Host-guest interactions based supramolecular prodrug self-assemblies for GSH-consumption augmented chemotherapy

Yang Bai[1#], Xihua Li[1#], Muqiong Li[2#], Qingqing Shang[1], Jing Yang[1], Li Fan[2*], Wei Tian[3*]*

[1]Shaanxi Key Laboratory of Chemical Additives for Industry, College of Chemistry and Chemical Engineering, Shaanxi University of Science and Technology, Xi'an 710021, China.

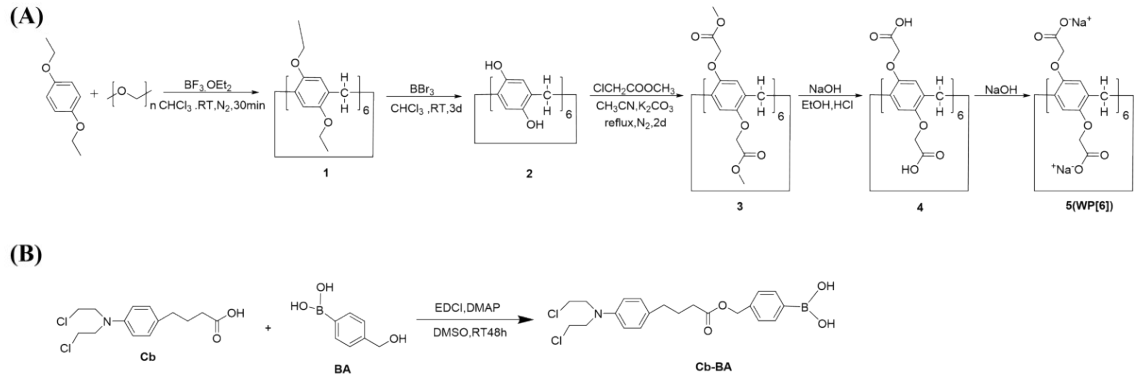
[2]School of Pharmacy, Air Force Medical University, Xi'an 710032, China.

[3]MOE Key Laboratory of Material Physics and Chemistry under Extraordinary Conditions, School of Chemistry and Chemical Engineering, Northwestern Polytechnical University, Xi'an, 710072, China.

1 Materials

4-Dimethylaminopyridine (DMAP, 99%) and chlorambucil (Cb, 98%) were purchased from J&K Chemicals. Curcumin (98%) and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl,99%) were obtained from Macklin Biochemical Co., Ltd. 4-(Hydroxymethyl)phenylboronic acid was purchased from Sarn Chemical Technology (Shanghai) Co., Ltd. Sodium bicarbonate ($NaHCO_3$), Anhydrous sodium sulfate (Na_2SO_4), Hexane (C_6H_{14}), Ethyl acetate ($C_4H_8O_2$), Dimethyl sulfoxide (DMSO) are analytical reagents. All chemical reagents mentioned above were purchased from commercial sources and used as received. Water-soluble pillar[6]arene was synthesized according to the reported procedure[1]. The details synthesis and characterization of WP[6] and Cb-BA was shown in ESI.

2 Synthesis of WP[6], Cb-BA and the preparation of SPSAs.



Scheme S1 Synthesis routes of WP[6] and Cb-BA.

2.1 Synthesis of compound 1

The water-soluble pillar[6]arene (WP[6]) was synthesized according to the literature.[S1] Briefly, 1,4-diethoxybenzene (3g, 18 mM) , paraformaldehyde (540 mg,18 mM) and 150 mL anhydrous $CHCl_3$ was stirred at room temperature under nitrogen atmosphere for 0.5 h. Then the boron trifluoride diethyl etherate (2.25 mL, 18 mM) was added as the catalyst. After being stirred for 0.5 h at room temperature, the reaction was quenched with 50 mL of water and the organic layer was washed with a saturated sodium bicarbonate solution. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was removed by rotary evaporation. After purification by column chromatography (Hexane /Ethyl acetate =10:1-40:1 v/v), compound **1** was obtained (215mg).

^{1}H NMR($CDCl_3$, TMS):δ=1.2-1.63 (36H-C*H*$_3$),δ=3.85-4.0 (24H, in Ar-C*H*$_2$- and -C*H*$_2$-), δ=6.73-6.75 (24H, in Ar).

MALDI-TOF-MS (DCM) found *m/z*:1068.1748 g/mol.

2.2 Synthesis of compound 2

In a 100 mL flask, compound 1 (427.74 mg, 0.4 mM) and BBr_3 (4.466 mL, 48 mM) were added to $CHCl_3$ (35 mL). After stirred over 3 days at room temperature, the water was added to quench the reaction in the ice bath. The precipitate was collected by centrifugation, and then washed by water. Then, the compound **2** was afforded after freeze-drying (269.54 mg).

^{1}H NMR (DMSO-*d*$_6$, TMS): δ=3.0-4.5 (24H, in Ar-C*H*$_2$-), δ= 6.41 (24H, in Ar), δ=8.3-8.5 (12H, in Ar-O*H*).

2.3 Synthesis of compound 3

Compound 2 (422 mg, 0.58mM) and K_2CO_3 (3800mg, 28 mmol) was dispersed in anhydrous CH_3CN (50 mL), then methyl chloroacetate (1.3 mL, 14 mM) was added into the mixture under N_2 atmosphere. The system was heated at the refluxing temperature for 48 h. After cooling to ambient temperature, the insoluble solid was removed by filtration, and the solvent was removed by rotary evaporation. Compound 3 (154mg, 16.6%) was obtained by column chromatography (CH_2Cl_2/ethyl acetate = 10:1, v/v).

^{1}H NMR (DMSO-d_6, TMS): δ=3.67 (36H, -C*H*$_3$), δ=3.82 (12H, Ar-C*H*$_2$-), δ=4.43 (24H, -C*H*$_2$-), δ=6.70 (12H,in Ar).

2.4 Synthesis of WP[6]

Compound 3 (481mg, 0.30 mM) and NaOH (8000mg, 200 mM) were dissolved in a mixture of ethanol and water (40 mL, v/v = 1: 1), and stirred at 78 °C overnight. The solution was concentrated by rotary evaporation and the pH of mixtures was adjust to 3 by HCl (1 mol/L). The precipitate was collected by filtration and washed by deionized water to obtain compound 4. Compound 4 and NaOH (the equivalent ratio of carboxyl group and sodium ion is 1:1) were dissolved together in 10 mL of deionized water and stirred overnight at room temperature. WP[6] was obtained by freeze-drying.

^{1}H NMR (D_2O): δ=3.8 (12H, Ar-C*H*$_2$-), δ=4.21 (24H, -C*H*$_2$-), δ=6.66(12H, in Ar).

2.5 Synthesis of Cb-BA

Chlorambucil (304.21mg, 0.001mmol), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (383.40 mg, 0.002mmol), 4-Dimethylaminopyridine (122.17mg, 0.001mmol) and 3mL DMSO was stirred at room temperature for 30 min. Then, 4-(Hydroxymethyl)phenylboronic acid (197.55 mg, 0.0013 mmol) dissolved in 2mL of DMSO was added dropwise at room temperature for 24h. Subsequently, 20 mL EA was added to the system and washed by sodium bicarbonate aqueous solution. Afterward, the organic phase was collected and dried over anhydrous sodium sulfate. Cb-BA was obtained by column chromatography (Hexane/Ethyl acetate = 10:1, v/v).

^{1}H NMR(DMSO-*d6*): δ=1.70-1.82 (2H, Ar - CH_2-C*H*$_2$-C*H*$_2$-), δ=2.29-2.36 (2H, Ar - CH_2-CH_2-C*H*$_2$-),δ=2.41-2.47(2H,Ar-C*H*$_2$-CH_2-CH_2-),δ=3.60-3.74 (8H, -C*H*$_2$-C*H*$_2$-Cl), δ=5.08 (2H, Ar-C*H*$_2$-O-), δ=6.67-7.04 (4H, in Ar of Cb), δ=7.24-7.83 (4H, in Ar of BA).

FT-IR (KBr): 1731 cm^{-1} (ν, C=O), 3711 cm^{-1} (ν, -OH).

ESI-MS (DMF) found *m/z*: 438.14 g/mol.

2.6 Preparation of supramolecular prodrug self-assemblies (SPSAs)

Cb-BA (2.63 mg, 0.006mmol) dissolved in 100 μL DMSO was slowly added to a solution of WP[6] (10.15 mg in 14 mL PBS (pH=9.0), 0.006mmol). After ultrasonic for 2h, SPSAs-1 was obtained. Cur (1.66 mg, 0.0045 mmol) dissolved in 500 μL of buffer (containing 10% methanol) at pH 9.0 was slowly added to a solution of SPSAs-1. After stirring for 12 h in dark, SPSAs-2 was obtained. The resulting solution was stored at 4 °C for the next use.

3 Characterization

Fourier transform infrared (FT-IR) spectra were recorded on a VECTOR-22 IR spectrometer, casting samples into thin films on KBr. Transition mode was used and the wavenumber range was set from 4000 cm^{-1} to 400 cm^{-1}. The 1H NMR and 2D NMR NOSEY spectra were obtained from a Bruker AV-400 spectrometer (Bruker BioSpin, Switerland), which operates at 400 MHz (1H) in $CDCl_3$, DMSO-*d6*, D_2O. UV-vis spectrophotometer measurement was performed on Shimadzu UV-2600 spectroscopy. Fluorescence emission spectroscopy was employed with a Hitachi F-7000 fluorescent spectrophotometer.

3.1 Measurement of the critical aggregation concentration for the supramolecular self-assemblies

The critical aggregation concentration (CAC) of the SPSAs-1 based on WP[6] and Cb-BA was determined by fluorescence spectroscopy. The SPSAs-1 were prepared at concentrations ranging from 0.1mg/mL to 1×10^{-5} mg/mL in pyrene aqueous solution (concentration: 6×10^{-6} M). Then, the fluorescence emission spectra of the above solutions were recorded from 350 nm to 550 nm by UV irradiation at 335 nm. The light intensity ratios (I_1/I_3) of the first and third electron vibration peaks in the emission spectrum were calculated. Finally, the concentration corresponding to the I_1/I_3 values in the higher concentration region and the lower concentration region was taken as the CAC of the SPSAs-1.

3.2 Investigation of the morphology and size of the supramolecular self-assemblies

The hydrodynamic diameter and diameter distribution of the self-assemblies were determined by a Brookhaven 90 Plus Zeta Dynamic Light Scattering Instrument (DLS). The light source was a He-Ne laser operating at 632 nm at an angle of 90°. The AFM measurements were carried out on a Park XE7 atomic force microscope in noncontact mode. The size and

morphology of the self-assemblies were revealed by TEM (FEI Tecnai G2 F20 S-TWIN, Accelerating voltage 150 kV). Samples were prepared by dropping 10 μL of the solutions on copper grids without staining.

3.3 In vitro drug release

The in vitro release profiles of both Cb and Cur from the SPSAs-2 were investigated under a series of simulated physiological conditions. A total of 2 mL of SPSAs-2 solution was added to a dialysis bag (cut-off Mn =500 Da) and immersed in 40 mL of phosphate buffer at different pH values (7.4 with 1% Tween 80, 5.0 with 1% Tween 80, w/v and 5.0 + 10mM H_2O_2 with 1% Tween 80, w/v). The mixture was then kept at 37 °C under constant shaking. At each predetermined time point, 3 mL of the release medium was withdrawn for characterization. The same volume of fresh phosphate buffer was added to ensure the same total volume. Each set of experiments was performed twice in parallel. The amount of Cb released was measured by HPLC, and the amount of Cur released was measured by UV-vis. The cumulative release of Cb and Cur was calculated by using Eq. (1) as follows:

$$\text{Cumulative Release}(\%) = \frac{100 \times (40.0C_n + 3.0\sum C_{n-1})}{W_0} \quad (1)$$

3.4 *In vitro* cellular experiment

3.4.1 *In vitro* cellular ROS investigation

The efficiency of ROS generation from different samples was investigated by performing in vitro cellular reactive oxygen species (ROS) experiments. HeLa cells were seeded on glass dishes at a density of 2×10^5 (1 ml of cell suspension was added to each dish) and incubated overnight at 37 °C in 5% CO_2. Cells from SPSAs-2 (cur concentration of 32 μg/ml) and controls were incubated at 37 °C for 6 h. At the end of the incubation, the cells were washed three times with cold PBS, labeled with dichlorofluorescein (DCF) decomposed from the ROS probe 2',7'- dichlorofluorescein diacetate (DCFH-DA) for 30 min and washed three times with cold PBS. Nuclei were stained with DAPI for 10 min, and finally the cells were washed three times with cold PBS for the CLSM observation.

3.4.2 *In vitro* cellular uptake study

HeLa cells were seeded on glass culture dishes (5×10^3/well, 1.5 ml cell suspension) and incubated overnight at 37°C, 5% CO_2.Then, the cells were incubated with SPSAs-2 (with an

equivalent concentration of Cb (32g/mL) and washed 3 times with cold PBS. After fixed with 4% (w/w) paraformaldehyde solution for 15 min, the cells were washed with cold PBS and labeled with DAPI s (10 min), and observed with a confocal laser scanning microscope (CLSM, instrument model:TCS-SP5, Leica, Germany).

3.4.3 *In vitro* cytotoxicity experiments

HeLa cells were seeded into 96 well plates (5×10^3/well, 100μL cell suspension) and incubated overnight at 37 °C with 5% CO_2. The cells were subsequently co incubated with a series of media containing different concentrations of CB, CB-BA, and $SPSA_S$ for 48 h. Then, the plate was washed by adding 10 μL CCK-8 solution to evaluate the cytotoxicity. After incubation for 2h, the absorbance at 450 nm was measured using a multiskan MK3 microplate reader (Thermo Scientific, USA), and the cell viability values were obtained after data treatment. The cell viability values were calculated by using Eq. (2) as follows:

$$\text{Cell Viability}(\%) = \frac{OD_{sample} - OD_{blank}}{OD_{control} - OD_{blank}} \times 100\% \quad (2)$$

3.4.4 Cellular GSH level studies

HeLa cells were seeded in 6-well plates (5×10^5/well, 1.5 ml cell suspension), placed at 37 °C and incubated with 5% CO_2 for 24 h. The fresh medium containing CB, CB-BA, Cur, SPSAs-1 and SPSAs-2 was added and incubated for 24 h. Then, the GSH amount in tumor cells is obtained using GSH/GSSG Kit. The GSH concentration is calculated as formula (3):

$$\text{GSH content}(\mu\text{mol/gprot}) = \frac{OD_{sample} - OD_{blank}}{OD_{standard} - OD_{blank}} \times \frac{\text{concentration of GSH standard solution}(20\text{mol/L})}{\text{total protein content}(\text{gprot/L})} \times \text{pretreatment dilution ratio}(2)$$

3.4.5 Cellular TrxR activity studies

HeLa cells were seeded in black 96-well plates at a density of 8,000 cells per well. After 24 h, various concentrations of nanoformulations were added and incubated for 6 h. The cellular TrxR probe (Cayman Chemical) was then added according to the manufacture's protocol and the fluorescent intensity or optical density (OD) value was measured using the microplate reader.

3.5. In vivo antitumor activity

B16 tumor model was firstly established. Briefly, B16 cells in 0.1 mL saline were subcutaneously inoculated into the right limb armpits of mouse. Tumors were allowed to grow for 2 weeks to reach proliferative phase with the size of ~100 mm^3. Tumor volume and body weights were measured for individual animals in all experiments. PBS, Cb-BA, Cur, Cb-BA+Cur, SPSAs-1 and SPSAs-2 at concentration of 5mg/kg were intravenously injected on day 0, 4, 8. The body weight and tumor growth relative volume were monitored for 20 days. Tumor progression and body weights of mice were monitored every other day. At day 20, all the mice were sacrificed. The major organs and tumors were dissected, collected, and used to evaluate systemic toxicity by H&E staining and TUNEL staining after sectioning into thin slices (10 μm).

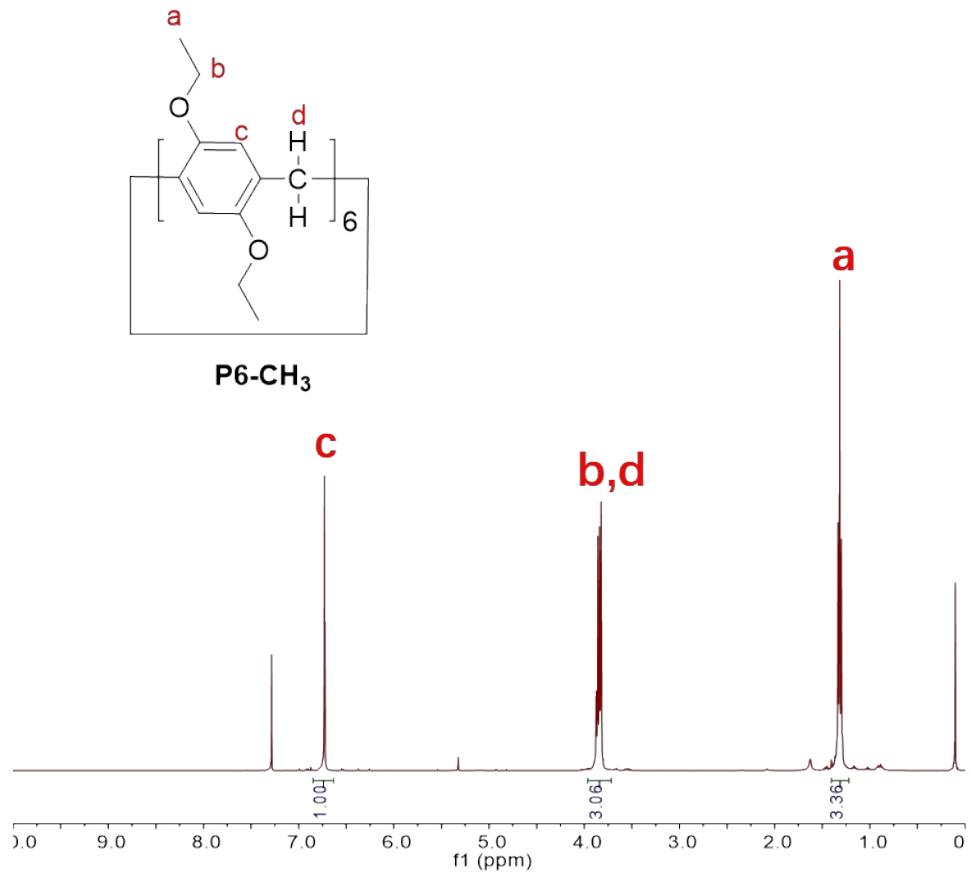


Fig. S1 ^{1}H NMR spectrum of P6-CH_3 in $CDCl_3$.

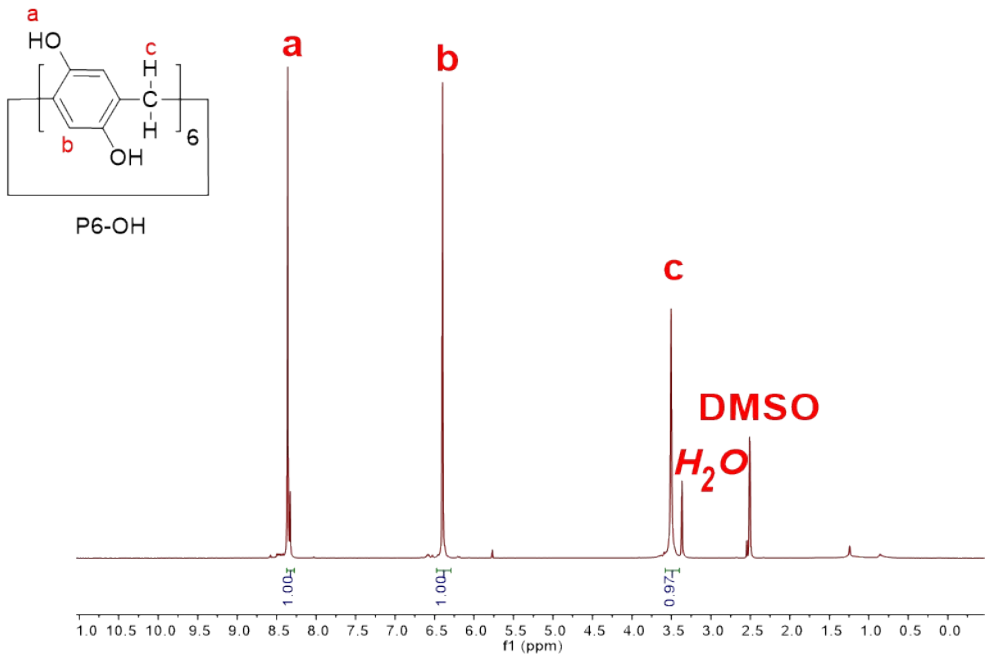


Fig. S2 ^{1}H NMR spectrum of P6-OH in DMSO-d_6.

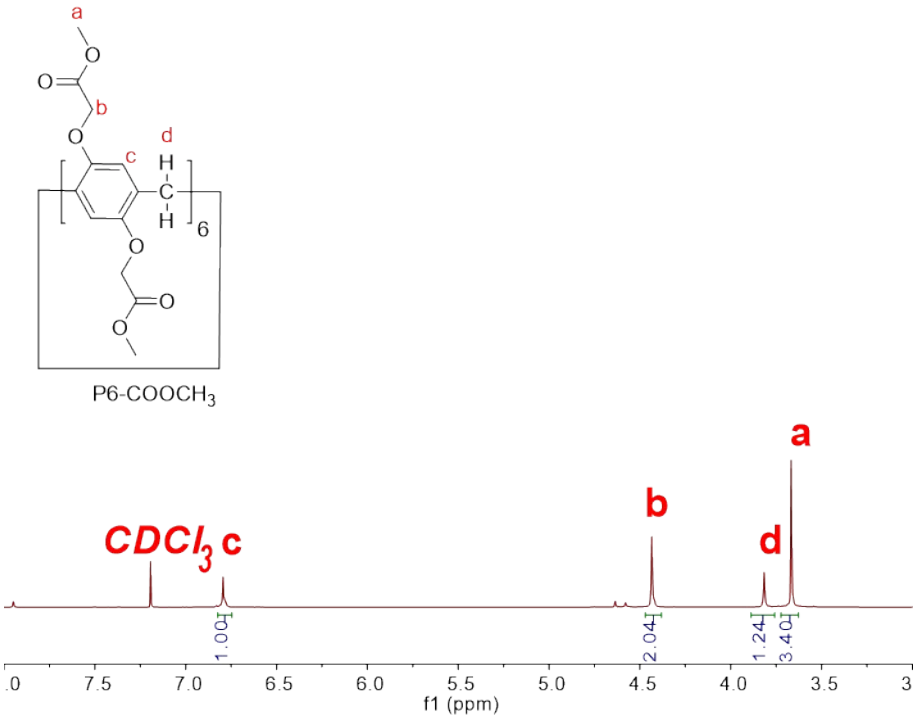


Fig. S3 ^{1}H NMR spectrum of P6-$COOCH_3$ in $CDCl_3$.

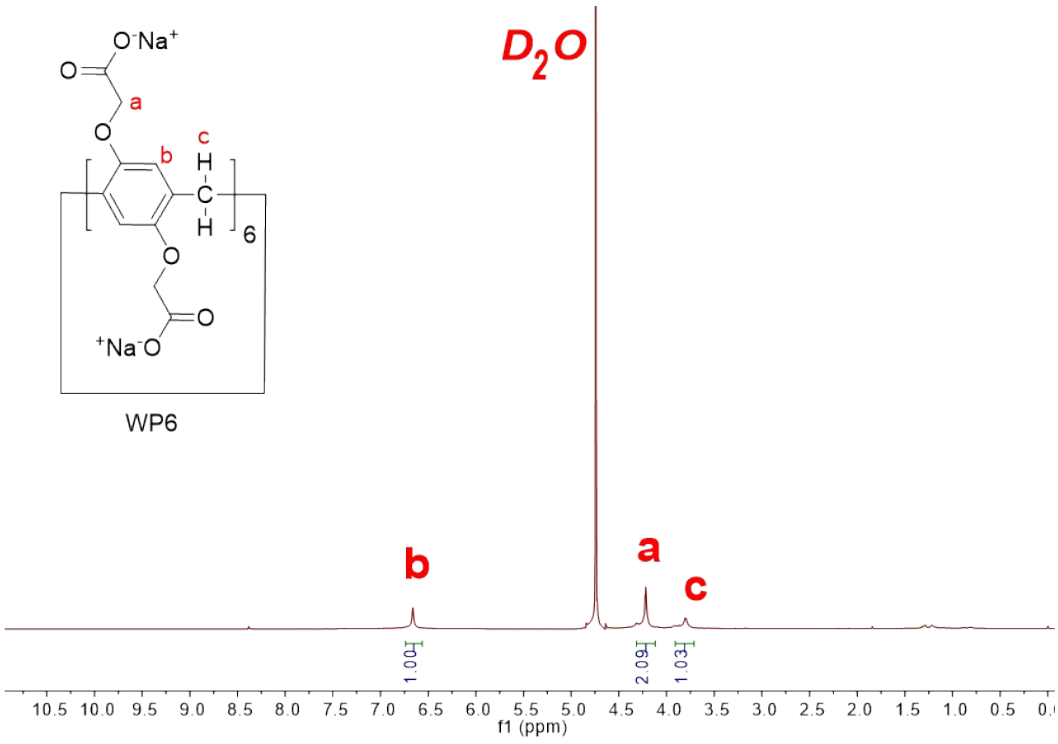


Fig. S4 ^{1}H NMR spectrum of WP6 in D_2O.

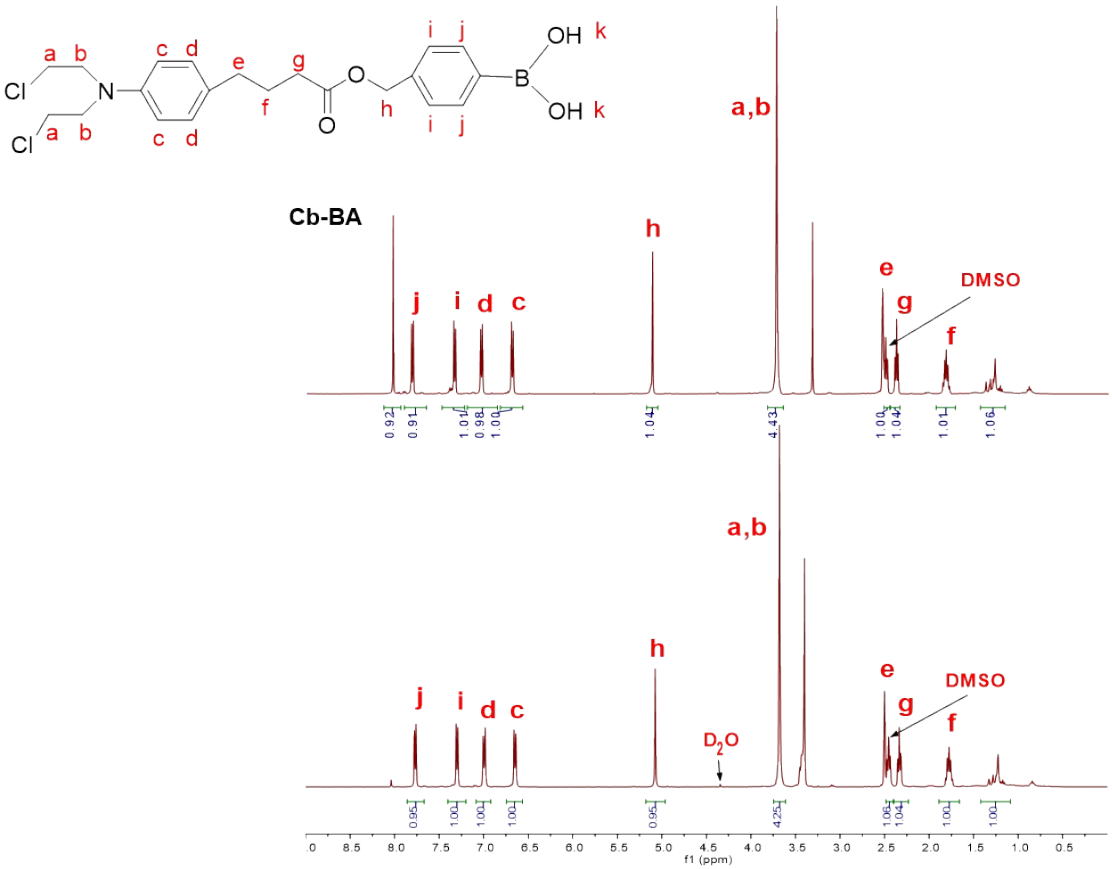


Fig. S5 ^{1}H NMR spectra of Cb-BA in DMSO-d6.

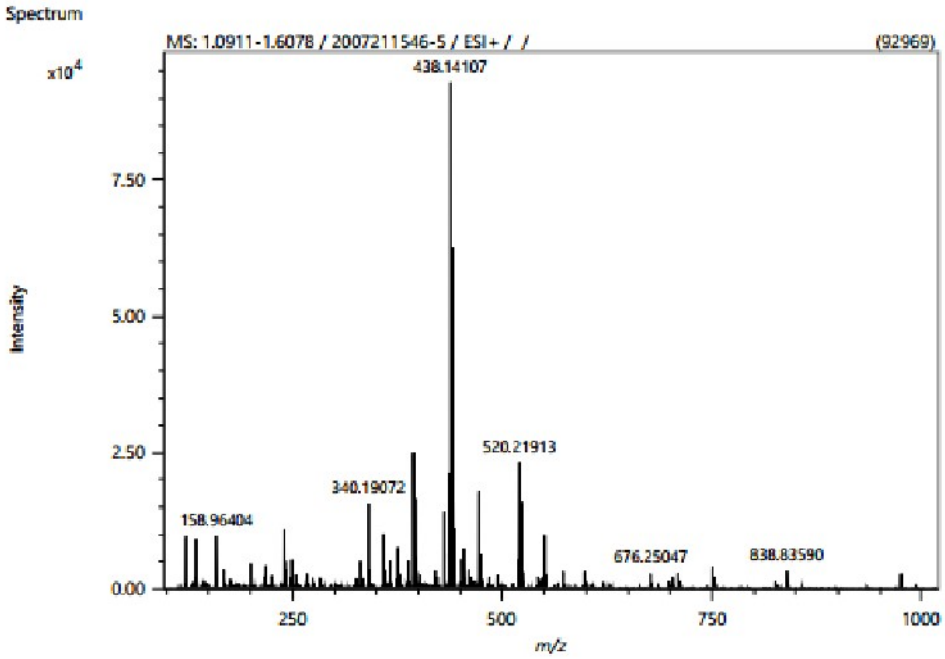


Fig. S6 ESI-MS spectrum of Cb-BA.

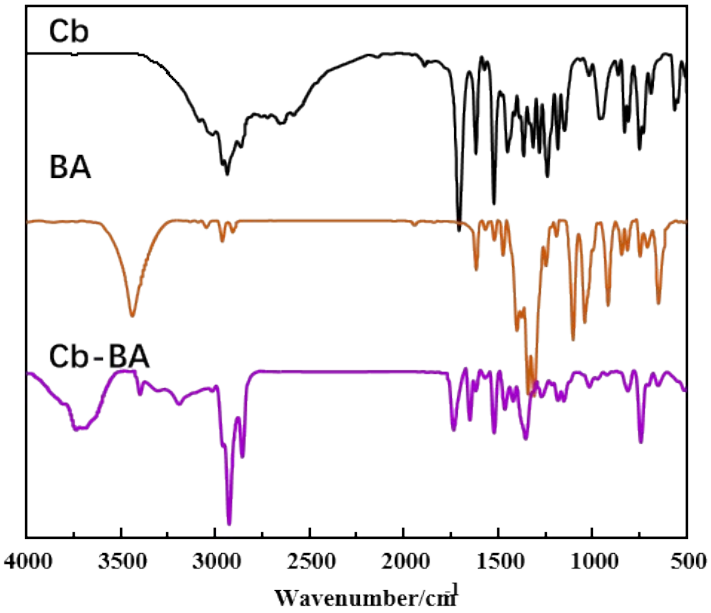


Fig. S7 The FT-IR spectra of Cb, BA and Cb-BA.

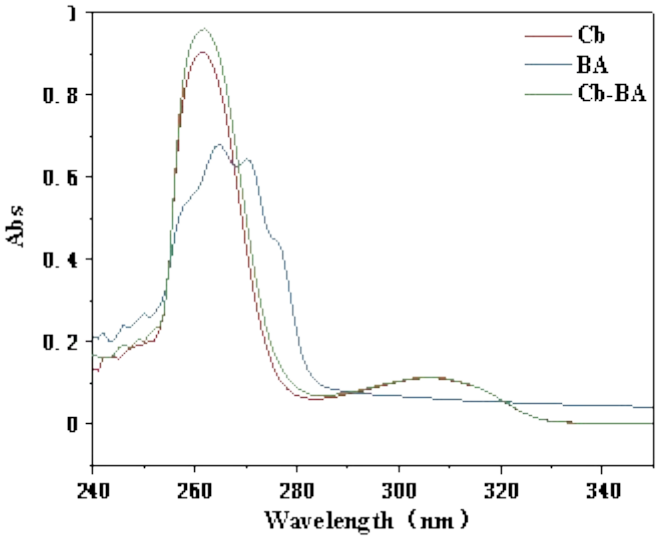


Fig. S8 UV/Vis absorption spectra of Cb,BA and Cb-BA.

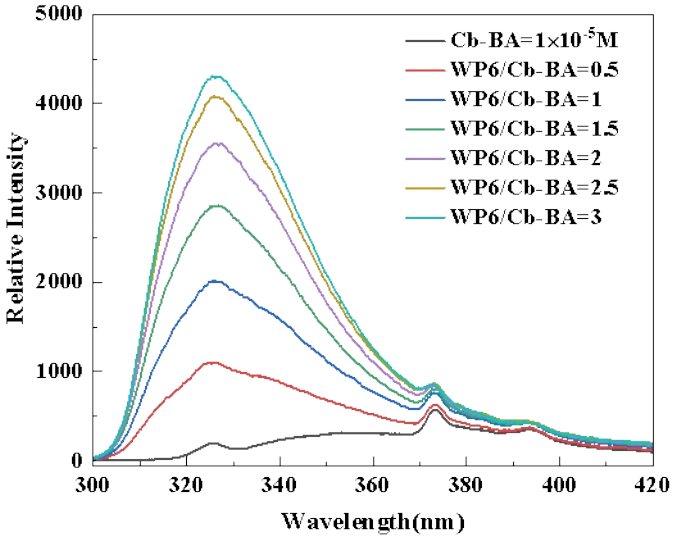


Fig. S9 Fluorescence spectra of Cb-BA.

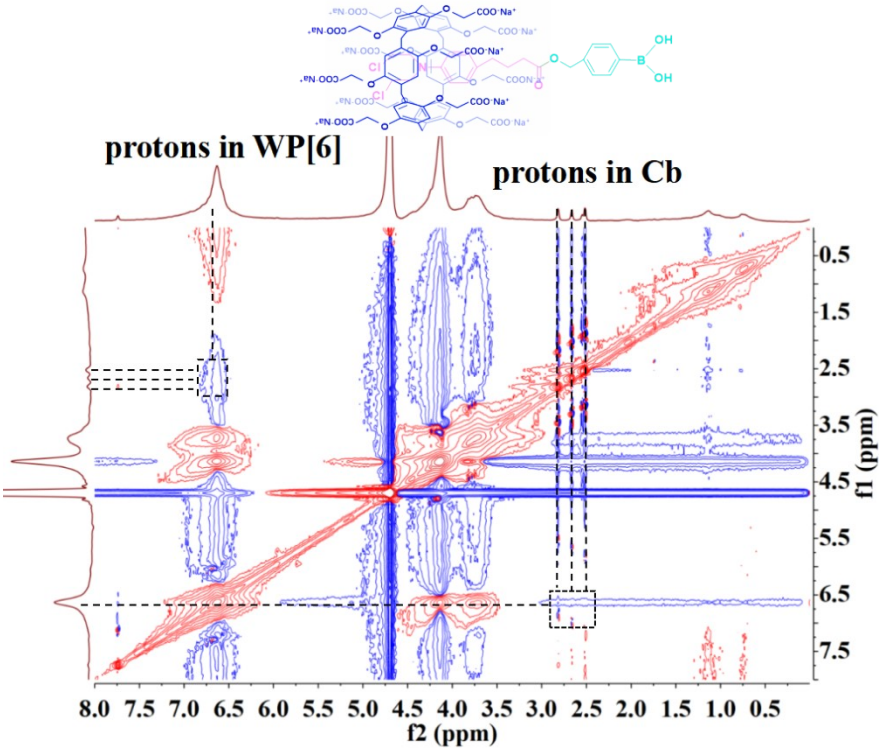


Fig. S10 The 2D NMR NOSEY spectra of WP[6]/Cb-BA mixtures in D_2O.

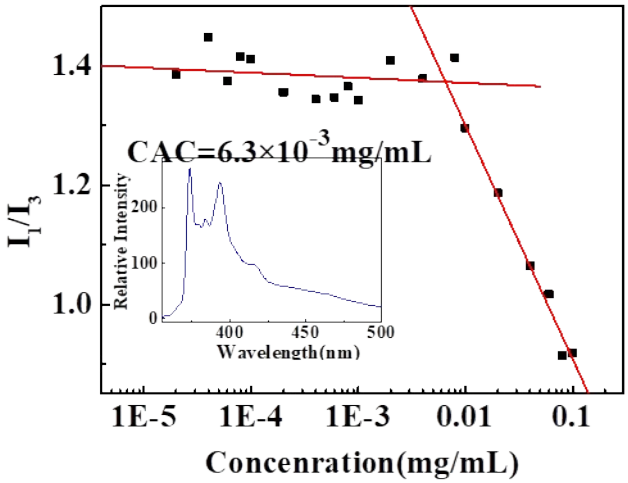


Fig. S11 The critical aggregation concentration (CAC) of SPSAs-1 measured with the method of pyrene as fluorescent probe.

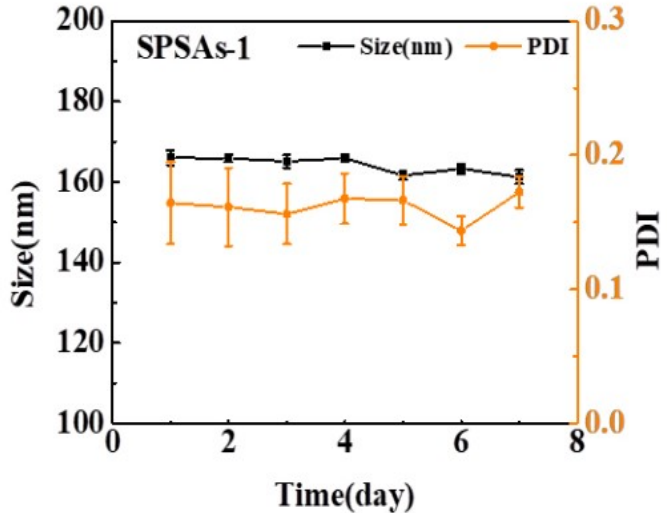


Fig. S12 The stability of SPSAs-1 monitored by DLS.

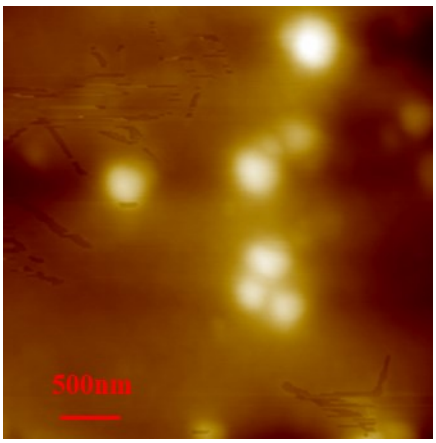


Fig. S13 The typical AFM image of SPSAs-1.

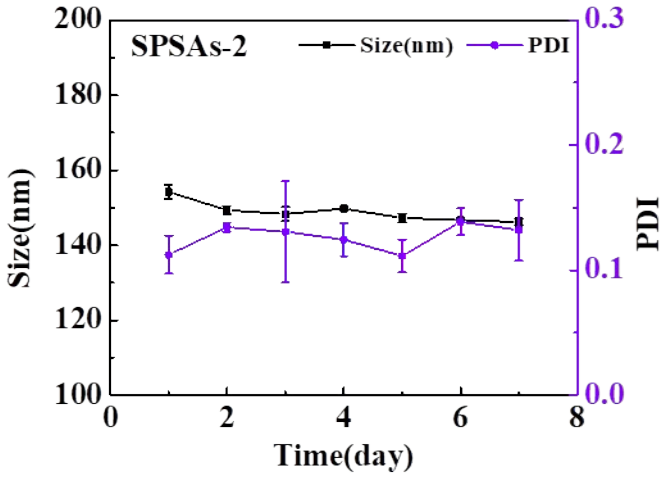


Fig. S14 The stability of SPSAs-2 monitored by DLS.

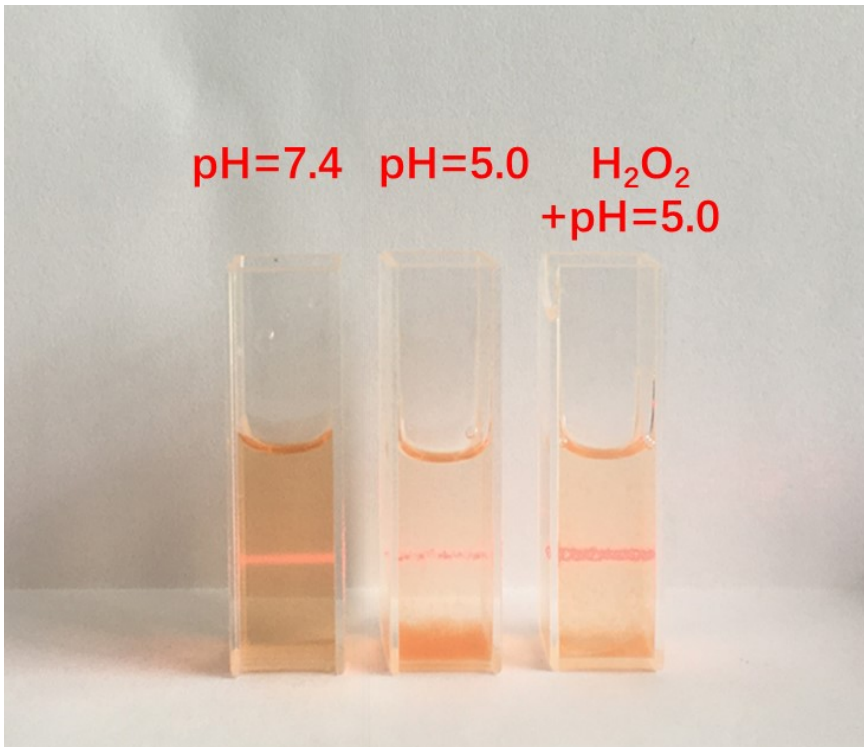


Fig. S15 The Tyndall images of SPSAs-2 at pH 7.4, pH 5.0 and pH 5.0 + H_2O_2 (from left to right).

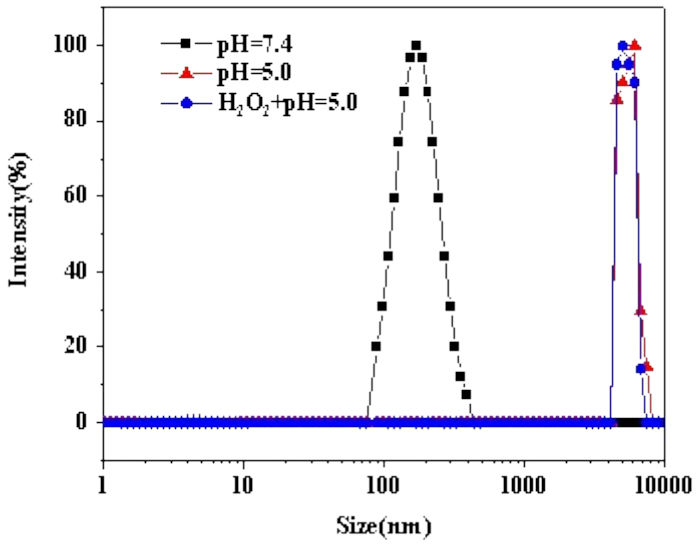


Fig. S16 DLS results of SPSAs-2 at pH 7.4, pH 5.0 and pH 5.0 + H_2O_2.

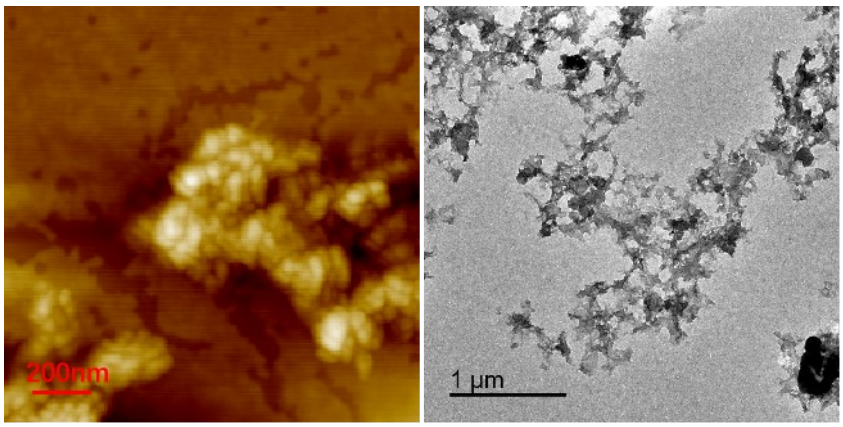


Fig. S17 Typical AFM image and TEM image of SPSAs-2 at pH 5.0.

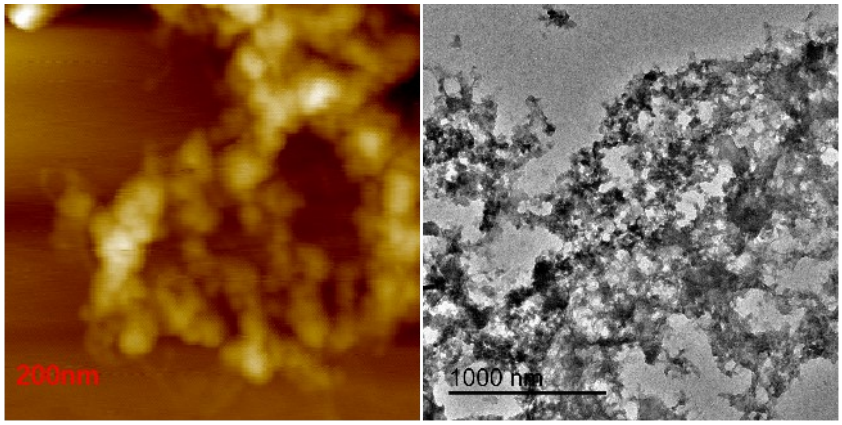


Fig. S18 Typical AFM image and TEM image of SPSAs-2 at pH 5.0 + H_2O_2.

4 References

[1] G. Yu, M. Xue, Z. Zhang, J. Li, C. Han, F. Huang, *J Am Chem Soc* **2012**, 134, 13248.