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Supporting Information

Dual Drug-Based Hyperbranched Polymer with Methotrexate and Chlorambucil Moieties for Synergistic Cancer Chemotherapy

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1. Methods

1.1 Cellular uptake of HBPMs by PC-3 cells

The cellular uptake behaviors were studied in PC-3 cells using confocal laser scanning microcopy (CLSM). For flow cytometry, PC-3 cells were seeded in 6-well plates at 2.0×10^5 cells per well in 3 mL of complete RPMI-1640 and cultured for 24 h. Then the solution of **HBPMs** was diluted with culture medium at a final concentration of 30μM. The diluted solution was added to different wells and the cells were incubated at 37 °C for 4 and 12h. Thereafter, culture medium was removed and cells were washed with PBS for three times and treated with trypsin. Data for 1.0×10^4 gated events were collected and analysis was performed by means of a beckman FC500 flow cytometer. For the CLSM study, HepG2 cells were seeded in 6-well plates at 2.0×10^5 cells per well in 1 mL of complete RPMI-1640 and incubated for 24h, followed by removing culture medium and adding **HBPMs** solutions (2 mLRPMI-1640 medium) at the concentration of 30 μM. After incubation at 37 °C for 4, 12 and 24h culture medium was removed, and cells were washed with PBS for three times and fixed with 4% (w/w) formaldehyde solution for 15 min. Subsequently, the cells were stained with Lyso Tracker Green DND-26 for 15 min at 37 °C, and the slides were rinsed with PBS three times. Then resulting slides were mounted and observed with a confocal laser scanning microscopy (TCS-SP5, Leica, Germany).

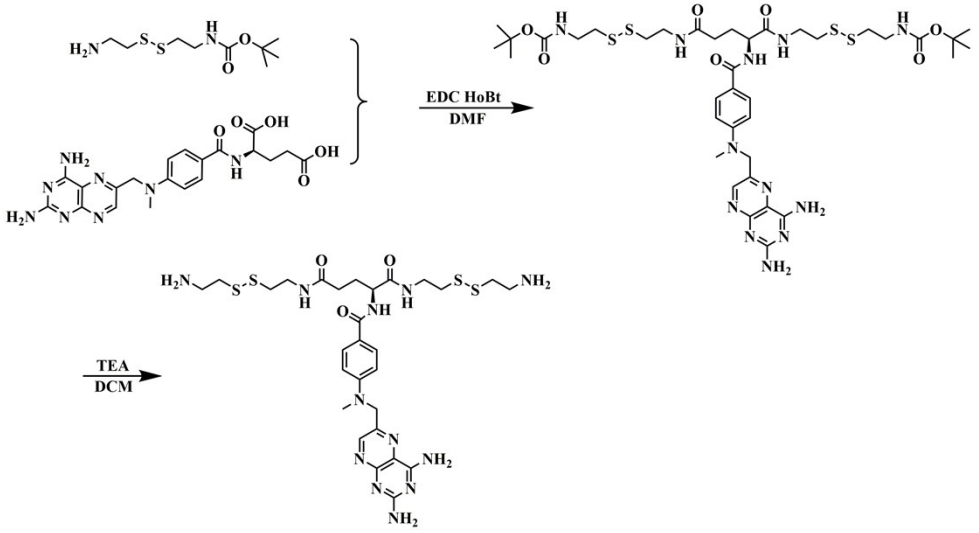
1.2 *In vitro* cytotoxicity studies of HBPMs

The PC-3 and MCF-7 were used to evaluate the anticancer activity of **HBPMs**. The free drug MTX and Cb and the mixture of MTX and Cb were used as control. The cells were seeded into 96-well plates at 2×10^5 cells per well in 200 μL of culture medium. The cells without the treatment were used as control. The cells were grown for another 72h with a series of varying concentrations of MTX, Cb, the mixture of MTX and Cb and HBPMs. Then, 20 μL of 5 mg mL^{-1} MTT assay stock solution in PBS was added to each well. After the cells were incubated for 1 h, the absorbance was measured at 490 nm using a Multiskan MK3 Microplate Reader (Thermo Scientific, USA). The blank was subtracted to the measured optical density (OD) values, and the cell viability was expressed as % of the values obtained for the untreated control cells.

1. 3 Cell apoptosis

PC-3 cells were seeded in 6-well plates at 6.0×10^5 cells per well in 2 mL of complete RPMI-1640 and cultured for 24 h. The cells were treated with free MTX, Cb, MTX/Cb and **HBPMs** at the same concentration (32μg/ml) for 24 h. For quantitative measurement of apoptosis, treated cells were harvested and washed twice with 4°C PBS, stained with FITC annexin V and PI according to the manufacturer's instructions. For cell cycle determination, treated cells were collected, washed for twice with 4°C PBS, fixed with 70% ethanol at 4 °C overnight, followed by PI/RNase staining for 15 min in the dark. Both cell apoptosis and cycle were analyzed by flow cytometry beckman FC500.

2 Synthesis of MTX-(SS-NH_2)$_2$



Scheme S1. Synthetic routes of MTX-(SS-NH_2)$_2$.

2.1 Synthesis of Boc-NH-SS-NH_2

Boc-NH-SS-NH_2 was synthesized according to previous report with slight change.[1]

^{1}H NMR (400 MHz, $CDCl_3$) δ = 5.22 (s, 1H), 3.35 -3.34 (m, 2H), 2.93 (t, *J*=6.2, 2H), 2.72-2.68 (m, 4H), 1.84 (s, 2H), 1.35 (s, 9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ = 156.20, 78.38, 41.61, 38.25, 28.81. HRMS (ESI-TOF) ($C_9H_{20}N_2O_2S_2$) m/z: calcd. for $[M+H]^+$ 253.1039; found 253.1036, error 1.2ppm.

2.2 Synthesis of MTX-(SS-Boc)$_2$

Methotrexate (0.18 g, 0.40 mmol), EDC • HCl (0.15 g 0.8 mmol) and HoBt (0.11 g, 0.81 mmol) were dissolved in DMF (20 mL) in a 100 mL round-bottom flask, and stirred at 0 ℃ for 0.5 h. Then, compound Boc-NH-SS-NH_2 (0.25 g, 1 mmol) as a solution in DMF (5.0ml) was added gradually. The mixture was reacted for 24 h at RT. After that, 5 mL deionized water was added to quench the reaction. Then, the solvent (DMF) was reduced via distillation and the product was dissolved by CH_2Cl_2. The reaction mixture was poured into saturated brine (50 mL)
and the resulting solution was extracted with dichloromethane (30 mL×3). The combined organic phase was concentrated and purified by flash column chromatography (CH_2Cl_2 /CH_3OH, 10:1 v/v) to afford compound MTX-(SS-Boc)$_2$ as a yellow solid (0.2 g, 54.7%).

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.55 (s, 1H), 8.07 (d, *J*=7.5, 1H), 7.98 (s, 2H), 7.74 (d, *J*=8.7, 2H), 7.43 (s, 1H), 6.96 (d, *J*=5.3, 2H), 6.81 (d, *J*=9.0, 2H), 6.61 (s, 2H), 4.78 (s, 2H), 4.32 (s, 1H), 3.21-3.17 (m, 8H), 2.73 (m, 8H), 2.14 (s, 2H), 1.93 (d, *J*=37.1, 2H), 1.36 (s, 18H). ^{13}C NMR (101 MHz, DMSO-d_6) δ = 172.39, 166.61, 163.20, 155.97, 151.31, 149.54, 146.88, 129.46, 121.65, 111.47, 78.24, 55.35, 53.61, 51.81, 38.39, 37.91, 37.50, 32.52, 28.67, 27.98. HRMS (ESI-TOF) ($C_{38}H_{58}N_{12}O_7S_4$) m/z: calcd. for $[M+H]^+$ 923.3507; found 923.3503, error 0.4ppm.

2.3 Synthesis of MTX-(SS-NH$_2$)$_2$

Compound MTX-(SS-Boc)$_2$ (0.15 g, 0.16 mmol) in anhydrous dichloromethane (25 mL) was added to trifluoroacetic acid (0.15 g, 1.3 mmol). The reaction mixture was stirred for 24 h, washed with saturated aqueous $NaHCO_3$ (25 mL), and dried with Na_2SO_4. The mixture was evaporated to obtain compound MTX-(SS-NH$_2$)$_2$ as a yellow solid (0.04 g, yield: 34.2%).

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.53 (s, 1H), 8.10 (s, 3H), 7.72 (d, *J*=8.8, 2H), 7.47 (d, *J*=8.3, 1H), 6.79 (d, *J*=9.2, 2H), 6.59 (s, 2H), 4.77 (s, 2H), 4.30 (s, 1H), 3.20-3.15 (m, 4H), 2.78-2.68 (m, 8H), 2.13 (s, 2H), 1.98 (d, *J*=6.0, 2H).^{13}C NMR (101 MHz, DMSO-d_6) δ = 172.93, 172.79, 166.58, 163.31, 163.16, 155.60, 151.32, 149.61, 146.54, 129.42, 121.98, 111.60, 54.99, 38.45, 38.26, 37.94, 37.55, 32.58, 28.30, 28.15. HRMS (ESI-TOF) ($C_{28}H_{42}N_{12}O_3S_4$) m/z: calcd. for $[M+Na]^+$ 745.2278; found 745.2236, error 5.5ppm.

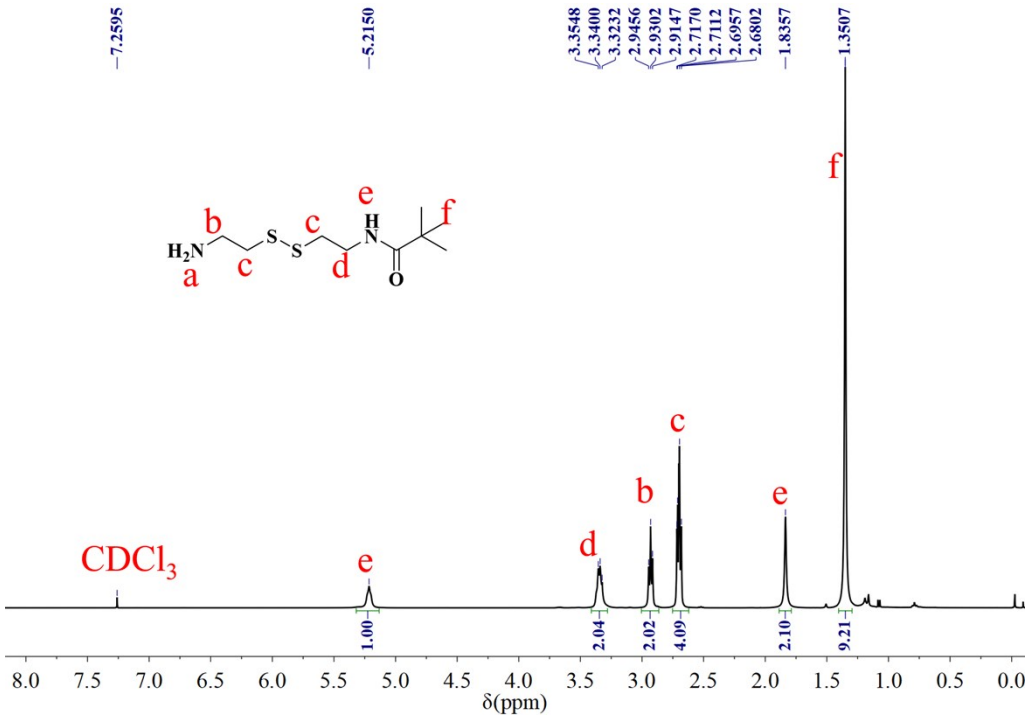


Figure S1. ^{1}H NMR spectrum recorded (400 MHz, $CDCl_3$, RT) for compound Boc-NH-SS-NH_2.

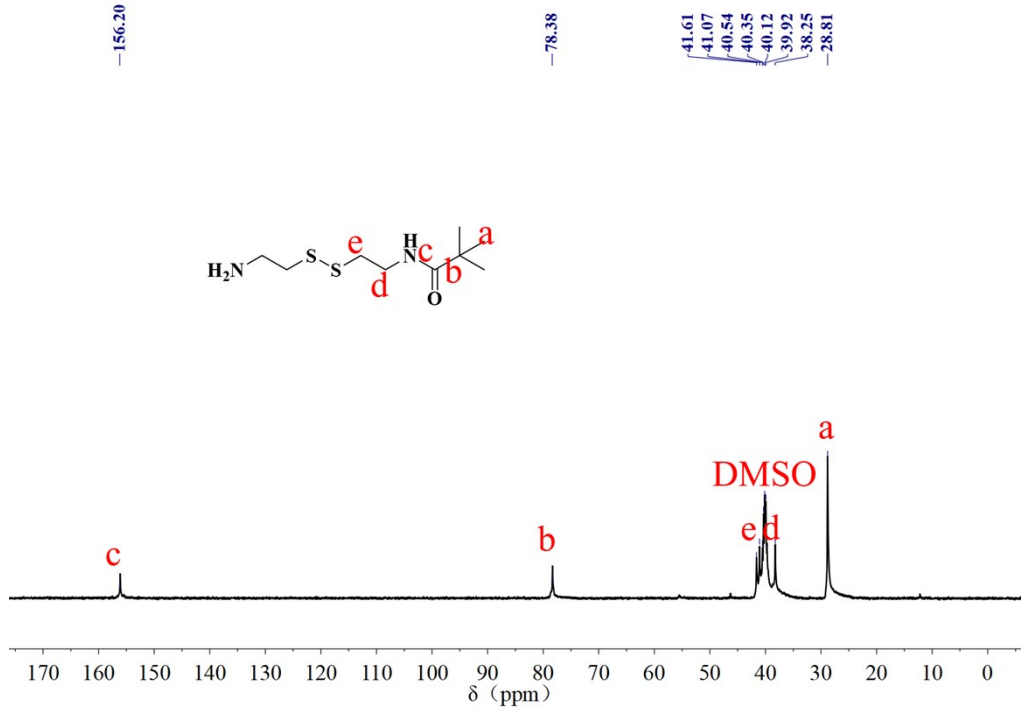


Figure S2. ^{13}C NMR spectrum recorded (100 MHz, DMSO-d_6, RT) for compound Boc-NH-SS-NH_2.

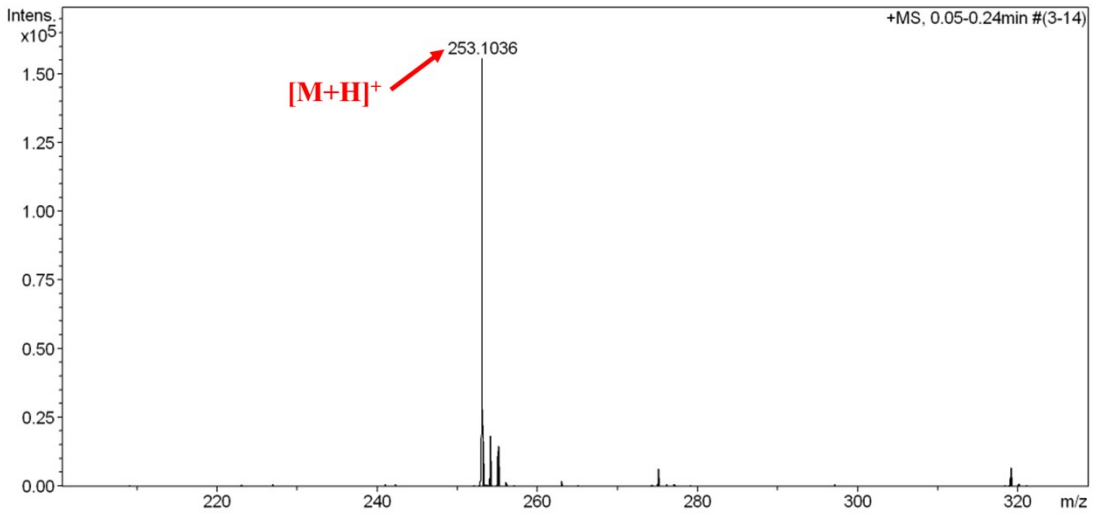


Figure S3. High-resolution electrospray ionization mass spectrum of compound Boc-NH-SS-NH_2.

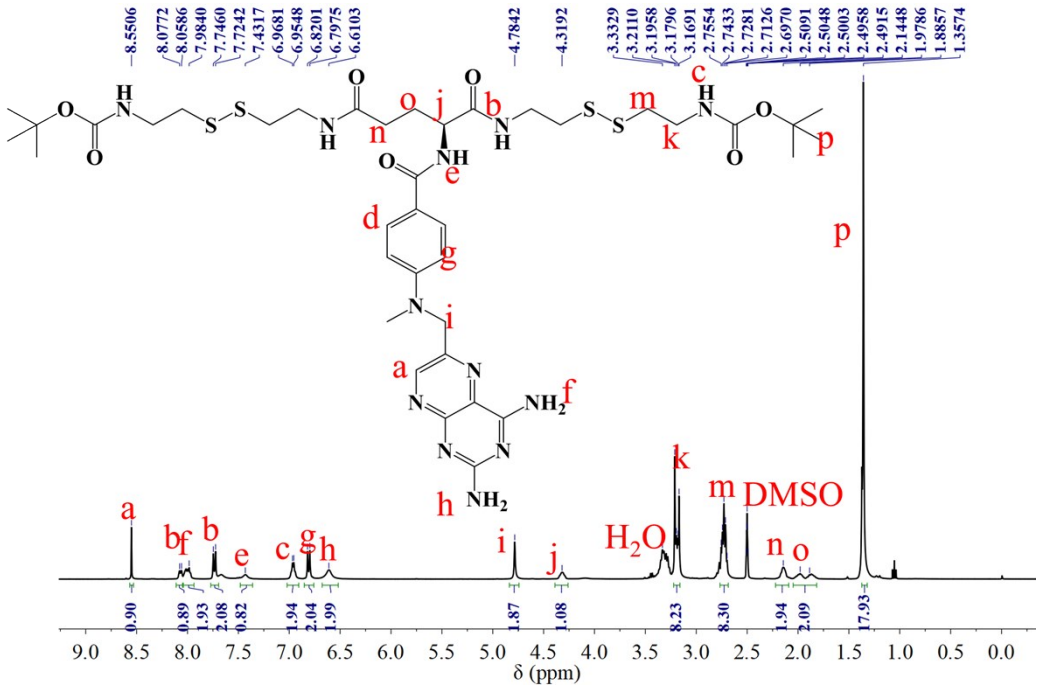


Figure S4. ^{1}H NMR spectrum recorded (400 MHz, DMSO-d_6, RT) for compound MTX-(SS-Boc)$_2$.

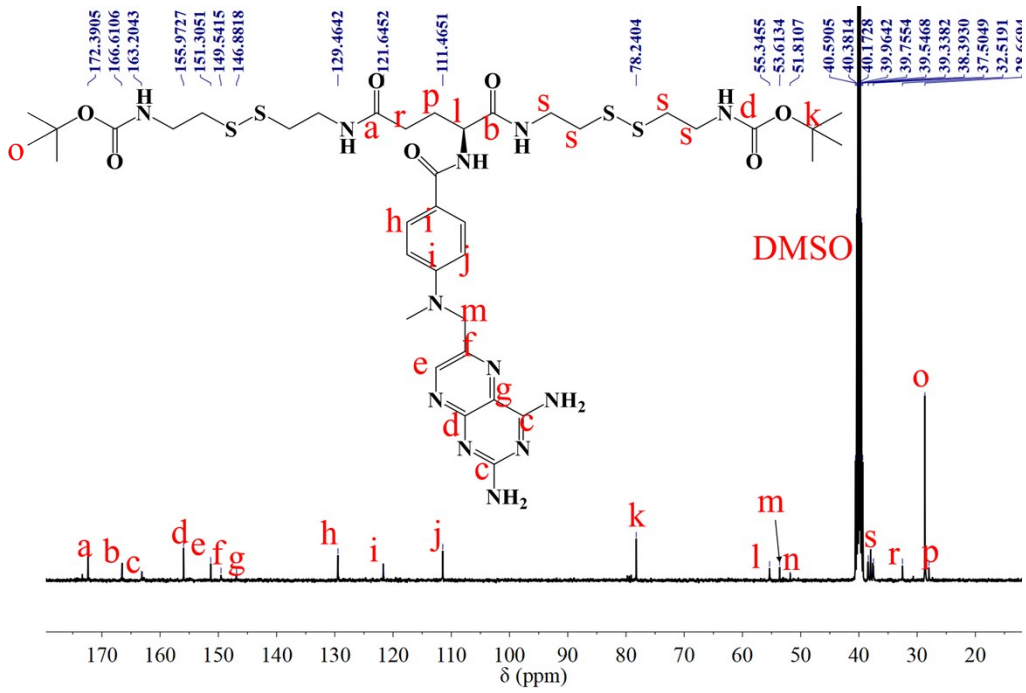


Figure S5. ^{13}C NMR spectrum recorded (100 MHz, DMSO-d_6, RT) for compound MTX-(SS-Boc)$_2$.

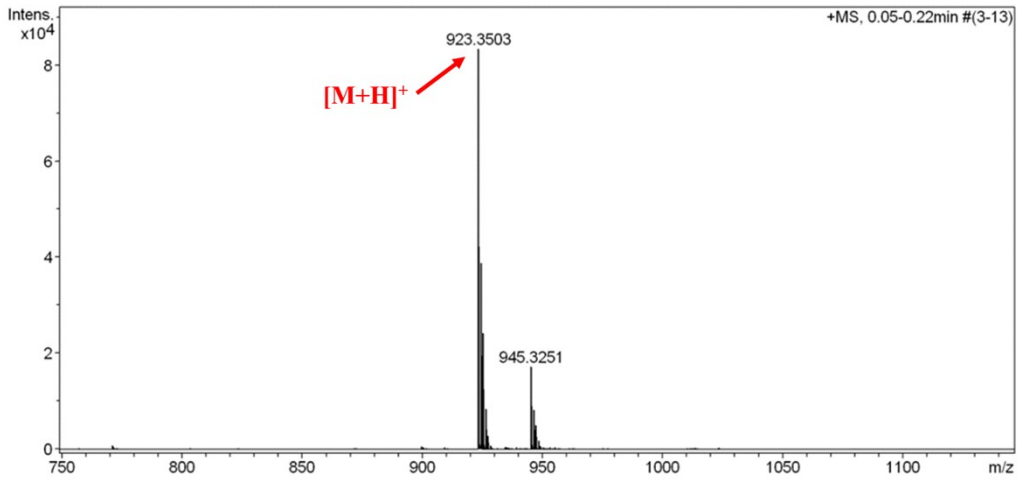


Figure S6. High-resolution electrospray ionization mass spectrum of compound MTX-(SS-Boc)$_2$.

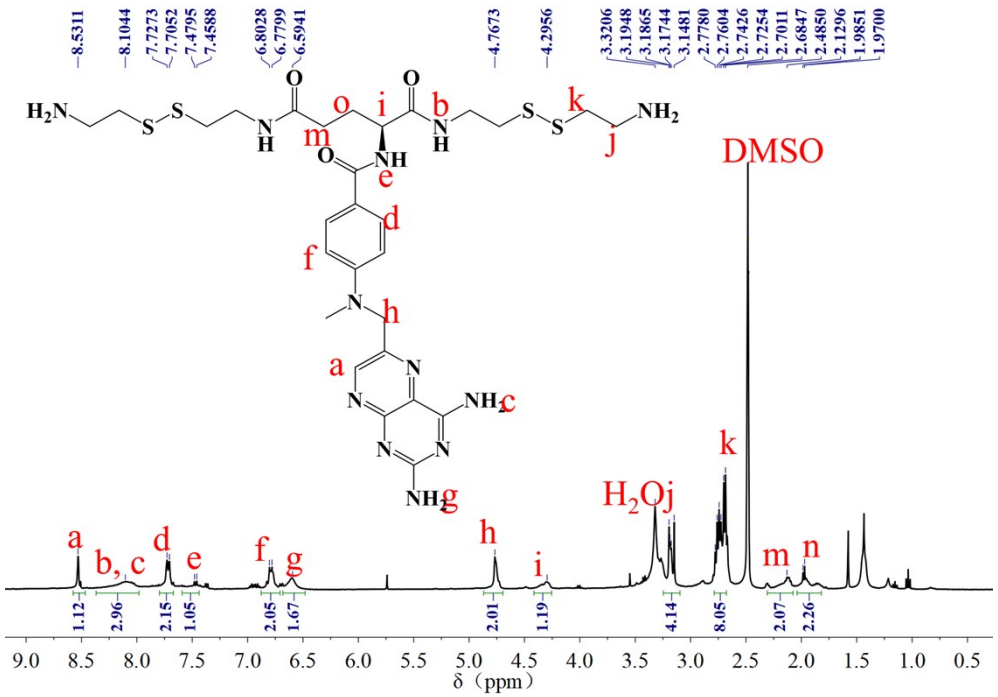


Figure S7. ^{1}H NMR spectrum recorded (400 MHz, DMSO-d_6, RT) for compound **MTX-(SS-NH$_2$)$_2$**.

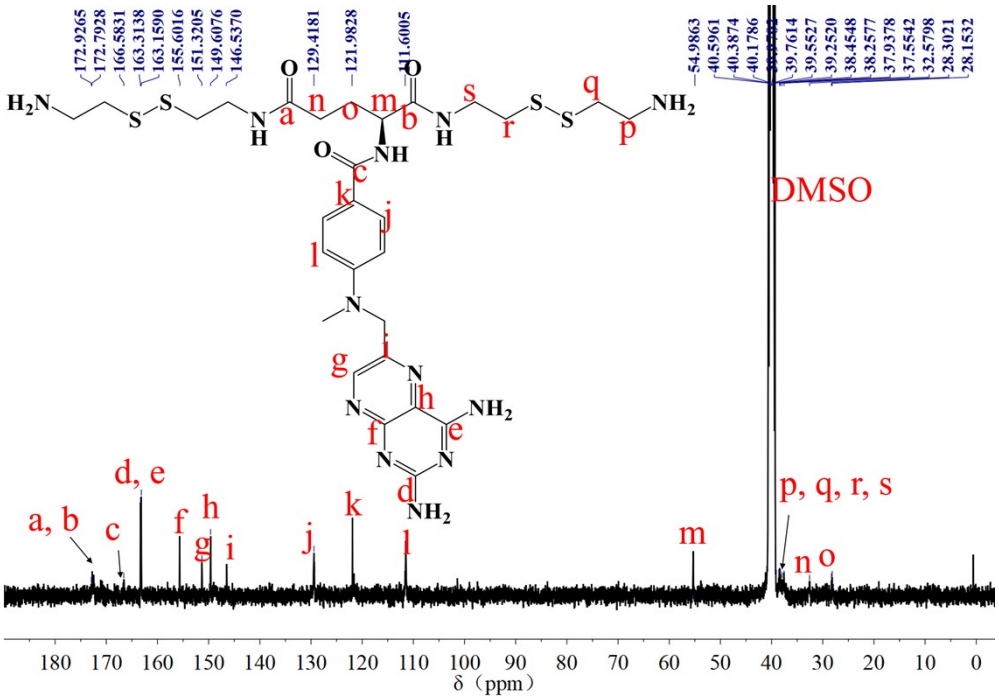


Figure S8. ^{13}C NMR spectrum recorded (100 MHz, DMSO-d_6, RT) for compound **MTX-(SS-NH$_2$)$_2$**.

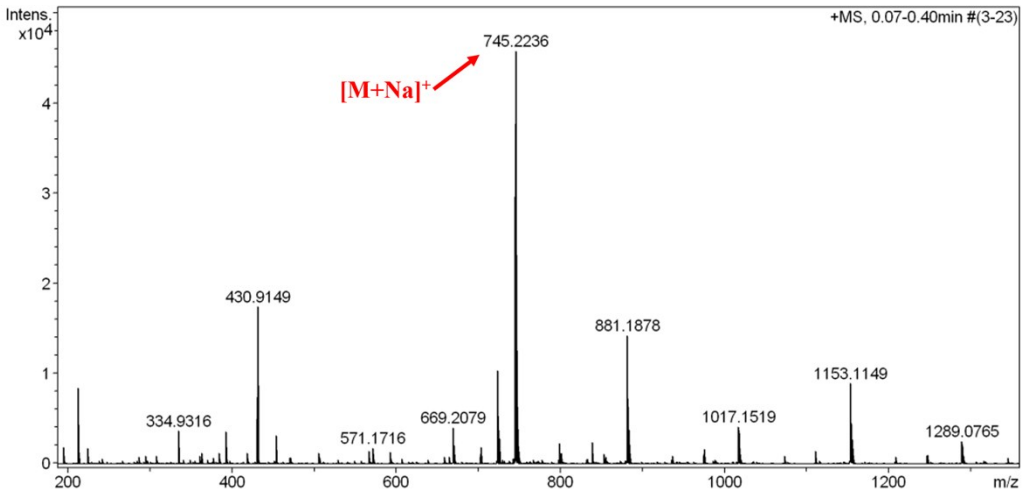
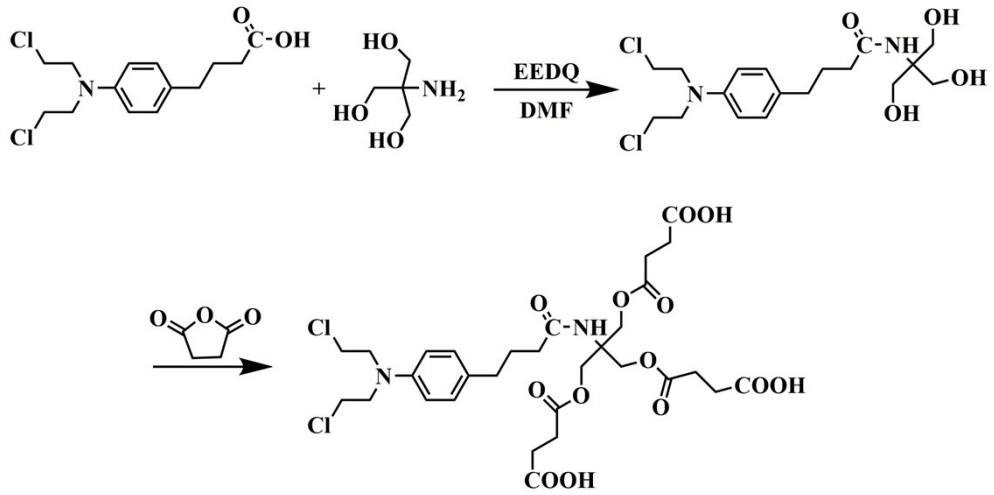


Figure S9. High-resolution electrospray ionization mass spectrum of compound **MTX-(SS-NH$_2$)$_2$**.

3 Synthesis of Cb-(COOH)$_3$



Scheme S2. Synthetic routes of Cb-(COOH)$_3$.

3.1 Synthesis of Cb-$(OH)_3$.

Chlorambucil (0.10 g, 0.33 mmol), TRIS (0.04 g, 0.36 mmol) and EEDQ (0.10 g, 0.40 mmol) were dissolved in 30 mL of EtOH and stirred at 60°C for 24h. After that, the reaction mixture was cooled down and the solvent was evaporated under vacuum. The reaction mixture was poured into saturated brine (50 mL) and the resulting solution was extracted with dichloromethane (30 mL×3). The combined organic phase was concentrated and purified by flash column chromatography (CH_2Cl_2 /CH_3OH, 40:1 v/v) to afford compound Cb-$(OH)_3$ as a white solid (0.07 g, 52.8%).

^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.13 (s, 1H), 7.02 (d, *J*=8.4, 2H), 6.66 (d, *J*=8.5, 2H), 4.51 (s, 3H), 3.69 (s, 8H), 3.52 (s, 6H), 2.44 (t, *J*=7.4, 2H), 2.13 (t, *J*=7.4, 2H), 1.71 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ = 173.76, 144.70, 130.32, 129.82, 112.19, 62.90, 61.22, 52.53, 41.43, 35.97, 33.40, 27.98. HRMS (ESI-TOF) ($C_{18}H_{28}Cl_2N_2O_4$) m/z: calcd. for $[M+Na]^+$ 429.1318; found 429.1331, error 3.0 ppm.

3.2 Synthesis of Cb-$(COOH)_3$

Compound Cb-$(OH)_3$ (0.24 g, 0.59mmol) and DMAP (0.043 g, 0.35 mmol) were dissolved in dry dichloromethane (50 mL), and stirred at 0 ℃ for 0.5 h. Then, succinic anhydride (0.29 g, 2.95 mmol) as a solution in THF (5.0 ml) was added gradually, and the mixture was reacted for 24 h at RT. Then, the solvent (THF) was reduced via distillation and the product was dissolved by CH_2Cl_2. The reaction mixture was poured into saturated brine (50 mL) and the resulting solution was extracted with dichloromethane (30 mL×3). The combined organic phase was concentrated and purified by flash column chromatography (CH_2Cl_2 /CH_3OH, 30:1 v/v) to afford compound Cb-$(COOH)_3$ as a white solid (0.21 g, 50%).

^{1}H NMR (400 MHz, DMSO-d_6) δ = 12.27 (s, 3H), 7.72 (s, 1H), 7.02 (d, *J*=8.6, 2H), 6.66 (d, *J*=8.6, 2H), 4.30 (s, 6H), 3.69 (s, 8H), 2.08 (t, *J*=7.3, 2H), 1.70 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ = 173.79, 173.46, 172.11, 144.86, 130.38, 129.77, 112.36, 62.27, 57.40, 52.71, 41.64, 35.51, 33.83, 29.15, 29.06, 27.66. HRMS (ESI-TOF) ($C_{30}H_{40}Cl_2N_2O_{13}$) m/z: calcd. for $[M+H]^+$ 707.1980; found 707.2017, error 5.1ppm.

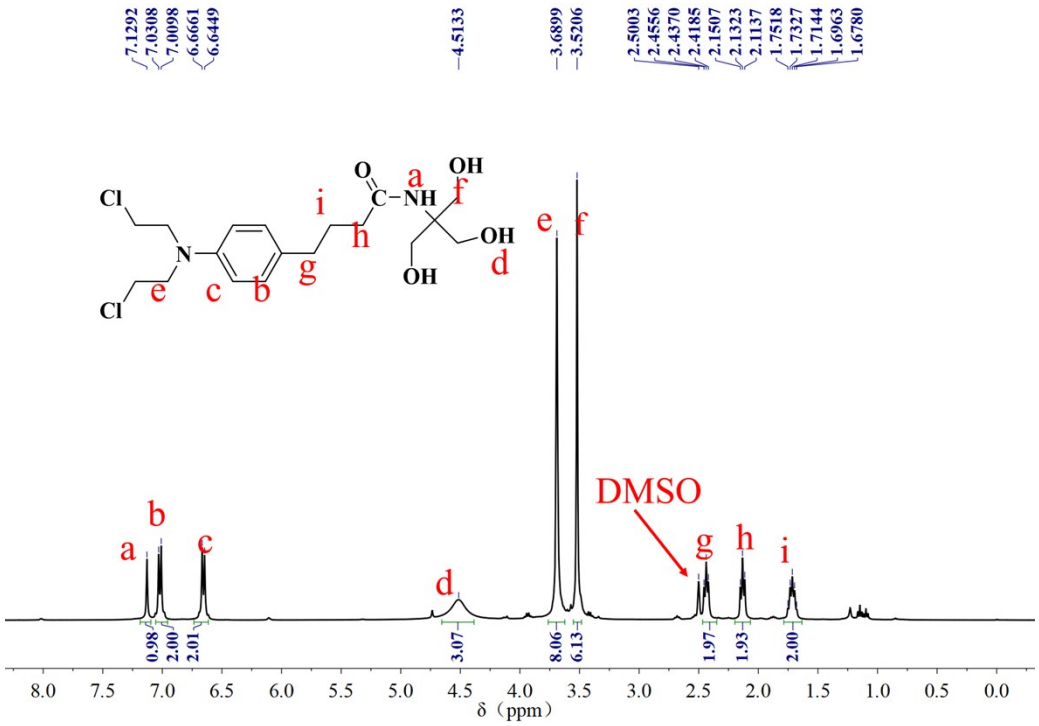


Figure S10. ^{1}H NMR spectrum recorded (400 MHz, DMSO-d_6, RT) for compound Cb-(OH)$_3$.

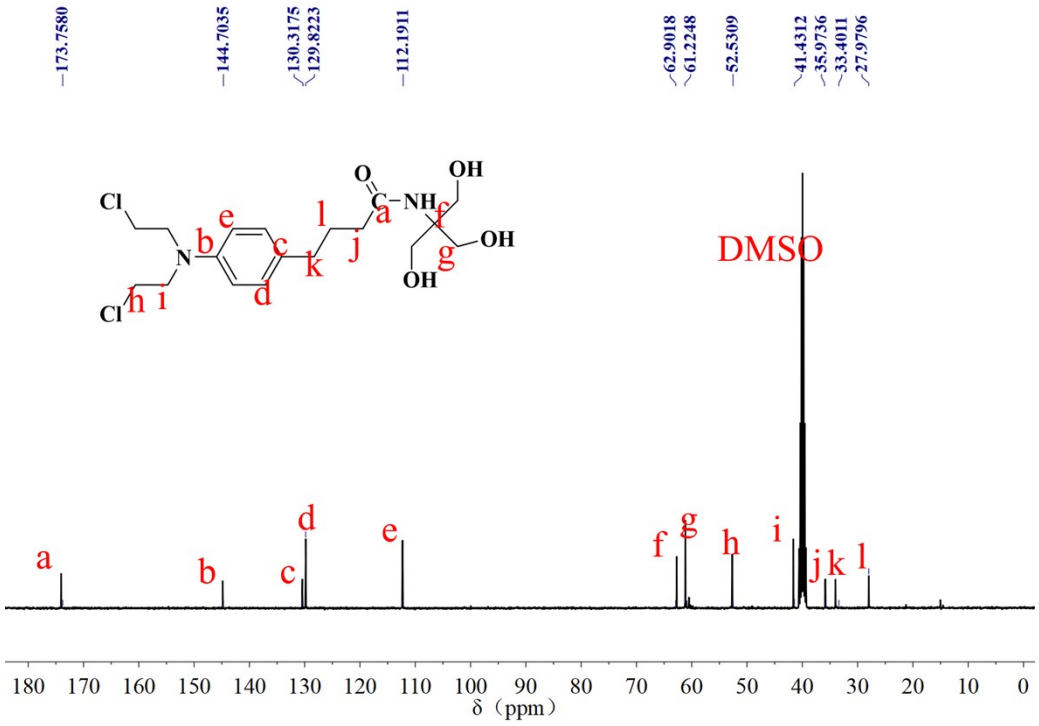


Figure S11. ^{13}C NMR spectrum recorded (100 MHz, DMSO-d_6, RT) for compound Cb-(OH)$_3$.

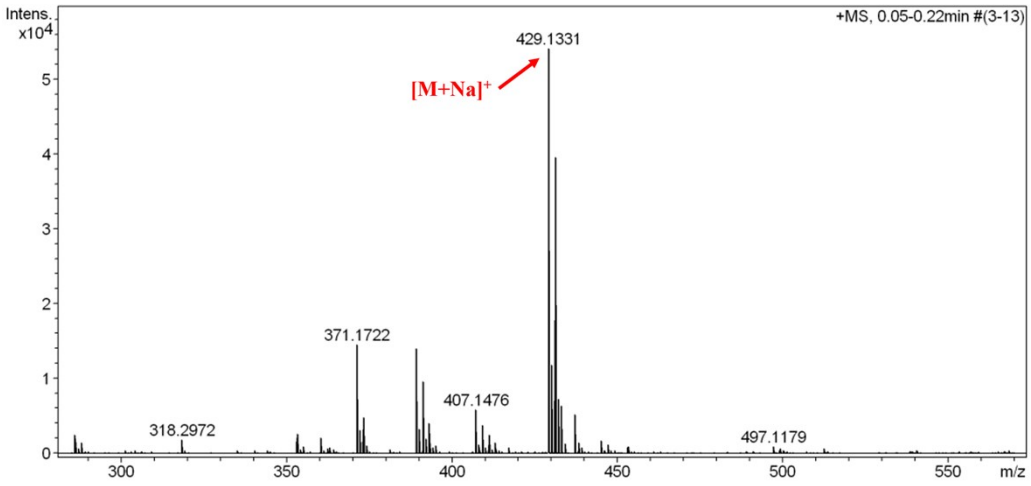


Figure S12. High-resolution electrospray ionization mass spectrum of compound Cb-$(OH)_3$.

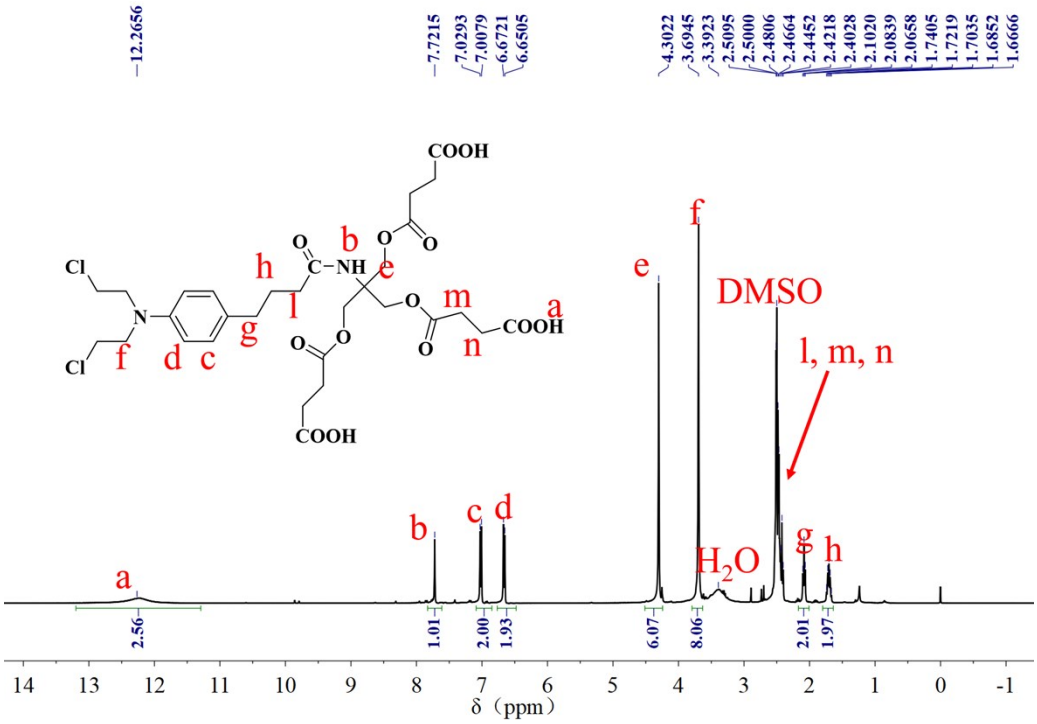


Figure S13. ^{1}H NMR spectrum recorded (400 MHz, DMSO-d_6, RT) for compound **Cb-$(COOH)_3$**.

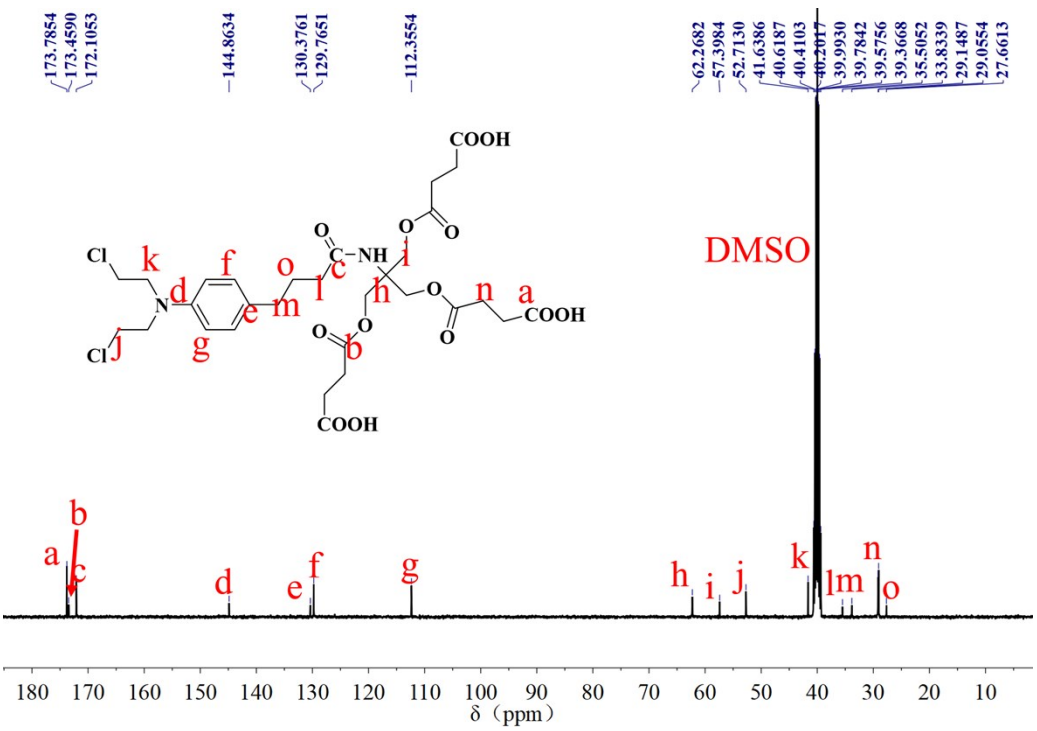


Figure S14. ^{13}C NMR spectrum recorded (100 MHz, DMSO-d_6, RT) for compound **Cb-(COOH)$_3$**.

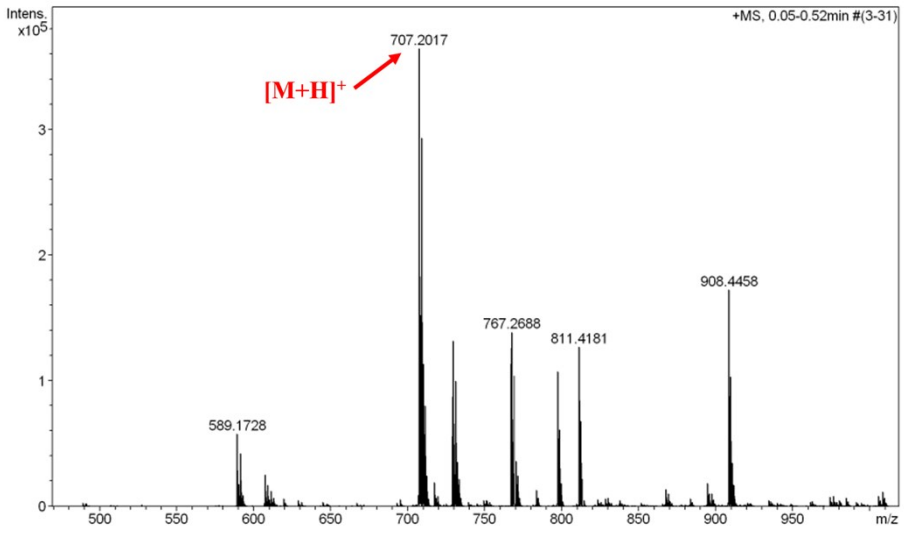
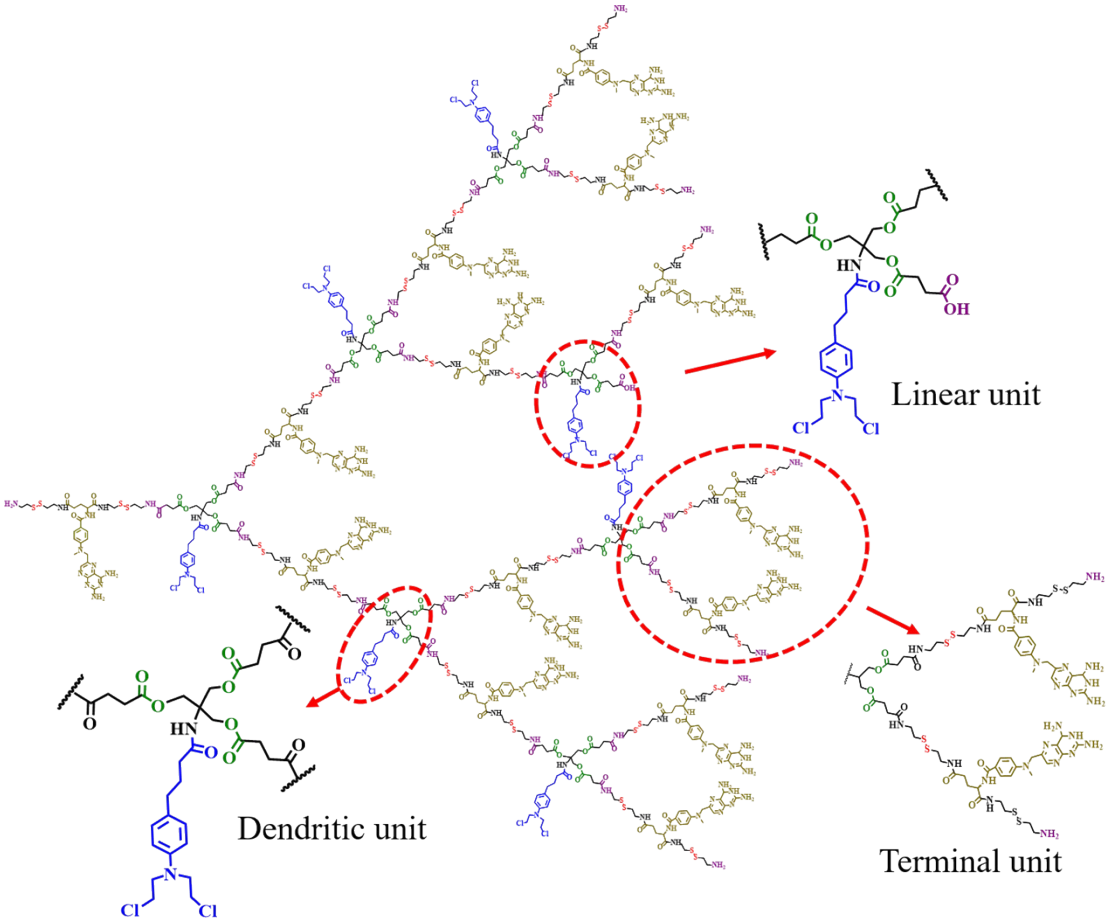


Figure S15. High-resolution electrospray ionization mass spectrum of compound **Cb-(COOH)$_3$**.

4 Synthesis of DHBP-*g*-PEG

4.1 Synthesis of DHBP.



Scheme S3. The structure of DHBP.

Cb-$(COOH)_3$ (40 mg, 0.057 mmol), EDC·HCl (22 mg, 0.12 mmol) and HoBt (15.4 mg, 0.12 mmol) were dissolved in DMF (20 mL) in a 100 mL round-bottom flask, and stirred at 0 ℃ for 0.5 h. Then, MTX-$(SS\text{-}NH_2)_2$ (123 mg, 0.17 mmol) as a solution in DMF (5.0 ml) was added gradually, and the mixture was reacted for 3 days at RT. After that, 5.0 mL deionized water was added to quench the reaction. Then, the solvent (DMF) was reduced via distillation and the residue was added dropwise into THF (70 ml). Then the solution was stirred for 2 h at RT and precipitated in yellow solid for another 5h sedimentation.

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.65, 8.11, 7.76, 7.00, 6.80, 6.65, 4.84, 4.29, 3.68, 2.73, 2.35, 2.14, 2.08, 1.97, 1.91, 1.69.

^{13}C NMR (101 MHz, DMSO-d_6) δ = 172.40, 171.29, 166.54, 163.15, 151.27, 149.48, 148.04, 144.82, 130.32, 129.76, 129.47, 122.12, 121.71, 112.30, 111.50, 62.26, 57.38, 55.33, 52.69, 41.64, 38.47, 37.51, 35.48, 33.84, 32.53, 30.18, 29.38, 29.06, 28.01, 27.69.

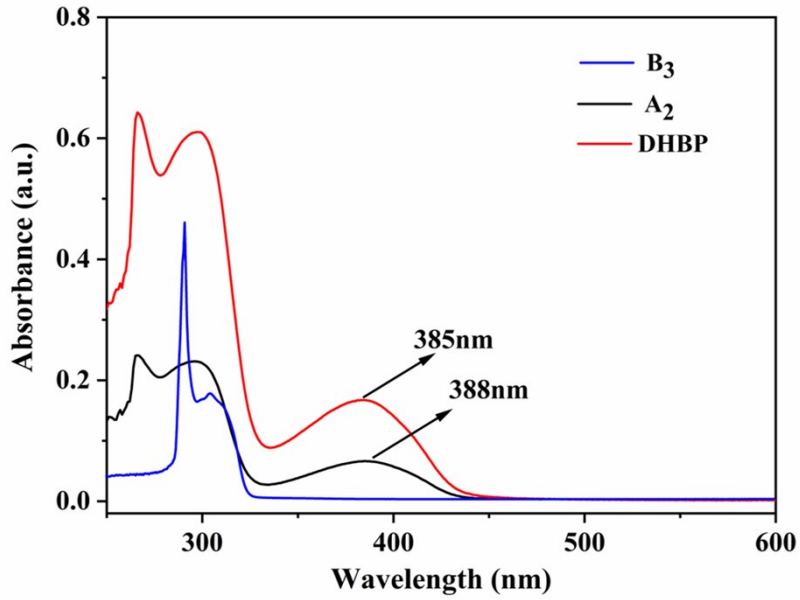


Figure S16. UV/Vis spectra of $\mathbf{A_2}$, $\mathbf{B_3}$ and **DHBP** in DMF.

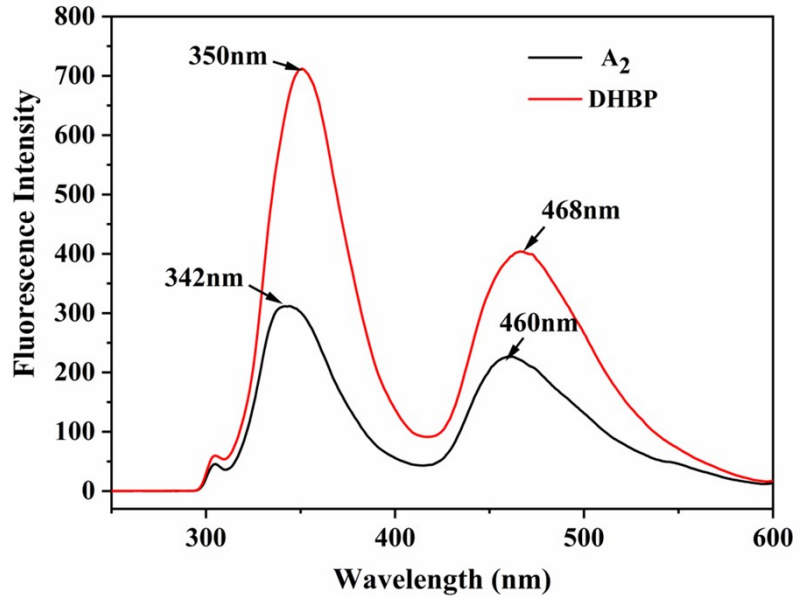
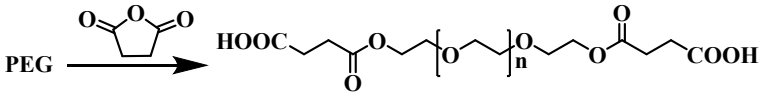


Figure S17. Fluorescence emission spectra of $\mathbf{A_2}$ (λ_{ex} = 300 nm, λ_{em} = 342 nm, 460nm) and **DHBP** (λ_{ex} = 300 nm, λ_{em} = 350 nm, 468nm) in DMF.

4.2 Synthesis of Carboxy-Terminated Poly (ethylene glycol) (HOOC-PEG-COOH).

HOOC-PEG-COOH was synthesized according to previous report.[2]

^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.23-4.21 (m, 4H), 3.62 (s, 182H), 2.61-2.59 (dt, 8H). ^{13}C NMR (101 MHz, $CDCl_3$) δ = 174.17, 172.14, 70.37, 68.85, 63.59, 29.00, 28.66.



Scheme S4. Synthetic routes of HOOC-PEG-COOH.

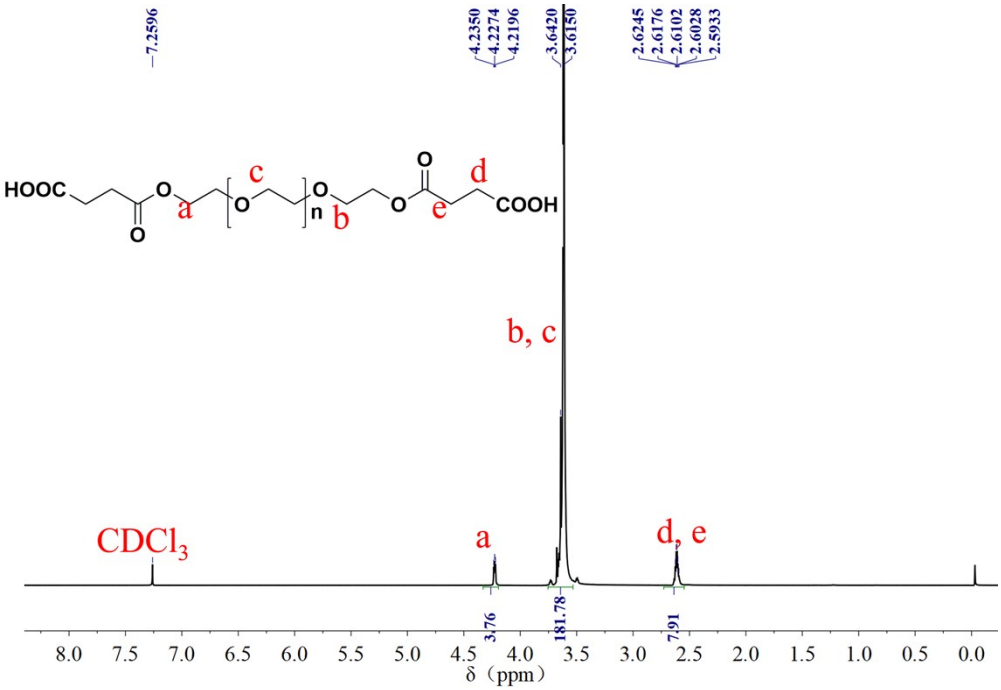


Figure S18. ^{1}H NMR spectrum recorded (400 MHz, $CDCl_3$, RT) for compound HOOC-PEG-COOH.

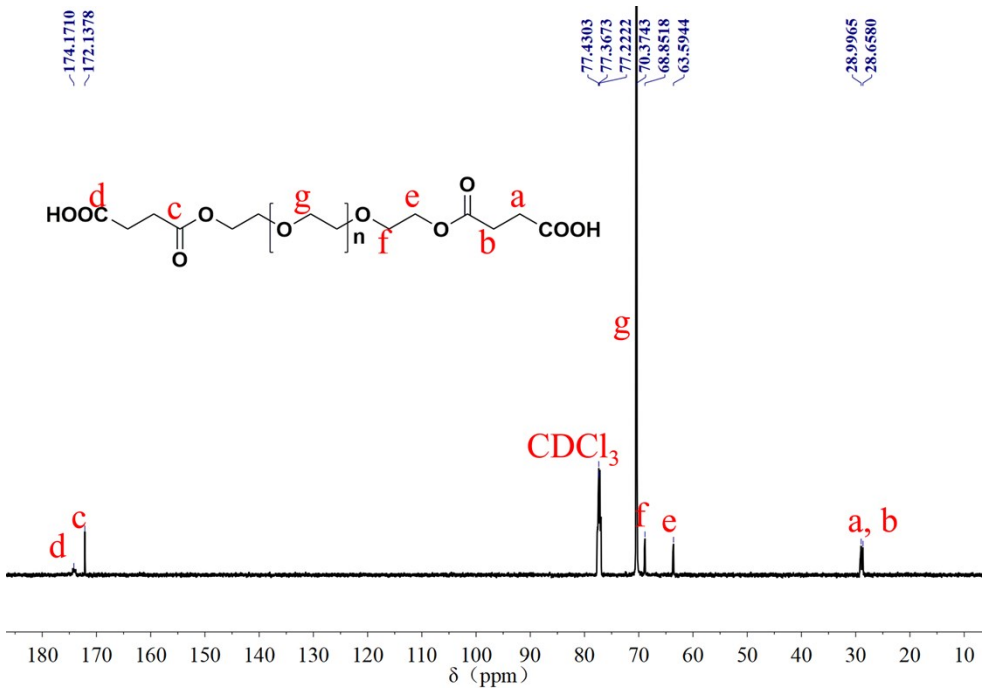
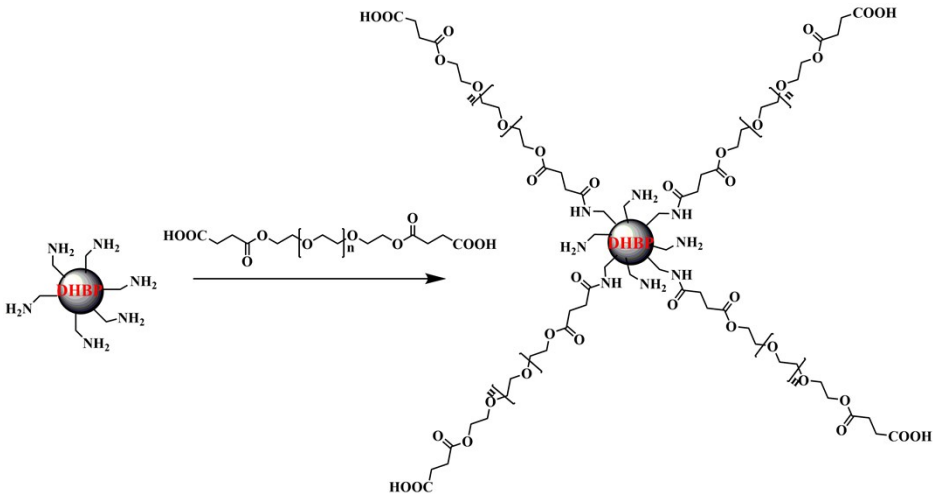


Figure S19. ^{13}C NMR spectrum recorded (100 MHz, $CDCl_3$, RT) for compound HOOC-PEG-COOH.

4.3 Synthesis of DHBP-*g*-PEG

HOOC-PEG-COOH (0.1 g, 0.05 mmol), EDC·HCl (17 mg 0.8 mmol) and HoBt(17 mg, 0.8 mmol) were dissolved in DMF (20 mL) in a 100 mL round-bottom flask, and stirred at 0 ℃ for 0.5 h. Then, **DHBP** (0.5 g, 0.01 mmol) as a solution in DMF (5.0 ml) was added gradually, and the mixture was reacted for 24h at RT. Then the reaction mixture was dialyzed (MWCO 2000) against deionized water for 3 days, with the water being renewed every 8 h. The obtained **DHBP-*g*-PEG** solution was then lyophilized to yellow solid.

^1H NMR (400 MHz, DMSO-d_6) δ = 7.00, 6.80, 6.63, 4.77, 4.29, 4.08, 3.67, 3.50.



Scheme S5. Synthetic routes of DHBP-*g*-PEG.

5. Self-assembly behavior of DHBP-*g*-PEG

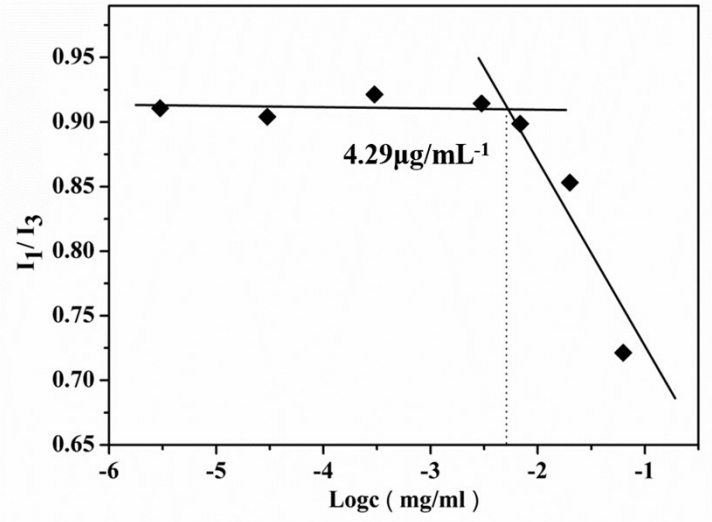


Figure S20. Relationship between the fluorescent intensity ratio (I_1/I_3) and self-assemblies concentration in water. The CAC value is about 4.29 μg mL^{-1}.

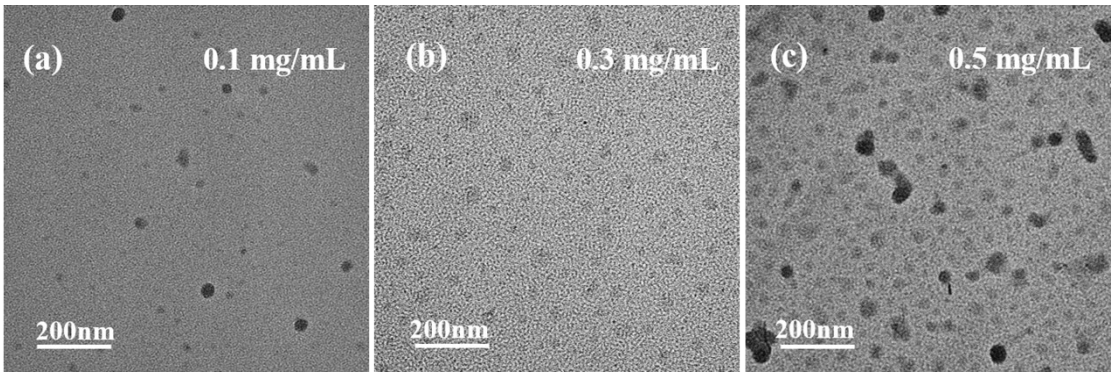


Figure S21. TEM images of **HBPM**s at different concentration.

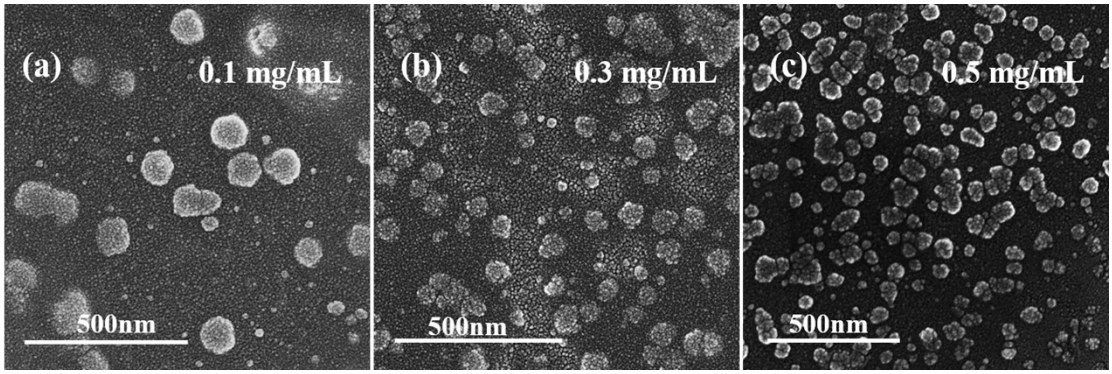


Figure S22. SEM images of **HBPM**s at different concentration.

6. Release kinetics of MTX and Cb from HBPMs.

The release mechanism of MTX and Cb from **HBPM**s was elucidated by studying the release kinetics with a simple semiempirical equation [Eq. (1)] and a modified equation [Eq. (2)].

$$M_t/M_\infty = kt^n \quad (1)$$

$$\ln r = \ln k + n \ln t, \mathrm{r} = M_t/M_\infty \quad (2)$$

in which M_t and M_∞ indicate the cumulative amounts released at time t and infinity, respectively; k describes the release constant; k' indicates the constant proportional to k; and n is the kinetic and release mechanism.

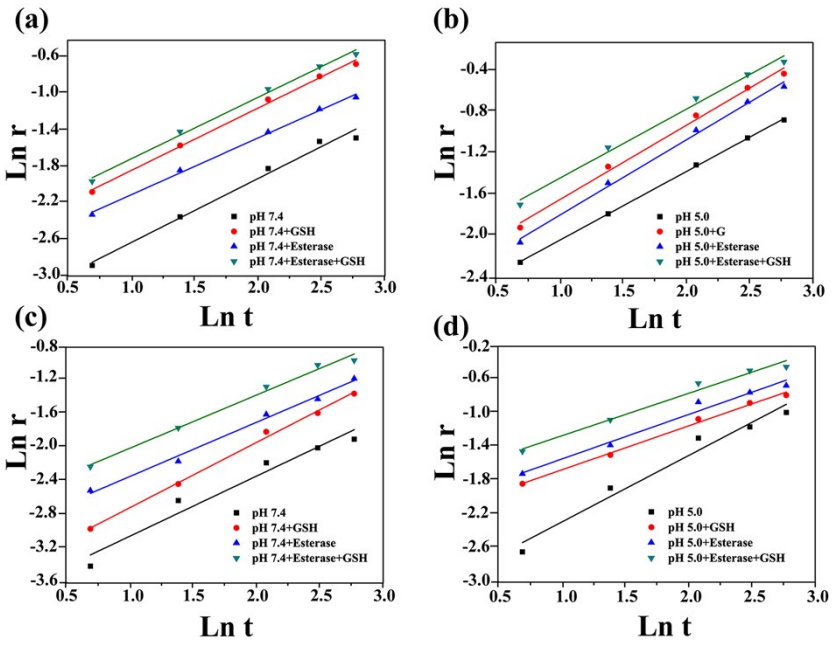


Figure S23. Cumulative release curves of active MTX (a, b) and Cb (c, d) from **HBPM**s as a function of time from 4 h to 16 h.

Table S1. Release kinetics parameters of active MTX from **HBPM**s fitted with Peppas' formula.[a]

Release experiment	Fitting equation	n [b]	k [b]	R^{2} [b]
pH7.4	$\ln r = 0.761 \ln t - 3.463$	0.761	0.031	0.981
pH7.4+GSH	$\ln r = 0.742 \ln t - 2.589$	0.742	0.075	0.995
pH7.4+Esterase	$\ln r = 0.673 \ln t - 2.808$	0.673	0.060	0.996
pH7.4+Esterase+GSH	$\ln r = 0.731 \ln t - 2.439$	0.731	0.087	0.993
pH5.0	$\ln r = 0.671 \ln t - 2.702$	0.671	0.067	0.991
pH5.0+GSH	$\ln r = 0.727 \ln t - 2.363$	0.727	0.094	0.995
pH5.0+Esterase	$\ln r = 0.737 \ln t - 2.524$	0.737	0.080	0.994
pH5.0+Esterase+GSH	$\ln r = 0.676 \ln t - 2.103$	0.676	0.122	0.988

[a] All experiments were conducted at 37℃. [b] Calculated by using Equation (2).

Table S2. Release kinetics parameters of active Cb from **HBPM**s fitted with Peppas' formula.[a]

Release experiment	Fitting equation	n [b]	k [b]	R^{2} [b]
pH7.4	$\ln r = 0.715 \ln t - 3.762$	0.715	0.023	0.965
pH7.4+GSH	$\ln r = 0.776 \ln t - 3.486$	0.776	0.031	0.995
pH7.4+Esterase	$\ln r = 0.644 \ln t - 2.983$	0.644	0.051	0.989
pH7.4+Esterase +GSH	$\ln r = 0.633 \ln t - 2.635$	0.633	0.072	0.986
pH5.0	$\ln r = 0.733 \ln t - 2.974$	0.733	0.051	0.990
pH5.0+GSH	$\ln r = 0.485 \ln t - 2.148$	0.485	0.117	0.992
pH5.0+Esterase	$\ln r = 0.491 \ln t - 2.035$	0.491	0.130	0.982
pH5.0+ Esterase +GSH	$\ln r = 0.469 \ln t - 1.756$	0.469	0.173	0.975

[a] All experiments were conducted at 37℃. [b] Calculated by using Equation (2).

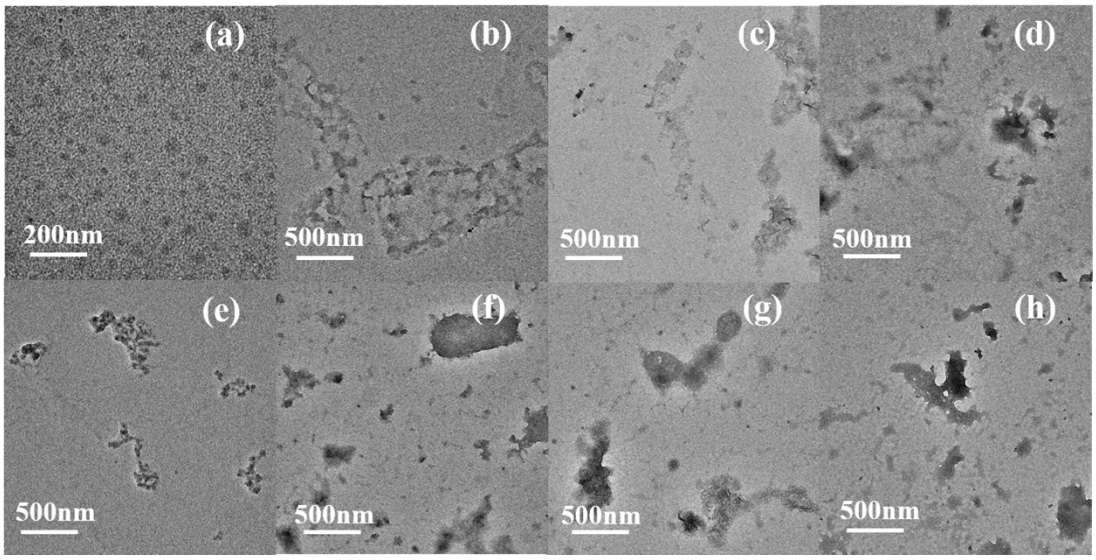


Figure S24. TEM images of **HBPM**s at (a) pH 7.4; (b) pH 7.4+GSH; (c) pH 7.4+Esterase; (d) pH 7.4+Esterase+GSH; (e) pH5.0; (f) pH5.0+GSH; (g) pH5.0+Esterase; (h) pH5.0+Esterase+GSH, respectively.

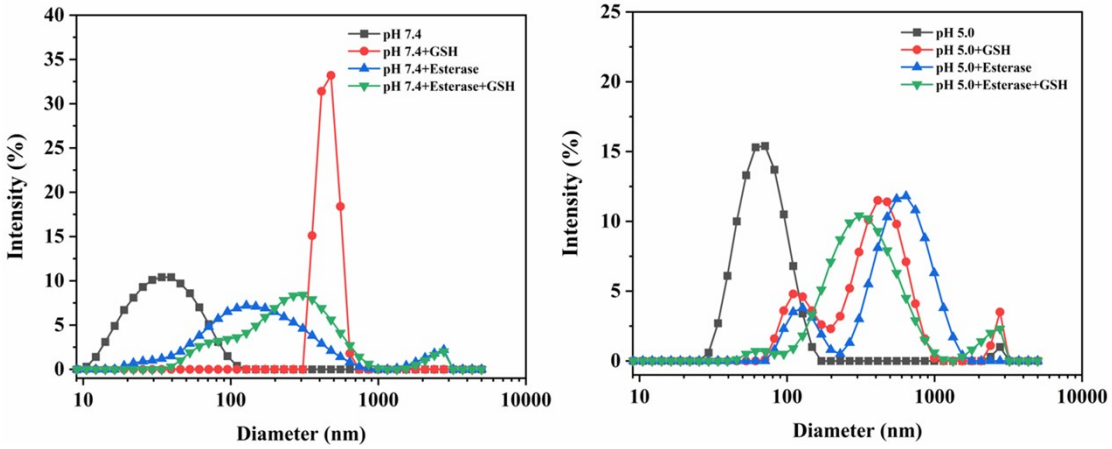


Figure S25. DLS images of **HBPM**s at (a) pH 7.4; (b) pH 7.4+GSH; (c) pH 7.4+Esterase; (d) pH 7.4+Esterase+GSH; (e) pH 5.0; (f) pH 5.0+GSH; (g) pH 5.0+Esterase; (h) pH 5.0+Esterase+GSH, respectively.

Synergistic Effect Evaluated by Combination Index

The degree of synergy between two drugs can be quantified by calculating the combination index (CI). The CI of methotrexate (MTX) and chlorambucil (Cb) when combined **HBPM**s was calculated from the IC_{50} values according to the equation: CI = A/Am1 + A/Am2, where A is the IC_{50} value of combination of drug 1 and drug 2, that in combination produce a certain level of cytotoxicity, and Am1 and Am2 are the IC_{50} values of the single drugs, respectively. CI values <1 indicate synergism, CI values equal to 1 indicate an additive effect, and CI values >1 indicate antagonism.

Table S3. IC_{50} of MTX, Cb, MTX/Cb mixture, and **HBPM**s in PC-3 and MCF-7. Error bars indicate SD (n = 3).

Sample	PC-3 (μg/ml)	MCF-7 (μg/ml)
MTX	22.52	21.57
Cb	40.55	49.81
MTX/Cb mixture	13.47	22.79
HBPMs	10.81	13.73

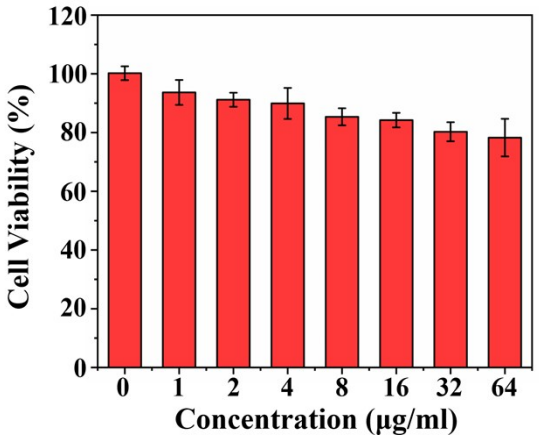


Figure S26. *In vitro* cytotoxicity of LO-2 incubated with **HBPM**s at different concentrations for 48 h.

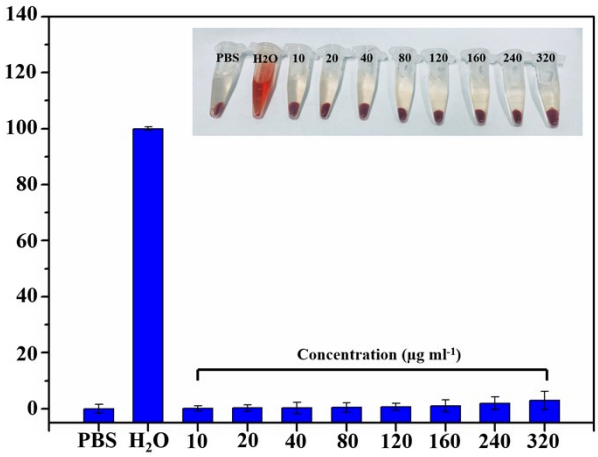


Figure S27. Hemolysis of **HBPM**s at different concentrations. Inset: digital photograph of hemolytic test, Error bar represent mean ± s. d., n=3.

7. References

[1] X. B. Chen, J. Yang, H. Liang, Q. Jiang, B. Ke and Y. Nie, *J. Mater. Chem. B.*, 2017, **5**, 1482.

[2] Y. Wang, L. Zhang, X. B. Zhang, X. Wei, Z. M. Tang and S. B. Zhou, *ACS Appl. Mater. Interfaces.*, 2016, **8**, 5833.