Redox-Responsive Amphiphilic Macromolecular [2]Pseudorotaxane Constructed from a Water-Soluble Pillar[5]arene and a Paraquat-Containing Homopolymer

Xiaodong Chi,[†] *Guocan Yu,*[†] *Xiaofan Ji,*[†] *Yang Li,*[‡] *Guping Tang*[‡] *and Feihe Huang**[,†]

[†]State Key Laboratory of Chemical Engineering, Department of Chemistry, Zhejiang University, Hangzhou 310027 (China)

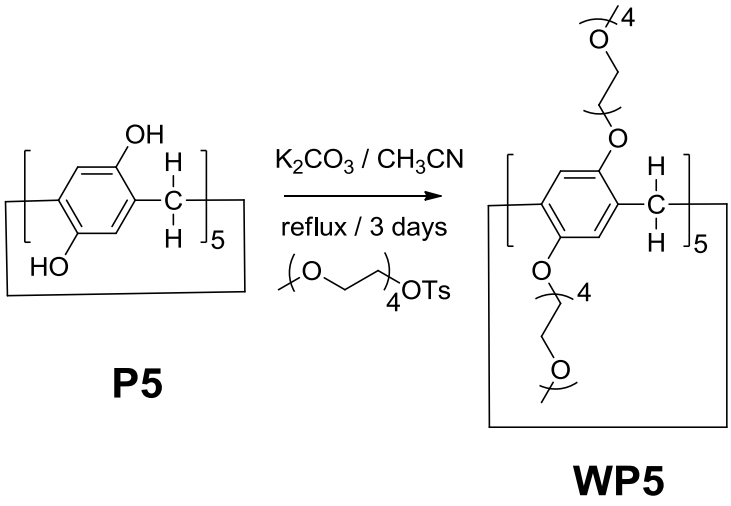
[‡]Department of Chemistry, Institute of Chemical Biology and Pharmaceutical Chemistry, Zhejiang University, Hangzhou 310027 (China)

Supporting Information

1. Materials and methods

All reagents were commercially available and used as supplied without further purification. Compound **P5** was prepared according to published procedures.[S1] NMR spectra were recorded on a Bruker Avance DMX 500 spectrophotometer or a Bruker Avance DMX 400 spectrophotometer using the deuterated solvent as the lock and the residual solvent or TMS as the internal reference. Low-resolution electrospray ionization mass spectra were recorded with a Bruker Esquire 3000 Plus spectrometer. High-resolution mass spectrometry experiments were performed with a Bruker Daltonics Apex III spectrometer. Molecular weights and molecular weight distributions were determined by gel permeation chromatography (GPC) with a Waters 1515 pump and Waters 1515 differential refractive index detector (set at 30 ℃); it used a series of three linear Styragel columns (HT2, HT4, and HT5) at an oven temperature of 45 ℃. The eluent was THF at a flow rate of 1.0 mL/min. A series of low polydispersity polystyrene standards were employed for the GPC calibration. Transmission electron microscopy investigations were carried out on a HITACHI HT-7700 instrument. Dynamic light scattering (DLS) measurements were carried out using a 200-mW polarized laser source Nd : YAG (λ = 532 nm). The polarized scattered light was collected at 90 °in a self-beating mode with a Hamamatsu R942/02 photomultiplier. The signals were sent to a Malvern 4700 submicrometer particle analyzer system. Isothermal titration calorimetric (ITC) measurements were performed on a VP-ITC micro-calorimeter (Microcal, USA). HepG2 (a human liver carcinoma cell line) and HUVEC (Human Umbilical Vein Endothelial Cells) were, respectively, cultivated in Dulbecco's modified eagle's medium and RPMI-1640 culture medium, and both with 10% fetal bovine serum, antibiotics penicillin (100 IU mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37 ℃ under a humidified atmosphere containing 5% CO_2.

*2. Synthesis of **WP5***



Scheme S1. Synthesis of **WP5**.

Compound **WP5**: *per*-Hydroxylated pillar[5]arene **P5** (0.300 g, 0.490 mmol)[S1] was dissolved in CH_3CN (60.0 mL). K_2CO_3 (1.30 g, 9.40 mmol) was added and the reaction mixture was stirred. Then tetra(ethylene glycol) monomethyl ether mono-*p*-tosylate (3.10 g, 9.80 mmol) was added and the reaction mixture was stirred under N_2 at reflux for 3 days. The solvent was evaporated and the residue was dissolved in CH_2Cl_2. The resultant solution was washed with H_2O and brine. The organic phase was collected, dried over anhydrous Na_2SO_4 and concentrated to give a crude liquid. Column chromatography (silica gel; CH_2Cl_2 : CH_3OH = 20 : 1) afforded a light yellow liquid (0.370 g, 30%). The ^{1}H NMR spectrum of pillar[5]arene **WP5** is shown in Figure S1. ^{1}H NMR (400 MHz, $CDCl_3$, room temperature) δ (ppm): 6.82 (s, 10H), 4.00 (t, J = 4 Hz, 20H), 3.82 (t, 4 Hz, 20H), 3.72 (t, J = 4 Hz, 30H), 3.64 (t, J = 4 Hz, 24H), 3.62–3.56 (m, 56H), 3.50–3.47 (m, 20H), 3.32 (s, 30H). The ^{13}C NMR spectrum of pillar[5]arene **WP5** is shown in Figure S2. ^{13}C NMR (125 MHz, $CDCl_3$, room temperature) δ (ppm): 149.82, 128.71, 115.31, 71.88, 70.72, 70.63, 70.52, 70.45, 70.20, 68.05, 58.95, 29.31. LRESIMS: *m*/*z* 2513.9 $[M + H]^+$ (58%), 2536.0 $[M + Na]^+$ (100%), *m*/*z* 2550.9 $[M + K]^+$ (60%). HRMALDIMS: *m*/*z* calcd. for $[M + K]^+$ $C_{125}H_{210}O_{50}K$ 2550.3527; found 2550.3515, error −0.5 ppm.

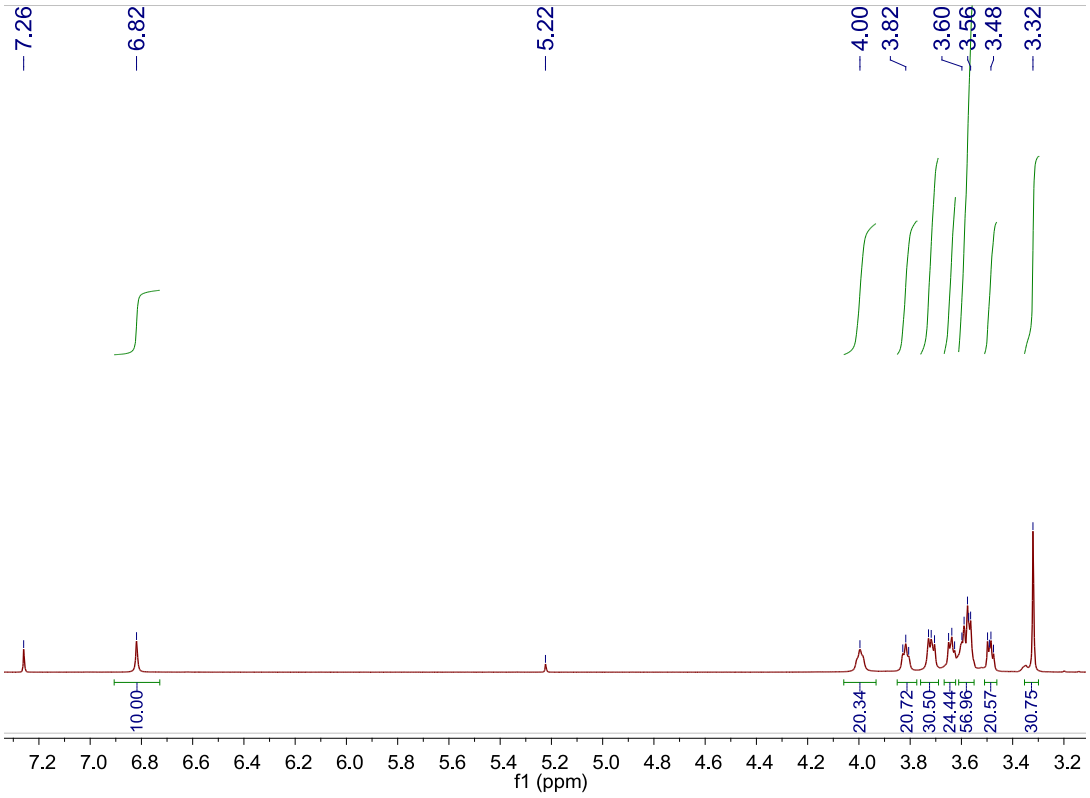


Figure S1. ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of **WP5**.

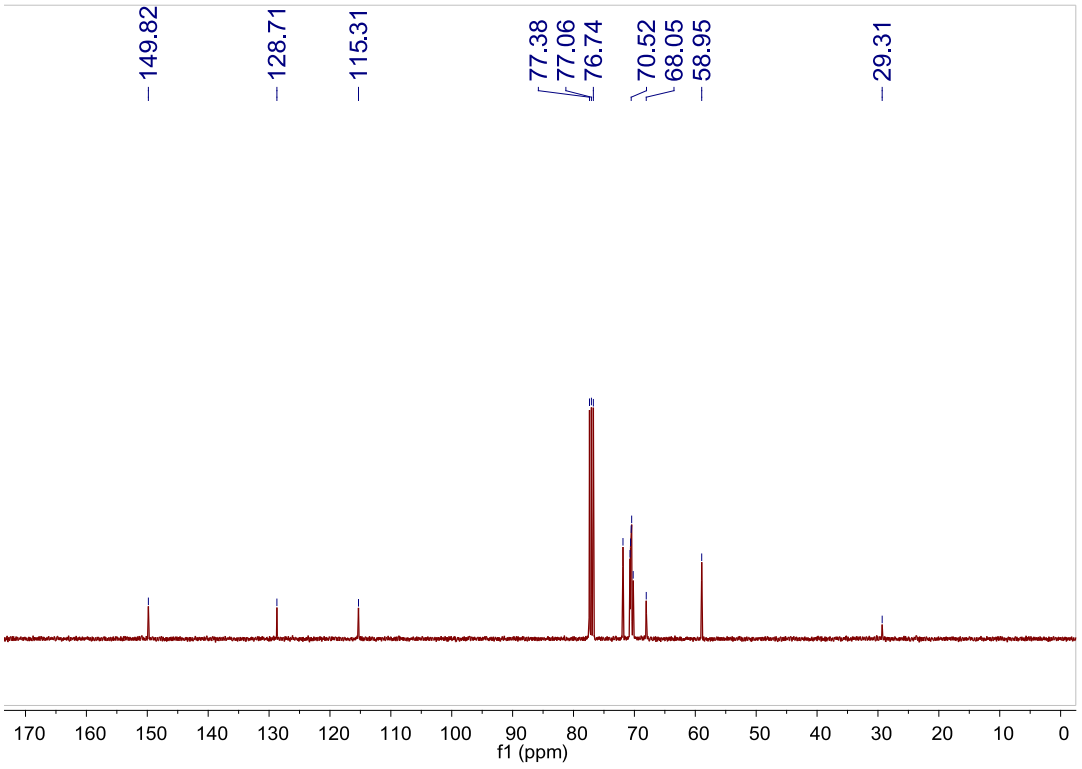


Figure S2. ^{13}C NMR spectrum (125 MHz, $CDCl_3$, 298 K) of **WP5**.

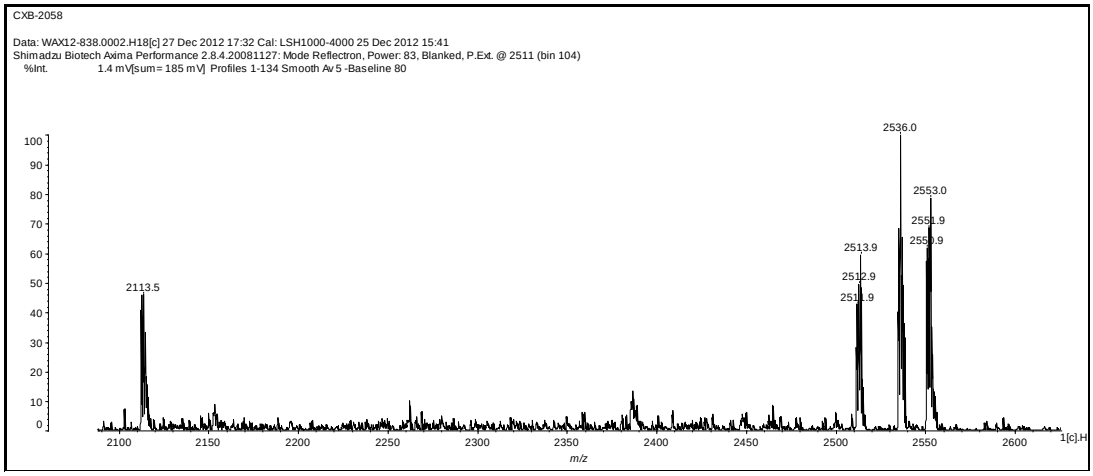
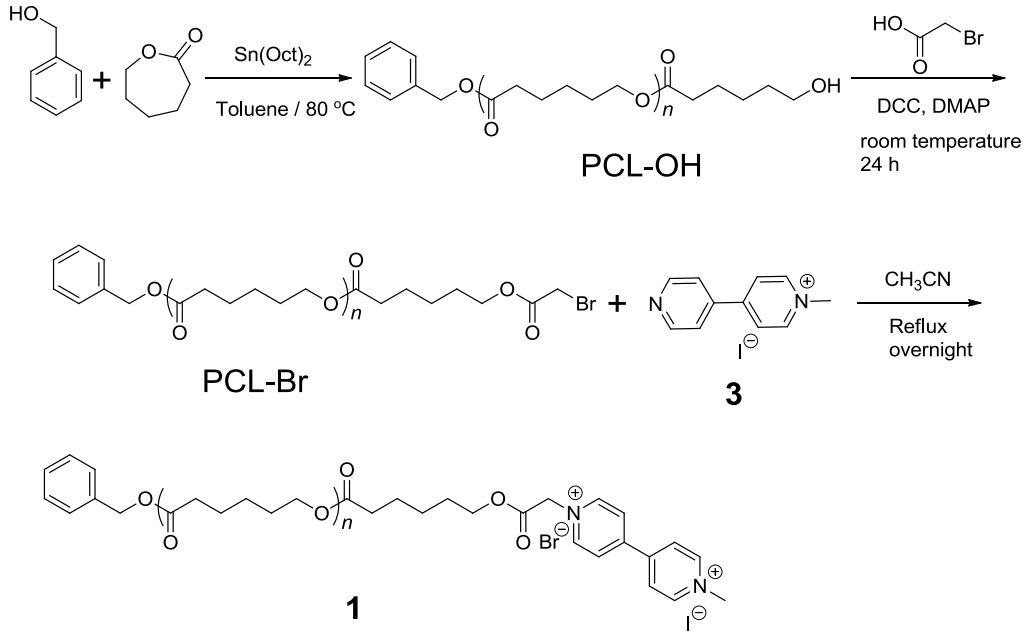


Figure S3. Electrospray ionization mass spectrum of **WP5**.

*3. Synthesis of polymer **1***



Scheme S2. Synthesis of polymer **1**.

PCL-OH: To a previously flamed Schlenk tube equipped with a magnetic stirring bar, benzyl alcohol (76.0 mg, 0.560 mmol), $Sn(Oct)_2$ in toluene (0.100 mol/L, 0.220 mL), CL (6.39 g, 56.0 mmol), and dry toluene (12.0 mL) were added. After removing ~5 mL of toluene under reduced pressure, the tube was carefully degassed by three freeze-pump-thaw cycles, sealed under vacuum, and placed in an oil bath at 80 ℃. After 24 h, the reaction mixture was dissolved in THF, and precipitated into an excess of diethyl ether. After filtration, the sediment was dissolved in THF and precipitated into an excess of diethyl ether; the above dissolution-precipitation cycle was repeated three times. After drying in a vacuum oven overnight at room temperature, PCL-OH was obtained as a white solid (4.82 g, yield: 65%; M_n, GPC = 4.9 kDa, M_w/M_n = 1.24). The actual DP (n+1) of the polymer was determined to be 30 by ^{1}H NMR analysis; M_n = 3.5 kDa. The ^{1}H NMR spectrum of PCL-OH is shown in Figure S4. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ (ppm): 7.34 (m, 5H), 5.10 (s, 2H), 4.05 (t, 8 Hz, 60H), 3.62 (t, 4 Hz, 2H), 2.30 (t, 8 Hz, 60H), 1.68–1.60 (m, 120H), 1.41–1.33 (m, 60H).

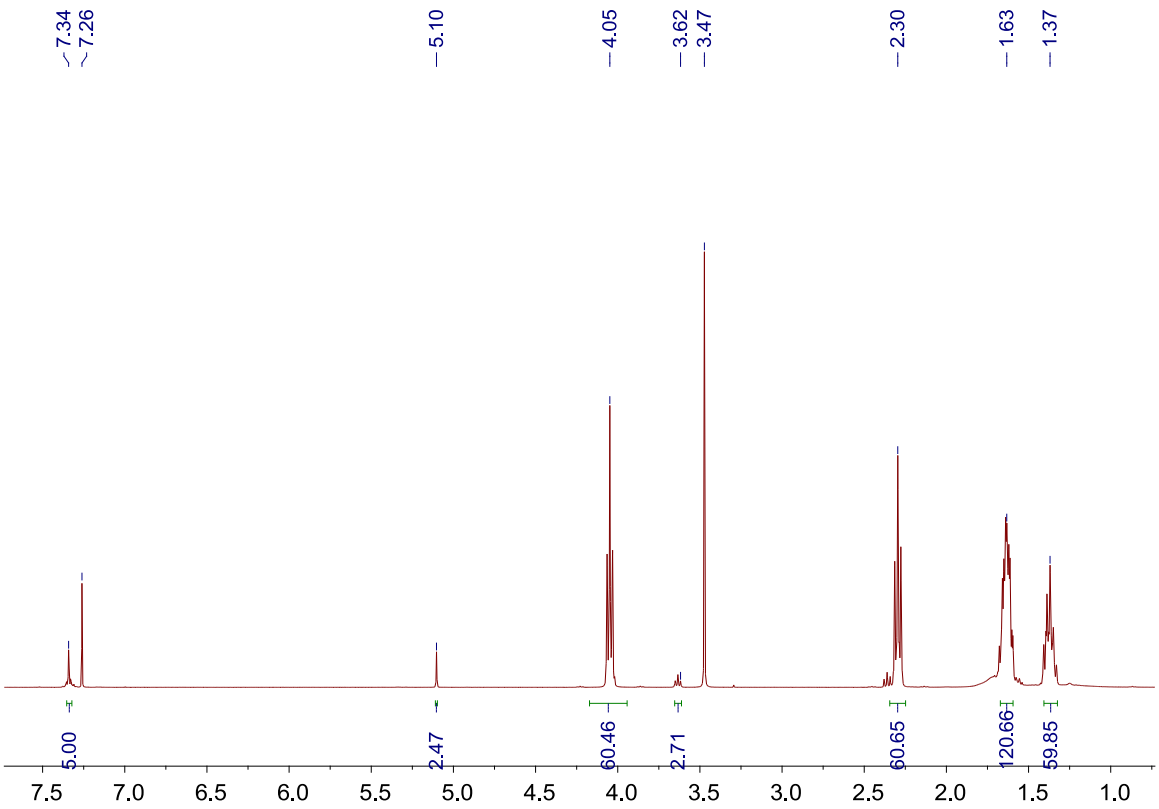
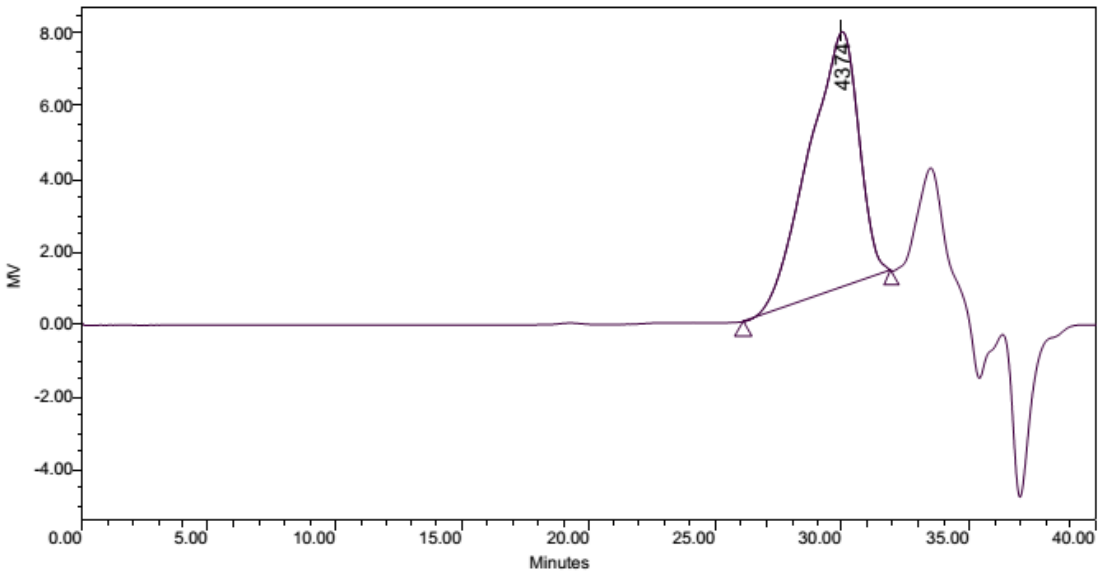


Figure S4. ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of PCL-OH.



Broad Unknown Relative Peak Table

	Distribution Name	Mn (Daltons)	Mw (Daltons)	MP (Daltons)	Mz+1 (Daltons)	Polydispersity	Mz/Mw	Mz+1/Mw
1		4930	6135	4374	9449	1.244426	1.251968	1.540184

Figure S5. GPC data for PCL-OH.

PCL-Br: A mixture of PCL-OH (2.50 g, 0.407 mmol), bromoacetic acid (1.13 g, 8.16 mmol), dicyclohexylcarbodiimide (DCC, 0.240 g, 1.16 mmol), and a catalytic amount of 4-dimethylaminopyridine (DMAP) in CH_2Cl_2 (50.0 mL) was stirred at room temperature overnight, then filtered and concentrated. The crude product was dissolved in CH_2Cl_2, and washed with water three times. The solvent was evaporated and the residue was dissolved in 2 mL of THF and precipitated into an excess of diethyl ether. The above dissolution-precipitation cycle was repeated three times. The solid was dried overnight in a vacuum to give a white solid (1.62 g, 64%). The ^{1}H NMR spectrum of PCL-Br is shown in Figure S6. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ (ppm): 7.34 (m, 5H), 5.10 (s, 2H), 4.05 (t, 8 Hz, 60H), 3.82 (t, 4 Hz, 2H), 2.28 (t, 8 Hz, 60H), 1.67–1.60 (m, 120H), 1.41–1.33 (m, 60H).

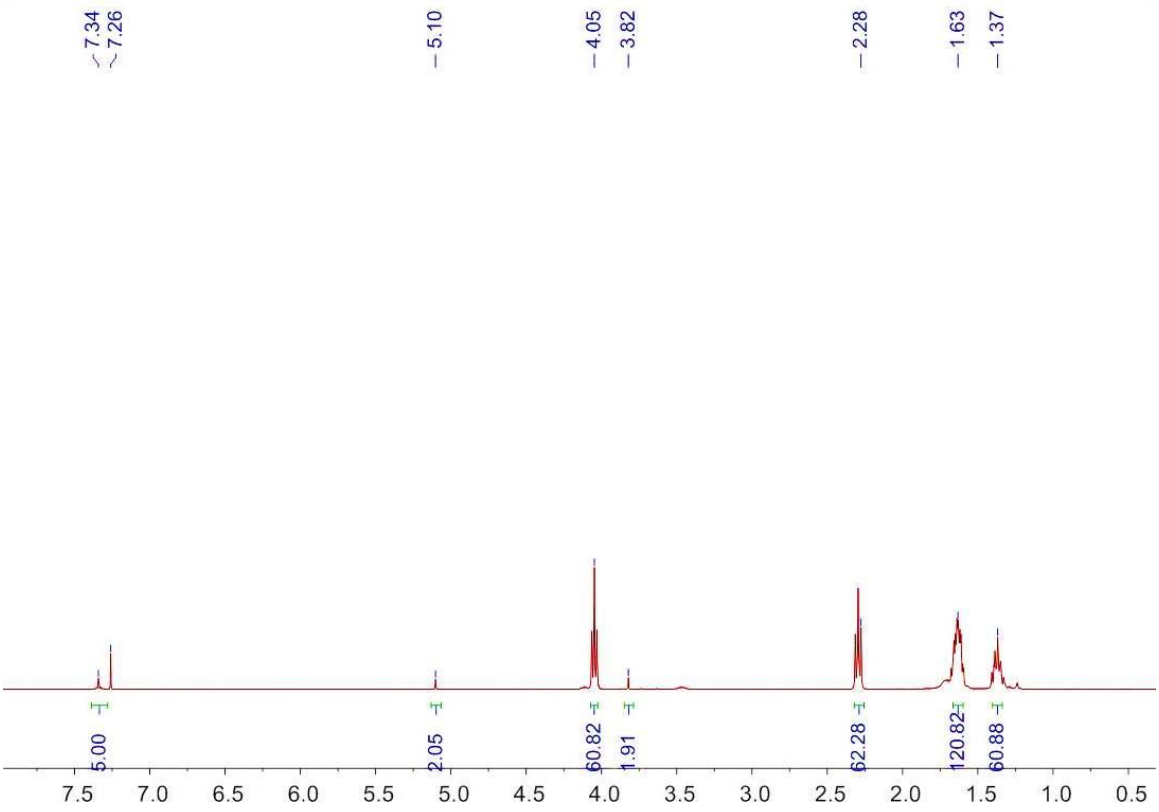


Figure S6. ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of PCL-Br.

Polymer 1: A mixture of PCL-Br (0.500 g, 0.0780 mmol) and **3** (0.237 g, 0.780 mmol) in CH_3CN (50 mL) was stirred at 85 °C overnight. After reaction, the solvent was evaporated and the residue was dissolved in CH_2Cl_2, and washed with water three times. The solvent was evaporated and the residue was dissolved in 2 mL of THF and precipitated into an excess of diethyl ether. The above dissolution-precipitation cycle was repeated three times. The solid was dried overnight in a vacuum to give a pale yellow power (0.352 g, 70%). The ^{1}H NMR spectrum of polymer **1** is shown in Figure S7. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ (ppm):

9.06 (d, 4 Hz, 2H), 8.78 (d, 4 Hz, 2H), 8.38 (m, 4H), 7.34 (m, 5H), 5.10 (s, 2H), 4.63 (m, 5H), 4.05 (t, 8 Hz, 60H), 2.28 (t, 8 Hz, 56H), 1.76–1.60 (m, 126H), 1.42–1.30 (m, 60H).

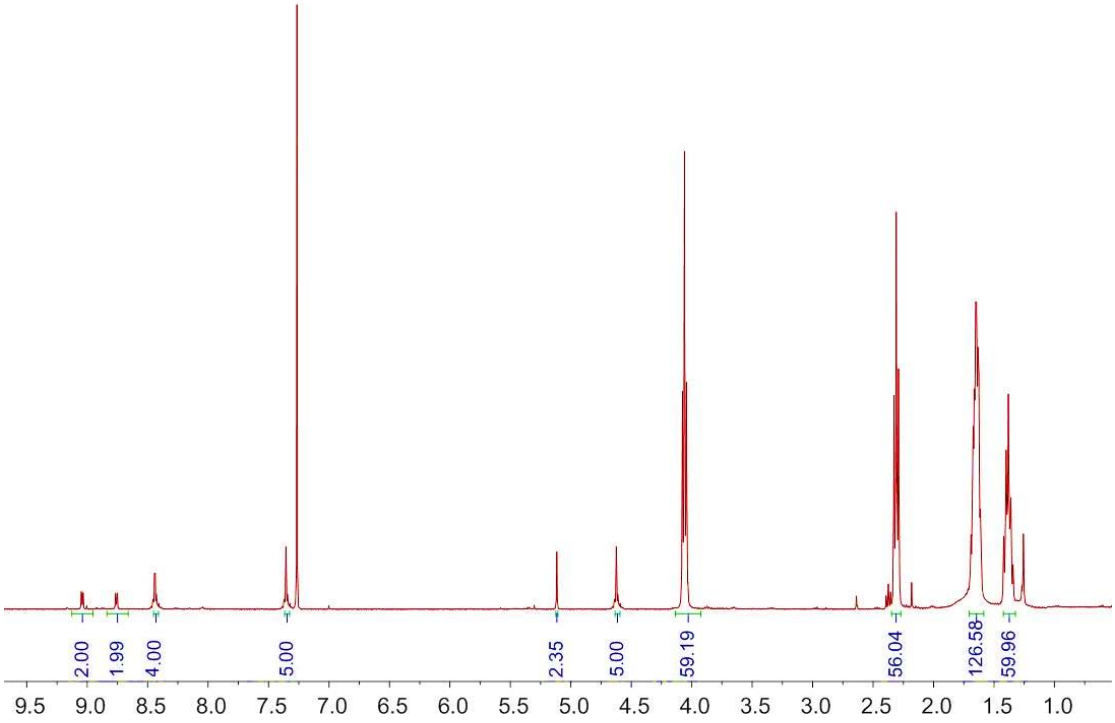


Figure S7. ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 298 K) of **1**.

*4. Electrospray ionization mass spectrometry of an equimolar mixture of **WP5** and **2***

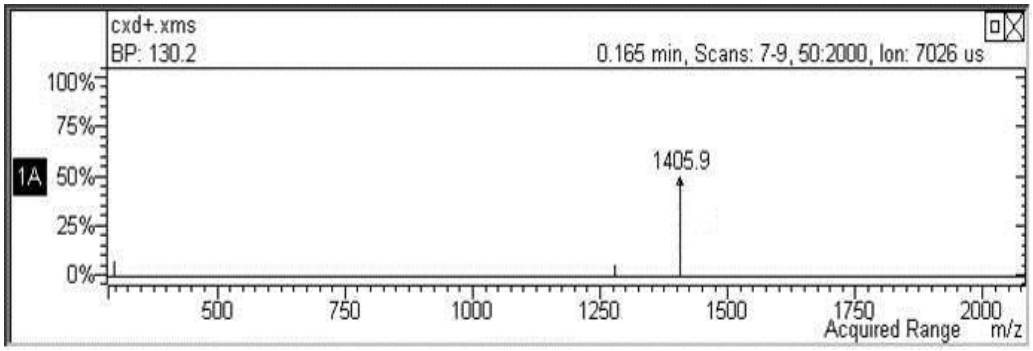


Figure S8. Electrospray ionization mass spectrum of an equimolar mixture of **WP5** and **2**.

5. NOESY NMR analysis of ***WP5⊃2***

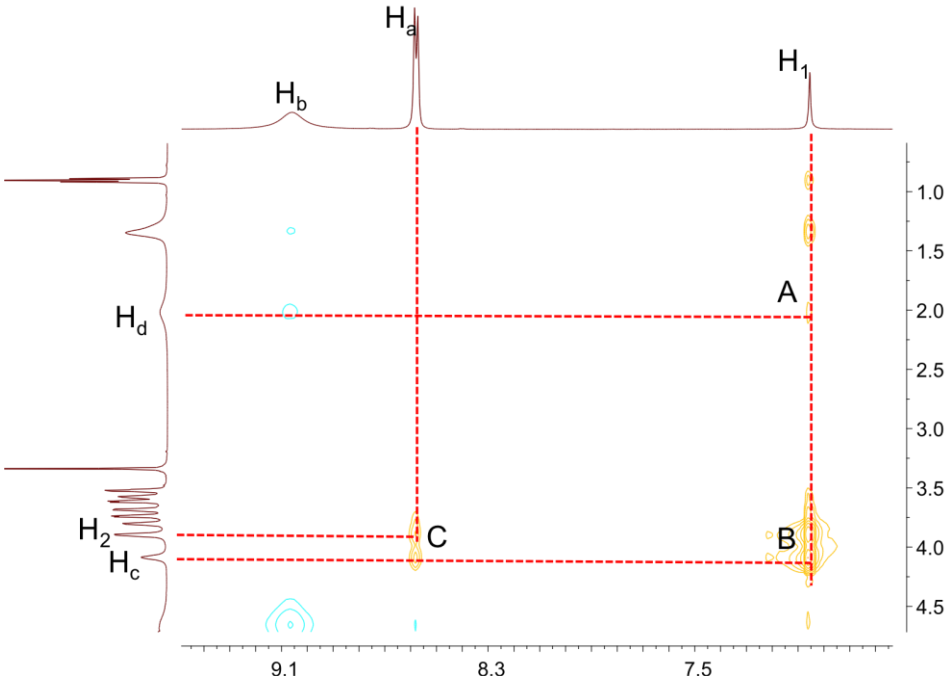


Figure S9. Partial NOESY NMR spectrum of **WP5⊃2** (5.00 mM) in D_2O with a mixing time of 800 ms (500 MHz, 298 K).

*6. Stoichiomestry and association constant determination for the complexation between **WP5** and **2***

Isothermal titration calorimetric measurements were performed on a VP-ITC micro calorimeter (Microcal, USA), which is composed of a reference cell and a sample cell of 1.43 mL. Stock solutions of host **WP5** (0.100 mM, 10.0 mL) and guest **2** (2.00 mM, 5.00 mL) in water were prepared using volumetric glassware. Before each titration, all the solutions were degassed and kept at constant temperature. In a typical run, a 250 μL syringe was full of guest (2.00 mM) and the cell was loaded with host (0.100 mM, 1.43 mL). The titration of the host with the guest was carried out at 298 K with a constant stirring rate of 307 rpm, 29 injections of 3.3 μL, a time interval of 240 s and a duration of 2 s per μL. The enthalpy change per mole of added **2** in the sample cell was recorded continuously. The control titration of **2** into water was also completed under the same conditions. The enthalpy changes of the titrations of the blank test were subtracted from the original titration. All the data were analyzed with Microcal Origin 7.0 software provided by the manufacturer. The final integration data obtained from the titration were fitted by the one set of binding site model.

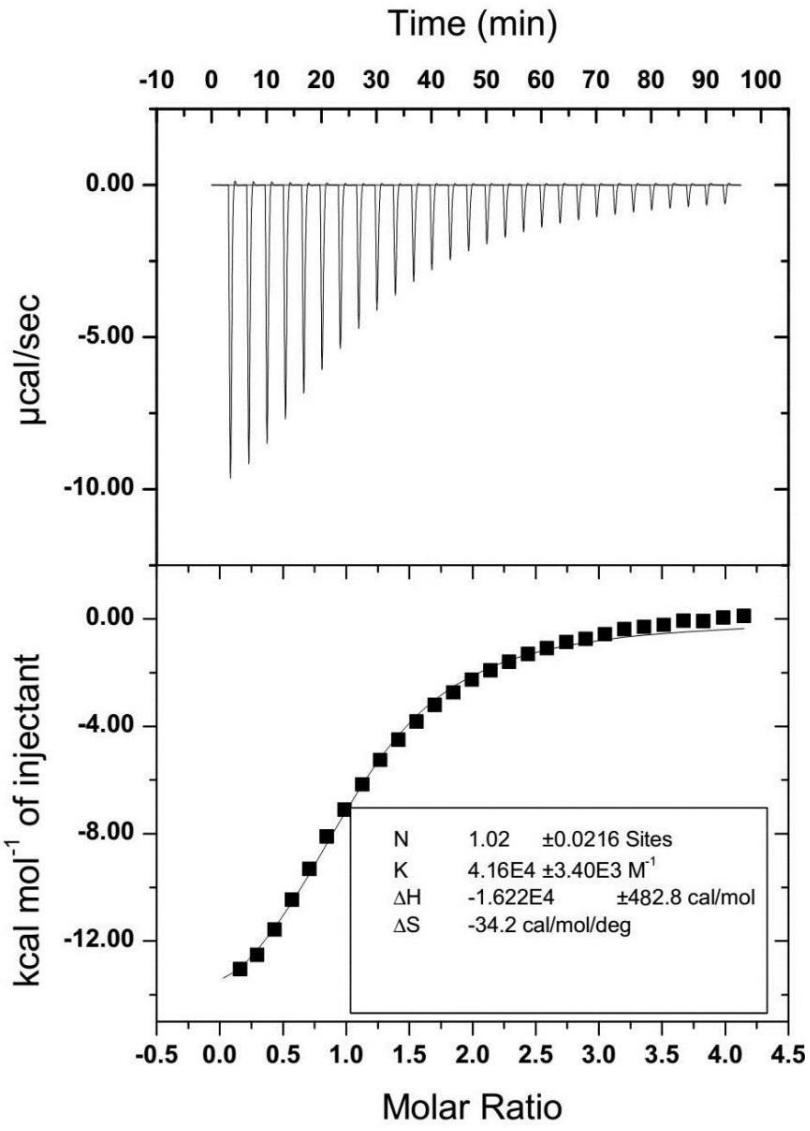


Figure S10. Titration of **WP5** (0.100 mM) with **2** (2.00 mM) in water at 298 K.

*7. UV-vis spectroscopy studies of the interactions between **WP5** and polymer **1***

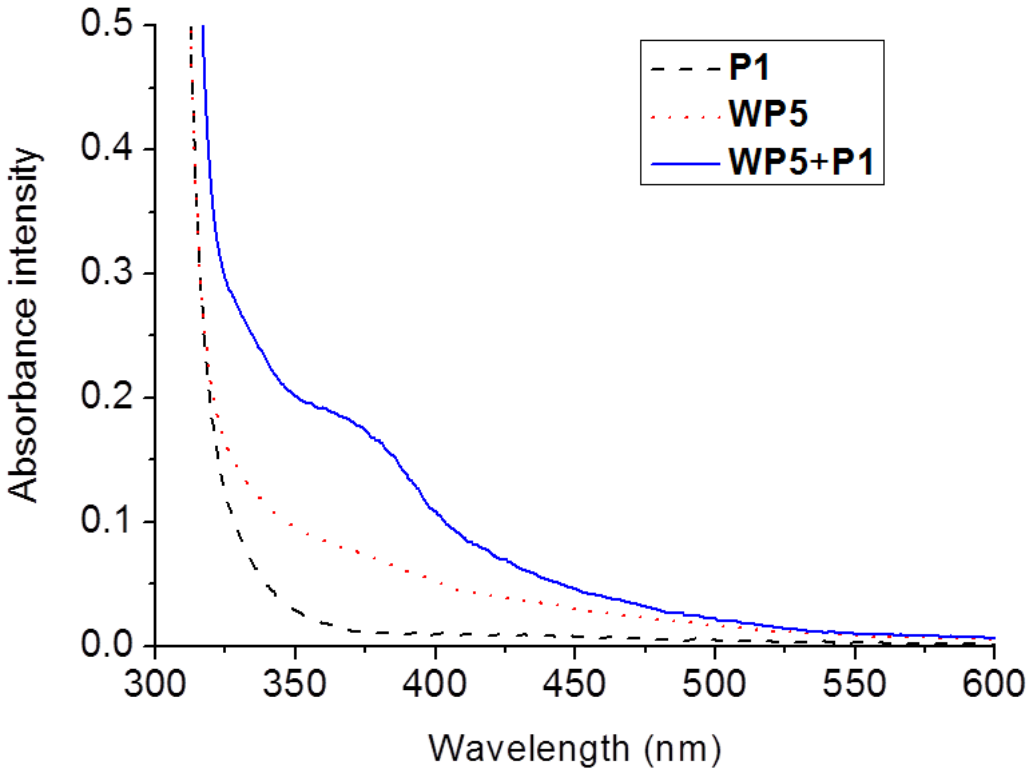


Figure S11. UV-vis spectra of polymer **1**, **WP5**, and polymer **1** in the presence of 1.0 equiv (1.50×10^{-5} M) of **WP5** in water.

8. Critical aggregation concentration (CAC) determination of ***WP5⊃1***

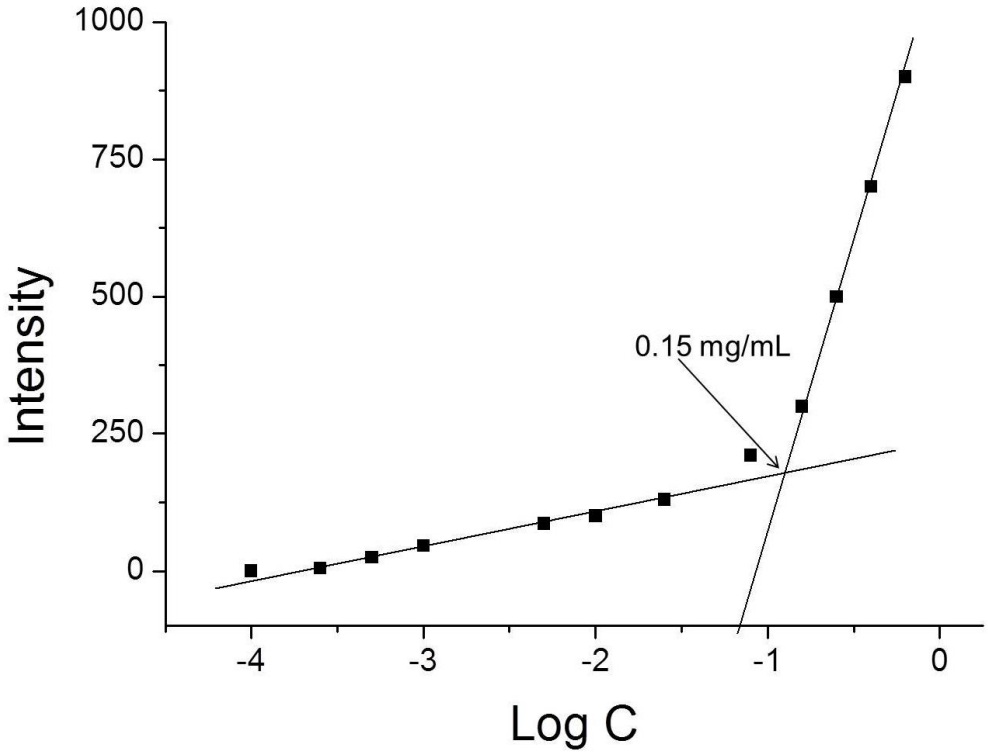


Figure S12. The plot of the fluorescence intensity at 601 nm versus the log of concentration to determine the CAC of **WP5⊃1**. Here we used Nile red as the probe.

9. DLS data of the DOX • HCl-loaded vesicles

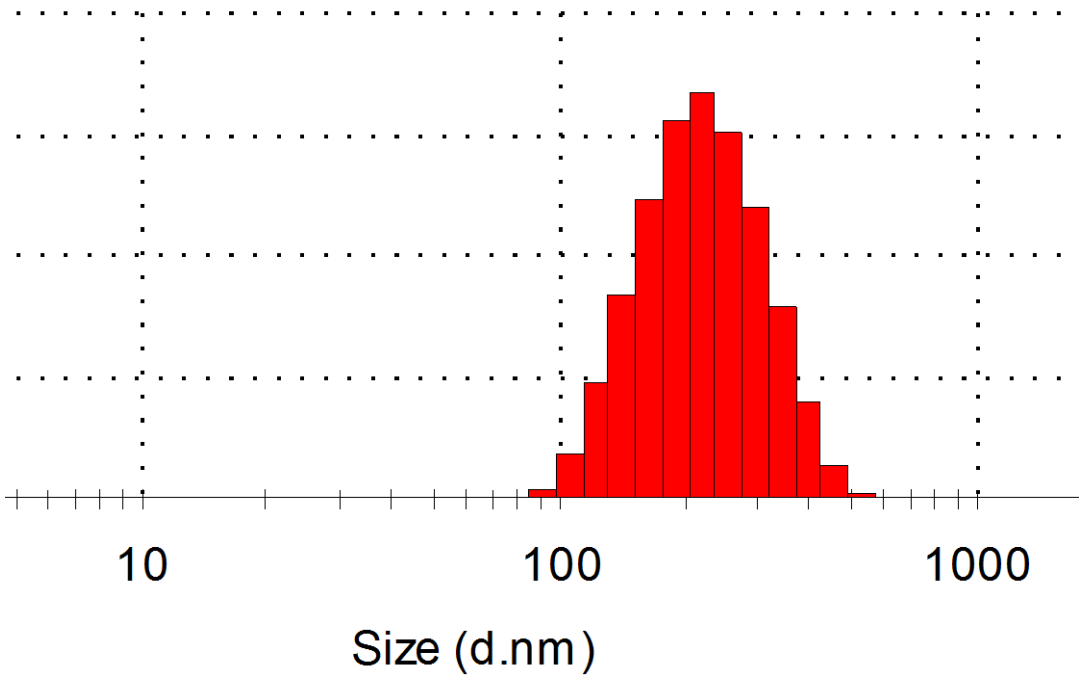


Figure S13. DLS data of the DOX • HCl-loaded vesicles.

10. Cell Culture and In Vitro Cytotoxicity Assay

MTT assay was used to determine the cytotoxicity of assemblies. Before the test, cells were seeded in a 96-well plate at 1.2×10^4 cells per well in 200 μL of the corresponding medium. After culture for 24 h, the medium was replaced with 200 μL of each of media containing assemblies of different concentrations (50–250 μg/mL). Cells were incubated for another 24 h followed by removing the culture medium and washing cells with PBS. Then, 180 μL of the culture medium and 20 μL of 5 mg/mL MTT solution in PBS were added into each well. After incubating for 4 h, the medium was removed and the cells were washed with PBS three times. The blue formazan crystals were dissolved in 150 μL of dimethyl sulfoxide, and the absorbance at a wavelength of 570 nm of each well was collected. To test the cytotoxicity of DOX • HCl-loaded assemblies, HepG2 cells were used.

11. Loading and triggered release of DOX • HCl

The DOX • HCl solution was added dropwise to 10 mL of 2 mg mL^{-1} assemblies under vigorous stirring. After stirring for another 6 h, the solution was dialyzed against deionized water for 1 day to remove unencapsulated DOX • HCl. To determine the drug loading content (DLC) and the drug loading efficiency (DLE), DOX • HCl-loaded assemblies after freeze-drying were dissolved in dimethylformamide (DMF) and analyzed by measuring the UV absorbance at the wavelength of 485 nm. The calibration curve was obtained with DOX in DMF at various concentrations. The DLC and DLE were calculated according to the following formula:

DLC (%) = (weight of loaded drug) / (weight of loaded drug + weight of polymer + weight of **WP5**) × 100%

DLE (%) = (weight of loaded drug) / (weight of drug in feed) × 100%

The release of DOX • HCl was conducted by dialysis against PBS at 37 ℃. 2 mL of each solution of DOX • HCl-loaded assemblies with different concentrations of $Na_2S_2O_4$ were added to a dialysis tube and then placed in 10 mL of PBS. At predetermined time intervals, 2 mL of the solution outside the dialysis tube was collected and replaced by 2 mL of fresh PBS. It should be noted that a solution of $Na_2S_2O_4$ with the same concentration as in the dialysis tube was also added to the PBS. The concentration of DOX • HCl was determined by measuring the UV absorbance at 485 nm.

*12. Cell viability tests of **WP5**, polymer **1** and **WP5⊃1** against HUVEC and HepG2 cells*

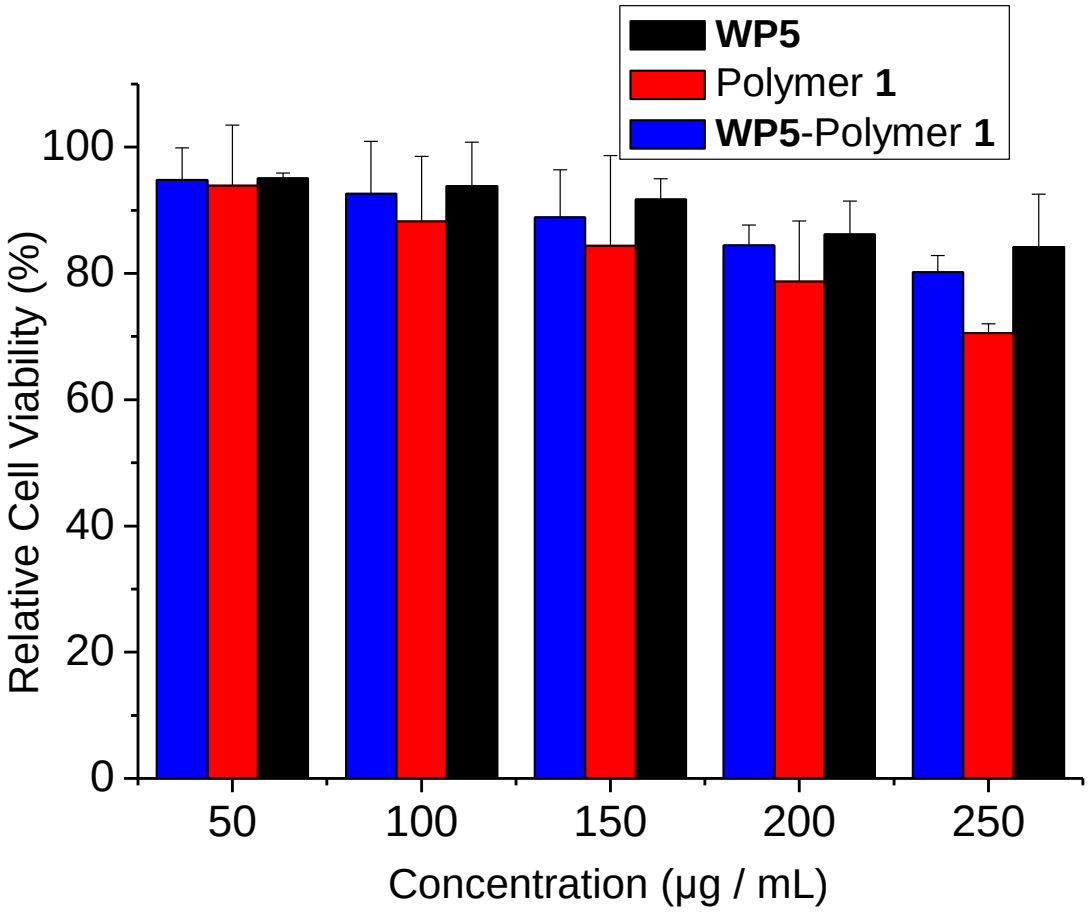


Figure S14. Cell viability tests of **WP5** (black column), homopolymer **1** (red column) and **WP5⊃1** (blue column) against HUVEC cells.

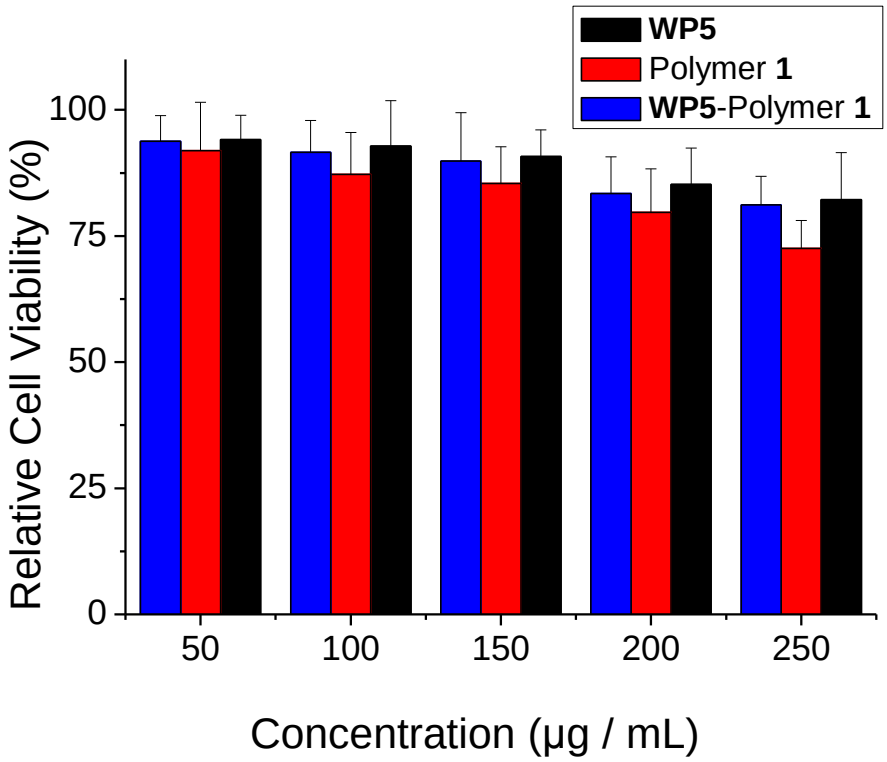


Figure S15. Cell viability tests of **WP5** (black column), homopolymer **1** (red column) and **WP5⊃1** (blue column) against HepG2 cells.

It is very important to evaluate the potential toxicity of polymeric materials for drug delivery applications. Thus, we selected two cell lines, HUVEC (Human Umbilical Vein Endothelial Cells) and HepG2 (a human liver carcinoma cell line) to evaluate the potential toxicity of the supramolecular assemblies by the 3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyltetrazolium bromide (MTT) cell survival assay. As shown in Figures S14 and S15, relative cell viability of HUVEC cells and HepG2 cells cultured with **WP5⊃1**, **WP5** and **1** showed minimal influence on cell viability at all concentrations, demonstrating that the supramolecular assemblies have very low cytotoxicity (Figures S14 and S15). This may be attributed to the excellent biocompatibility of the oligo(ethylene glycol) decorated pillar[5]arene shell. We believe the good performance on biocompatibility will make the assemblies very attractive for practical bio-related applications.

13. Electrochemical experiments

The electrochemical experiments were performed with a BAS 100 B/W electrochemical analyzer (Bioanalytical Systems, West Lafayette, IN). A glassy carbon working electrode (0.08 cmZ), a Pt counter electrode, and a home-made sodium chloride saturated calomel electrode (SSCE) were utilized in a single-compartment cell. The experiments were conducted in 0.1 M phosphate buffer solutions (pH = 7) prepared with purified water. All solutions were deoxygenated by purging with nitrogen gas and maintained under an inert atmosphere during the electrochemical experiments. The concentration of **2** was 1.00 mM. The host was added to the solution in the electrochemical cell to yield the required concentrations.

14. Determination of K_2 and K_3

The values of K_2 and K_3 were determined by the potential shifts of cyclic voltammetry.[S2]

$$G^{m+} + ne^- \longrightarrow G^{(m-n)+} \quad \triangle G_1 = -nFE^0{}_1 \quad \text{a}$$

$$G^{m+} + H \longrightarrow H/G^{m+} \quad \triangle G_2 = -RT\ln K_1 \quad \text{b}$$

$$G^{(m-n)+} + H \longrightarrow H/G^{(m-n)+} \quad \triangle G_3 = -RT\ln K_2 \quad \text{c}$$

a + c - b

$$H/G^{m+} + ne^- \longrightarrow H/G^{(m-n)+} \quad \triangle G_4 = -nFE^0{}_1 + RT\ln(K_1/K_2)$$

$$\triangle G_4 = -nFE^0{}_2$$

$$E^0{}_2 = E^0{}_1 - RT\ln(K_1/K_2)/nF$$

$$E_p = E^0 - RT\ln(D_o/D_r)^{1/2}/nF - 1.109\,RT/nF$$

$$E_{p,2} - E_{p,1} = -(\ln(K_1/K_2) + \ln(D_1/D_2)^{1/2})\,RT/nF$$

	$E_{1/2,p}$[a]	$E_{1/2,p}$[b]	D (cm · s^{-1})
2	-0.707	-1.012	7.5×10^{-6}
WP5 + 2	-0.768	-1.158	5.6×10^{-6}

Table S1. Voltammetric parameters for **2** in the absence of **WP5** and presence of equimolar **WP5** in 0.1 M phosphate buffer (pH 7.0) solution at 25 ℃. [a] Half-wave potential for the first reduction process expressed in volts *vs.* saturated calomel electrode. [b] For the second reduction process.

K_1	K_2	K_3
$(4.2 \pm 0.3) \times 10^4$	$(4.3 \pm 0.3) \times 10^3$	$(1.6 \pm 0.2) \times 10^2$

Table S2. Association constants (M^{-1}) for the inclusion of paraquat derivative **2** in **WP5** in different redox states. In this system, E^0 is the formal potential, E_p is the half-peak potential, D is the diffusion coefficient, F is the Faraday constant, T is the absolute temperatuere, n is the number of moles of electrons transferred in half-reaction, and K is the association constant. It is assumed that **WP5**⊃**2** has the same diffusion coefficient in the different redox states.

15. Cell uptake and intracellular drug release experiments

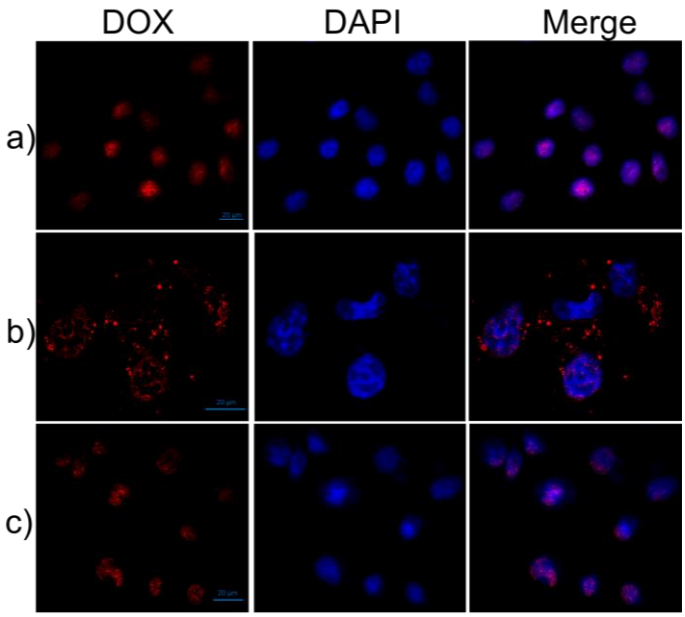


Figure S16. Cell uptake and intracellular drug release of the DOX • HCl-loaded vesicles followed by CLSM. The DOX • HCl concentration was 8 μg / mL. (a) Free DOX • HCl, 4 h incubation; (b) DOX • HCl-loaded vesicles, 4 h incubation; (c) DOX • HCl-loaded vesicles, 24 h incubation. DAPI: 4',6-diamidino-2-phenylindole.

16. The anticancer efficiency of DOX • HCl-loaded vesicles

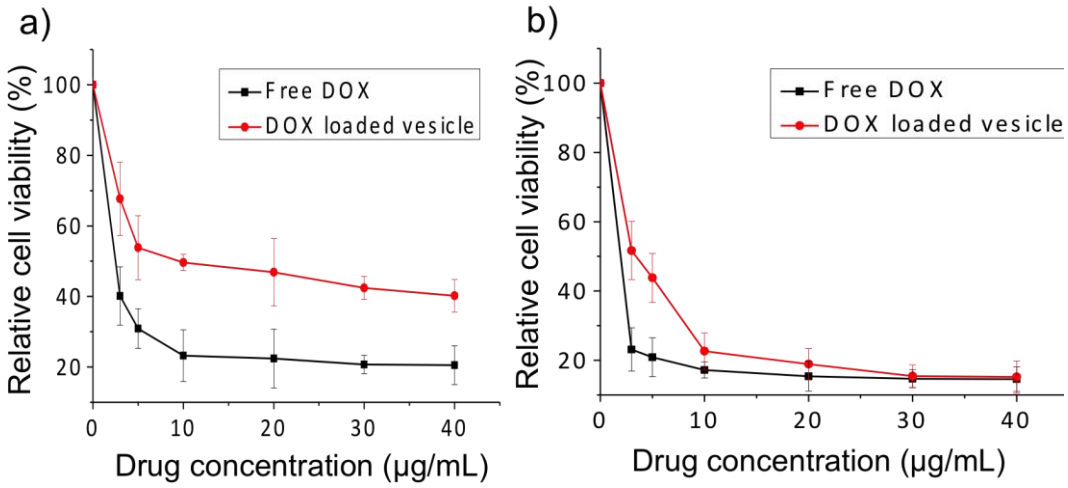


Figure S17. *In vitro* inhibition to HepG2 cell proliferation after (a) 24 h and (b) 48 h by DOX • HCl loaded vesicles and DOX • HCl in the free form at various doses.

*17. TEM images of polymer **1***

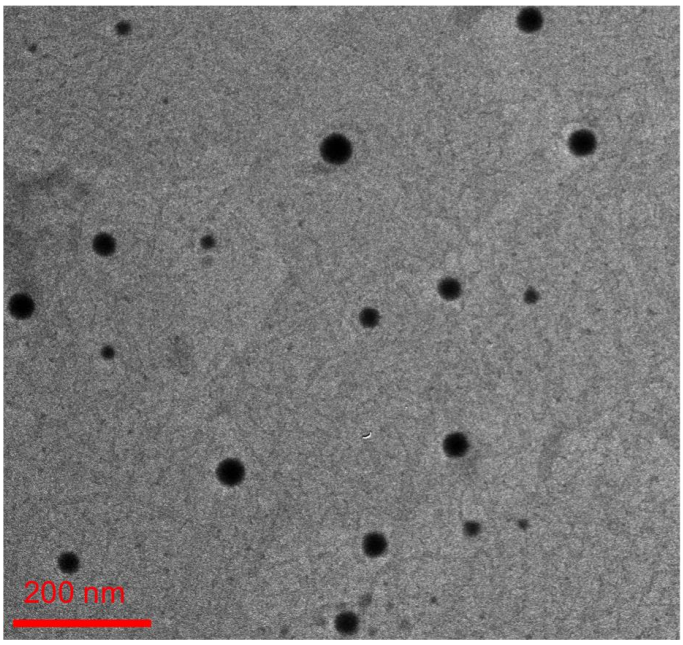


Figure S18. TEM image of aggregates self-assembled from **1**.

18. References

S1. Ogoshi, T.; Shiga, R.; Yamagishi, T.-a. *J. Am. Chem. Soc*. **2012**, *134*, 4577–4580.

S2. Kim, H.-J.; Jeon, W. S.; Ko, Y. H.; Kim, K. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 5007–5011