Enzyme-responsive pillar[5]arene-based polymeric macrocyclic amphiphile: synthesis, self-assembly in water, and application in controlled drug release

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1. Materials and methods

All reagents were commercially available and used as supplied without further purification. Compounds **2**[S1], **3**[S1]and **4** [S2] were prepared according to published procedures. NMR spectra were recorded with a Bruker Avance DMX 500 spectrophotometer using the deuterated solvent as the lock and the residual solvent or TMS as the internal reference. UV/Vis spectra were obtained on UV-2550PC. Transmission electron microscopic investigations were carried out on a HT-7700 instrument. Dynamic light scattering was carried out on a Malvern Nanosizer S instrument.

*2. Syntheses of compound **1** and **3***

*2.1. Synthesis of compound **3***

K_2CO_3 (2.78 g, 20.2 mmol), KI (0.400 g, 2.40 mmol), methyl 3,4,5-trihydroxybenzoate (1.00 g, 5.05 mmol), and **2** (7.63 g, 15.1 mmol) were added to a 500 mL round-bottom flask. The flask was vacuumed and nitrogen was introduced. After this process was repeated thrice, DMF (250 mL) was added. The solution was stirred at reflux for 24 h. Then the mixture was filtered and the filtrate was concentrated under vacuum. The crude product was stirred with sodium hydroxide solution (1M) in ethanol for about 5 hours at 50 °C. When the reaction was finished, the mixture was concentrated. The residue was washed with water and CH_2Cl_2. The organic phase was combined, dried over anhydrous Na_2SO_4 and then concentrated to give **3** as a yellow oil (5.80 g, 70%). The compound was characterized by proton NMR (Figure S1) and GPC (Figure S2). ^{1}H NMR (500 MHz, CD_3COCD_3, 298 K) δ (ppm): 7.35 (2H, s), 4.26–3.29 (-OC***H***$_2$C***H***$_2$O- of PEG), 3.29 (9H, s, -OC***H***$_3$ of PEG).

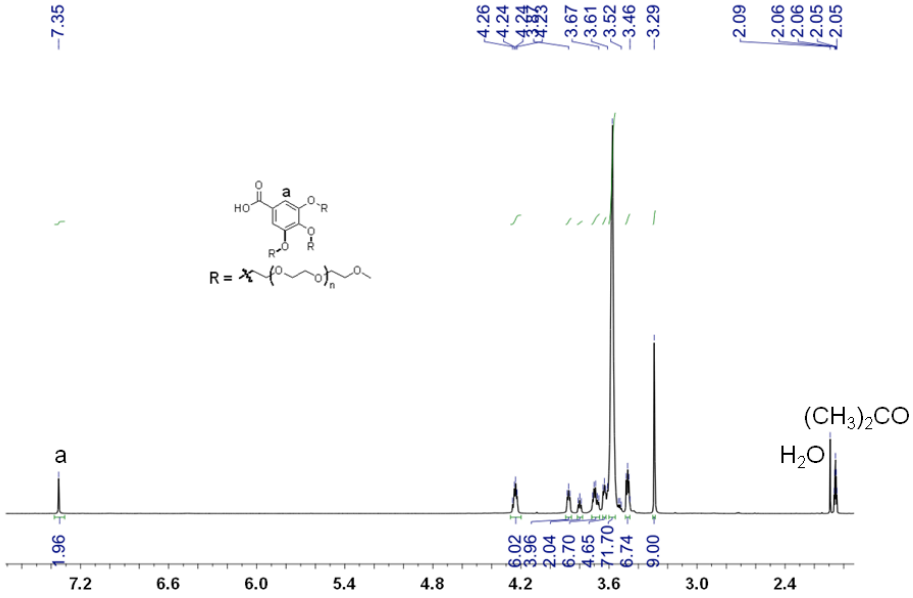
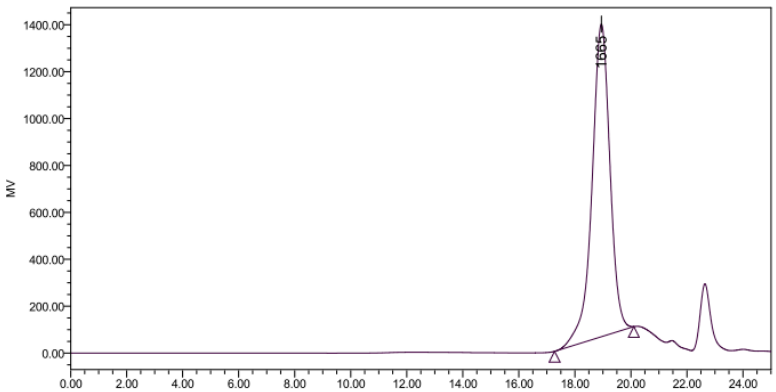


Figure S1. ^{1}H NMR spectrum (500 MHz, CD_3COCD_3, 298 K) of **3**.



M_n	M_w	M_p	M_z	M_{z+1}	PDI
1645	1738	1665	1845	1971	1.056538

Figure S2. GPC analysis of **3** using conventional calculations, with PS as the standards and THF as the solvent. M_n and polydispersity of **3** were 1.6 kDa and 1.06, respectively.

*2.2. Synthesis of compound **1***

A mixture of **3** (73.2 mg, 0.062 mmol), **4** (50.0 mg, 0.062 mmol), 4-dimethylaminopyridine (DMAP, 12.0 mg, 0.124 mmol) and 1-(3'-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, 24.0 mg, 0.124 mmol) were stirred in dichloromethane (15 mL) for 8 h. The solution was evaporated under vacuum and the residue was purified by flash column chromatography on silica gel (dichloromethane/methanol = 10/1, *v*/*v*) to afford **1** as a yellow oil (102 mg, 84%). The compound was characterized by proton NMR (Figure S3) and GPC (Figure S4). ^{1}H NMR (500 MHz, $CDCl_3$, 298 K) δ (ppm): 7.11 (2H, s), 6.78–6.74 (10H, m), 3.86–3.53 (-OC***H***$_2$C***H***$_2$O- of PEG, -PhCH_2Ph- and -PhOC***H***$_3$), 3.37 (9H, s, -OC***H***$_3$ of PEG).

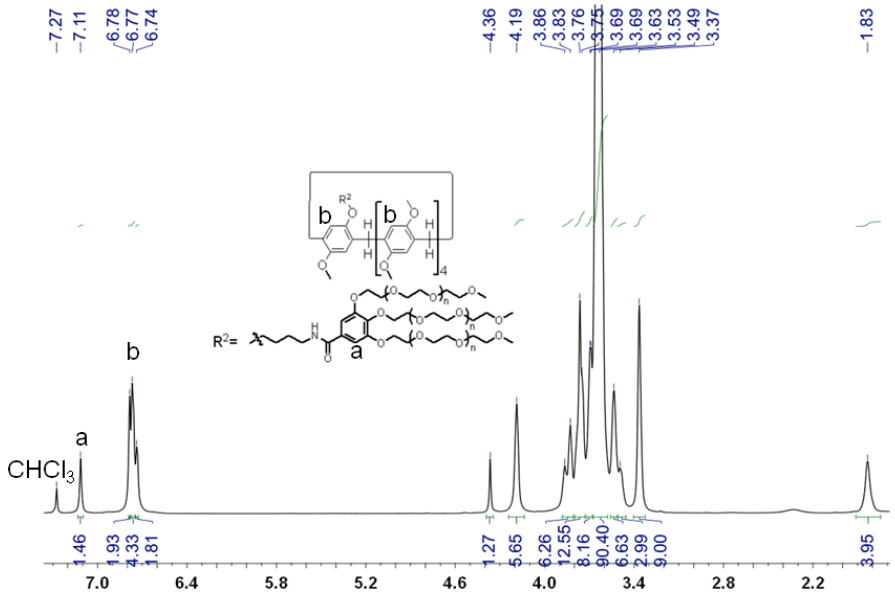
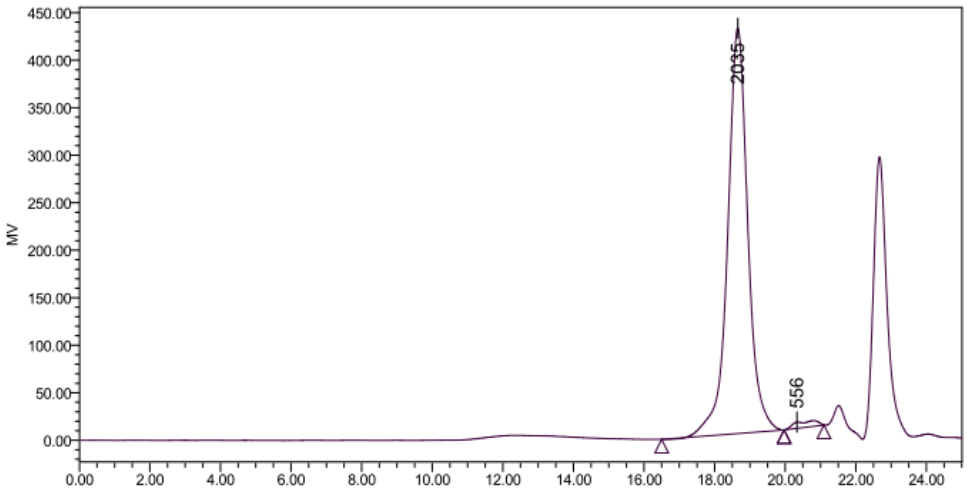


Figure S3. ^{1}H NMR spectrum (500 MHz, $CDCl_3$, 298 K) of **1**.



M_n	M_w	M_p	M_z	M_{z+1}	PDI
1984	2080	2035	2186	2307	1.048388

Figure S4. GPC analysis of **1** using conventional calculations, with PS as the standards and THF as the solvent. M_n and polydispersity of **1** were 2.0 kDa and 1.05, respectively.

*3. ^{1}H NMR spectroscopy of compound **1** in water*

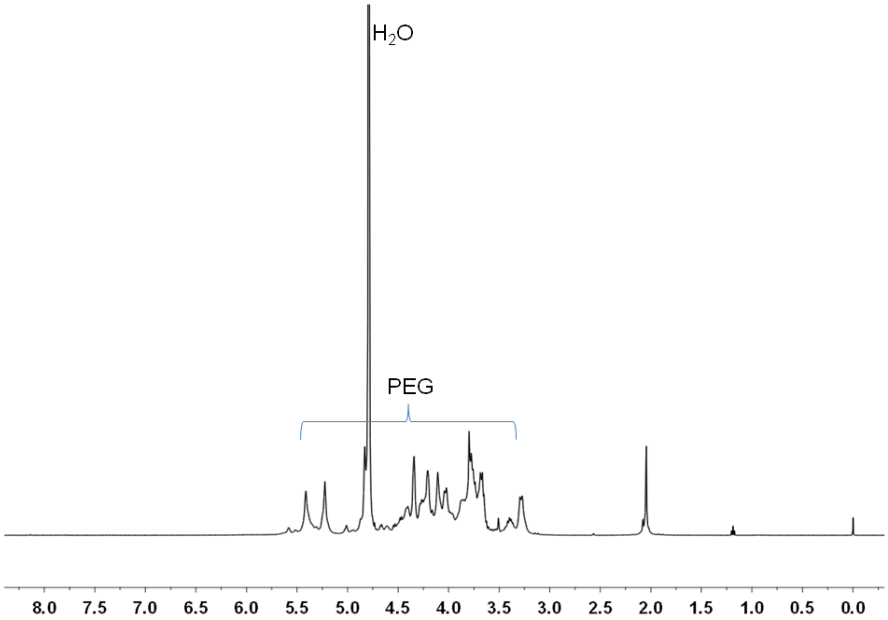


Figure S5. ^{1}H NMR spectrum (500 MHz, D_2O, 298 K) of **1**.

As shown in Figure S5, the ^{1}H NMR spectrum in deuterium oxide only showed the proton resonances of PEG segments, which meant the pillar[5]arene part was shielded. According to the literature[S3] and the structure of **1**, the compound may form a micellar-type structure. Combined with the results of TEM images (Figure 1a), we considered that it could form micelles in water.

*4. Critical micelle concentration (CMC) of **1***

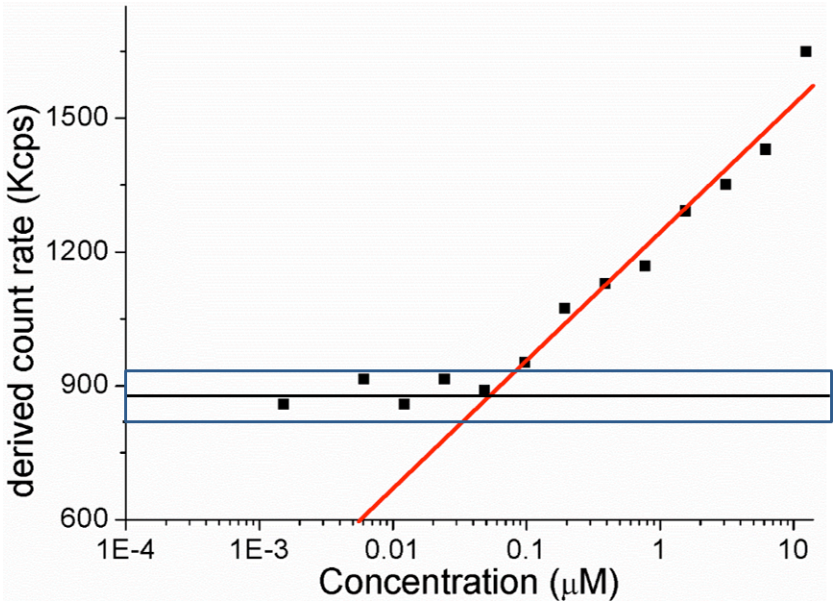


Figure S6. Dynamic light scattering of the solution of **1**. The background count rate of the pure water is given by the horizontal full line. The blue box represents the standard deviation for 4 measurements. At low concentrations, below 0.05 μM, the scattered count rate is in the water background. Above 0.05 μM, the count rate of the diffused light is quasi-linear.[S4] From the results, we could obtain that the CMC of **1** was approximately in the range of 0.05-0.06 μM in water.

*5. ^1H NMR spectroscopy of the isolated white precipitate generated from the addition of enzyme L-ASP to compound **1***

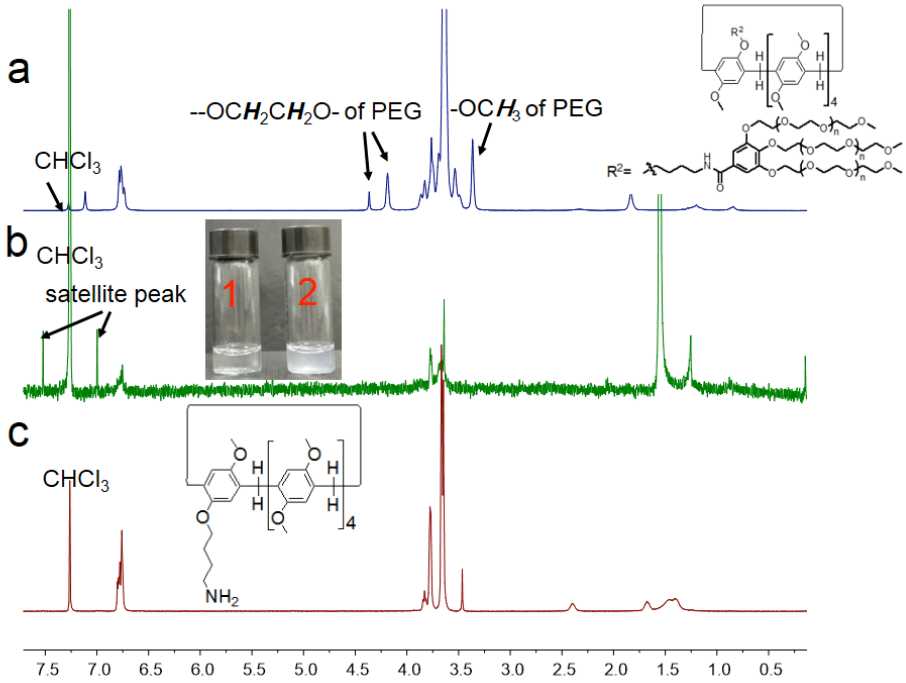


Figure S7. ^{1}H NMR spectra (500 MHz, $CDCl_3$, 298 K): a) **1**; b) the isolated white precipitate generated from the addition of enzyme L-ASP to compound **1** [Inset: direct observation of white flocculent deposit gradually appeared after the addition of L-ASP (2) compared with the blank micelle solution **1** (1)]; c) copillar[5]arene reported earlier[S2].

In Figure S8b, the integration of the isolated white precipitate mainly matched the copillar[5]arene frame reported earlier (Figure S7c). Thus, we considered that the enzymatic treatment of the micellar solution resulted in the cleavage of amide linkage on **1**. Subsequently, the pillar[5]arene-based core precipitated out from the solution due to its insolubility in water. Besides, the enzyme-responsive disassembly was further supported by the direct observation of the white flocculent deposit gradually after the addition of L-ASP.

6. UV/Vis absorption spectra of the solutions of DOX, ***M1*** *and* ***DM1*** *and the characterization of* ***DM1***

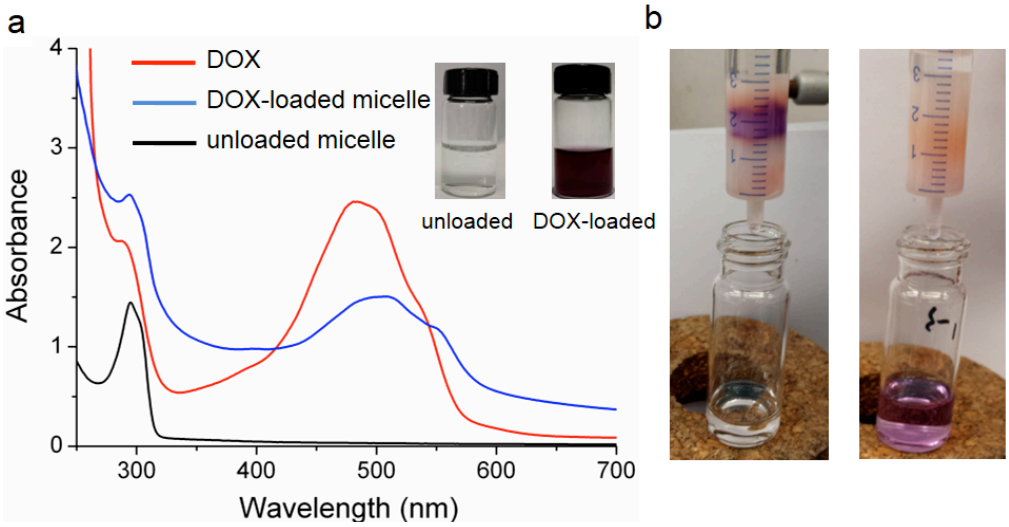


Figure S8. a) UV/Vis absorption spectra of the solutions of DOX, unloaded micelle **M1** and DOX-loaded micelle at 37 °C in aqueous buffer. Inset: color change of DOX-loaded micelle (right) compared with unloaded micelle (left). b) Pictures of the DOX-loaded micelles during the separation by glucan gel column chromatography.

Table S1. Characterization of DOX-loaded micelles.[a]

DLC (wt.%)		DLE (%)
Theory	Determined	
16.7	12.0	72.0
33.3	24.5	73.4
50.0	41.0	82.0

[a] Determined by UV measurements.

DLC (wt.%) = (weight of loaded drug/total weight of polymer and loaded drug) × 100%.

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

7. Confocal Laser Scanning Microscopy (CLSM)

MCF-7 cells were seeded on microscope slides in a 24-well plate (1.0×10^4 cells/well) using 1640 culture medium containing 10% FBS. After 24 h incubation, the medium was replaced by 450 μL of fresh culture medium and 50 μL of prescribed amounts of **DM1** or free DOX. After incubation for 2h, 4 h and 8 h, respectively, the culture medium was removed and the cells were washed twice with PBS. The cells were fixed with 4% paraformaldehyde for 20 min, and washed with PBS containing 0.1% triton X-100 for three times. The cytoskeleton was stained with fluorescein isothiocyanatelabeled phalloidin (phalloidin-FITC, green) for 50 min, and washed three times with PBS. The cell nuclei were stained with DAPI for 20 min, and washed three times with PBS. Fluorescence images of cells were observed with Confocal Laser Scanning Microscope (Leica, Germany) and analyzed by Leica 2.6.0 software.

8. MTT assay

The cytotoxicity of blank **M1** and DOX-loaded micelles **DM1** was studied by MTT assay using MCF-7 and DOX-resistant MCF-7 cells (MCF-7/ADR cells). For culture of MCF-7/ADR cells, DOX at a concentration of 58 ng/mL was added to maintain the drug resistance of the cells. One week before the experiments, the culture medium without DOX was added as replacement. MCF-7 and MCF-7/ADR cells were separately seeded into a 96-well plate at a density of 1.0×10^4 cells per well in 90 μL of 1640 culture medium containing 10% FBS, and incubated at 37 °C with 5% CO_2. After 24 h, 10 μL of **DM1** samples or free DOX at different concentrations in PB (10 mM, pH 7.4) were added. The cells were incubated for another 48 h, and then 10 μL of MTT solution (5 mg/mL) was added. The cells were incubated for 4 h, and the medium was replaced by 150 μL of DMSO to dissolve the resulting purple crystals. The optical densities were measured by a microplate reader at 570 nm. The experiments were conducted in triplicate and the results were presented as the average ± standard deviation.

References:

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