using CH-DL-Glu-NCA and tyrosine-NCA (molar ratio at 27:3) as the monomer (Yield: 72 %).

Synthesis of bacteria activated antibacterial polypeptides. PCHT and NaI (3 equiv of chlorine groups) was dissolved in DMF and acetonitrile (v/v=1:1). The mixture was transferred to a 25 mL Schlenk tube into which *N*,*N*-dimethyloctylamine (3 equiv of chloro groups) was added. The mixture was stirred at 80 ºC for 48 h. NaCl aqueous solution (1.0 M, 3 mL) was then added. The solution was then stirred at room temperature for 3 h to promote ion exchange. The product was purified by dialysis (MWCO = 1 kDa) against distilled water for 1 day. White solid powder was obtained after lyophilization. (Yield: PHOT-1: 56 %, PHOT-2: 46%, PHOT-2-2: 43%, PHOT-2-3: 42%). DL-PHOT-2 was synthesized similarly using DL-PCHT-2 as the starting material (Yield: 63 %).

PHOT (20 mg) were dissolved in anhydrous *N*-Methyl pyrrolidone at 0 °C. Anhydrous triethylamine (100 μL) and phosphoryl chloride (100 μL) were then added. The reaction was stirred over night and sodium bicarbonate solution were then added and the reaction was allowed to stir for another two hours. PHOPT was obtained by dialysis (MWCO = 1 kDa) against distilled water for 1 day and lyophilization. (Yield: PHOPT-1: 71 %, PHOPT-2: 82%, PHOPT-2-2: 78%, PHOPT-2-3: 81%). DL-PHOPT-2 was synthesized similarly using DL-PHOT-2 as the starting material (Yield: 83 %).

Synthesis of PHLG-MMO. Poly(γ-6-chlorohexyl-L-glutamate) and NaI (3 equiv of chloro groups) was dissolved in DMF/acetonitrile (v/v=1:1). The mixture was transferred to a 25 mL Schlenk tube into which *N*,*N*-dimethyloctylamine (3 equiv of chloro groups) was added. The mixture was stirred at 80 ^{o}C for 48 h. After most solvent was removed under vacuum, NaCl aqueous solution (1.0 M, 3 mL) was added. The solution was then stirred at room temperature for 3 h to promote ion exchange. The product was obtained by dialysis (MWCO = 1 kDa) against distilled water for 3 days and lyophilization.

Minimal inhibition concentration (MIC). ATCC11778, ATCC12608 and NRS384 were grown in LB medium at 37 °C. For determination of the MIC, polypeptides were dissolved in media using serial dilutions from a stock solution. Into each well of a 96-well plate was added 200 μL of each concentration and 2 μL of bacteria (1×10^8 CFU (colony forming units)) in medium. The plate was incubated at 37 °C. The optical density readings of microorganism solutions at 600 nm were measured after 24 h incubation. The MIC was considered as the lowest concentration of peptide where no

visual growth of bacteria was detected.

Hemolysis assay. Fresh rabbit blood (HemoStat Laboratories, Inc, US) was obtained and subjected to 25 times dilution with PBS buffer to reach a concentration of approximately 4% (in volume) of the blood cells. 150 μL of PBS solution containing a polymer at various concentrations was placed in a 1.5 mL microfuge tube, followed by the addition of an equal volume (150 μL) of red blood cell suspension. The mixture was incubated at 37 °C for 1 h to allow for the hemolysis process to take place. At the end of the incubation time, the non-hemolyzed red blood cells were separated by centrifugation at 1000 rpm for 5 min. Aliquots (100 μL) of the supernatant were transferred to a 96-well plate, and hemoglobin release was measured by UV-absorbance at 576 nm using a microplate reader (TECAN, Switzerland). Two controls were provided in this assay: an untreated red blood cell suspension in PBS solution was used as the negative control; a solution containing red blood cells lysed with 1% Triton-X was used as the positive control. Percentage of hemolysis was calculated using the following formula: Hemolysis (%) = [(O.D. 576 nm of the treated sample-O.D. 576 nm of the negative control)/(O.D. 576 nm of positive control- O.D. 576 nm of negative control)]×100%.

MTT Assay. Raw 264.7 cells, HEK-293 cells, MCF-10A cells and IMR-90 cells were seeded in 96-well plates with density of 10,000 cells/well and grown in DMEM/10% FBS overnight. The medium was removed, and PHOT-2 or PHOPT-2 at various concentrations diluted in DMEM was added in triplicates. After 24 h incubation, 20 μL of the MTT solution (5 mg/mL in PBS) was added to each well, and the plates were

incubated for 2 h in 5% CO_2 incubator at 37 °C. The MTT-containing medium was then removed by aspiration, and the blue formazan product generated was dissolved by the addition of 100 µL of 100% DMSO per well. The plates were gently swirled for 10 min at room temperature to dissolve the precipitate. The absorbance was monitored at 570 nm.

Degradation of Polypeptides under the Function of Bacterial Phosphatase. Fmoc(Tyr-PO_3H_2)-OH (0.4 mM) was incubated with bacterial phosphatase (1.25 UN/mL, alkaline from *E. coli*) in Tris HCl buffer (pH 7.4) under 37 °C for 10 min. The degradation products of Fmoc(Tyr-PO_3H_2)-OH under the function of phosphatase were then analyzed by HPLC with C18 column and UV absorption at 266 nm. The degradation of PHOPT-2 was also monitored by HPLC with SEC column and UV absorption at 225 nm (0.9% sodium chloride as the eluent). PHOPT-2 (8.2 µM) was incubated with bacterial phosphatase (0.06 UN/mL) in Tris HCl buffer (0.02 mM, pH 7.4) under 37 °C. At predetermined time, the degradation was monitored by HPLC.

ATCC12608 cells (10^8 CFU) were incubated with PBS for 24 h. The bacterial cells were removed by filtration through 0.22 µm filter. PHOPT-2 (4.1 µM) was incubated with the medium and the degradation was monitored by HPLC using the same method.

Degradation of Polypeptides under the Function of mammalian cell culture medium. DMEM (Dulbecco's Modified Eagle Medium) cell culture medium (with 10% fetal bovine serum) of Raw 264.7 cells were taken out after two days' incubation, and the medium was filtrated with 0.22 µm filter before incubation with PHOPT-2 (82 µM)

under 37 ^{o}C. At predetermined time, 20 μL of samples were taken out and diluted in 400 μL PBS solution for HPLC analysis.

Liposome Dye-Leakage Assay. Calcein dye was dissolved in Tris buffer (pH = 7) to achieve a concentration of 40 mM. To a clean round-bottom flask, appropriate volumes of lipid stocks were added to make up 1 mL of $CHCl_3$ (3:1 POPE/POPG vesicles, POPE (130 μL, 25 mg/mL $CHCl_3$) and POPG (115 μL, 10 mg/mL $CHCl_3$) were used). The solvent was removed by a stream of nitrogen gas to obtain a thin lipid film, which was then hydrated by 1 mL of calcein solution. The mixture was left to stir for 1 h, after which it was subjected to 10 freeze−thaw cycles (using dry ice/acetone to freeze and warm water to thaw). The suspension was extruded 20 times through a polycarbonate membrane with 400 nm pore diameter. The excess dye was removed using Sephadex G-50 column as the eluent. The dye-filled vesicle fractions were diluted 2000 times with Tris buffer (final lipid concentration: ~5.0 mM). This suspension (90 μL) was subsequently mixed with polymer stock solutions (10 μL) on a 96-well black microplate (Greiner, flat bottom). Tris Buffer (10 μL) and Triton-X (0.1% v/v, 10 μL) were employed as the negative and positive controls, respectively. After 30 min, the fluorescence intensity in each well was recorded using the microplate reader with excitation and emission wavelengths of 490 and 515 nm, respectively. The percentage of leaked calcein dye in each well was determined as follows: leakage (%) = $[(F-F_0)/(F_{TX}-F_0)]\times100\%$ where F is the fluorescence intensity recorded in the well, F_0 is the intensity in the negative control well, and F_{TX} is the intensity in the positive control well.

SEM. Bacteria cells grown in LB with or without polypeptides treatment were performed using a similar protocol as MIC measurements but with a 30-min incubation time. All the samples were collected into a microfuge tube and pelleted at 4000 rpm for 5 min, and then washed twice with phosphate-buffered saline. Subsequently, bacteria were fixed with paraformaldehyde solution (4%) for 1 h before proceeding, followed by washing with DI water. Dehydration was performed with a series of graded ethanol solution (10%, 25%, 50%, 75%, 95%, and 100%). The dehydrated samples were dried under vacuum overnight before being mounted on carbon tape and coated with gold-platinum for imaging using a Hitachi S-4700 High Solution SEM (Japan).

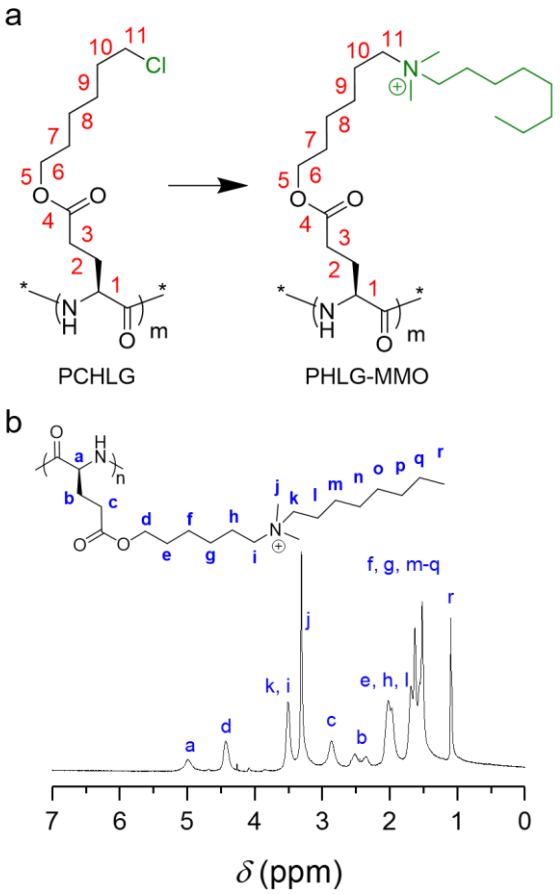

Figure S1. The synthesis scheme of PHLG-MMO (a). The ^{1}H NMR spectra of PHLG-MMO (b) in TFA-d.

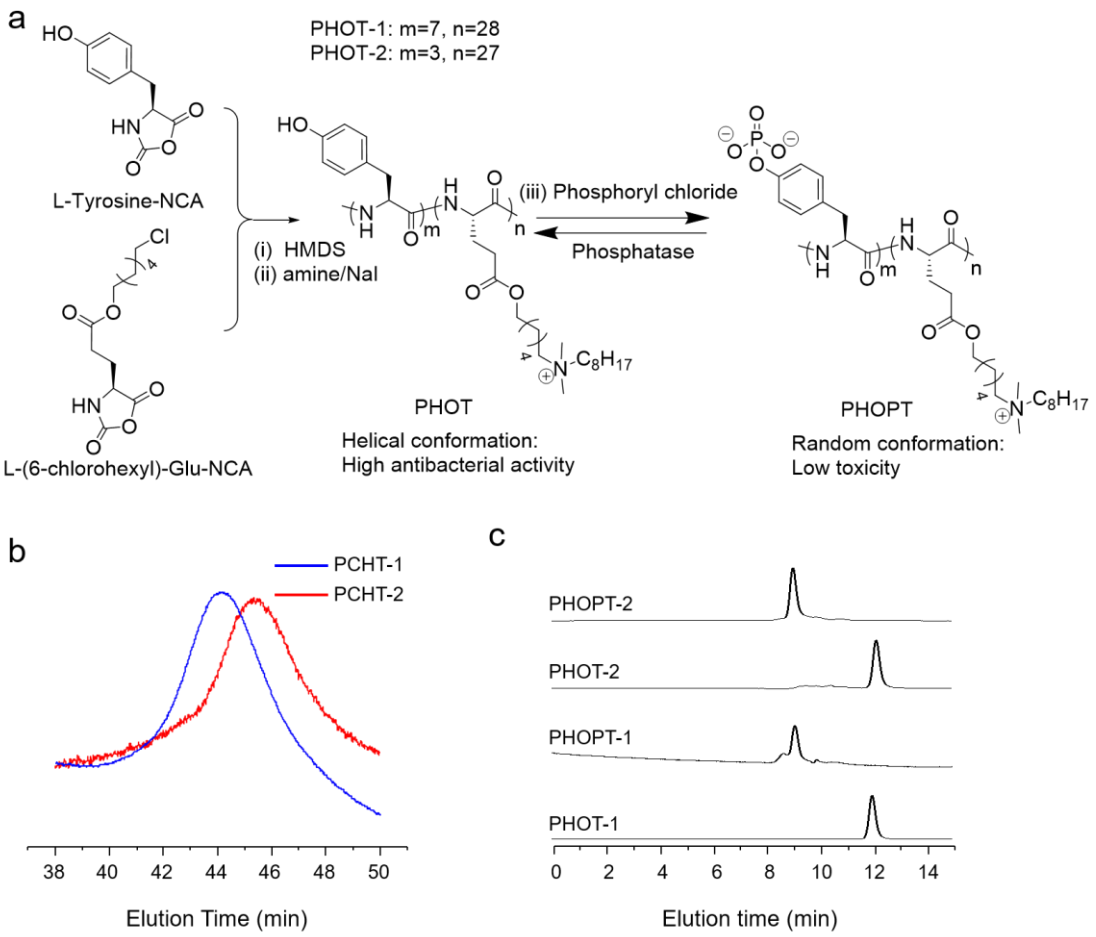

Figure S2. (a) Synthesis route of PHOPT polypeptides. (b) GPC spectra of PCHT-1 and PCHT-2. The molecular weight for PCHT-1 and PCHT-2 is 9600 and 6900, respectively. The poly dispersity index (PDI) for PCHT-1 and PCHT-2 is 1.25 and 1.10, respectively. (c) HPLC analysis of PHOT-1, PHOT-2, PHOPT-1 and PHOPT-2 with SEC column and PDA detector.

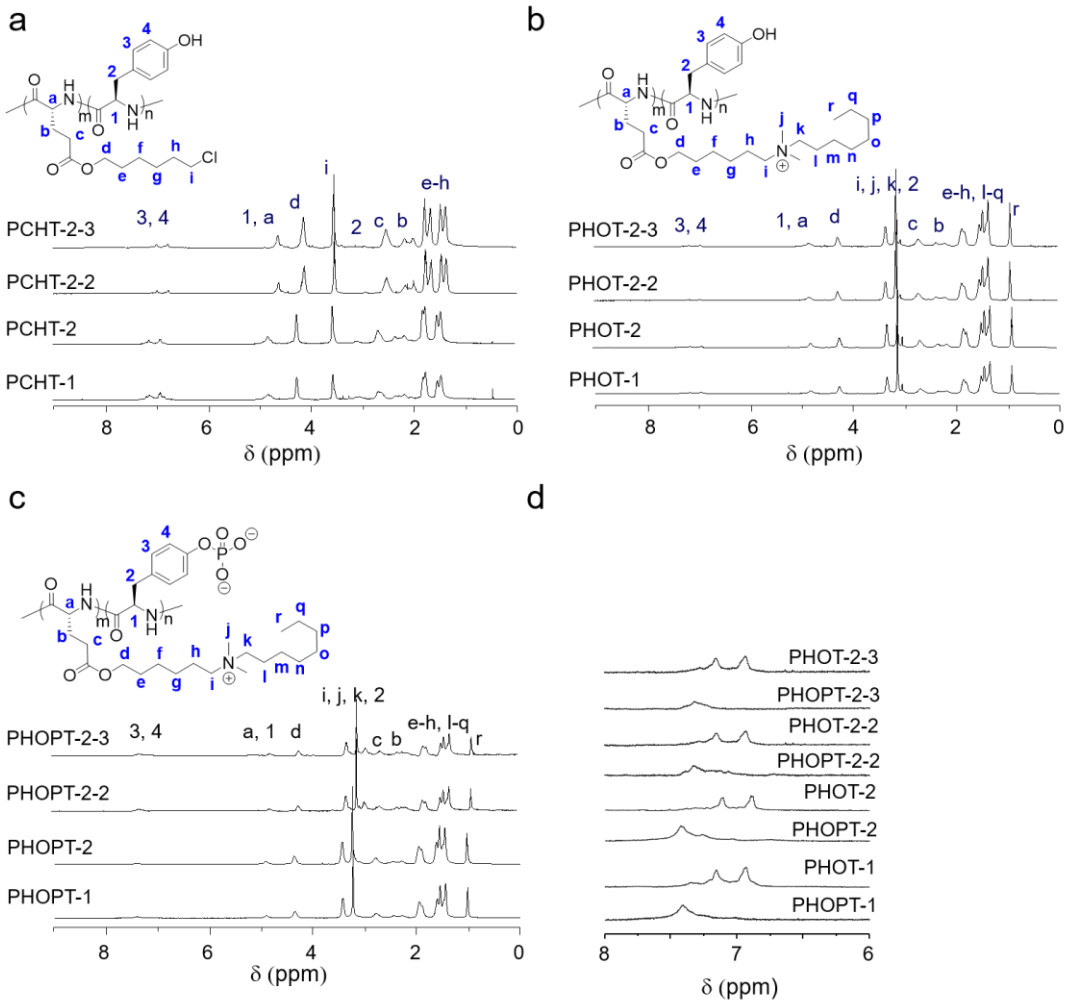

Figure S3. The ^{1}H NMR spectra of PCHT-1, PCHT-2, PCHT-2-2, and PCHT-2-3 (a), PHOT-1, PHOT-2, PHOT-2, and PHOT-3 (b), and PHOPT-1, PHOPT-2, PHOPT-2-2, and PHOPT-2-3 (c) in TFA-d. (d) The expansion of ^{1}H NMR spectra of PHOT-1, PHOPT-1, PHOT-2, PHOPT-2, PHOT-2, PHOPT-2-2, PHOT-3, and PHOPT-2-3 from 6.0 ppm- 8.0 ppm.

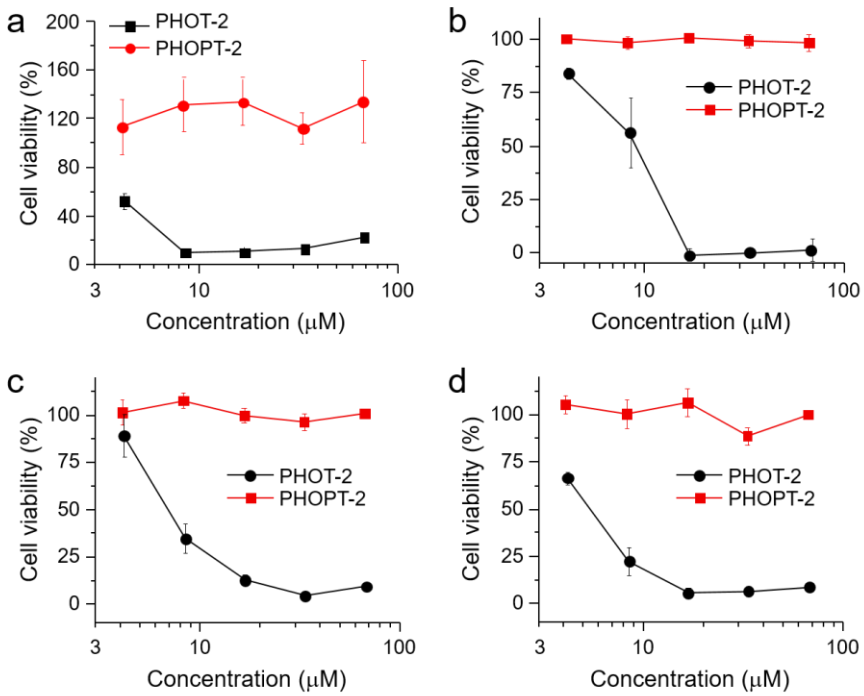

Figure S4. Toxicity of PHOT-2 and PHOPT-2 against Raw 264.7 cells (a), HEK-293 cells (b), MCF-10A cells (c), and IMR-90 cells (d) after 24-h incubation as measured by the MTT assay (n=3).

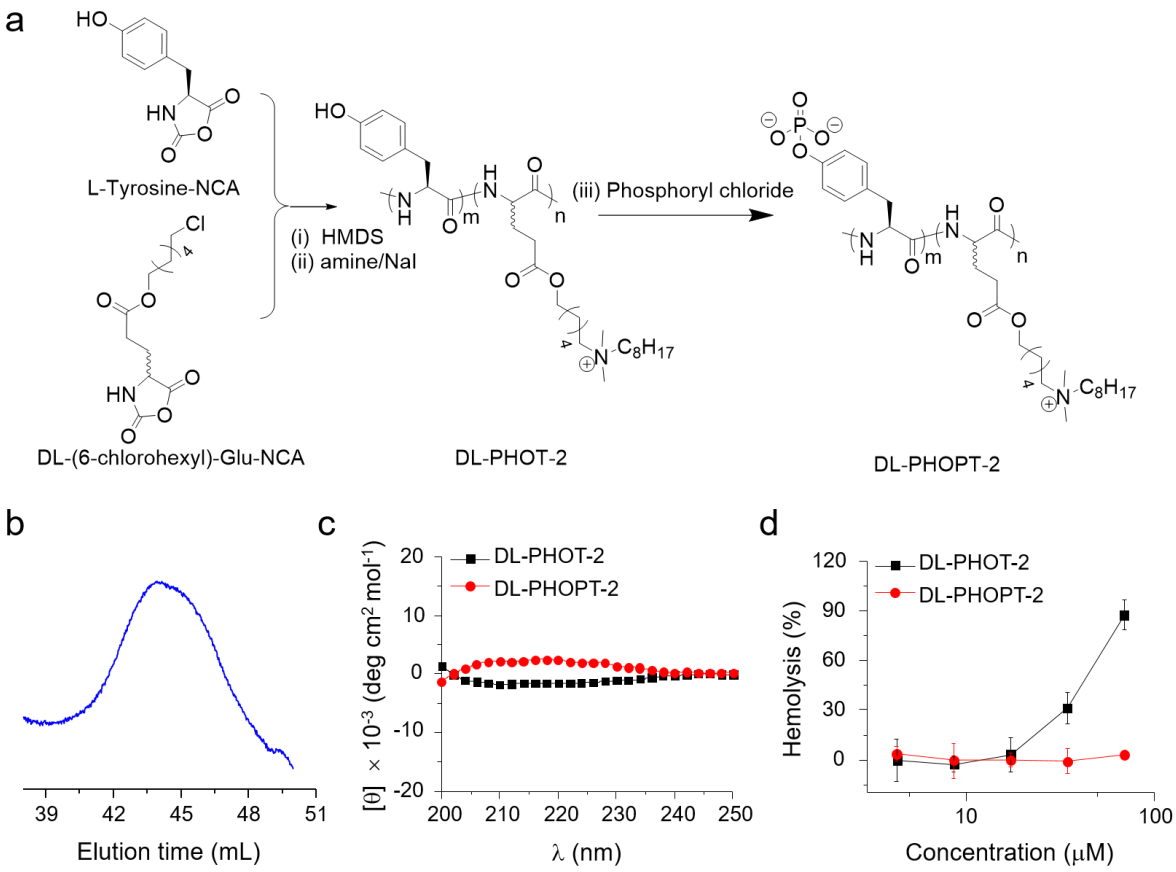

Figure S5. (a) Synthesis route of DL-PHOPT polypeptides. (b) GPC trace of DL-PCHT-2. The molecular weight and PDI of DL-PCHT-2 are 6500 and 1.17, respectively (m=2.5, n=25). (c) CD spectra of DL-PHOT-2 and DL-PHOPT-2. (d) Hemolytic activity of DL-PHOT-2 and DL-PHOPT-2.

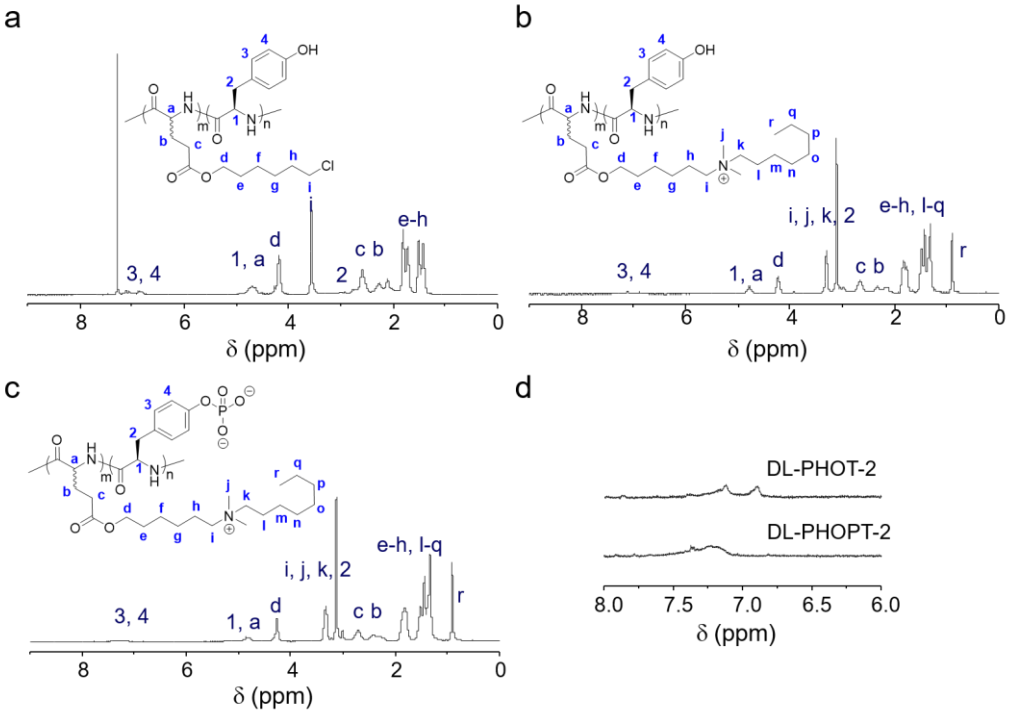

Figure S6. ^{1}H NMR spectra of DL-PCHT-2 (a) in $CDCl_3$ and d-TFA (v/v=9/1), DL-PHOT-2 (b) and DL-PHOPT-2 (c) in TFA-d. (d) The expansion of ^{1}H NMR spectra of DL-PHOT-2 and DL-PHOPT-2 from 6.0 ppm- 8.0 ppm.

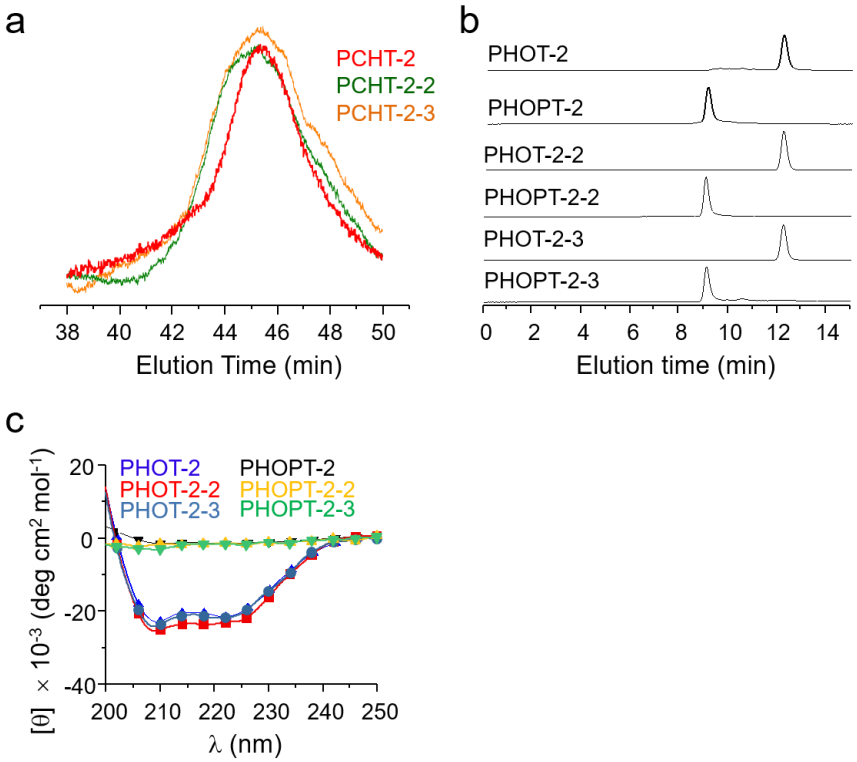

Figure S7. (a) GPC trace of PCHT-2, PCHT-2-2, and PCHT-2-3. The molecular weight for PCHT-2, PCHT-2-2 and PCHT-2-3 is determined to be 6900, 7000, and 6400 Da, respectively. The polydispersity index (PDI) for PCHT-2, PCHT-2-2, and PCHT-2-3 is 1.10, 1.17, and 1.28, respectively. (b) HPLC analysis of PHOT-2, PHOT-2-2, PHOT-2-3, PHOPT-2, PHOPT-2-2, and PHOPT-2-3 with SEC column and PDA detector. (c) CD spectra of PHOT-2, PHOT-2-2, PHOT-2-3, PHOPT-2, PHOPT-2-2, and PHOPT-2-3.

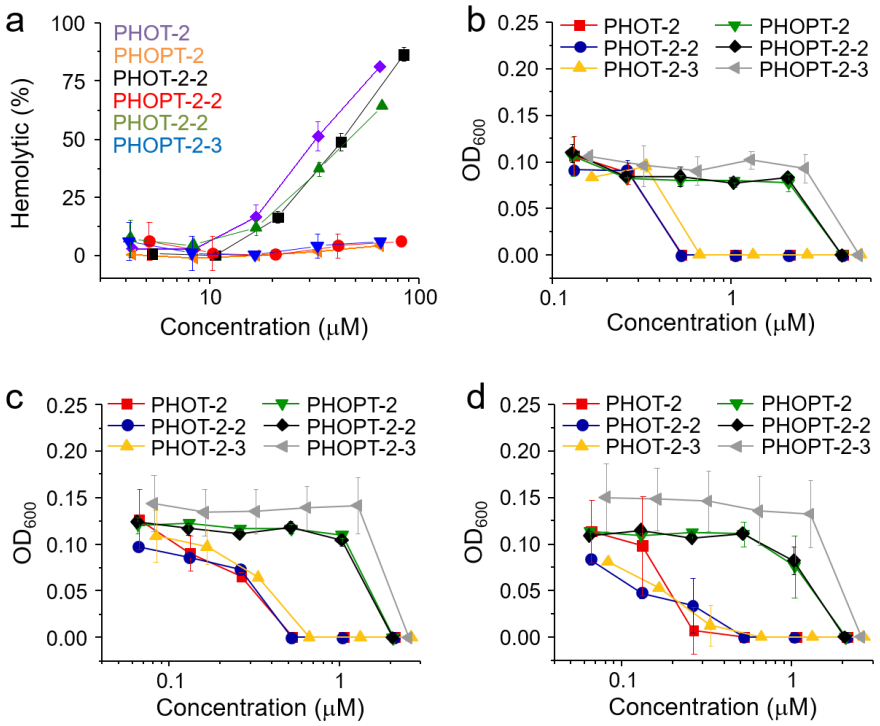

Figure S8. (a) Hemolytic activity of PHOT-2, PHOPT-2, PHOT-2-2, PHOT-2-3, PHOPT-2-2, and PHOPT-2-3. The antibacterial activity of PHOT-2, PHOT-2-2, PHOT-2-3, PHOPT-2, PHOPT-2-2, and PHOPT-2-3 against ATCC12608 (b), ATCC11778 (c), and NRS384 (d) at various concentrations. The optical density at 600 nm (OD_{600}) of bacteria was analyzed to determine the growth of bacteria.

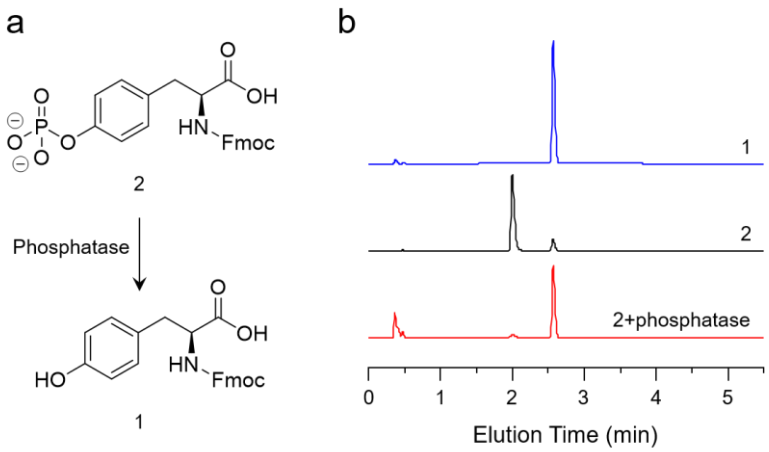

Figure S9. (a) The structures of Fmoc(Tyr-PO_3H_2)-OH (2) and Fmoc(Tyr)-OH (1). (b) HPLC analysis of Fmoc(Tyr-PO_3H_2)-OH and its degradation product under the function of phosphatase for 10 min. Fmoc(Tyr-PO_3H_2)-OH (200 mg/L) was incubated with phosphatase (1.25 UN/mL, alkaline from *E. coli*) in Tris HCl buffer (pH 7.4).

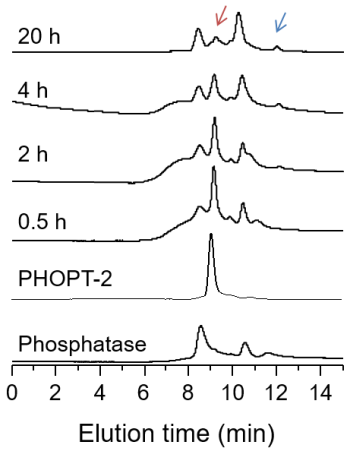

Figure S10. HPLC analysis PHOPT-2, and its degradation product under the function of bacterial phosphatase. PHOPT-2 (8.2 μM) was incubated with bacterial phosphatase (0.06 UN/mL) in Tris HCl buffer (pH 7.4). The red arrow represents for PHOPT-2, and the blue arrow represents for PHOT-2.

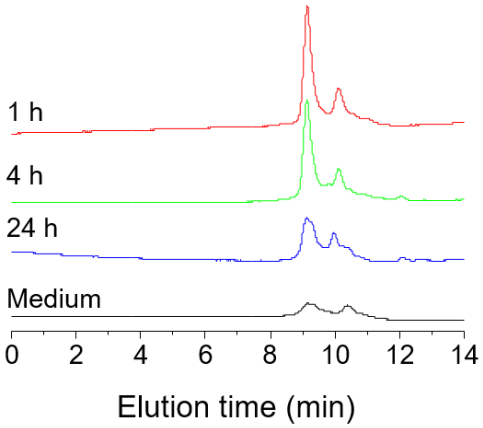

Figure S11. HPLC analysis PHOPT-2, and its degradation product under the function of bacterial incubation medium. ATCC12608 cells (10^8 CFU) were incubated with PBS for 24 h. The bacterial cells were removed by filtration with 0.22 μm filter. PHOPT-2 (4.1 μM) was incubated with the medium and the degradation was monitored by HPLC.

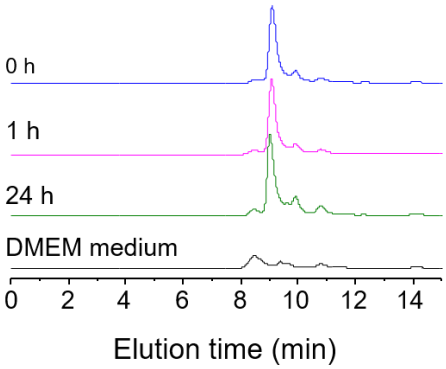

Figure S12. HPLC analysis PHOPT-2 and its degradation product under the function of Raw 264.7 cell culture medium. Briefly, the DMEM cell culture medium of Raw 264.7 cells were taken out after two days' incubation, and filtrated with 0.22 μm filter before incubation with PHOPT-2 under 37 ^{o}C. At predetermined time, 20 μL of samples were taken out and diluted in 400 μL PBS solution for HPLC analysis.

[1] Z. Y. Song, N. Zheng, X. C. Ba, L. C. Yin, R. J. Zhang, L. Ma, J. J. Cheng, *Biomacromolecules* **2014**, *15*, 1491.