Supporting Information

Optimization of Bronic Ester Based Amphiphilic Copolymers for ROS-Responsive Drug Delivery

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Material and methods

Chemicals were purchased from commercial sources and used as received. H_2O (18.2 MΩ-cm) was purified with a Barnstead International NANOpure DIamond Analytical system. NMR spectra were recorded with a Zhongke Niujin QUANTUM-I-400MHz. GPC was performed with a Phenomenex Phenogel 5-μm MXM column (7.8 × 300 mm) operated with a Malvern TDA-305 system in THF at a flow rate of 0.7 mL min^{-1}. DLS measurements were performed with a Malvern Zetasizer Nano ZS ZEN3600 apparatus. Absorption and emission spectra were recorded with ThermoFisher Varioskan LUX. Fluorescence images were recorded with a Nikon A1 confocal laser-scanning microscope.

Doped Polymer Nanoparticles. MeOH solutions of Cur or BODIPY (0.1 mM, 1–1000 μL) and one of the polymers (10 mg mL^{-1}, 50 μL) were combined in a glass vial. The solvent was distilled off under reduced pressure, the residue was dispersed in PBS (sterile, 1 mL) and the mixture was sonicated for 5 min. The dispersion was then passed through a syringe filter with a pore size of 0.22 μm and the filtrate was used for the spectroscopic and cellular experiments without further purification.

FOX assay. The FOX reagent was prepared by mixing xylenol orange and ferrous ions in a sulfuric acid solution. The samples, including the Cur-loaded micelles, were then incubated with H_2O_2 to eliminate ROS. After reacting for 3 hours, the sample (5 μL) was added to FOX agent (200 μL) and incubated at room temperature for 1 hour. The absorbance was measured at 560 nm using a spectrophotometer. A standard curve of H_2O_2 concentration was used to quantify the ROS levels. The results were normalized against the standard curve to assess the ROS scavenging efficacy of each polymer formulation.

Cell cytotoxic assays. The cell viability was evaluated using the standard Cell Counting Kit-8 (CCK-8) method. RAW 264.7 cells (5×10^4 cells mL^{-1}) were inoculated into Petri dishes with DMEM, 15% fetal bovine serum, 1% penicillin, and 1% streptomycin and cultured in 96-well plates for 24 h, and then different concentrations of the polymers were introduced and further incubated with the cells for 24 h at 37 ℃. Cells were then washed with PBS buffer thrice before being treated with CCK-8 solutions (100 μL, 10%) for 2 h at 37 ℃. Finally, the cell viability was obtained by measuring the absorbance at 450 nm.

Lysosome co-localization. Cells were seeded into 24-well plates, each well fitted with a cell culture coverslip, at a density of 5×10^4 cells and incubated for 48 hours to allow for cell adhesion and growth. Cells were treated with BODIPY (5 μM) encapsulated in P1b or P2, and incubated for 30 minutes to permit cellular uptake. Subsequently, Lyso-Tracker Red was added to the culture medium to achieve a final concentration of 50 nM and incubated for an additional 30 minutes. Cells were then fixed with 4% paraformaldehyde for 15 minutes to preserve cell morphology and ensure the retention of the fluorescent labels. After fixation, an anti-fading agent containing DAPI was applied to the cells to stain the nuclei, and the coverslips were sealed to protect against photobleaching. The cells were then examined under a confocal laser scanning microscope. The degree of co-localization between BODIPY and lysosomal compartments was quantitatively analyzed using ImageJ.

Endocytosis. RAW 164.7 cells (1×10^4 cells mL^{-1}) were inoculated into Petri dishes with DMEM, 15% fetal bovine serum, 1% penicillin, and 1% streptomycin and cultured in 24-well plates for 24 h. Then the culture medium was replaced by fresh medium mixed with 4b or 6 micelles (0.5 mg mL^{-1}, encapsulating 5 μM compound BODIPY). The cells were further incubated with different periods of time at 37 ℃. Then the cells were washed with PBS twice and fixed with 4% paraformaldehyde. The nucleus was stained with DAPI and washed with PBS twice. Finally, the cells were imaged by a confocal laser-scanning microscope.

ROS elimination *in vitro*. RAW 264.7 cells (1×10^5 cells mL^{-1}) were inoculated into Petri dishes with DMEM, 15% fetal bovine serum, 1% penicillin, and 1% streptomycin and cultured in 24-well plates for 24 h. The culture medium of the control group was replaced by fresh one and the rest were replaced by fresh medium mixed with H_2O_2 (1 mM). After incubation for 12 h, all the cells were washed and the culture medium were replaced by P1b or P2 (0.5 mg mL^{-1}, encapsulating 0.1 mM Cur) mixed with fresh medium. All the cells were further incubated for different time before being stained with DAPI and washed with PBS twice. Finally, the cells were imaged by a confocal laser-scanning microscope.

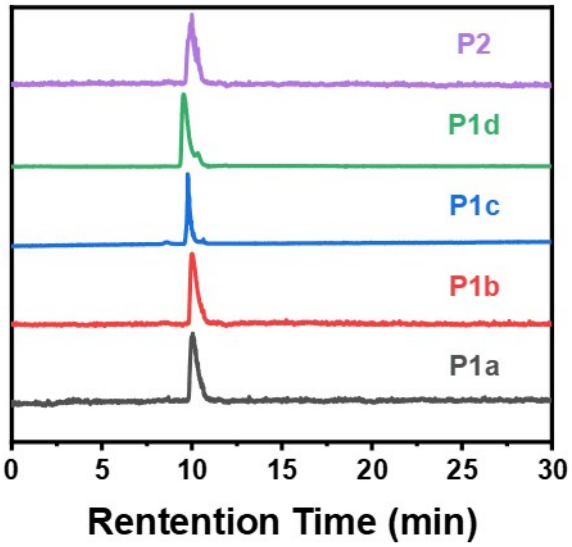


Figure S1. GPC traces of the synthesized polymers.

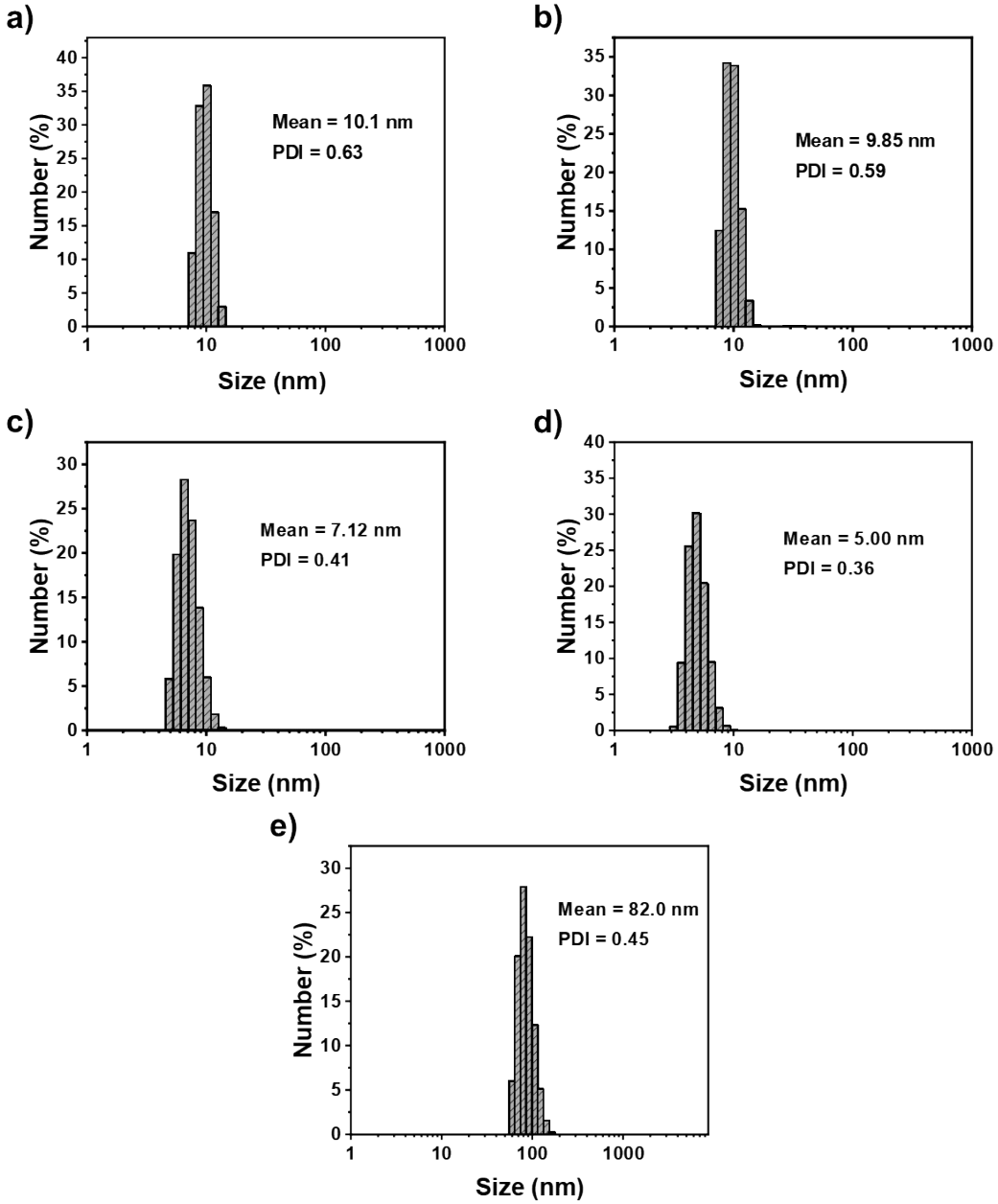


Figure S2. Statistical distributions of size for micelles formed by P1a (a), P1b (b), P1c (c), P1d (d) or P2 (e) (0.5 mg mL^{-1}) in PBS, determined by DLS in PBS at 22 °C.

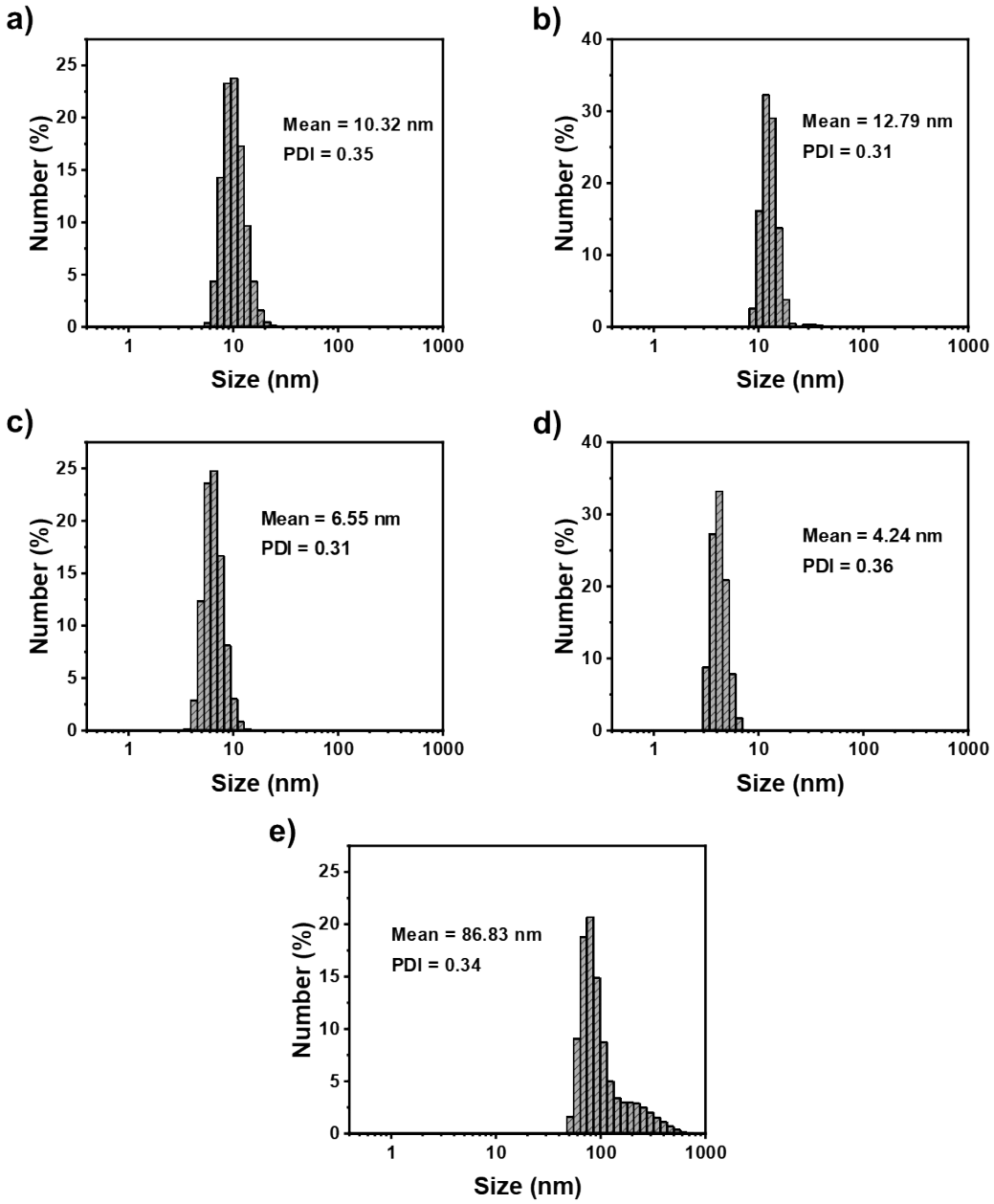


Figure S3. Statistical distributions of size for micelles formed by P1a (a), P1b (b), P1c (c), P1d (d) or P2 (e) (0.5 mg mL^{-1}) in DMEM, determined by DLS in PBS at 22 °C.

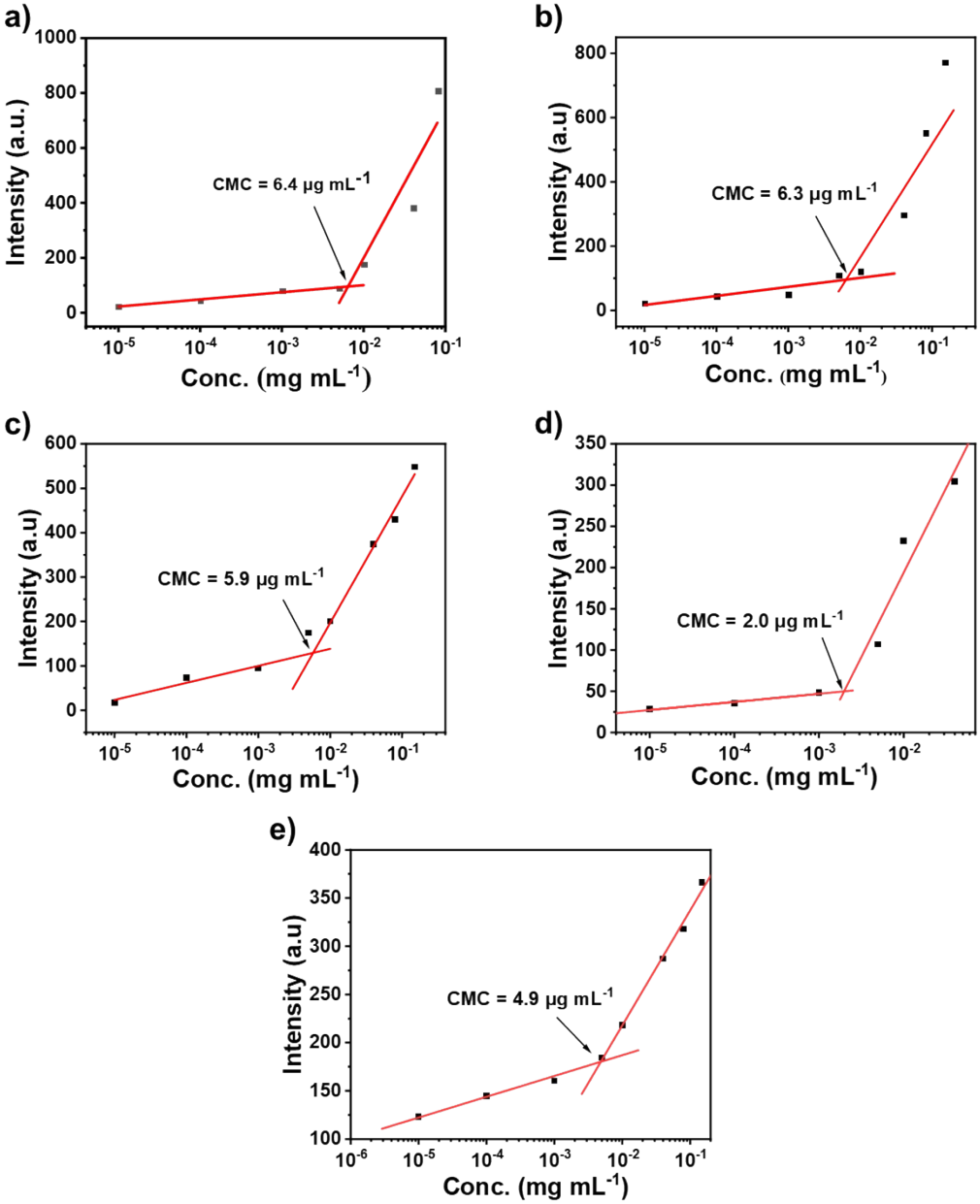


Figure S4. Plots of the emission intensity (λ_{Ex} = 494 nm, λ_{Em} = 512 nm), recorded at 25 °C after combining MeOH solutions of BODIPY (1 mM, 50 μL) and P1a (a), P1b (b), P1c (c), P1d (d) or P2 (e), distilling the solvent off under reduced pressure, dispersing the residue in PBS (1 mL) and passing the resulting dispersion through a 220 nm membrane, against the polymer concentration.

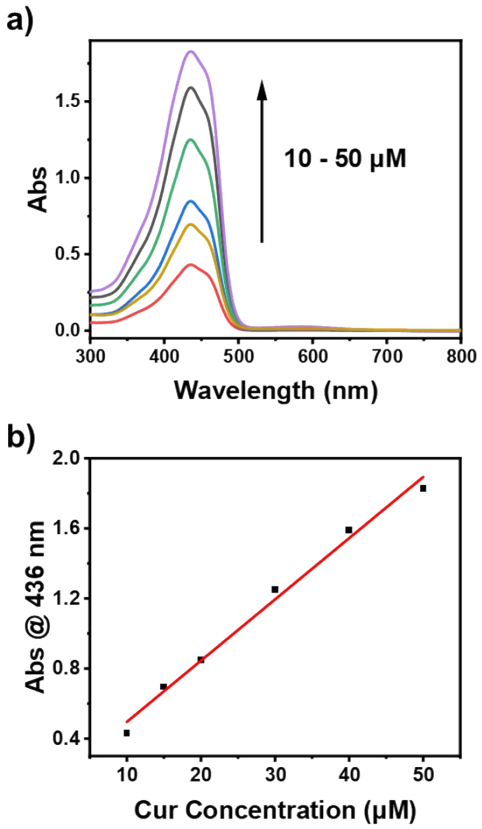


Figure S5. (a) Absorption spectra of Cur (10, 15, 20, 30, 40 and 50 µM) in DMSO, recorded at 25 °C. (b) Plots of the absorption recorded at 436 nm of Cur against concentration.

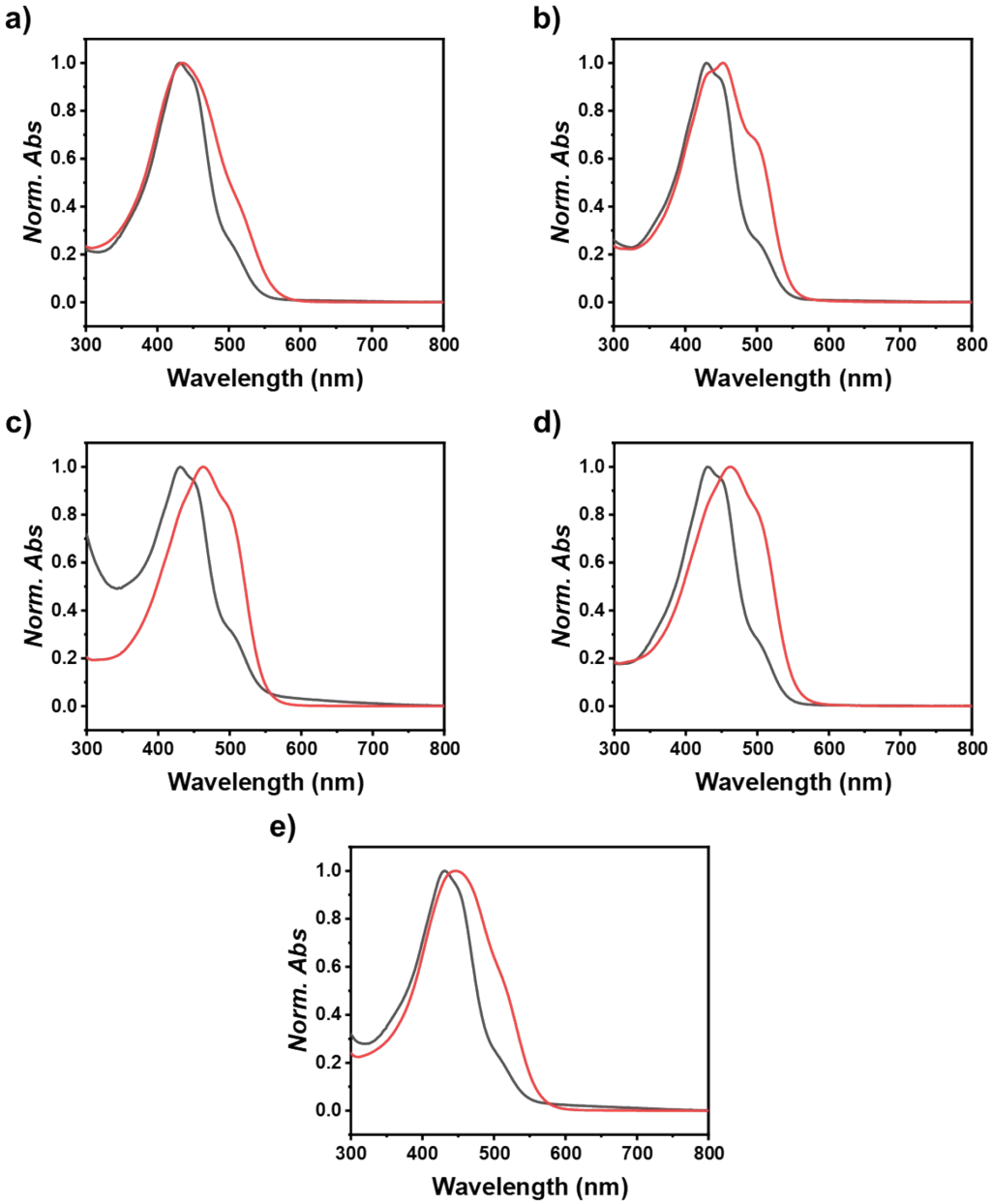


Figure S6. Normalized absorption spectra of Cur (10 μM, grey line, 50μM, red line) within polymeric micelles of P1a-P2 (0.5 mg mL^{-1},a-e) in PBS, recorded at 25 ℃.

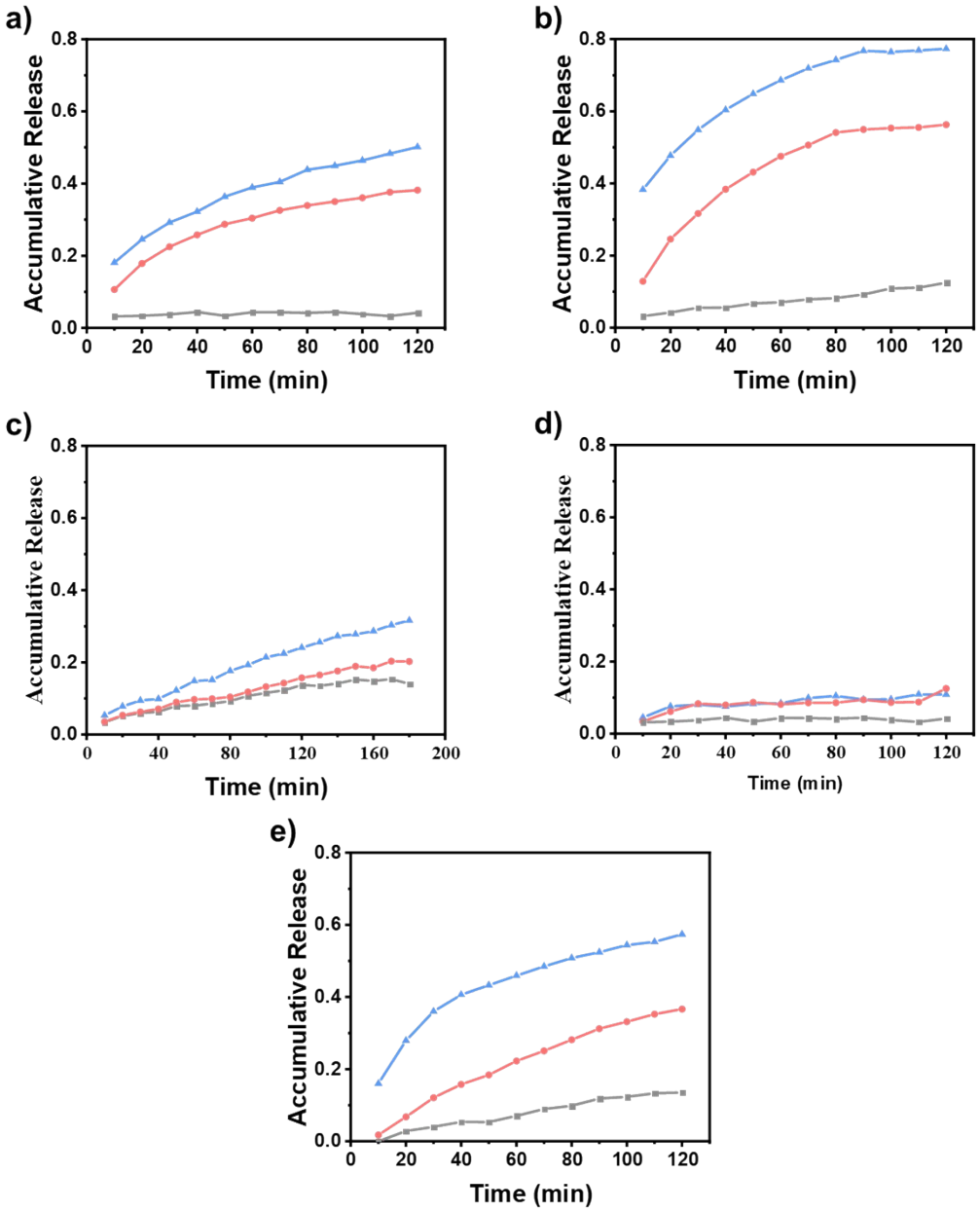


Figure S7. Release kinetic of BODIPY within P1a with different concentrations of H_2O_2 with different time. The accumulative release was calculated by 1-I/I_0. I stand for the emission intensity (λ_{Ex} = 494 nm, λ_{Em} = 512 nm).

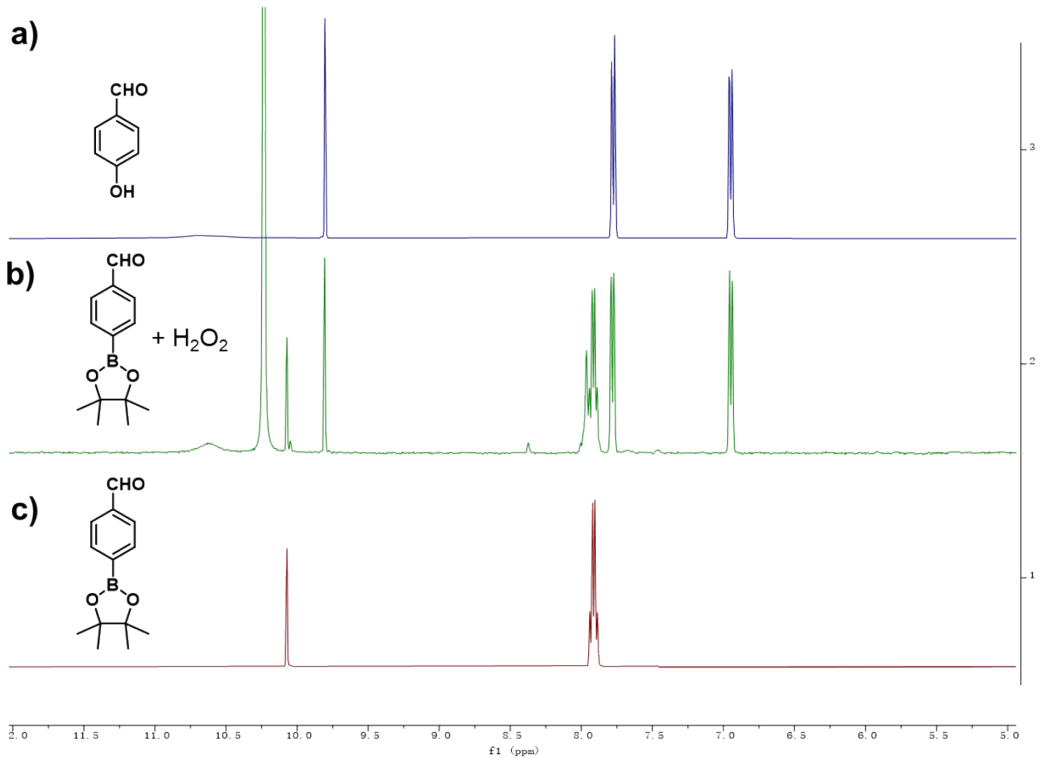


Figure S8. ^{1}H NMR spectra (400 MHz, DMSO-d6, 25 °C) of 4-hydroxybenzaldehyde (a) and (4-formylphenyl)boronic acid pinacol ester before (c) and after (b) adding H_2O_2.[1]

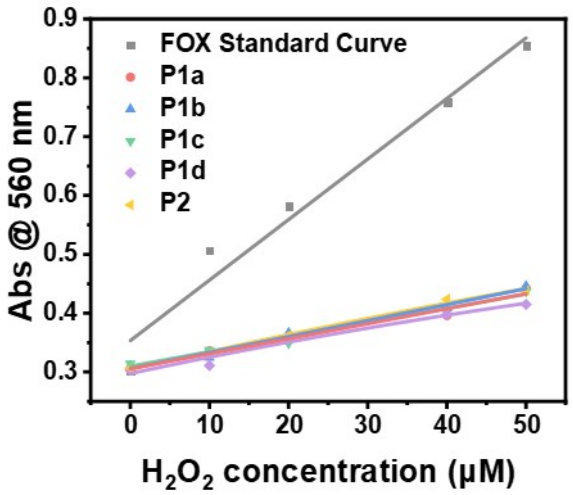


Figure S9. FOX assay of H_2O_2 solutions with or without Cur encapsulated within different polymers in PBS. The absorption at 560 nm was recorded at 25 °C after combining 200 μL FOX solution with 5 μL reaction results from different concentrations of H_2O_2 with Cur (10 μM) within each polymer (0.5 mg mL^{-1}) for 3 h.

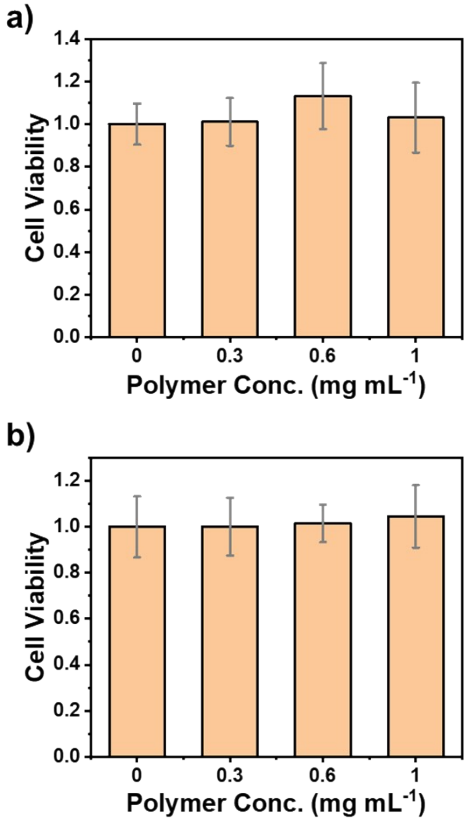


Figure S10. In vitro toxicity of micelles of either P1b (a) or P2 (b) with RAW 264.7 cells after 24 h incubation with various polymer concentrations (CCK-8 assay).

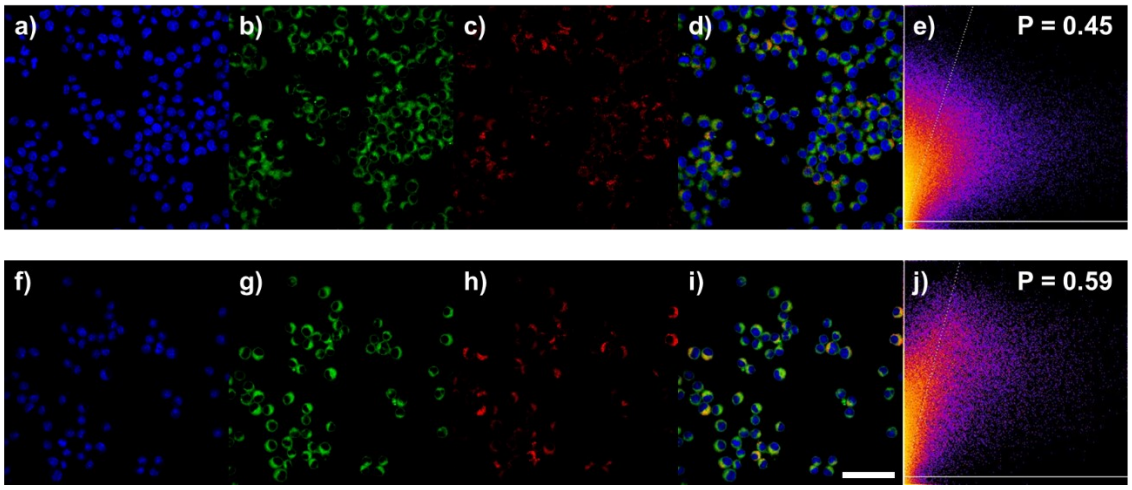


Figure S11. Co-localization images of lysosomes (Red) and BODIPY (Green) in P1a (a-e) or P2 (f-j) in RAW 264.7 cells, along with Pearson coefficient analysis. The nuclei of the cells were stained with DAPI. Blue Channel: λ_{Ex} = 405 nm, detection window: 425 nm – 475 nm; Green Channel: λ_{Ex} = 488 nm, detection window: 500 nm – 550 nm; Red Channel: λ_{Ex} = 640 nm, detection window: 650 nm – 720 nm. Scale Bar: 50 μm.

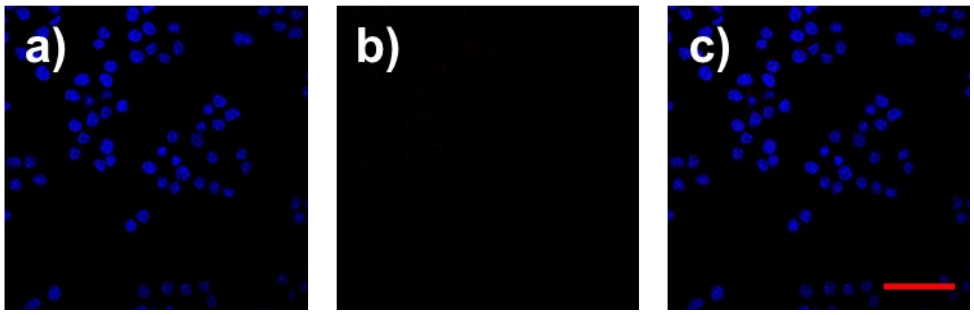
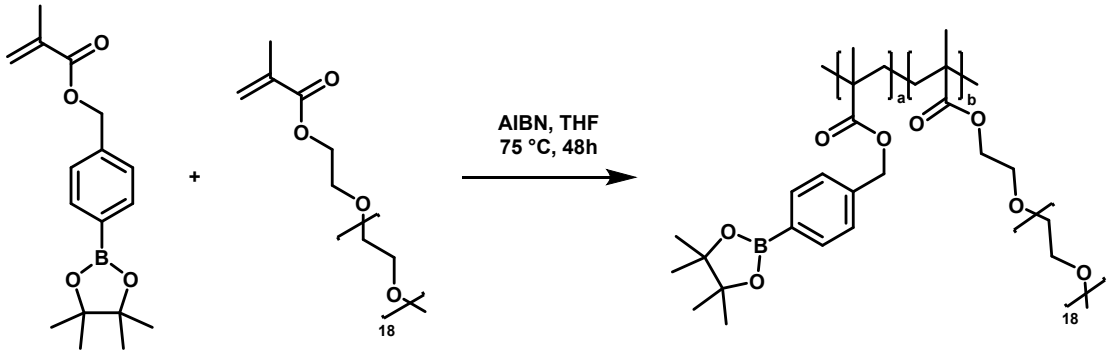


Figure S12. Fluorescent imaging of RAW 264.7 cells incubated with culture media mixed for 12 h and then culture media mixed Deep Red for 30 min. The nuclei of the cells were stained with DAPI. (a) Blue Channel: λ_{ex} = 405 nm, detection window: 425 nm – 475 nm; (b) Red Channel: λ_{ex} = 640 nm, detection window: 650 nm – 720 nm. (c) Merged image of (a) and (b). Scale Bar: 50 μm.



Synthesis of the polymers. A solution of 4-boronic acid pinacol ester benzyl methacrylate (3 mmol for P1a, 2 mmol for P1b, 1 mmol for P1c, 0.5 mmol for P1d, 0.33 mmol for P2), poly (ethylene glycol) methyl ether methacrylate (Mw = 950, 1 mmol for P1a-P1d, Mw = 2000, 1 mmol for P2), and AIBN (5 mg, 0.03 mmol) in degassed THF (8 mL) was heated at 75 °C for 48 h in a sealed tube. After cooling down to ambient temperature, the solvent was distilled off under reduced pressure. The residue was purified by gel permeation chromatography [LH-20, CH_2Cl_2/MeOH (1:1, v/v)] to give the corresponding polymer as a transparent viscous liquid.

P1a. ^{1}H NMR (400 MHz, Chloroform-*d*): δ = 7.81 (s, 6H,), 7.33 (s, 6H), 5.20 – 4.77 (s, 6H), 3.95 – 4.24 (s, 1H), 3.83 – 3.52 (m, 79H), 3.41 (s, 3H), 2.16-1.66 (s, 24H), 1.45-1.21 (s, 39H). Mn = 6.8 kDa, Mw = 11.1 kDa, PDI = 1.64.

P1b. ^{1}H NMR (400 MHz, Chloroform-*d*): δ = 7.81 (s, 3H,), 7.33 (s, 3H), 5.20 – 4.77 (s, 3H), 3.95 – 4.24 (s, 2H), 3.83 – 3.52 (m, 86H), 3.41 (s, 3H), 2.16-1.66 (s, 12H), 1.45-1.21 (s, 24H). Mn = 6.6 kDa, Mw = 10.2 kDa, PDI = 1.54.

P1c. ^{1}H NMR (400 MHz, Chloroform-*d*): δ = 7.81 (s, 2H,), 7.33 (s, 2H), 5.20 – 4.77 (s, 1H), 3.95 – 4.24 (s, 2H), 3.83 – 3.52 (m, 99H), 3.41 (s, 3H), 2.16-1.66 (s, 14H), 1.45-

1.21 (s, 13H). Mn = 7.0 kDa, Mw = 10.3 kDa, PDI = 1.48.

P1d. ^{1}H NMR (400 MHz, Chloroform-*d*): δ = 7.81 (s, 0.5H,), 7.33 (s, 1H), 5.20 – 4.77 (s, 1H), 3.95 – 4.24 (s, 1H), 3.83 – 3.52 (m, 89H), 3.41 (s, 3H), 2.16-1.66 (s, 6H), 1.45-1.21 (s, 4H). Mn = 8.2 kDa, Mw = 11.8 kDa, PDI = 1.44.

P2. ^{1}H NMR (400 MHz, Chloroform-*d*): δ = 7.81 (s, 3H,), 7.33 (s, 3H), 5.20 – 4.77 (s, 3H), 3.83 – 3.52 (m, 200H), 3.41 (s, 3H), 2.16-1.66 (s, 4H), 1.45-1.21 (s, 24H). Mn = 8.2 kDa, Mw = 11.9 kDa, PDI = 1.45.

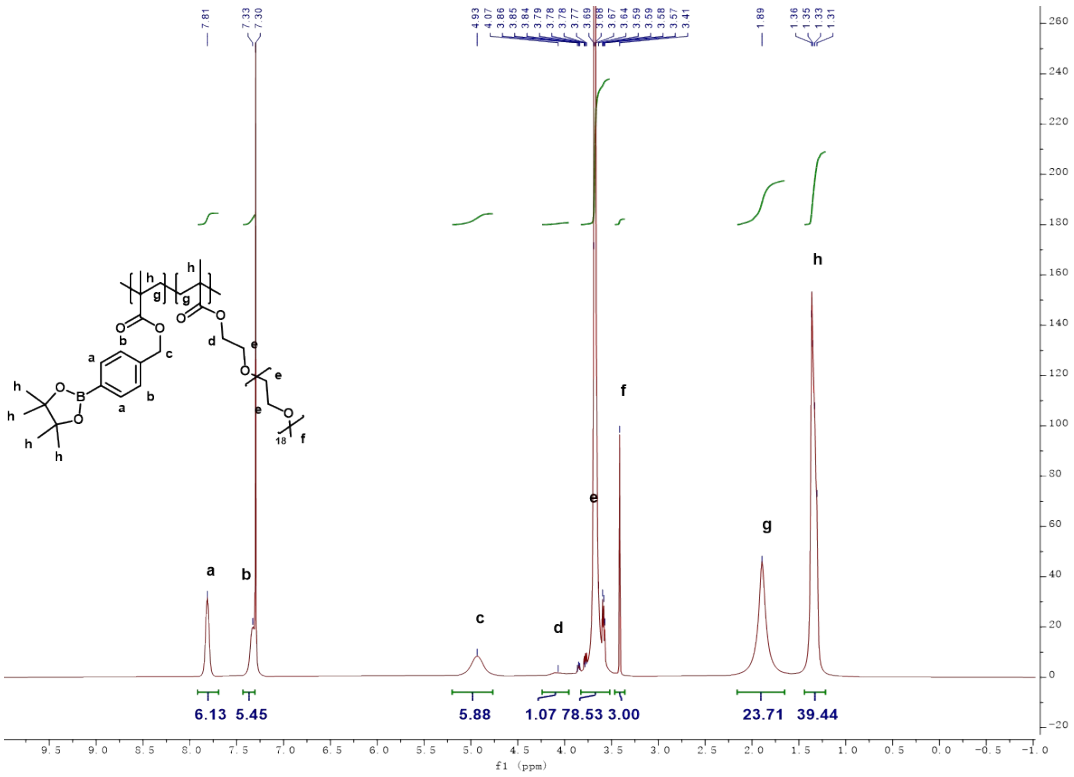


Figure S13. ^{1}H NMR of P1a.

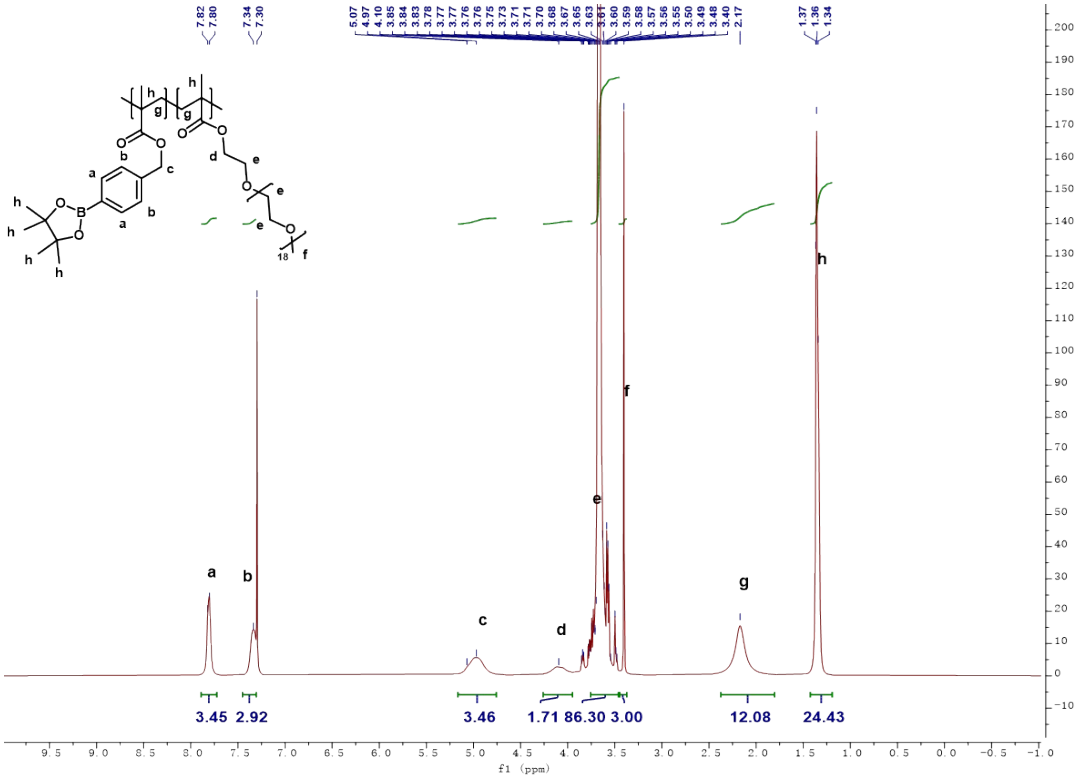


Figure S14. ^{1}H NMR of P1b.

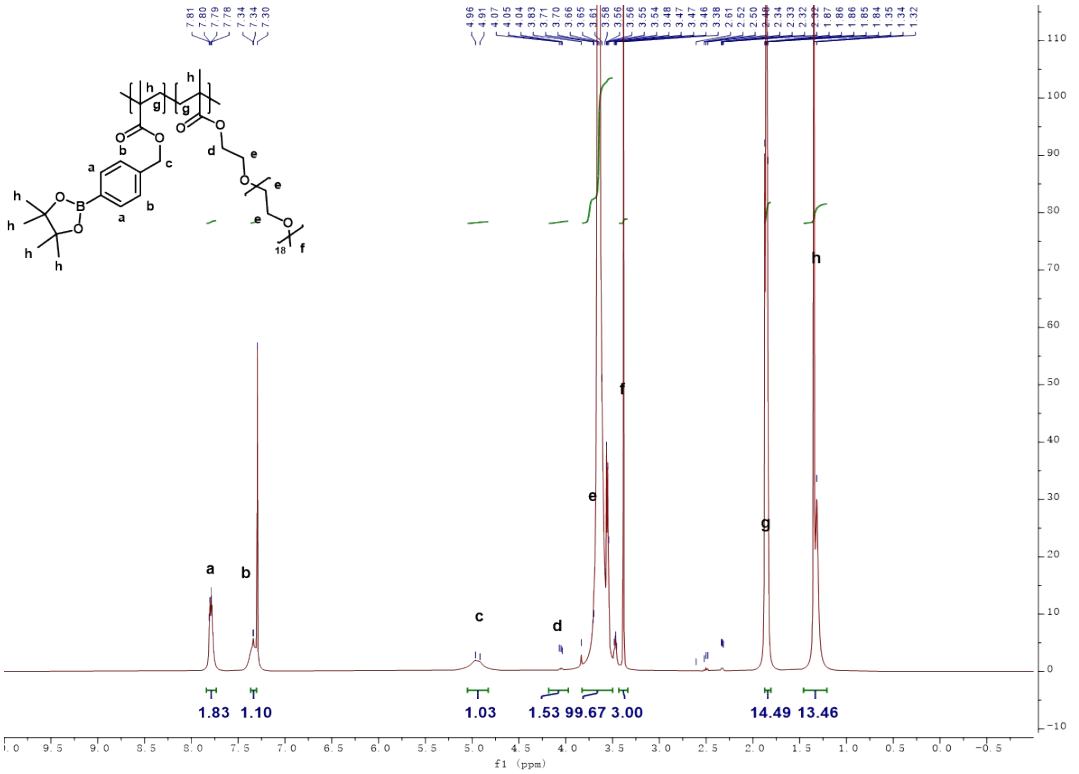


Figure S15. ^{1}H NMR of P1c.

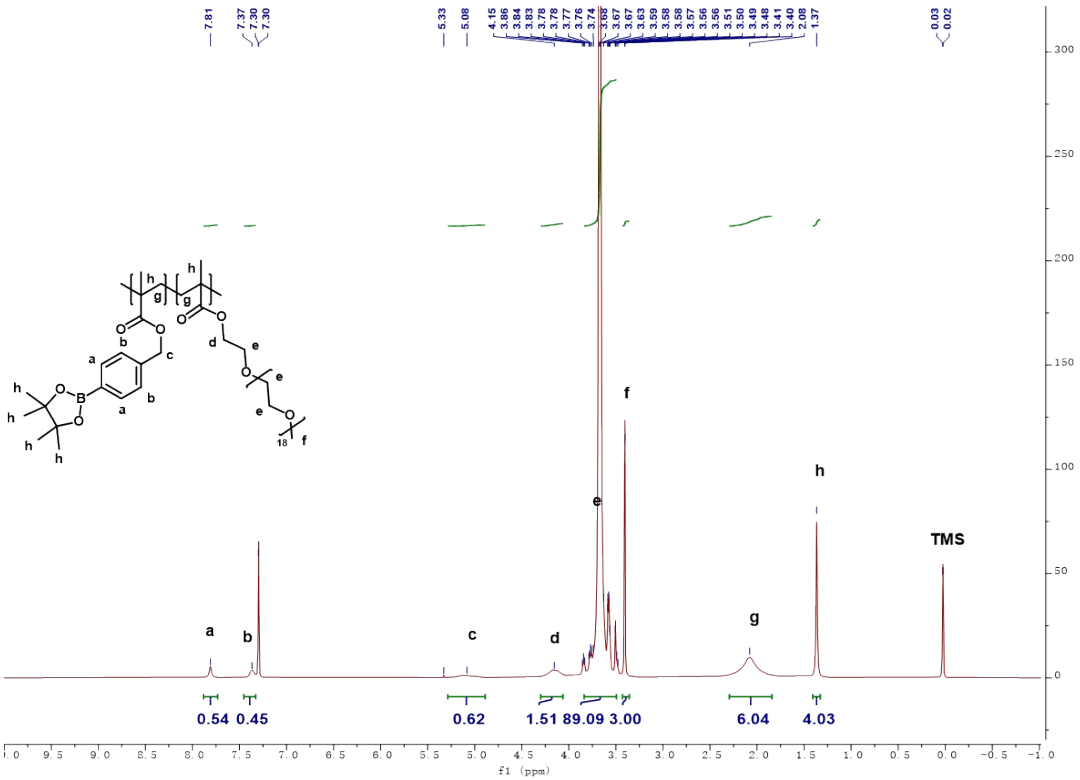


Figure S16. ^{1}H NMR of P1d.

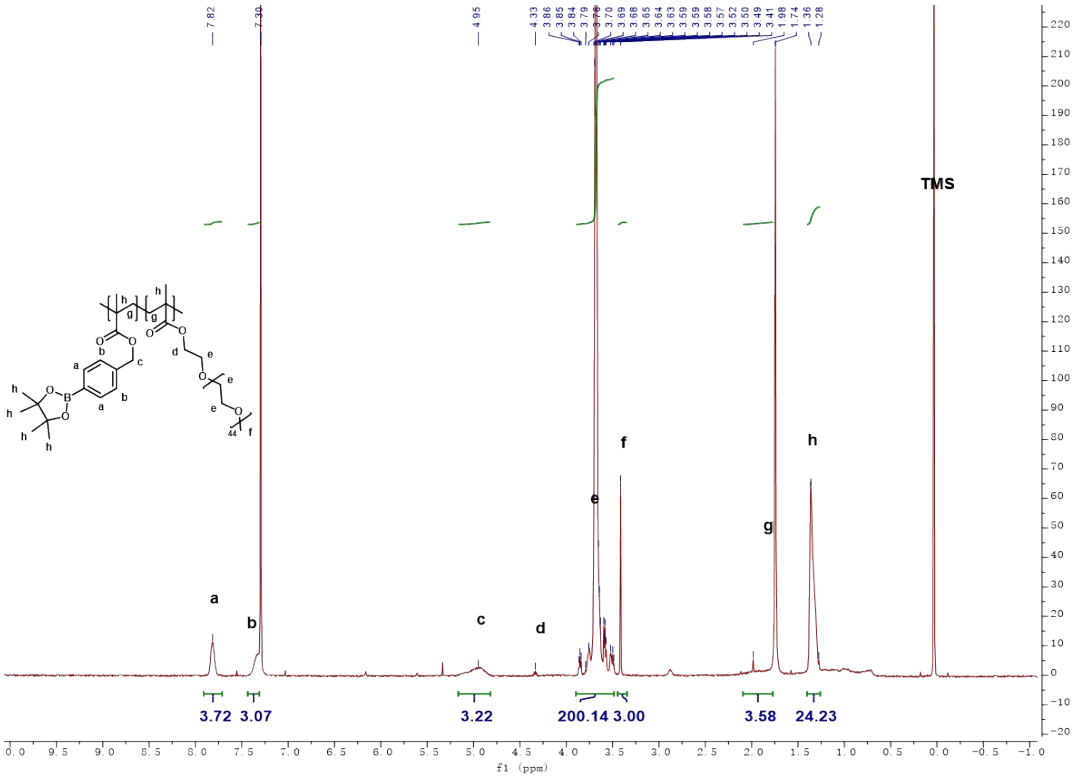


Figure S17. ^{1}H NMR of P2.

Reference

1. S. Tang, Y. Gao, W. Wang, Y. Wang, P. Liu, Z. Shou, R. Yang, C. Jin, X. Zan and C. J. A. M. L. Wang, 2023, **13**, 58-64.