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Nitric oxide-containing supramolecular polypeptide nanomedicine based on [2]biphenyl-extended-pillar[6]arenes for drug resistance reversal

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Materials

Anhydrous dimethylformamide (DMF, 99.8%, J&K), doxorubicin hydrochloride (DOX·HCl, 98%, Macklin), 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC, 97%, Adamas), 4-dimethylaminopyridine (DMAP, 99%, Adamas), tert-butyl nitrite (TBN, 95%, J&K), and indocyanine green (ICG, Macklin) was used as received. S-(onitrobenzyl)-L-cysteine-N-carboxyanhydride ($_L$-NBC-NCA) monomer and OHBpP6 were synthesized following the procedure described previously [1, 2]

Synthesis of AWBpP6

OHBpP6 (3 g, 3.6 mmol), K_2CO_3 (21 g, 0.21 mol), KI (50 mg, 0.3 mol) and acetonitrile (300 mL) were added in a 500 mL round-bottom flask. The reaction mixture was stirred at 20 °C for 20 min. Upon addition of $BrCH_3COOCH_3$ (10 mL), the reaction mixture was stirred at 65 °C for 48 h. After cooling the reaction to room temperature, ammonium chloride was added and the reaction was quenched by addition of water. The resulting crude product was subjected to silica gel chromatography to give the intermediate product methoxycarbonyl-substituted BpP6 (5.12 mg, 83.7% yield). The ^{1}H NMR spectrum of methoxycarbonyl-substituted BpP6 was shown in **Fig. S1**.

Subsequently, the methoxycarbonyl-substituted BpP6 (1.4 g, 1 mmol) and NaOH (1.5 g, 37.5 mmol) were dissolved in water/ethanol (200 mL, *v*/*v* = 1/1), and stirred at 60 °C overnight. After cooling the reaction to room temperature, the solid was obtained by filtration, and then dissolved in ammonium hydroxide (20 mL). Finally, the solution was concentrated to give product AWBpP6 (71.8 mg, 50.5% yield). The ^{1}H NMR and ^{13}C NMR spectrum, and electrospray ionization mass spectrum of AWBpP6 were shown in **Fig. S2-4**, respectively. HRMS: $[C_{70}H_{59}O_{24}]^-$: 1283.33963. Found: 1238.33875.

Synthesis of polymer PPC

The polypeptide PPC was prepared by ring-opening polymerization (ROP). The

pyridinium-terminal-modified poly(ethylene oxide) (PPC) was synthesized by the ROP of $_L$-NBC-NCA using 1-(4-aminobutylpyridine hexafluorophosphate (Py-NH_2) as an initiator in DMF solution at room temperature. Briefly, $_L$-NBC-NCA (200.0 mg, 0.025 mmol) was dissolved in 2.0 mL DMF under N_2 atmosphere, and then a degassed solution of Py-NH_2 (7.5 mg, 0.71 mmol) in DMF was added. The resulting solution was stirred vigorously at room temperature for 48 h and then precipitated dropwise into a large excess of diethyl ether (16 mL). The white precipitate was centrifuged and dried under vacuum at 35 °C to give 178.5 mg of PPC (yield: 86%). The ^{1}H NMR spectrum of PPC was shown in **Fig. S5**.

Synthesis of polymer PPNC

The PPC solution (0.5 mg/mL) in 100 mL of DMF/CH_3CN (v : v = 4 : 1) was irradiated under a high pressure mercury lamp (λ = 365 nm, 150 W) for 12 h to achieve complete photocleavage of NB groups. The solution was concentrated to about 2 mL and then precipitated dropwise into a large excess of ether (16 mL). The brown precipitate was centrifuged and dried under vacuum at 35 °C to give the intermediate product (42 mg, 84% yield). Then, 80 μL TBN was added into 2 mL DMF solution of the 120 mg intermediate product and kept stirring in dark at room temperature. After stirring for 24 h, the resulting solution was precipitated dropwise into diethyl ether. The orange precipitate was centrifuged, rapidly dried in high vacuum, and finally stored in dark to give 99.6 mg of PPNC (yield: 84%). ^{1}H NMR spectrum and GPC trace of PPNC was shown in **Fig. S6** and **S7**. M_n = 927, M_w/M_n = 1.2.

Fabrication of supramolecular polypeptide nanomedicine BPC/DOX-ICG

Generally, PPNC (3.5 mg), DOX (1.5 mg) and ICG (1.5 mg) were dissolved in DMF (0.5 mL), and then stirred for 6 h in the dark. Thereafter, AWBpP6 (1.35 mg) solution was added into the above solution under vigorous stirring overnight. Then, the mixture solution was transferred into dialysis tube (MWCO 3500 Da) by dialysis against distilled water for 24 h to give the supramolecular polypeptide nanomedicine BPC/DOX-ICG. Similarly, the BPC/DOX and BPC/ICG were fabricated without ICG and DOX, respectively.

***In vitro* drug release**

The solution of BPC/DOX-ICG (2.0 mL, 0.5 mg/mL) was placed into dialysis tube (MWCO 3500 Da), followed by dialysis against PBS (20 mL, pH 5.0 or 7.4). As for the groups with irradiation, the above samples were irradiated with 808 nm laser (1.0 W/cm^2) for 5 min at selected time intervals. The original dialysate was replaced with fresh PBS at predetermined time. The collected dialysate was analyzed by UV-Vis spectroscopy to calculate the amount of released DOX.

***In vitro* cytotoxicity**

MCF-7 or MCF-7/ADR cells suspension in DMEM was placed in a 96-well plate at a density of 1×10^4 cells/well (200 μL) and incubated for 24 h. Subsequently, the medium in each well was replaced with fresh medium (pH 6.5 or 7.4) containing DOX, BPC/DOX, BPC/ICG or BPC/DOX-ICG with different concentrations. After incubation for 4 h, the irradiation groups were irradiated with 808 nm NIR (1.0 W/cm^2) for 5 min. Following culture for another 48 h, the cytotoxicity was determined by using MTT assays by a Microplate Reader (Elx800, BioTek Company).

In vitro cell internalization

The cell internalization of free DOX, BPC/DOX, BPC/ICG or BPC/DOX-ICG was investigated by confocal laser scanning microscope (CLSM). MCF-7 or MCF-7/ADR cells was seeded into 6-well tissue culture plate at a density of 5.0×10^5 cells/well, and incubated for 24 h. Next, the fresh medium (pH 6.5 or 7.4) containing free DOX, BPC/DOX, BPC/ICG or BPC/DOX-ICG with same drug concentration (5 μg/mL DOX equiv. and 3.51 μg/mL ICG equiv.) was added to each well for selected time with or without the 808 nm NIR irradiation (1.0 W/cm^2, 5 min). Then, the medium containing drugs was removed, and the cells were washed with PBS, stained with Hoechst33342 for 15 min, and respectively imaged by CLSM for cell internalization.

In vivo parmacokinetics analysis

The Laboratory Animal Centre of Nantong University performed the experiments using animals with approval from Nantong University. The pharmacokinetics analysis was performed using SD rats that were randomly divided into two groups (n = 4). The free DOX, BPC/DOX and BPC/DOX-ICG at equivalent DOX concentrations (8 mg/kg) were injected via the tail vein. The orbital vein blood (0.3 mL) was obtained at a predetermined time, harvested by centrifugation, and frozen at -20 °C. The DOX level in blood was measured by fluorescence spectroscopy.

In vivo antitumor activity

The MCF-7/ADR tumor-bearing mice with a tumor volume (60-80 mm^3) were spontaneously assigned into six groups (n = 4), and then intravenously injected at 0 day with PBS, free DOX, BPC/DOX, BPC/ICG or BPC/DOX-ICG at DOX equivalent dose

of 5 mg/kg and ICG dose of 3.51 mg/kg. As for the BPC/ICG+NIR and BPC/DOX-ICG+NIR groups, the tumor sites were irradiated with 808 nm NIR laser (1.0 W/cm^2, 5 min) at 12 h post-injection. The tumor volume (V) is calculated according to the following equation: V = 1/2 × length× width^2. The tumor inhibitory rates (TIR) is calculated by the equation: TIR (%) = 100 × (mean tumor volume of the PBS group - mean tumor volume of others)/(mean tumor volume of the PBS group). At the end of the treatment, all tumors and major organs (heart, liver, spleens, lung, and kidneys) were dissected for histological examination by H&E staining and TUNEL assays.

Statistical analysis

The data was expressed as mean ± standard deviations (S.D) using GraphPad Prism software 5.0. The two-tailed analysis of variance and the Student's t-test were used to determine statistical significance. A probability (P) value < 0.01 was indicated to be significant, and P < 0.001 was highly significant.

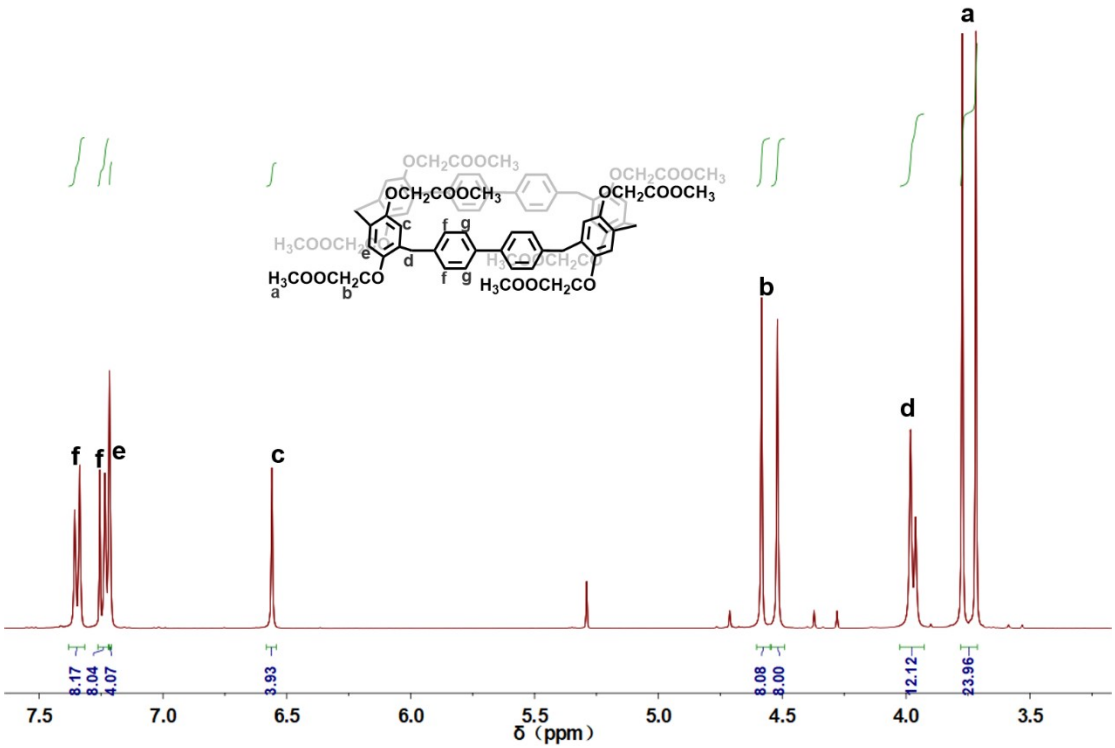

Fig. S1 ^{1}H NMR spectra of methoxycarbonyl-substituted BpP6 ($CDCl_3$).

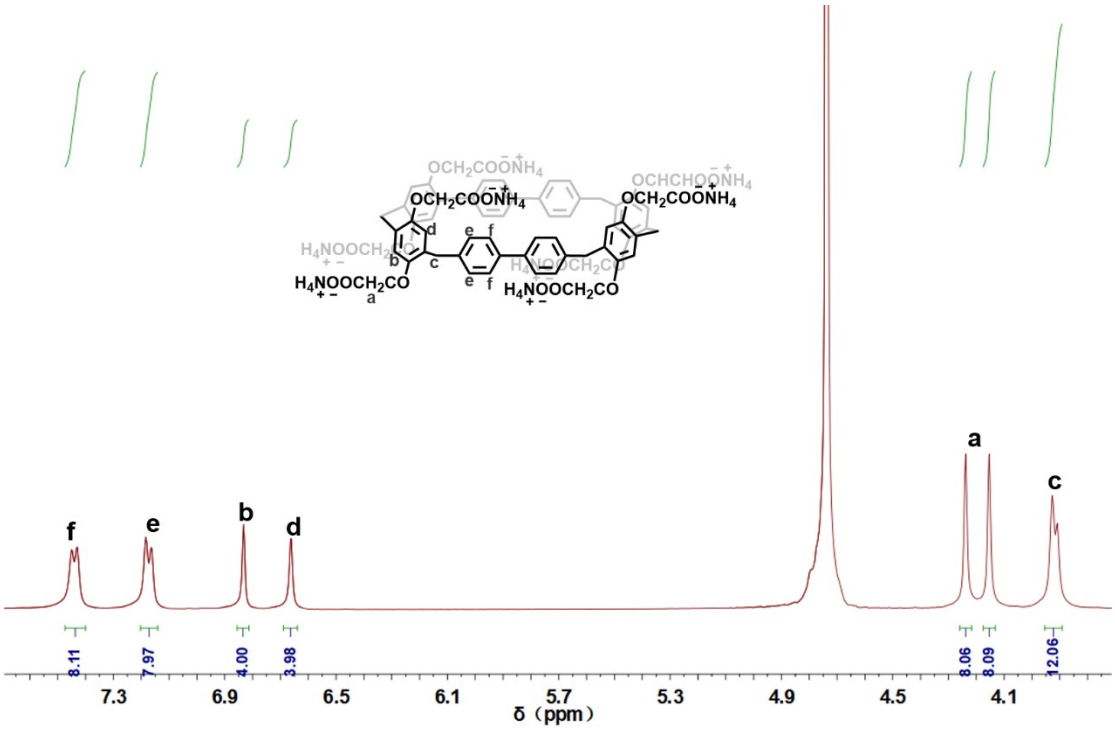

Fig. S2 ^{1}H NMR spectra of AWBpP6 (D_2O).

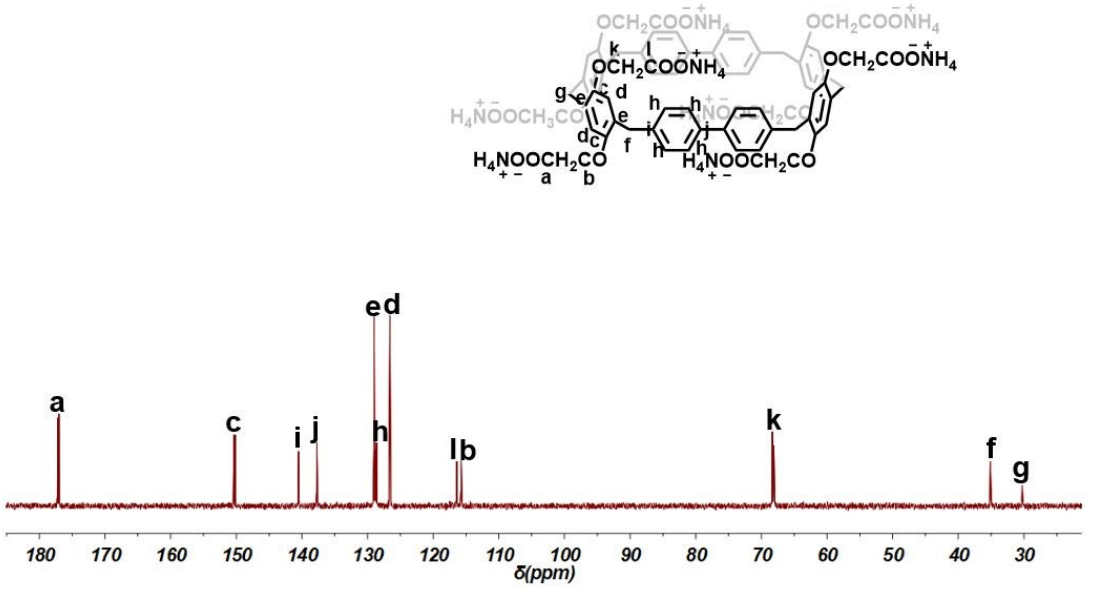

Fig. S3 ^{13}C NMR spectra of AWBpP6 (D_2O).

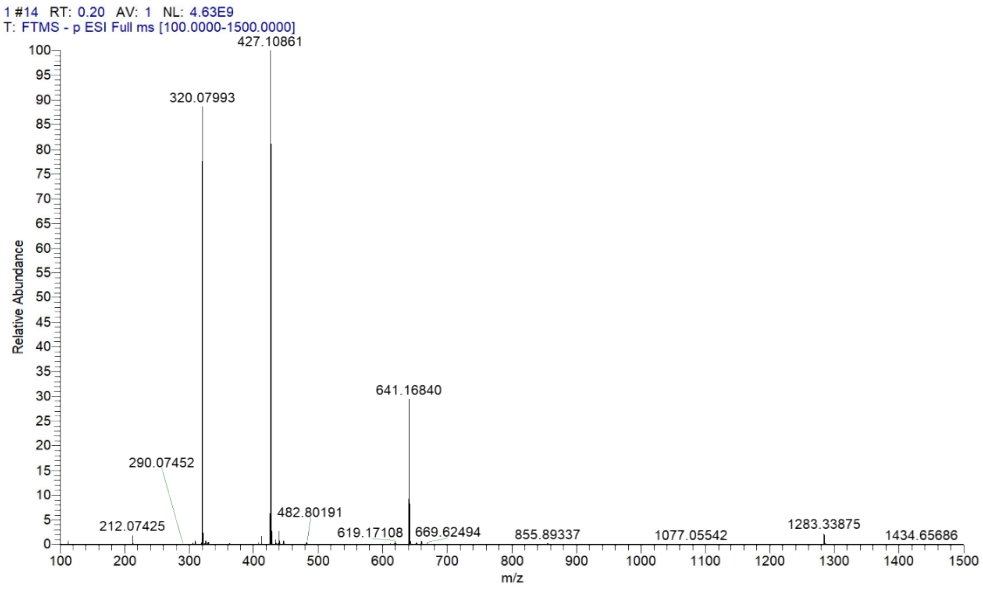

Fig. S4 Electrospray ionization mass spectrum of AWBpP6.

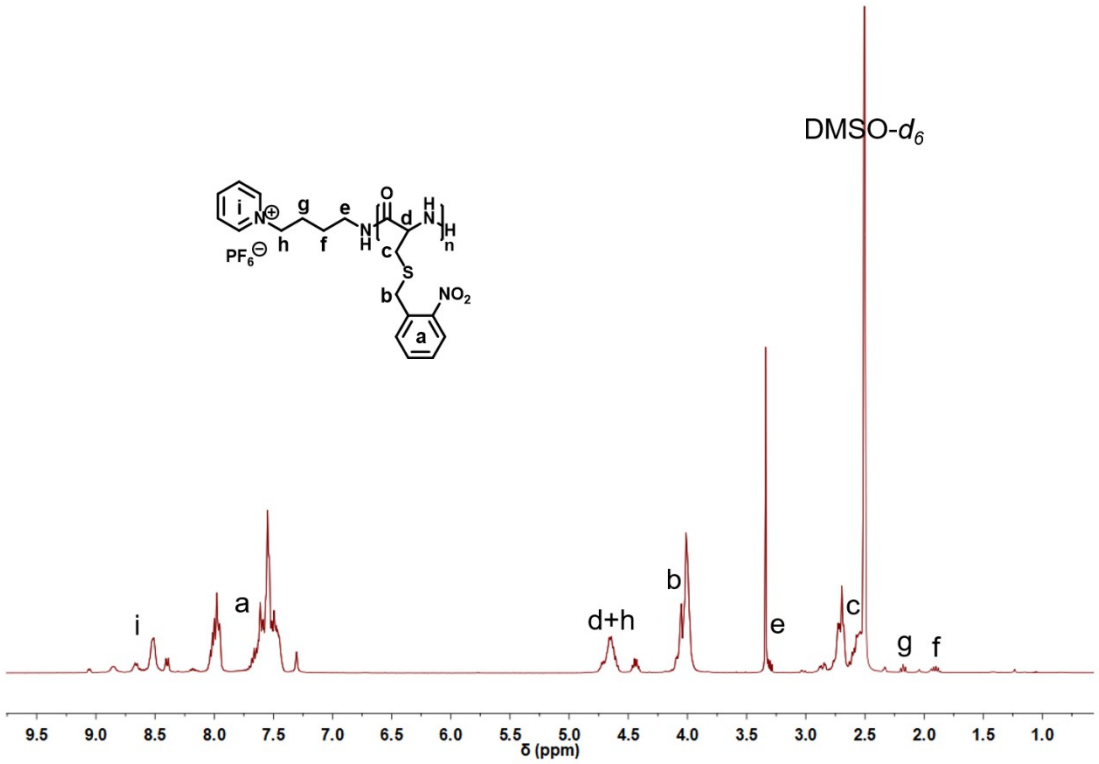

Fig. S5 ^{1}H NMR spectra of PPC (DMSO-d_6).

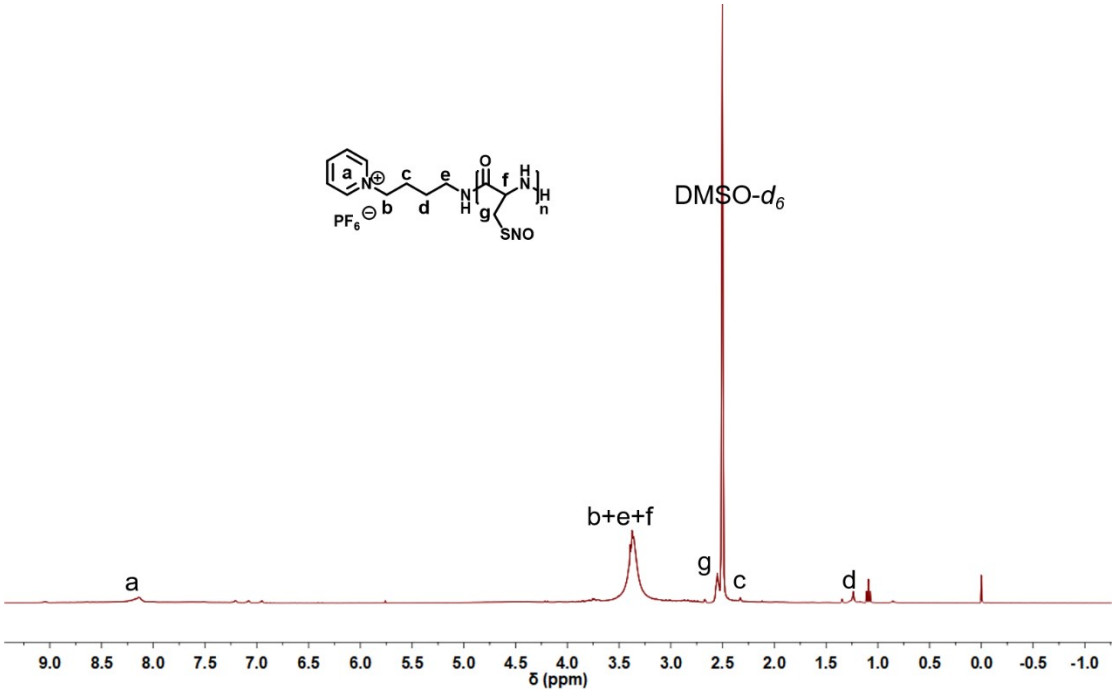

Fig. S6 ^{1}H NMR spectra of PPNC (DMSO-d_6).

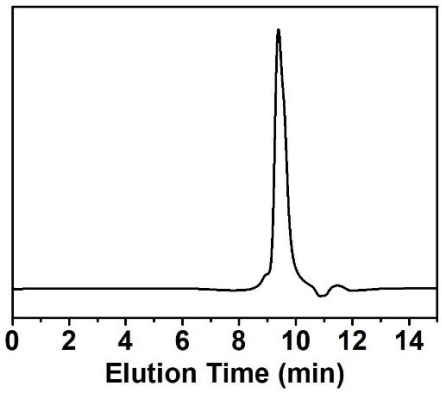

Fig. S7 GPC trace of PPNC.

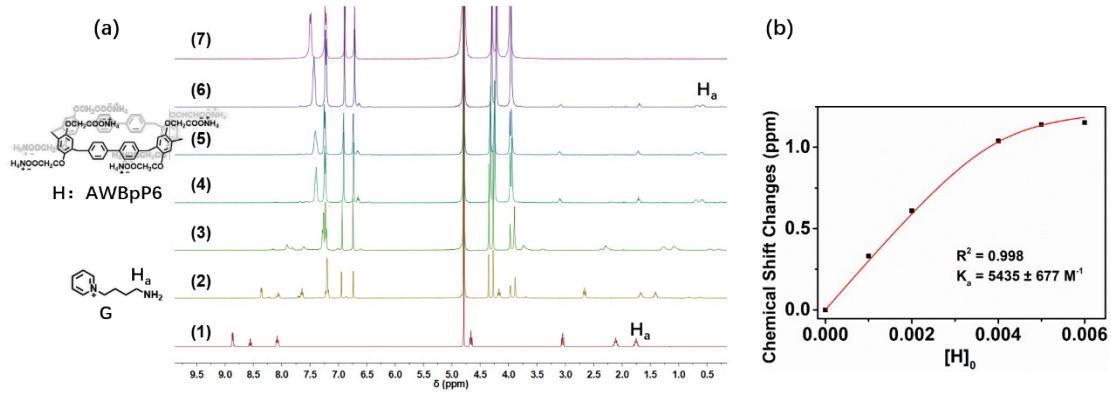

Fig. S8 (a) ^{1}H NMR spectra (D_2O, 293K, 400MHz) of model guest **G** at a concentration of 4 mM upon different concentration of host **H** (AWBpP6) (1) 0.0 mM, (2) 1.0 mM, (3) 2.0 mM, (4) 4.0 mM, (5) 5.0 mM, and (6) 6.0 mM, and (7) host **H** at a concentration of 4 mM without **G**. (b) The chemical shift changes of H_a on **G** upon addition of **H**. The red solid line was obtained from the non-linear curve-fitting.

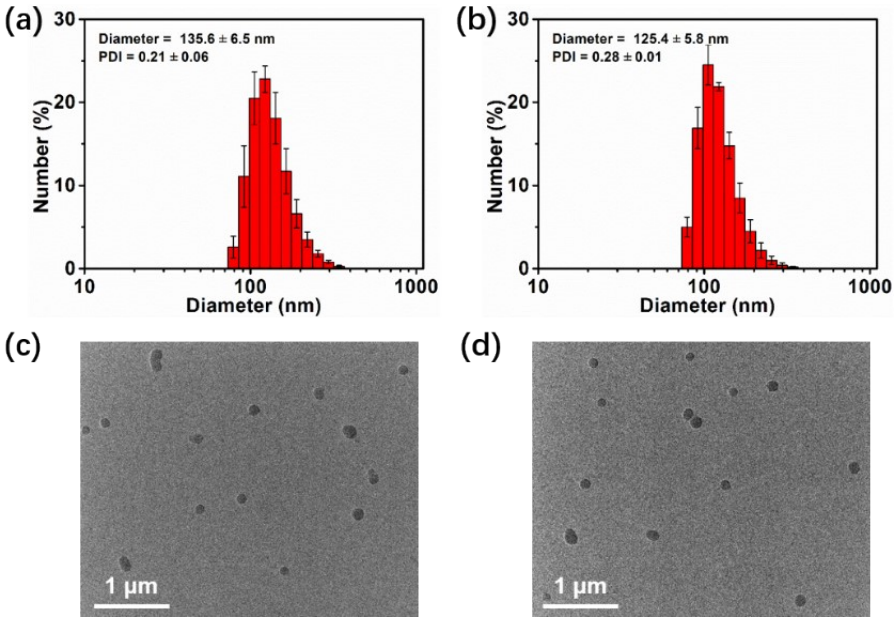

Fig. S9 DLS data and TEM image of BPC/DOX (a, c) and BPC/ICG (b, d).

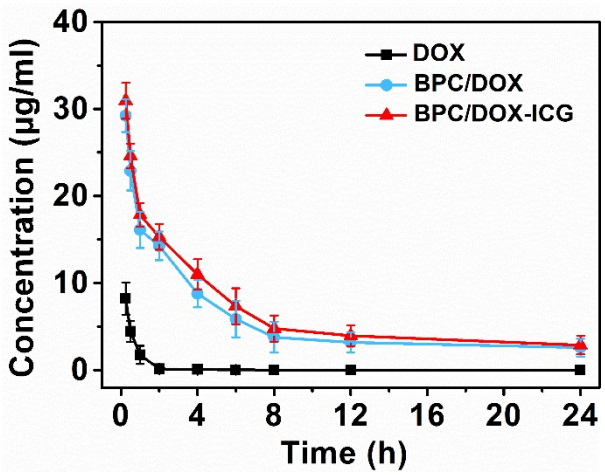

Fig. S10 Representative plasma concentration-time profiles of free DOX, BPC/DOX and BPC/DOX-ICG.

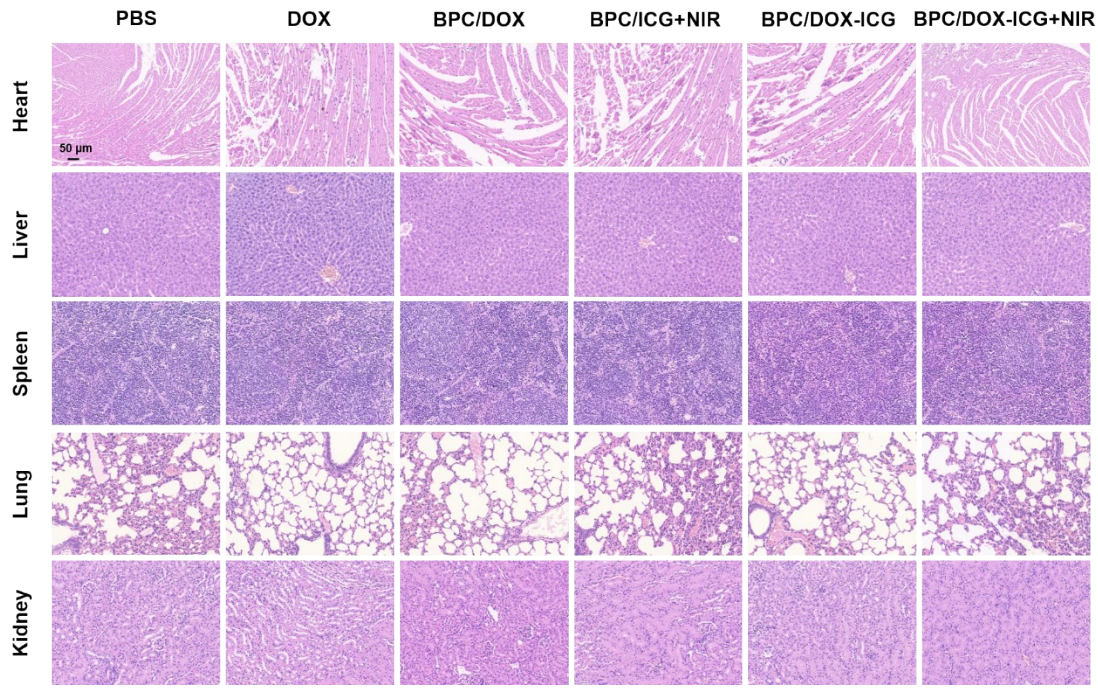

Fig. S11 H&E-stained tissue sections from the major organs (heart, liver, spleen, lung, and kidney) dissected after 20 day treatments (magnification × 400).

Table S1 Characterizations of BPC/DOX, BPC/ICG and BPC/DOX-ICG.

Sample	Diameter[a] (nm)	Zpotential[a] (mV)	IC_{50} (PTT-NO)[b] (μg/mL)	IC_{50} (CT)[b] (μg/mL)	IC_{50} (PTT-NO-CT)[c] (μg/mL)	CI[d]
BPC/DOX	135.6 ± 6.5	1.2 ± 0.2	--(MCF-7) -- (MCF-7/ADR)	1.92 31.76	--	--
BPC/ICG	125.4 ± 5.8	-7.2 ± 0.3	0.99 (MCF-7) 1.08 (MCF-7/ADR)	--	--	--
BPC/DOX-ICG	175.9 ± 2.1	-0.7 ± 0.1	--(MCF-7) -- (MCF-7/ADR)	--	0.14 + 0.49 (DOX) 0.18 + 0.61 (DOX)	0.58 0.56

[a]The mean diameter of nanomedicine is determined by DLS;

[b]IC_{50} is calculated from Fig. 4a, b by GraphPad Prism 6 software;

[c]As for PTT-NO-CT, the former value represents the combination PTT-NO and the latter one is the combination CT;

[d]CI represents the combination index.

Supplementary References

[1] Y. Ding, C. Du, J. Qian, et al., Polym. Chem. 9 (2018) 3488-3498.

[2] J.-R. Wu, C.-Y. Wang, Y.-C. Tao, et al., Eur. J. Org. Chem. 11 (2018) 1321-1325.