Synthesis of self-reporting polymeric nanoparticles for *in situ* monitoring endocytic microenvironmental pH

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Materials

Propargylamine (PA), poly(ethylene glycol) diacrylate (average M_n 700, PEGDA) and Nile red (NR) were purchased from Aldrich-Sigma Chemical Corporation. Cell counting kit-8 assay (CCK-8, Beyotime Institute of Biothechnology, China) and LysoTracker Green DND-26 (Invitrogen Co.) were used without further purification. Bis-pyrene (BP) was prepared as described before.[1] Peptide c(RGDfC) (>95%) was ordered from GL Biochem Ltd (Shanghai, China). U87 cell line was purchased from cell culture center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Other solvents and reagents were used as received.

Synthesis of copolymers P1-P3

The poly (PA-*co*-PEGDA) copolymers (**P1-P3**) were prepared by Michael addition and all synthetic procedures were carried out under a nitrogen atmosphere. Take **P3** as

an example. PA (0.055 g, 1 mmol) and PEGDA (0.735 g, 1.05 mmol) were all dissolved into 2 mL dimethyl sulfoxide (DMSO), and then bubbled over N_2 for 15 min under stirring. The mixture was allowed to react for 5 days at 50 ^oC in dark, and then the final reaction solution was dialyzed against deionized water (MWCO: 3500 Da). The polymer solution was lyophilized to obtain a yellow solid. **P1** and **P2** were synthesized with the similar procedures.

Synthesis of RGD-P3 conjugates

For the synthesis of RGD-**P3** conjugates (**P**), peptide c(RGDfC) (11.6 mg, 0.02 mmol) and **P3** (0.128 g, 0.01 mmol) were added into 1 mL DMSO, and then bubbled with N_2 for 15 min under stirring. The mixture was allowed to react for 24 h at 50 ^oC in dark and dialyzed against deionized water (MWCO: 3,500 Da). The polymer solution was lyophilized to obtain a pale yellow solid.

Synthesis of BP

5-(dodecyloxy)isophthalic acid (1.0 g, 2.9 mmol), 10 mL of $SOCl_2$ and 0.2 mL DMF were put into a 50 mL, round bottomed flask under N_2 atmosphere and the mixture was refluxed for 12 h. The solvent was removed in vacuum. The residue and pyrene (1.4 g, 7.0 mmol) were dissolved in 30 mL dry methylene chloride, and the mixture was cooled to 0 °C. After the portion-wise addition of $AlCl_3$ (1.0 g, 7.0 mmol), the mixture was allowed to react overnight at room temperature. The mixture was poured into ice-water and stirred until the color of the organic phase turned from black to yellow. The aqueous phase was extracted with methylene chloride, the combined

organic phase was dried with $MgSO_4$ and the solvent was evaporated. The residue was purified through the silica gel column chromatography rinsed by a mixture eluent of methylene chloride/ethyl acetate with the gradient from 100: 0 to 95: 5 (v/v). The solvent was evaporated to give yellow solid product, 0.9 g, yield: 56%. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): δ = 8.38 (d, J = 10.0 Hz, 2H); 8.27-8.23 (m, 4H); 8.16-8.12 (m, 4H,); 8.09-7.97 (m, 8H); 7.78 (s, 1H); 7.67 (s, 2H). MS (MALDI-TOF) calculated for $C_{40}H_{22}O_3$, 550.15 m/z, found 550.05.

Synthesis of BP-Br

1 (550 mg, 1 mmol), 1,6-dibromohexane (244 mg, 1 mmol) and K_2CO_3 (280 g, 2 mmol) were dissolved in dry acetone (50 mL). The reaction mixture was refluxed for 48 h. Then the solvents were removed under vacuum and the residue was dissolved in methylene chloride (150 mL), washed with saturated brine (3 × 50 mL). The extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was purified through the silica gel column chromatography rinsed by a mixture eluent of methylene chloride/petroleum ether. The solvent was evaporated to give yellow solid product BP-Br (**2**), 356 mg, yield: 50%. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): ^{1}H NMR: 8.36 (d, 2H), 8.23 (t, J = 7.5 Hz, 4H), 8.12 (dd, 4H), 8.06-7.94 (m, 8H), 7.73 (s, 3H), 4.04 (t, 2H), 3.36 (t, 2H), 1.83-1.76 (m, 4H), 1.46-1.42 (m, 4H). MS (MALDI-TOF) calculated for $C_{46}H_{33}BrO_3$ 712.16, found 712.17.

Synthesis of BP-N_3

2 (71.2 mg, 0.1 mmol) and NaN_3 (13.0 mg, 0.2 mmol) were dissolved in dry DMF (50

mL). The reaction mixture was heated to 80 °C for 12 h. Then the solvents were removed under vacuum and the residue was dissolved in methylene chloride (100 mL), washed with saturated brine (3 ×50 mL). The extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was purified through the silica gel column chromatography rinsed by a mixture eluent of methylene chloride/petroleum ether. The solvent was evaporated to give yellow solid product BP-N_3 (**3**), 41.8 mg, yield: 62%. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K, TMS): ^{1}H NMR: 8.36 (d, 2H), 8.23 (t, J = 7.5 Hz, 4H), 8.12 (dd, 4H), 8.06-7.95 (m, 8H), 7.74 (s, 3H), 4.06 (t, 2H), 3.24 (t, 2H), 1.81-1.77 (m, 2H), 1.59-1.63 (m, 2H), 1.46-1.42 (m, 4H). MS (MALDI-TOF) calculated for $C_{46}H_{33}N_3O_3$ 675.25, found 675.21.

Synthesis of P-BP conjugates

For the synthesis of **P-BP** conjugates, P (15 mg, 1 μmol) and BP-N_3 (3.4 mg, 5 μmol) were dissolved in 1 mL DMSO, and then $CuSO_4$•$5H_2O$ (0.3 mg) dissolved in 50 μL deionized water was added into the reaction solution dropwisely. The solution was bubbled with N_2 for 15 min under stirring, and then sodium ascorbate (1 mg) was added rapidly, followed by bubbling with N_2 for another 10 min. The mixture was allowed to react for 24 h at 50 ^{o}C and dialyzed against deionized water (MWCO: 3,500 Da). Finally, the solution was lyophilized to obtain a yellow solid.

Characterization of copolymers

The chemical structures of copolymers were measured by NMR measurements. ^{1}H NMR spectra (400 MHz) of the copolymers in D_2O and d^6-DMSO were recorded on a

Bruker ARX 400 MHz spectrometer. The molecular weight and its distribution were determined using gel permeation chromatography (GPC) equipment (Shimadzu LC-20A). The measurements were performed at 40 ^{o}C in DMF at a flow rate of 1.0 mL min^{-1}. A family of narrow dispersed polystyrenes was used as the standards for calibration.

Particle size measurements

The **P-BP** conjugates were dissolved into buffer solutions and then sonicated for 10 min to obtain the **P-BP** nanoparticles. The hydrodynamic diameters of the copolymer micelles were measured on a dynamic light scattering (DLS) analyzer (Zetasizer Nano ZS). The nanoparticle dispersion (0.8 mg/mL, pH 7.4 PB) was passed through syringe filters (0.45μm, Millipore) before measurements. The measurements of the nanoparticles in buffer solutions (10 mM PB, pH 7.4 or 50 mM acetate buffer, pH 5.0) were performed at 25 ^{o}C.

Transmission electron microscopy (TEM)

The morphologies of **P-BP** nanoparticles were observed by TEM (Tecnai G2 20 S-TWIN) with an acceleration voltage of 200 kV. The **P-BP** nanoparticle dispersion (10 μL) was dropped onto a copper mesh, and then most of the liquid was dried by a filter paper after 2 min. The samples were stained with 10 μL of uranyl acetate solution for 40 s followed by drying the spare liquid with the filter. Finally, 10 μL of deionized water was used to wash the copper mesh, which was blotted after 30 s and dried at room temperature.

pH titration of P-BP copolymers

The copolymer **P-BP** was dissolved in deionized water at a concentration of 1.6 mg mL^{-1} and the pH was adjusted to 3. The solution was titrated with a 0.1 M NaOH aqueous solution at an increment of 10–20 μL. Finally, the pH increases of the solution were monitored with a pH meter at room temperature.

Fluorescence measurement of P-BP

The **P-BP** conjugates were dissolved into buffer solutions (1.6 mg/mL) with different pHs (pH 7.4, 6.5, 5.5 and 5.0). The solution was sonicated for 10 min and equilibrated for 24 hours at ambient temperature. An F-280 spectrometer was used for the fluorescence measurements of solution with different concentrations. The excitation and emission slit widths of the fluorometer were set at 5 nm and 5 nm, respectively. The excitation wavelength was set up at 350 nm, and the emission spectra were recorded from 360 to 650 nm at PMT of 700 V.

Nile Red (NR) loading and fluorescence measurement

NR in ethanol (1.0×10^{-3} mol/L) was added into 1 mL **P-BP** nanoparticle dispersion (0.8 mg/mL, pH 7.4, 10 mM PB). The micelle dispersions were sonicated for 10 min at room temperature, and then the measurements were performed on an F-280 fluorometer with the excitation wavelength of 350 nm. The emission spectra were recorded from 400 to 680 nm.

pH-dependent fluorescence of P-BP/NR.

For the fluorescent measurement of **P-BP/NR** at different pHs, the pH of nanoparticle

dispersion was adjusted to 7.0. 6.8, 6.5, 6.0, 5.5 and 5.0 by adding acetate buffer gradually, and then the fluorescence spectra of the dispersions were measured at the excitation wavelength of 350 nm.

Lysosome colocalization observation

U87 cells were cultured in MEM containing 10% FBS and 1% penicillin-streptomycin at 37 ^{o}C in a humidified atmosphere containing 5% CO_2 for 24 h. The U87 cells were first treated with **P-BP/NR** nanoparticles (0.08 mg/mL for polymers, 20 μM for BP) for 10 min, 30 min and 90 min, respectively, and then washed with PBS three times. The cells were incubated with LysoTracker Green DND-26 (10 mM) for 30 minutes, and then washed with PBS three times. After replacement of medium, cells were imaged using a Zeiss LSM710 confocal laser scanning microscope (CLSM) with a 63× objective lens. For BP and NR fluorescence observation, the excitation wavelength was 405 nm, and the emissions of 410-430 nm (blue channel) and 625-645 nm (red channel) were observed. For LysoTracker Green observation, the excitation wavelength was 488 nm, and the emissions of 515-535 nm (green channel) were observed.

***In situ* fluorescence observation**

U87 cells were first treated with **P-BP/NR** nanoparticles (0.08 mg/mL for polymers, 20 μM for BP) for 10 min, and then washed with PBS three times. After replacement of medium, the cells were imaged *in situ* at 10 min, 20 min, 60 min and 90 min at 37 ^{o}C by a Zeiss LSM710 confocal laser scanning microscope (CLSM) with a 63×

objective lens. The excitation wavelength was 405 nm, and the emissions of 410-430 nm (blue channel) and 625-645 nm (red channel) were observed.

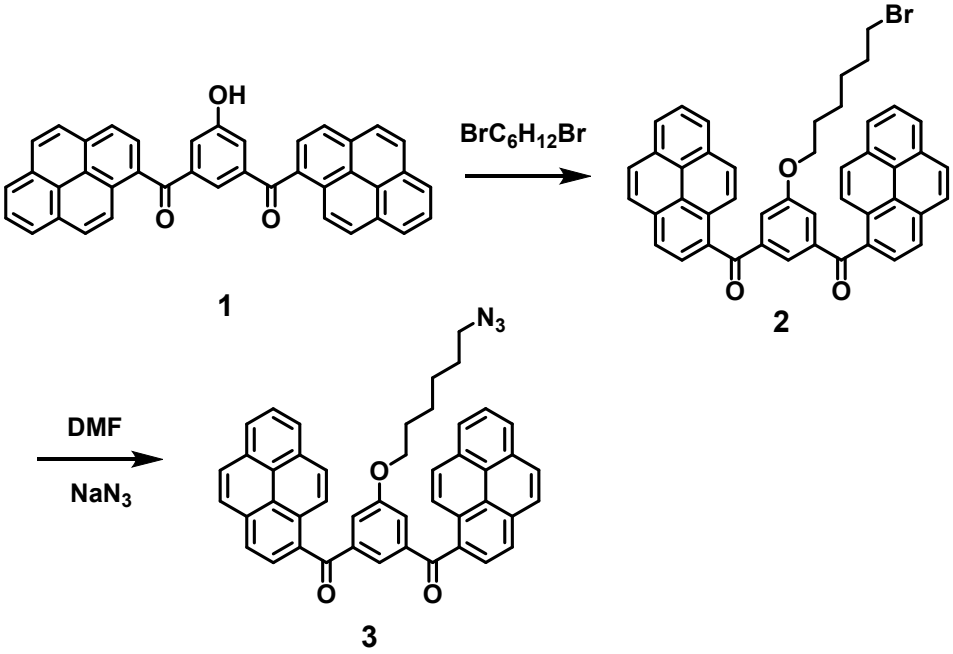
Cell cytotoxicity assay

U87 cells were used to evaluate the cytotoxicity of **P-BP/NR** nanoparticles by the CCK-8 assay. **P-BP/NR** nanoparticles were dispersed in PBS (10 mM, pH 7.4) with a series of different concentrations. A density of 5×10^3 cells per well were seeded in the 96-well plates in MEM and then cultured at 37 ^{o}C in a humidified atmosphere with 5% CO_2 for 24 h. 10 μL of the sample solutions with different concentrations were added to each well, and the cells were incubated for additional 24 h, followed by washing with PBS twice. Then 10 μL of CCK-8 solutions was added to each well and cultured for another 4 h. The UV-Vis absorptions of sample wells (A_{sample}) and control wells ($A_{control}$) were measured using a Microplate reader at a test wavelength of 450 nm and a reference wavelength of 690 nm, respectively. Cell viability (%) was equal to ($A_{sample}/A_{control}$) ×100. All the experiments were performed in triplicate.

Table S1. Synthesis and characterization of **P1-P3** copolymers

	PEGDA:PA [a]	DP [b]	M_n [c]	M_w [c]	PDI [c]
P1	1.2:1	8	17900	27700	1.54
P2	1.1:1	11	19600	29200	1.49
P3	1.05:1	17	22900	33200	1.45

[a] Molar feed ratio. [b] The degree of polymerization (DP) of copolymers determined by ^{1}H NMR spectra. [c] Number-averaged molecular weight (M_n), weight-averaged molecular weight (M_w) and polydispersity index (PDI) determined by GPC with DMF as an eluent and monodisperse polystyrenes as the standards.



Scheme S1. The synthesis route of BP-N_3.

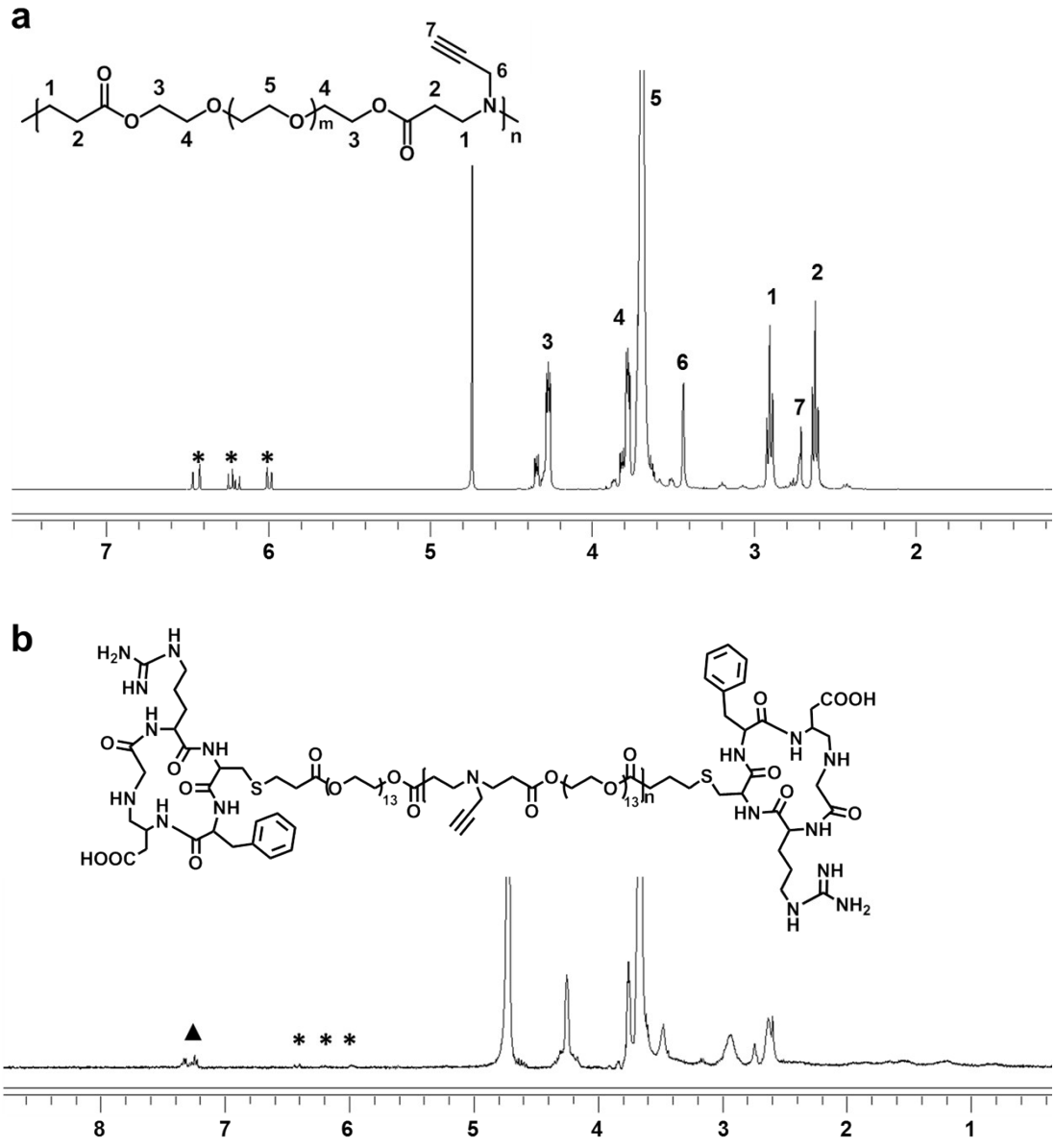


Fig. S1 ^{1}H NMR spectra of (a) poly(PEGDA-*co*-PA) (**P3**) and (b) RGD-**P3** conjugates (**P**) in D_2O.

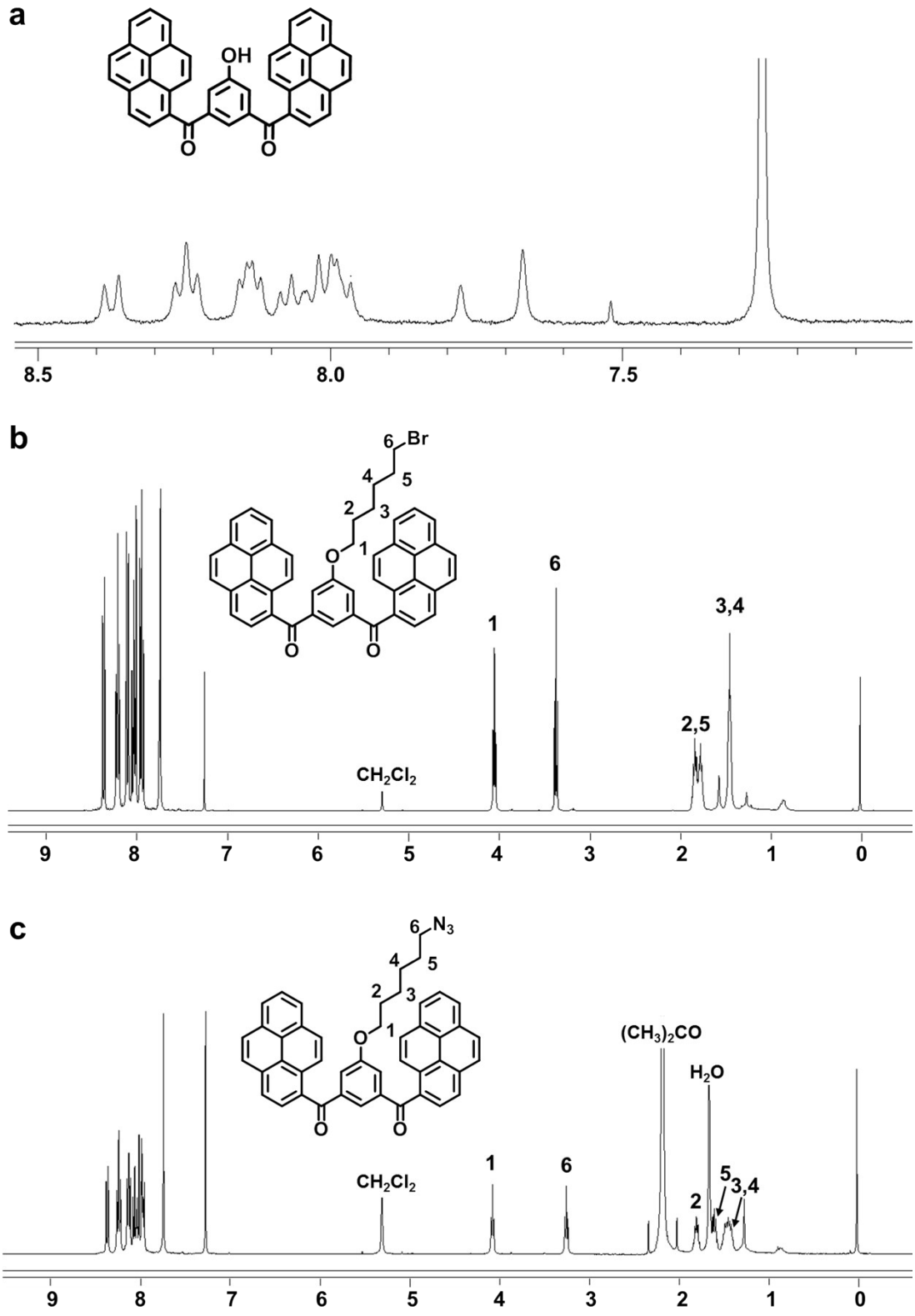


Fig. S2 ^{1}H NMR spectra of (a) BP (**1**), (b) BP-Br (**2**) and (c) BP-N_3 (**3**) in $CDCl_3$.

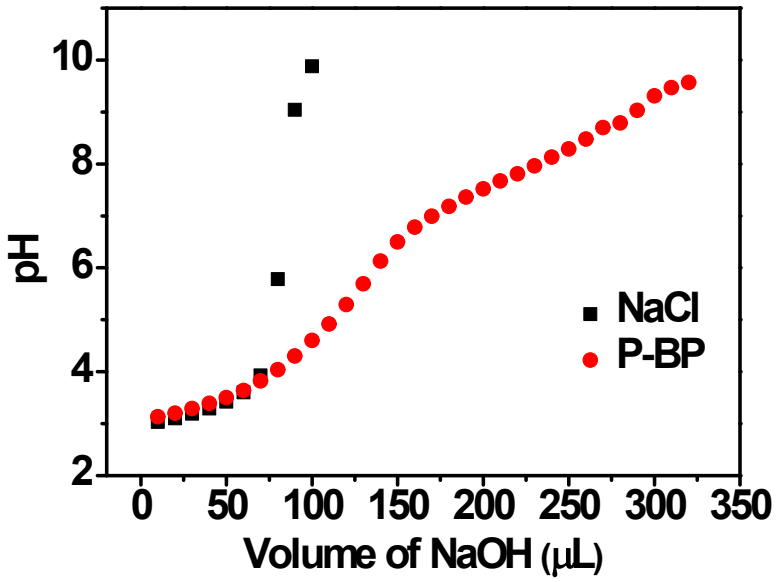


Fig. S3 Titration curve of copolymers **P-BP** (1.6 mg mL^{-1}) obtained by adding 0.1 M NaOH at room temperature.

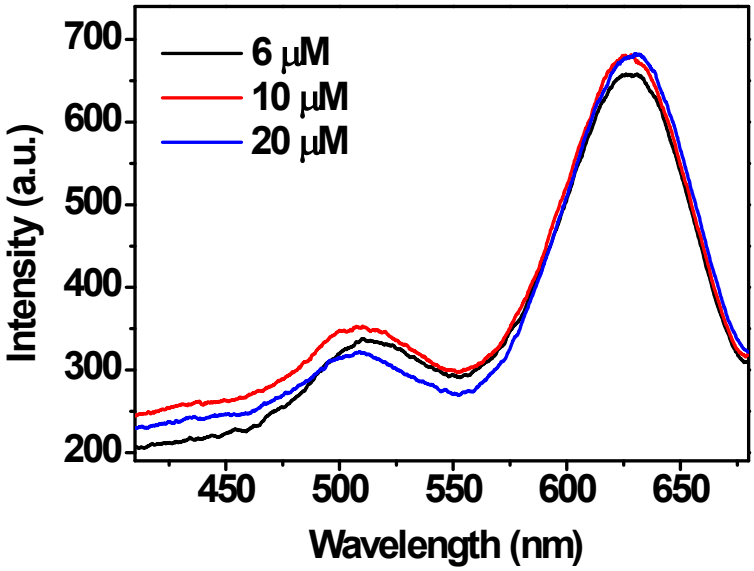


Fig. S4 Fluorescence emission spectra of **P-BP/NR** upon gradual addition of NR (6, 10 and 20 μM) in PB solutions (10 mM, pH 7.4).

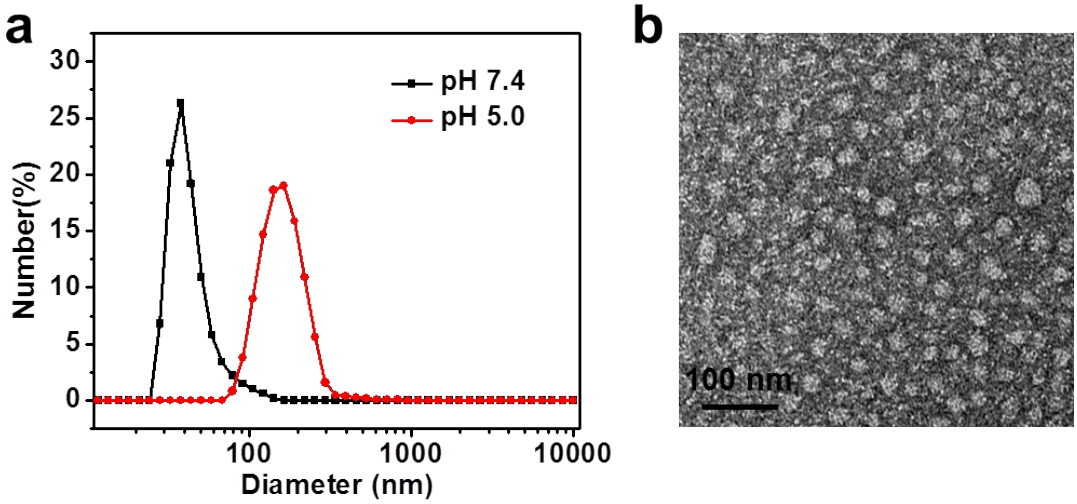


Fig. S5 (a) Number size distribution of **P-BP/NR** nanoparticles (0.8 mg mL^{-1}) in PB solutions (10 mM, pH 7.4) and acetate buffer (50 mM, pH 5.0) measured by DLS. (c) TEM images of **P-BP/NR** in aqueous solutions at pH 7.4.

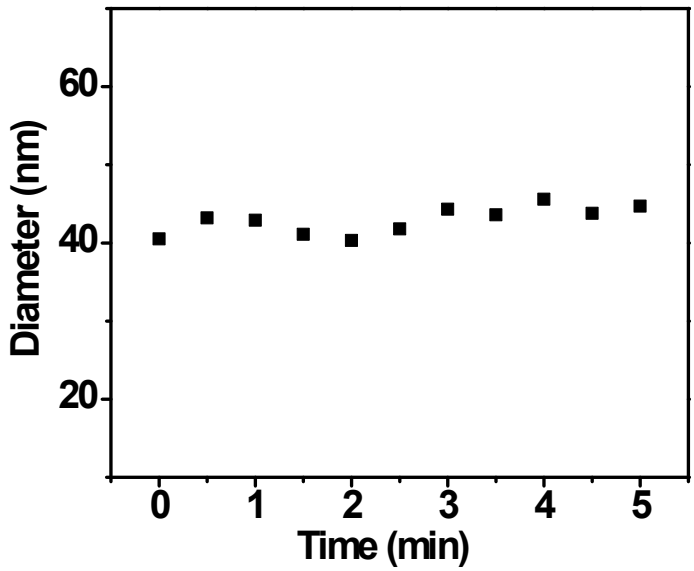


Fig. S6 Hydrodynamic diameter vs. time plots of **P-BP/NR** nanoparticles in PBS solution (pH 7.4) at 37 °C. Copolymer concentration: 0.8 mg mL^{-1}.

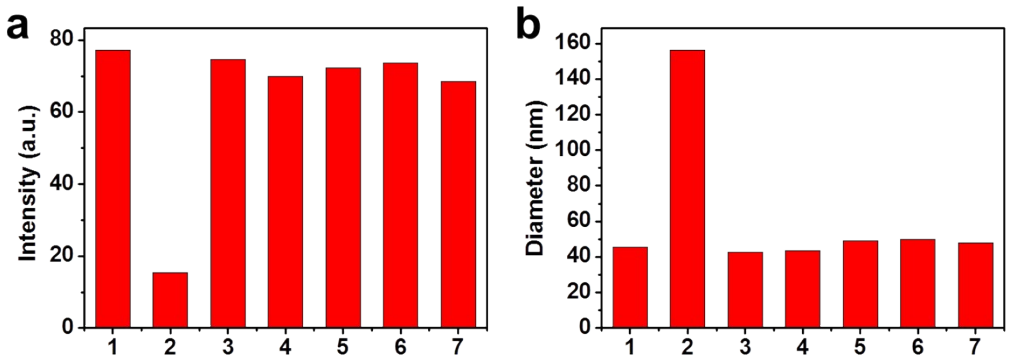


Fig. S7 (a) Fluorescence intensity at 635 nm and (b) hydrodynamic diameter of **P-BP/NR** nanoparticles in different conditions in 2 h. 1: pH 7.4, PB solution; 2: pH 5.0, PB solution; 3: pH 7.4, PBS solution; 4: pH 7.4, BSA (10 mg/mL); 5: pH 7.4, 37 ^{o}C PB solution; 6: pH 7.4 10% serum; 7: pH 7.4, 37 ^{o}C, 10% serum.

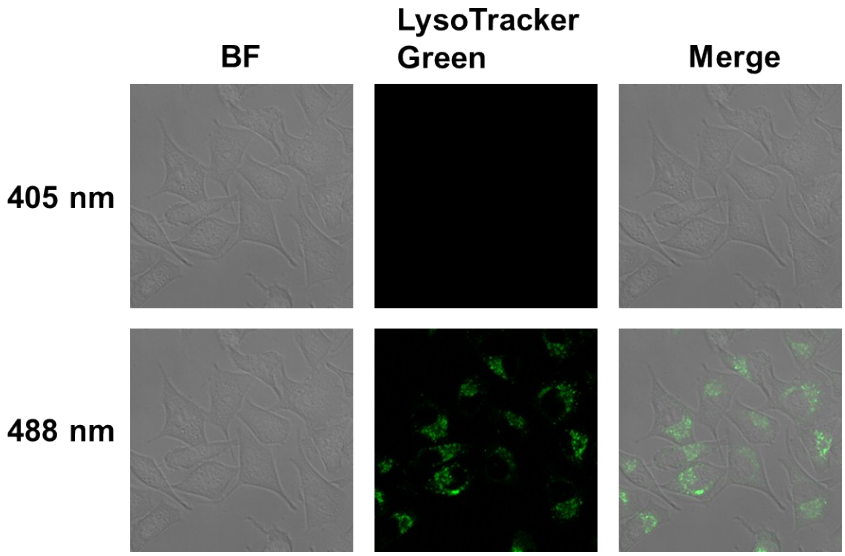


Fig. S8 CLSM microscopy of living U87 cells that were labeled with LysoTracker Green DND-26 for 30 min before imaging.

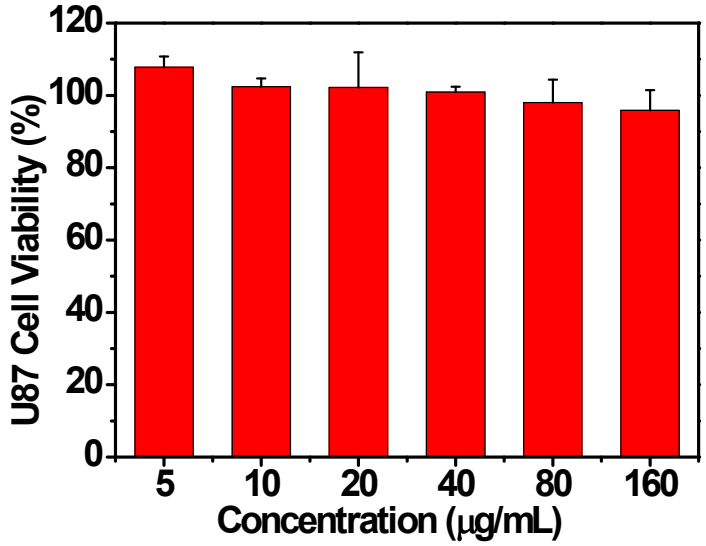


Fig. S9 U87 cell viability of **P-BP/NR** nanoparticles at various concentrations measured by CCK-8 assay. Results are presented as the mean ± SD in triplicate.

References

1. L. Wang, W. Li, J. Lu, Y.-X. Zhao, G. Fan, J.-P. Zhang and H. Wang, *J. Phys. Chem. C*, 2013, **117**, 26811-26820.