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Integration of porous coordination network and black phosphorus nanosheets for improved photodynamic therapy of tumor

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Experimental Procedures

Materials. The black phosphorus crystals were purchased from XFNANO Materials Tech Co., Ltd. N-Hydroxysuccinimide (ethylene glycol) folic acid (NHS-PEG-FA, Mw 2000) and fluoresceine isothiocyanate-ethylene glycol-folic acid (FITC-PEG-FA Mw 2000) was purchased from Shanghai Ponsure Biotech, Inc. Tanespimycin (17-AAG), 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), Methyl 4-formylbenzoate, benzoic acid, pyrrole, and propionic acid were purchased from Sigma-Aldrich. Tetrahydrofuran (THF), potassium hydroxide (KOH), and N, N′-dimethylformamide (DMF) were purchased from Shanghai Chemical Co. (Shanghai, China) and used without further treatment. (China). Propidium iodide (PI) were purchased from Beyotime Biotechnology (China). 2′, 7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from R&D Systems. Calcein was obtained from Shanghai Yisheng Biological Technology Co., Ltd. (China). Dulbecco's phosphate buffered saline (PBS), trypsin, Dulbecco's modified Eagle's medium (DMEM), RMPI 1640, fetal bovine serum (FBS), 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium-bromide (MTT), and penicillin-streptomycin were purchased from Lonza Group Ltd. (Switzerland). All other reagents and solvents were analytical grade and used directly.

Apparatus. SEM images were obtained by using scanning electron microscope (Sigma). TEM images were obtained from Tecnai G^2 20 TWIN transmission electron microscope. The UV-vis absorbance was measured by UV-vis spectroscopy (Lambda Bio40). Zeta potentials and particle size were measured by a zeta sizer (Nano ZS, Malvern Instruments). TGA was measured by Pyris1 thermogravimetric analyzer

(PerkinElmer). Electron Paramagnetic Resonance (EPR) spectrum was conducted by Bruker EMX ESR spectrometer (Billerica, MA). Confocal laser scanning microscope (CLSM) images were obtained on a super-resolution laser confocal (Leica) scanning confocal microscope. Flow cytometry (BD FACSAria TM III) was used to analyze the cell death. The *in vivo* imaging experiments were conducted by using IVIS imaging systems (PerkinElmer). Blood routine was measured by Auto Hematology Analyzer (MC-6200VET). Blood biochemistry analysis was examined by biochemical auto analyzer (MNCHIP, Tianjin, China).

Synthesis of ligand. The tetrakis (4-carboxyphenyl) porphyrin (H_2TCPP) ligand was synthesized according to our previous work.[S1] Firstly, 6.9 g of methyl *p*-formylbenzoate was added into a three necked round-bottom flask containing 100 mL of propionic acid, and 3.0 g of pyrrole was instilled in the flask after the mixture were refluxed, and then the solution was refluxed for 12 h in darkness. After the reaction mixture was cooled to room temperature, crystals were collected by centrifugation and washed with DMF for several times to obtain purple crystals. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 8H), 8.43 (d, 8H), 8.28 (d, 8H), 4.11 (s, 12H), -2.82 (s, 2H) (Figure S1). Ulteriorly, the obtained ester was dispersed in mixed solvent consisting of 25 mL THF and 25 mL MeOH, and then a solution of 2.6 KOH in 25 mL H_2O was added. The mixture was refluxed for 12 h in dark. The solvent was removed after cooling down to room temperature, and 200 mL water was poured into the mixture and the mixture was heated until the solid was fully dissolved, then the homogeneous solution was acidified with 1 M HCl until no further precipitate was detected. The brown solid was collected by centrifugation,

washed with water for several times and dried in vacuum. ^{1}H NMR (400 MHz, DMSO-d6) δ 13.21 (s, 4H), 8.86 (d, 8H), 8.33 (d, 16H), -2.82 (s, 2H) (Figure S2). ESI-MS m/z (%): 791.4 [M+1] (Figure S3).

Synthesis of porphyrinic Zr-MOFs (PCN). Zr (IV)-based porphyrinic metal-organic frameworks were synthesized according to previous research.[S2,S3] 100 mg tetrakis (4-carboxyphenyl) porphyrin (TCPP), 300 mg $ZrOCl_2 \cdot 8H_2O$ and 2.2 g benzoic acid were dissolved in 100 mL N, N-dimethylformamide (DMF). Then the mixture was stirred at 90 ℃ for 5 h. After the reaction was completed, the PCN nanoparticles were collected by centrifugation of 11000 rpm for 30 min and washed by fresh DMF for three times. The resulting PCN nanoparticles were dispersed in DMF for following experiment.

Preparation of 17-AAG loaded PCN. Before loading the HSP90 inhibitor, tanespimycin (17-AAG), PCNs were washed by ethanol for several times and dispersed in ethanol (2 mg mL^{-1}), and then 40 mg tanespimycin was added into 20 mL brown vial containing 5 mL ethanol, and after that 20 mg PCN dissolved in 10 mL ethanol were added into the vial. The mixture was stirred in room temperature for 24 h. The drug-loaded PCNs were collected by centrifugation at 11000 rpm for 30 min. The unbonded 17-AAG was removed by washing with fresh ethanol for several times. Then, the drug-loaded PCNs were washed by distilled water for several times and dispersed in distilled water for the next step.

Preparation of plack phosphorus nanosheets (BP). BP nanosheets were obtained from bulk black phosphorus in ethanol by ultrasound in ice bath for 16 h. The resulting brown suspensions was centrifuged at 5000 rpm for 10 min to remove the residual

unexfoliated black phosphorus crystals, and the supernatant was collected and centrifuged again at 11000 rpm for 10 min to obtain the BP nanosheets. After that the BP nanosheets were dispersed in ethanol and stored at 4℃ for further use.

Synthesis of APCN/BP. The 20 mg drug loaded PCN in ethanol and 5 mg BP in ethanol were poured in the brown vial, and the mixture solution was stirred at room temperature. After stirring for 24 h, the mixture solution was centrifuged at 11000 rpm for 10 min and washed with distilled water, and then dispersed in distilled water and stored at 4℃ for further use.

Preparation of FA coated APCN/BP. Before coating FA, 20 mg PEI in distilled water and 25 mg NHS-PEG-FA in distilled water were poured into the vial and stirred for 24 h. After dialysis and lyophilization, the resulting PEI-PEG-FA in distilled water was mixed with APCN/BP in distilled water. The mixture solution was stirred in a brown vial for 24 h. After centrifugation at 11000 rpm for 10 min, the FA coated APCN/BP was washed for several times and dispersed in distilled water and stored at 4℃ for the following experiment.

Degradation of PCN and drug release evaluation. The nanoplatforms were biodegradable owing to the degradable capacity of BP that release phosphate anion, which could expedite the degradation of PCN and resulted in internal drug release. Firstly, the degradation of nanoplatforms in aqueous solution with pH 7 or pH 5 were observed by TEM observation for 1, 3, 5, 7, and 15 days respectively. Then the degradation of BP was revealed by photographing the aqueous solution of BP at pH 7 and pH 5 for four days and recording the corresponding UV optical absorption.

Secondly, the degradable ability of the nanoplatforms were conducted by photographing the aqueous solution of APCN/BP-FA in pH 7 and pH 5 for seven days and recording the corresponding UV optical absorption. Then, pH-dependent drug release was carried out in suspending APCN/BP-FA in the pH 7 and pH 5 aqueous solution (10 mL) and incubated in 37 °C, respectively. The UV optical absorption of the supernatant of incubation solution was measured by a Lambda Bio40 UV/vis spectrometer (Perkin-Elmer) within given time intervals.

***In vitro* ROS evaluation by DCFH.** The ROS generation produced by BP, PCN, and PCN/BP under illumination with 660 nm was evaluated by the fluorescence of DCFH. Firstly, the 2 μL DCFH-DA (10 mM) was added into a 2 mL vial containing 80 μL NaOH (0.01 M) solution and incubated for 30 min for complete hydrolysis, then 918 μL PBS was added to the mixture and then a 20 μM of DCFH aqueous solution was obtained. Then, four capped vials were marked with A, B, C, and D (A represented the blank group, B was the group of BP, C was the group of PCN, and D represented the group of PCN/BP). The blank group consisted of 980 μL deionized water and 20 μL DCFH stock solution. The vial B, C, D consisted of 980 μL BP (20 μg mL^{-1}), PCN (20 μg mL^{-1}) and PCN/BP (20 μg mL^{-1}), respectively. The fluorescence of DCFH was measured after the irradiation at 660 nm for 0 s, 2 s, 10 s, 20 s, 30 s, and 60 s respectively.

***In vitro* ROS evaluation by ESR spectroscopy.** The generated ROS were detected by ESR measurements, conducted by Bruker EMX ESR spectrometer (Billerica, MA) at room temperature. Briefly, three clean vials were marked as the control group, the PCN

group, and the PCN/BP group. The group of BP was not included due to its negligible $^1O_2$ generation. 20 mM TEMPO was employed as the $^1O_2$ trapping agent for $^1O_2$ generation evaluation. Similarly, 980 μL deionized water and 20 μL TEMPO were add to the vial control group; 980 μL 50 μg mL^{-1} PCN and 20 μL TEMPO were add to the vial PCN group; 980 μL 50 μg mL^{-1} PCN/BP and 20 μL TEMPO were add to the vial PCN/BP group. The ESR spectra were recorded after 660 nm irradiation for 5 mins.

Cell culture. Colon adenocarcinoma cells (CT26), murine mammary carcinoma cells (4T1), and human cervix carcinoma cells (HeLa) were incubated in RMPI 1640 medium containing 10% FBS and 1% antibiotics (penicillin-streptomycin, 1×10^4 U mL^{-1}). African green monkey kidney fibroblast cells (COS7) and human breast cancer cells (MCF-7) were incubated in DMEM medium with 10% FBS and 1% antibiotics (penicillin-streptomycin, 1×10^4 U mL^{-1}) . All cells were cultured at 37 °C in a humidified atmosphere containing 21% O_2 and 5% CO_2.

Cellular uptake. To evaluate the cumulative degree of APCN/BP-FA, MCF-7 cells in DMEM were seeded into petri dish and incubated for 24 h (37 °C, 5% CO_2). After that, PCN (50 μg mL^{-1}), APCN/BP (50 μg mL^{-1}), and APCN/BP (50 μg mL^{-1}) in DMEM medium were respectively added to the petri dish to replace the medium and incubated with the cells for 6 h. Then, the cells were washed three times with PBS and observed via CLSM and analyzed by flow cytimetry (BD FACSAria TM III).

The evaluation of *in vitro* ROS production. The ROS production in living cells was also assessed. MCF-7 cells were seeded into Petri dishes and incubated for 24 h. Then, the medium was respectively replaced by fresh DMEM containing PCN (50 μg mL^{-1})

and PCN/BP (50 μg mL^{-1}) and incubated for 5 h. Then, the cells were washed with PBS and incubated with DCFH-DA (5 μM) for 30 min, after which the cells were incubated shielded from light or irradiated for 5 min (660 nm LED light, 29.8 mW cm^{-2}). Then, the treated cells were observed by CLSM.

***In vitro* HSP90 expression.** To evaluate the expression of HSP90 after PDT treatment, MCF-7, 4T1, Hela, CT26 and COS7 cells were seeded into two 6-well plates with a density of 5 × 10^5 for each well, respectively. After incubation for 24 h at 37 °C with a suitable atmosphere (21% O_2 and 5% CO_2), the cells were co-incubated with PCN (50 μg mL^{-1}) for 5 h. Then one of the 6-well plates was treated with 660 nm for 5 min while the other one was kept in darkness. After that, before western blot analysis, the cells were incubated for another 6 h. To evaluate the HSP90 inhibition capacity of 17-AAG after PDT treatment, MCF-7 cells were seeded into 6-well plates with a density of 5 × 10^5 for individual well, respectively. After incubation for 24 h under the same conditions, the cells were co-incubated with fresh DMEM, DMEM containing APCN/BP (50 μg mL^{-1}), containing PCN (50 μg mL^{-1}), or containing PCN/BP (50 μg mL^{-1}) for 5 h. After exposed to 660 nm irradiation for 5 min, the cells were collected for western blot analysis after another 6 h incubation. Besides, to evaluate the expression of HSP90 and HSP90 inhibition capacity of the nanoplatforms after PDT treatment, MCF-7 cells were seeded into 6-well plates with a density of 5 × 10^5 for individual well, respectively. After incubation for 24 h, the medium was replaced by fresh DMEM containing BP (50 μg mL^{-1}), PCN (50 μg mL^{-1}), PCN/BP-FA (50 μg mL^{-1}), APCN (50 μg mL^{-1}), or APCN/BP-FA (50 μg mL^{-1}) for 5 h and then treated with

660 nm for 5 min. After another 6 h incubation, the cells were collected for immunofluorescent staining.

HSP90 related evading apoptosis. To evaluate the evading apoptosis capacity of HSP90, firstly, MCF-7, 4T1, CT26, and COS7 cells were seeded into two 96-well plates with a density of 5×10^3 cells per well. After incubation for 24 h, the cells were co-incubated with PCN (44 μg mL^{-1}) for 5 h. Then, one of the 96-well plates was treated with 660 nm for 5 min while another one was kept in darkness for evaluating the phototoxicity and dark toxicity of PCN. Then, all cells were incubated for 24 h. Subsequently, MTT (20 μL, 5mg mL^{-1}) was added into each well. After incubation for 4 h, the MTT containing culture medium was replaced with 150 μL DMSO. The absorbance was measured using a microplate reader (BIO-RAD 550). The cell viabilities (%) were calculated as follows: 100 × OD (samples)/OD (control), where OD (control) and OD (samples) represent the absorbance at 570 nm in the absence and presence of samples, respectively. In addition, to further investigate the expression of evading apoptosis protein, such as AKT, IGF-1R, and Survivin, MCF-7 cells were seeded into 6-well plates with a density of 5×10^5 for individual well, respectively. After incubation for 24 h, the cells were treated with DMEM, DMEM containing PCN (50 μg mL^{-1}), or DMEM containing APCN (50 μg mL^{-1}) for 5 h. After 660 nm irradiation for 5 min, the cells were collected for western blot analysis after another 6 h incubation.

Live/dead cell staining assay. MCF-7 cells were seeded into 6-well plates with a density of 5×10^5 in a well, respectively. After incubation for 24 h at 37 °C surrounding

a suitable atmosphere with 21% O_2 and 5% CO_2, the cells were co-incubated with BP (50 μg mL^{-1}), APCN (50 μg mL^{-1}), or APCN/BP-FA (50 μg mL^{-1}) for 5 h. With the treatment of PDT, the cells were irradiated at 660 nm laser for 5 min with a power intensity of 1 W cm^{-2}. The control group was the cells without any treatment. After that, all cells were treated with Calcein-AM (2 μM) and PI solutions (5 μM) in PBS buffer solution (1 mL per well) for 15 min at 37 °C with 5% CO_2. Then, all cells in 6-well plates were washed with PBS three times and pictured by CLSM ((FV1000-IX81, Leica, Germany). With the excitation of 488 nm (Calcein-AM) and 543 nm (PI), the corresponding emission spectra were collected using two different ranges of wavelength namely 510-540 nm (green) and 570-620 nm (red).

In vitro **cytotoxicity.** The cytotoxicity was measured by MTT assay. All cells were seeded into 96-well plates at a density of 5×10^3 cells per well. After incubation for 24 h, the cells were co-incubated with BP, APCN, APCN-BP and APCN-BP-FA at the concentration of 100 μg mL^{-1}. The cells were irradiated for 5 min (660 nm LED light, 29.8 W cm^{-2}) to evaluate the laser induced cytotoxicity. The cells without irradiation treatment were used as the control. Then, all cells were incubated for 24 h. After that, MTT (20 μL, 5mg mL^{-1}) was added into each well. After incubation for 4 h, the MTT containing culture medium was replaced with 150 μL of DMSO. The absorbance was measured using a microplate reader (BIO-RAD 550). The cell viabilities (%) were calculated as follows: 100 × OD (samples)/OD (control), where OD (control) and OD (samples) represent the absorbance at 570 nm in the absence and presence of samples, respectively.

***In vivo* image.** MCF-7 cancer bearing Balb/c nude mice were intravenously injected with PCN (100 μL, 1 mg mL^{-1}), PCN/BP (100 μL, 1 mg mL^{-1}), and PCN/BP-FA (100 μL, 1 mg mL^{-1}). After that, the fluorescence was observed at 0h, 2 h, 4 h, 6 h, 8 h, 12 h, 24 h, 36 h, 48 h, and 60 h using IVIS imaging systems. Moreover, the cancer tissues and the organs (heart, liver, spleen, lung, and kidney) were obtained from the sacrificed mice and observed by IVIS imaging systems (PerkinElmer).

The evaluation of the antitumor effect *in vivo*. When MCF-7 tumors volume grew to approximately 100 mm^3, the tumor-bearing mice were divided into five groups with five mice in each group. The mice were treated with 1) PBS, 2) APCN/BP, 3) BP+660nm, 4) APCN+660nm, 5) APCN/BP-FA+660nm. After tail vein injection for 36 h, PDT treatment groups (3-5) were irradiated with 660 nm light (1 mW cm^{-2}) for 3 minutes at tumor regions. To evaluate the antitumor effect, the relative tumor volume and relative body weight of all mice were measured every other day. The tumor volume was calculated by using the formula: $V = W^2L/2$, where W and L represented the shortest and longest diameters of tumors, respectively. After 14 days, all experimental mice were sacrificed, the tumor tissues and main organs (heart, liver, spleen, lung, and kidney) were removed for histological analysis.

Blood routine and blood analysis. BALB/c mice were tail vein injected with PCN, APCN-BP, and APCN/BP-FA (100 μL, 1 mg mL^{-1}), respectively. After 24 h, the blood was collected and analyzed by Auto Hematology Analyzer (MC-6200VET) and Blood Biochemistry Analyzer (MNCHIP POINTCARE). The mice without any treatment were used as the control.

Statistical analysis. The values are presented as mean ± standard deviation (SD), and the data were obtained based on at least three independent experiments. The statistical significance was obtained by two-tailed Student's tests. *P < 0.05, **P < 0.01, and ***P < 0.001.

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Tab. S1 Blood routine analysis of various groups treated with PBS, or BP, or PCN, or APCN/BP-FA.

	PBS	BP	PCN	APCN/BP-FA	Units
WBC	3.4	3.2	3.3	3.6	10^9 L^{-1}
Lymph	2.4	1.9	2	2.6	10^9 L^{-1}
Mid	0.5	0.4	0.4	0.3	10^9 L^{-1}
Gran	0.5	0.9	0.9	0.7	10^9 L^{-1}
RBC	8.91	7.78	8.25	6.12	10^12 L^{-1}
HGB	109	94	105	78	g L^{-1}
MCV	51.9	47.9	47.3	49.2	fL
MCH	12.2	12.1	12.7	12.7	pg
MCHC	235	252	268	259	g L^{-1}
HCT	46.2	37.3	39	30.1	%
PLT	830	770	817	596	10^9 L^{-1}

Tab. S2 Blood biochemical analysis various groups treated with PBS, or BP, or PCN, or APCN/BP-FA.

	PBS	BP	PCN	APCN/BP-FA	Units
TP	45.6	43.4	43.7	42.8	g L^{-1}
ALB	21.7	21.4	23.9	23	g L^{-1}
GLO	23.9	22	19.8	19.8	g L^{-1}
A/G	0.9/1	1.0/1	1.2/1	1.2/1	
TBIL	7.5	5	4.3	2.9	umol L^{-1}
ALT	97	88	57	46	U L^{-1}
AST	253	207	195	195	U L^{-1}
UREA	8.64	7.52	8.7	9.06	U L^{-1}
GLU	4.91	3.85	5.49	5.35	mmol L^{-1}

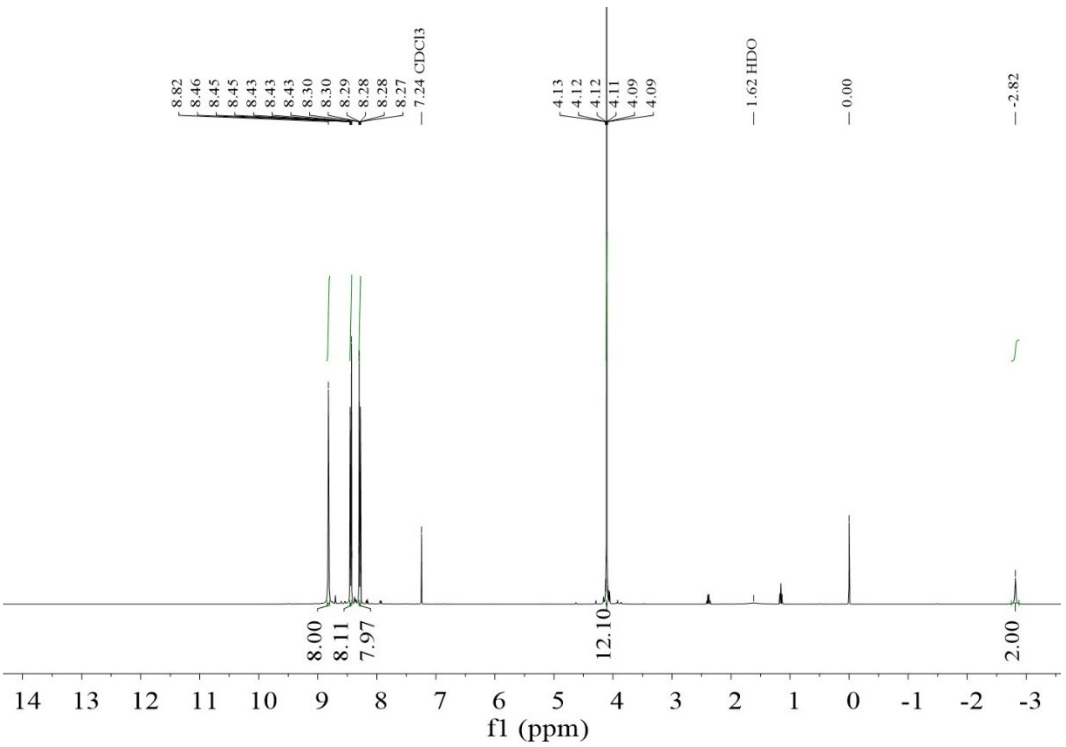

Fig. S1 ^{1}H-NMR of tetrakis (4-methoxycarbonylphenyl) porphyrin.

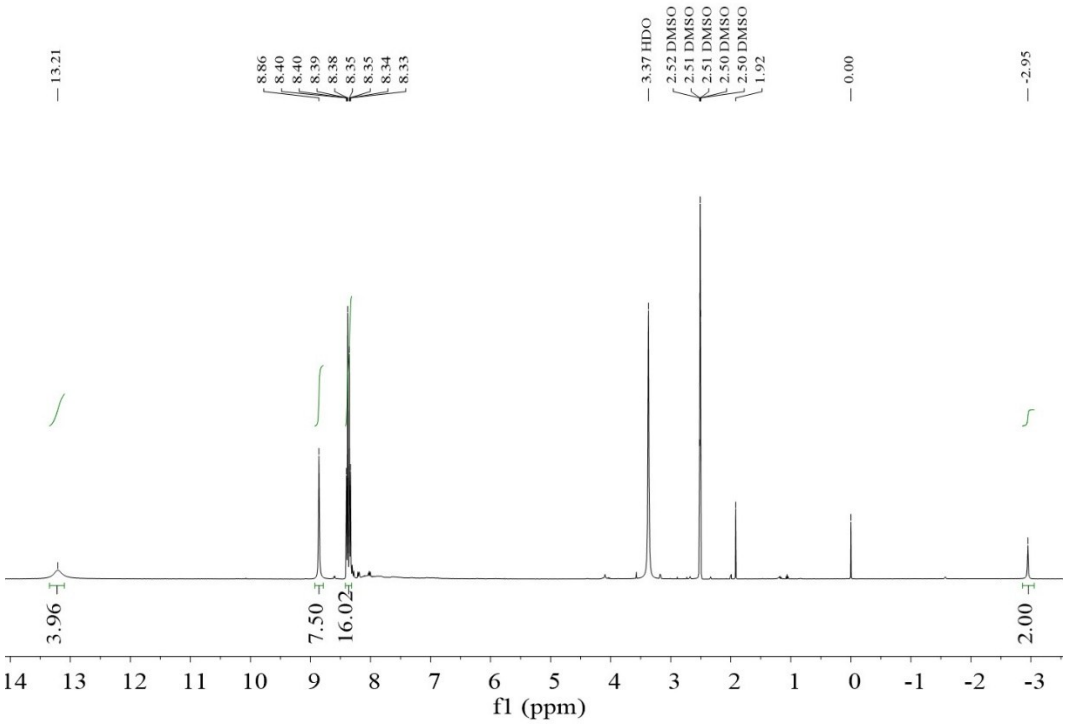

Fig. S2 ^{1}H-NMR of tetrakis(4-carboxyphenyl)porphyrin.

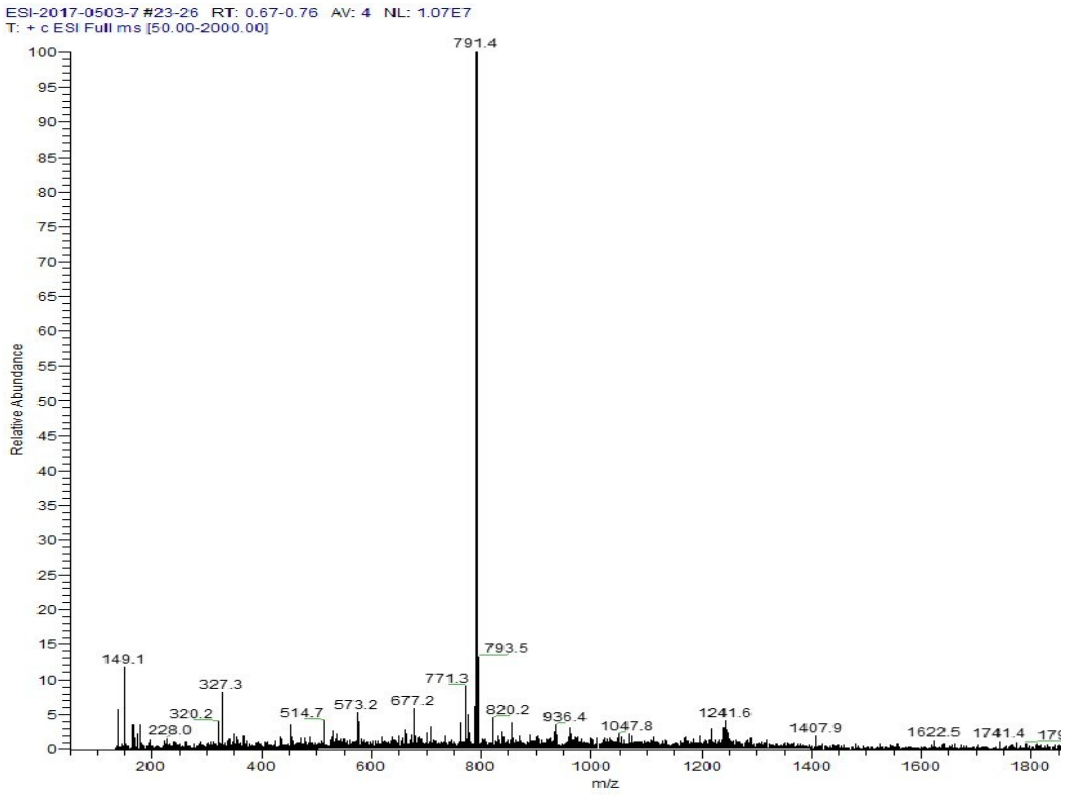

Fig. S3 ESI-MS of tetrakis(4-carboxyphenyl)porphyrin.

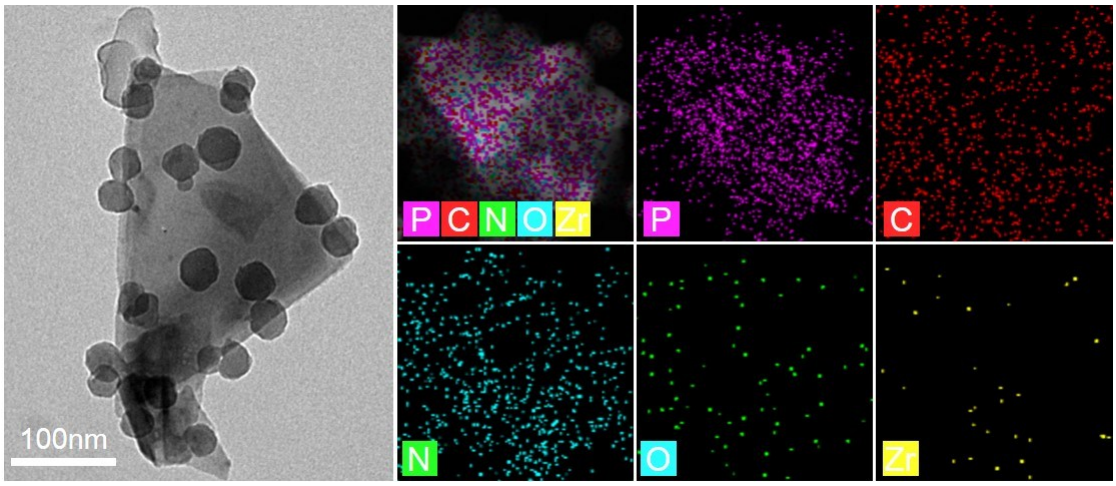

Fig. S4 HAADF-STEM image and the corresponding element mapping of PCN/BP.

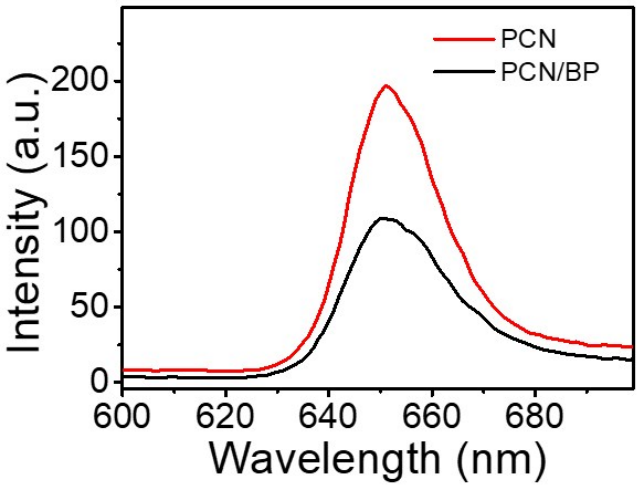

Fig. S5 The fluorescence of PCN and PCN/BP.

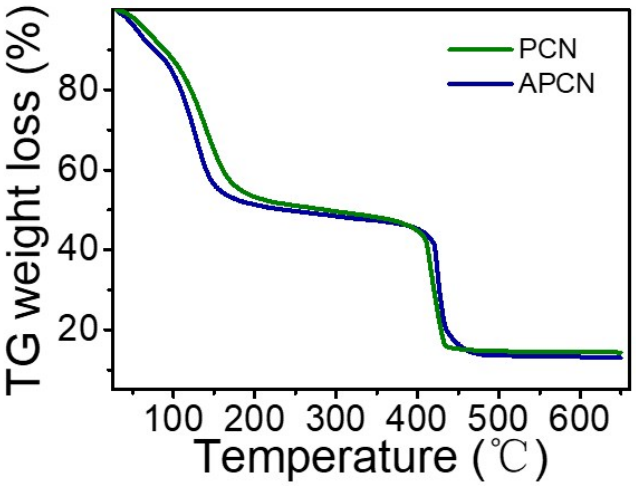

Fig. S6 TG weight loss profiles of PCN and APCN.

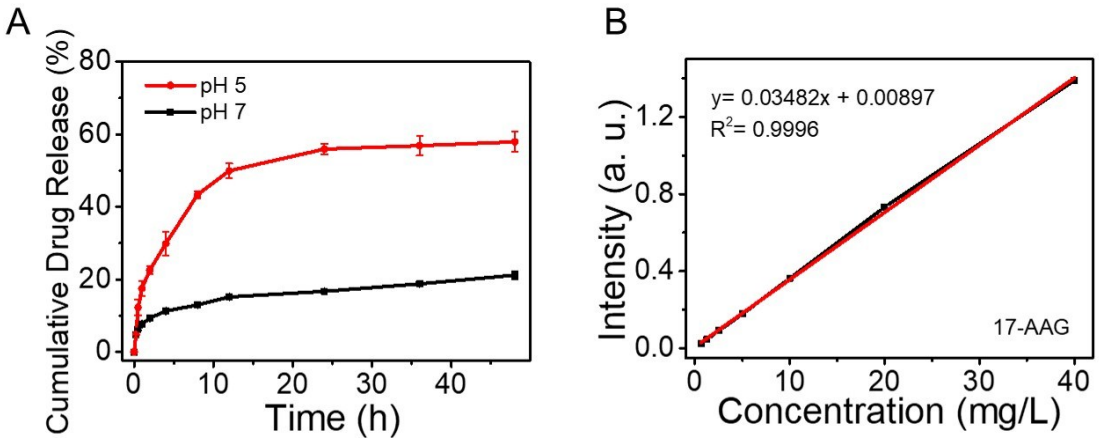

Fig. S7 (A) pH mediated drug release and (B) the standard curve of 17-AAG.

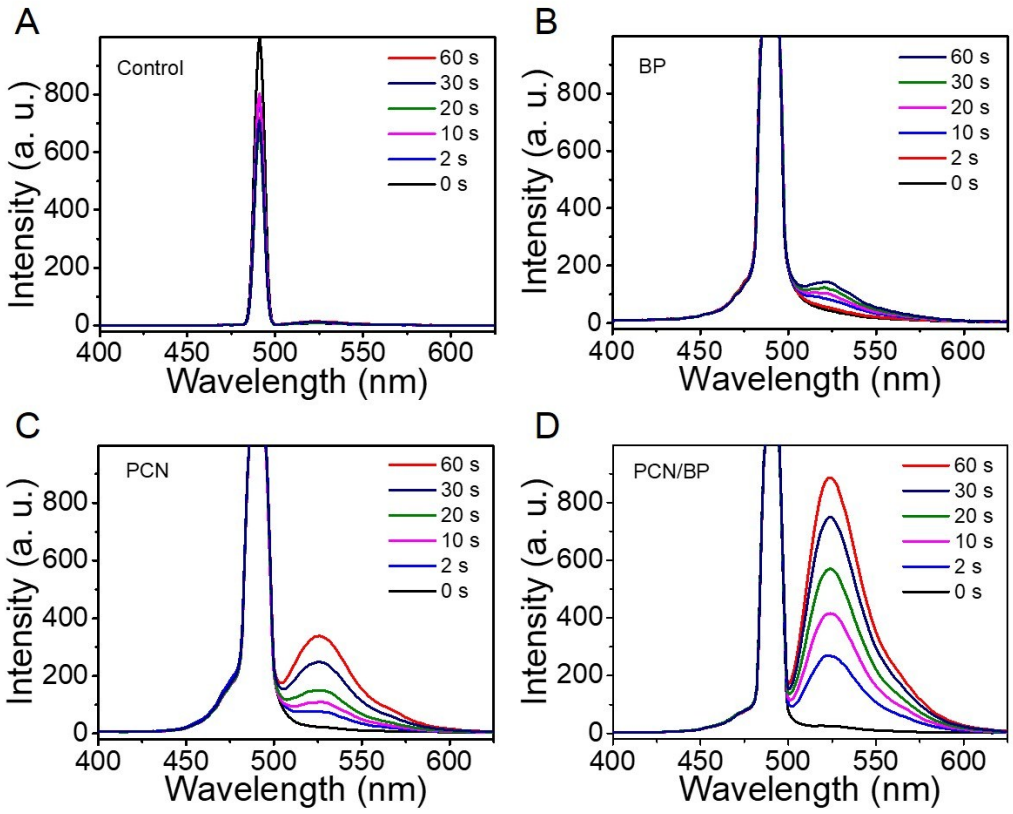

Fig. S8 Fluorescence spectra of DCFH solution containing (A) PBS (control), (B) BP, (C) PCN, and (D) PCN/BP under 660 nm irradiation for 2 s, 10 s, 20 s, 30 s and 60 s.

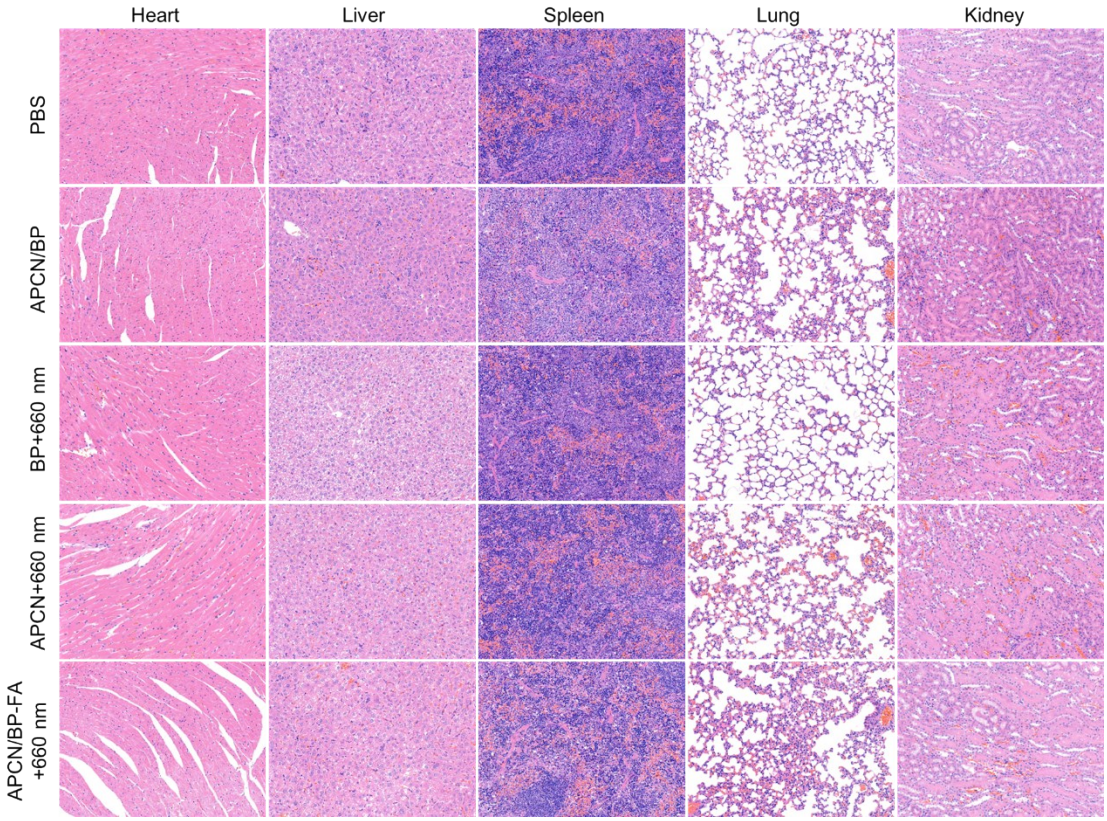

Fig. S9 H&E staining images of heart, liver, spleen, lung and kidney tissues after various treatments by tail vein injection on MCF-7 tumor-bearing mice. Different treated groups: PBS, APCN/BP, BP+660 nm, APCN+660 nm and APCN/BP-FA.