Supporting Information

Aptamer-functionalized hollow carbon nanospheres for targeted chemo-photothermal therapy of breast tumor

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Materials and methods

Materials

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide Hydrochloride (EDC•HCl), N-Hydroxysuccinimide (NHS), resorcinol (99.0%) and formaldehyde were purchased from Aladdin Reagent Co., Ltd. Hydrofluoric (HF) acid aqueous solution (40.0%), tetrapropyl orthosilicate (TPOS, 98.0%), DOX, $NH_3 \cdot H_2O$ and ethanol were obtained from Macklin Reagent Co., Ltd. COOH-PEG-COOH (Mw 4000) were acquired from Shanghai Yare Biotechnology Co., Ltd. Roswell Park Memorial Institute-1640 (RPMI-1640) medium and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Biosharp Reagent Co., Ltd. Fetal bovine serum (FBS) was obtained from Gibco Reagent Co., Ltd. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Penicillin-Streptomycin were acquired from Sigma Aldrich Co., Ltd. Calcein AM/PI cytotoxicity assay kit, Annexin V-FITC/PI apoptosis assay kit, Lyso-Tracker Green assay kit and Hoechst 33342 were acquired from Shanghai Beyotime Biotechnology Co., Ltd. Apt sequences (Table 1) were synthesized by Shanghai Sangon Biotech Co., Ltd. All chemicals were used without further purification. The Deionized water was obtained from a Millipore water purification system.

Table 1. Apt sequences.

Oligonucleotide	Sequence (5' to 3')
Apt-M	NH_2-$(CH_2)_6$-GCAGTTGATCCTTTGGATACCCTGGTTTTTTTTTT
Apt-Control (Apt-C)	NH_2-$(CH_2)_6$-ATTGCACTTACTATATTGCACTTACTATATTGCAC
Apt-M-FAM(Apt-MF)	NH_2-$(CH_2)_6$-GCAGTTGATCCTTTGGATACCCTGGTTTTTTTTTT-(6-FAM)
Apt-C-FAM(Apt-CF)	NH_2-$(CH_2)_6$-ATTGCACTTACTATATTGCACTTACTATATTGCAC-(6-FAM)

Preparation of HCNs

HCNs were prepared according to the previous report with slight modifications[36]. First, ethanol (17.5 mL),

$NH_3 \cdot H_2O$ (0.75 mL, 25 wt%) and deionized water (2.50 mL) were mixed and stirred at 30°C for 5 min, then TPOS (0.91 mL) was added and stirred for 15 min. Then, resorcinol (0.10 g) and formaldehyde (0.14 mL, 37 wt%) were added and stirred for 24 h. Subsequently, the resulting mixture was centrifuged and the precipitation was washed and dried in the air. Then, carbonization of the resulting mixture was performed at 700°C under nitrogen for 3 h. Finally, the SiO_2 was completely removed by HF (Please take personal protection when using this hazards reagent) etching for 4 h to obtain HCNs.

Preparation of HCNs/DOX

HCNs (5.0 mL, 1.0 mg mL^{-1}) was mixed with DOX aqueous solution (5.0 mL, 2 mg mL^{-1}) and stirred for 1 day in the dark. The mixture was centrifugally washed to remove excess unbound DOX. Finally, the supernatant was collected and the absorbance of DOX in the supernatant at 480 nm was determined. DOX loading efficiency (LE) was calculated by the equation:

$$LE\ (\%) = (W_a - W_b) / W_c * 100\%$$

In which W_a was the added mass of DOX, W_b was the mass of DOX in the supernatant, and W_c was the mass of HCNs.

Preparation of HCNs/DOX/PEG

HCNs/DOX (3.0 mg) were dispersed in 10.0 mL of deionized water under sonication, and then PEG (6.0 mg) was added into the solution under stirring for 6 h. Next, the mixture was centrifuged and washed to remove the excess unbound PEG.

Fabrication of HCNs/DOX/PEG/Apt-M

HCNs/DOX/PEG (2.0 mL, 1.0 mg mL^{-1}) were activated by EDC•HCl (500 μL, 500 mM) and NHS (500 μL, 100 mM) under stirring for 30 min. Meanwhile, the Apt-M (6 OD) was denatured at 85°C for 10 min, and then refolded in ice bath for 10 min. After that, the activated HCNs/DOX/PEG reacted with Apt-M under shaking for 5 h. Finally, HCNs/DOX/PEG/Apt-M were obtained by centrifugation and washed with water for 3 times.

Characterizations

Scanning electron microscopy (SEM) images were recorded using TESCAN MAIA3 microscope. The transmission electron microscopy (TEM) images, Energy Dispersive Spectroscopy (EDS) spectra and element mapping images were obtained using a JEOL JEM-2100F transmission electron microscope. Brunauer-Emmett-Teller (BET) surfaces area and pore size measurements were conducted using a Micromeritics ASAP 2020 PLUS HD88 specific surface area analyzer. X-ray photoelectron spectroscopy (XPS) analysis was carried out using a Thermo Scientific K-Alpha XPS energy spectrometer. Raman spectra were measured by a Renishaw Qontor spectrometer. X-ray diffraction (XRD) spectra were measured on a Rigaku Smart Lab SE diffractometer. Hydrated size distribution and zeta potential were obtained by Malvern Zetasizer Nano ZS90 nanometer particle size analyzer. Photothermal performances of the sample were detected and recorded using a FOTRIC 323pro infrared thermal imager. The confocal laser scanning microscopy (CLSM) images were recorded by an Olympus FV1200V inverted CLSM. Flow cytometry experiments were conducted on a Beckman Coulter CytoFLEX flow cytometer. UV-VIS absorption spectra were recorded by PerkinElmer Lambda35 spectrometer.

Photothermal performance

To measure the photothermal effect of HCNs/DOX/PEG/Apt-M, the HCNs/DOX/PEG/Apt-M suspensions with different concentrations were irradiated by 808 nm laser for 10 min. Meanwhile, HCNs/DOX/PEG/Apt-M was irradiated by 808 nm laser with different power densities for 10 min. To measure the photothermal stability of HCNs/DOX/PEG/Apt-M, the suspensions of HCNs/DOX/PEG/Apt-M (100 μg mL^{-1}) were examined by six cycles of laser irradiation and natural cooling. The temperature variation and thermal images were recorded by infrared thermal imager. The photothermal conversion efficiency (η) was calculated according to Roper's report[37].

pH/NIR-triggered release of DOX

To investigate the DOX release behavior, HCNs/DOX/PEG/Apt-M (1 mL, 1.0 mg mL^{-1}) was dispersed in PBS (pH 5.0 and 7.4) with shaking at 37°C in the dark. For NIR-triggered test groups, the mixture was irradiated with 808 nm laser (1.5 W cm^{-2}) at 2 h and 5 h time points, respectively. At given time intervals, the release percentage of DOX from different groups was measured by UV-VIS spectrometer.

Biosafety of HCNs/DOX/PEG/Apt-M

The MCF-7 cell were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% Penicillin-Streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The HepG2 cell were cultured in high glucose DMEM medium supplemented with 10% FBS and 1% Penicillin-Streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

The biocompatibility of HCNs/PEG/Apt-M (unloaded DOX) was evaluated by MTT assay. Briefly, MCF-7 and HepG2 cells were seeded in 96-well plates and treated with HCNs/PEG/Apt-M at different concentrations for 24 h or 48 h. Then, MTT solution (10 μL) was added to each well and the cells were incubated for 4 h. In the end, the medium was replaced with 150 μL DMSO, and the optical density (OD) was recorded at 570 nm with a microplate reader. Untreated tumor cells served as controls.

Hemolysis assay was performed to assay the biosafety of HCNs/DOX/PEG/Apt-M *in vivo*. Briefly, blood samples from healthy mice were centrifuged at 8000 rpm for 5 min to obtain the isolated red blood cells (RBCs). RBCs sample was resuspended with PBS (negative control), water (positive control) and HCNs/DOX/PEG/Apt-M solution with various concentrations under gentle shaking and kept at room temperature for 4 h followed by centrifugation at 11000 rpm for 10 min. Last, the absorbance of obtained supernatants at 541 nm were tested by UV-VIS spectrometer. The hemolysis percentage was obtained based on the following equation:

$$\text{Hemolysis percentage} = \frac{A_{\text{sample}} - A_{\text{negative}}}{A_{\text{positive}} - A_{\text{negative}}} \times 100$$

Where A_{sample}, A_{negative} and A_{positive} represent the absorbance of the tested samples and the negative and positive controls, respectively.

Aptamer affinity analysis

To evaluate the targeting ability of Apt-M, MCF-7 and HepG2 cells were collected and incubated with Apt-MF or Apt-CF for 2 h respectively. Then, the cells were analyzed by flow cytometry.

Cellular uptake and targeting property of HCNs/DOX/PEG/Apt-M

To investigate the cellular uptake of the HCNs/DOX/PEG/Apt-M, MCF-7 cell was seeded in 6-well plates and

CLSM dishes and incubated with HCNs/DOX/PEG/Apt-M for different time. The intracellular DOX fluorescence of cells was tested by flow cytometry. After Hoechst 33342 and Lyso-Tracker Green staining, CLSM was used to detect fluorescence signals of cells.

To verify the targeting specificity, MCF-7 and HepG2 cells were incubated with different nanomaterials for 4 h. In the competing experimental group, cells were incubated with Apt-M or Apt-C first, and then HCNs/DOX/PEG/Apt-M was added for incubation for 4 h. Finally, the cells were analyzed by flow cytometry.

***In vitro* cytotoxicity assay**

To investigate tumor cells killing ability of HCNs/DOX/PEG/Apt-M, the MCF-7 and HepG2 cells were seeded in 96-well plates, 12-well plates, confocal culture dishes and treated with different nanomaterials (100 μg mL^{-1}) for 4 h. Subsequently, the original medium was replaced with fresh medium. For the NIR irradiation groups, the cells were irradiated by 808 nm laser (1.5 W cm^{-2}) for 5 min. The cell viability of MCF-7 and HepG2 cells was measured by MTT assay upon incubation for 8 h. MCF-7 cell in 12-well plates were collected and stained with Annexin V-FITC/PI kit for the flow cytometric analyze. MCF-7 cell in confocal culture dishes were stained with Calcein-AM/PI kit and analyzed with CLSM.

***In vivo* therapy effect assessment**

All mice experiments were performed under the permission of experimental animal ethics committee of Shanxi Datong University. Four-week-old female Balb/c nude mice were obtained from Beijing Huafukang Bioscience Co., Ltd. The xenograft model was established by subcutaneously injection of MCF-7 cell suspension into the groin region of mice. The volume of tumors and the weight of mice were monitored every two days. The volume of tumors was calculated using the following formula: V (mm^3) = L × W^2 × 1/2. Where V, L and W represent the volume, length and width of tumor, respectively. When the volume of tumors reached ~100 mm^3, the tumor bearing mice were divided randomized into six groups (five mice each group) for different treatments: PBS, DOX (3.0 mg kg^{-1}), HCNs/PEG + L (10.0 mg kg^{-1}), HCNs/DOX/PEG + L (10.0 mg kg^{-1}), HCNs/DOX/PEG/Apt-C + L (10.0 mg kg^{-1}) and HCNs/DOX/PEG/Apt-M + L (10.0 mg kg^{-1}). For NIR involved groups, after nanomaterials were injected via the tail vein for 6 h, the tumor sites of mice were irradiated with 808 nm laser (1.5 W cm^{-2}) for 5 min. The temperature changes and thermal images of mice tumor sites were recorded by infrared thermal imager. After 19

days of treatment, the mice were humanely executed to obtain tumor tissues and main organs (heart, liver, spleen, lung and kidney). The tumors were sectioned into slices and stained with hematoxylin–eosin (H&E), TDT-mediated dUTP nick-end labeling (TUNEL) and Ki-67 for observing histological variations. The main organs of mice were collected and used for H&E staining.

Statistical analysis

One-way analysis of variance (ANOVA) statistical method was utilized to evaluate the experimental data. The criterion of statistical significance was considered at *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

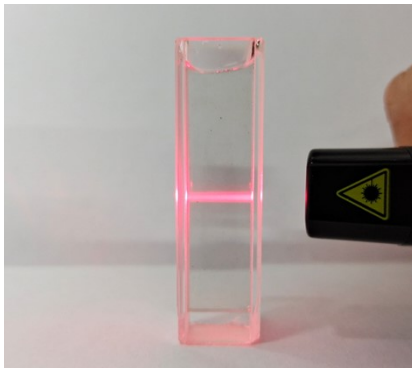

Fig. S1 Digital photo of HCNs suspension's Tyndall effect.

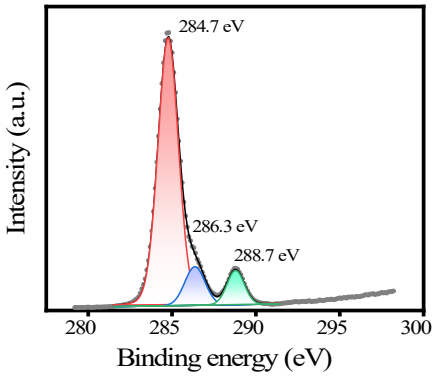

Fig. S2 Fitted XPS spectra of C 1s.

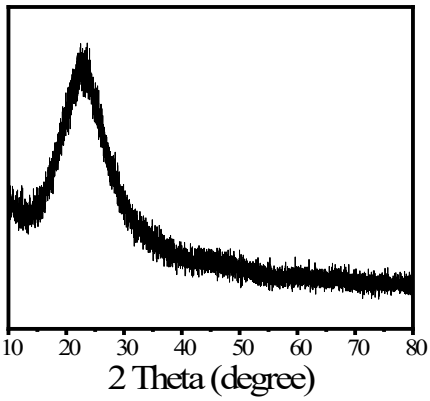

Fig. S3 XRD pattern of HCNs.

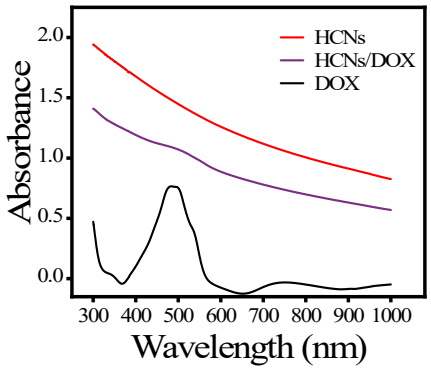

Fig. S4 UV-VIS spectra of HCNs, HCNs/DOX.

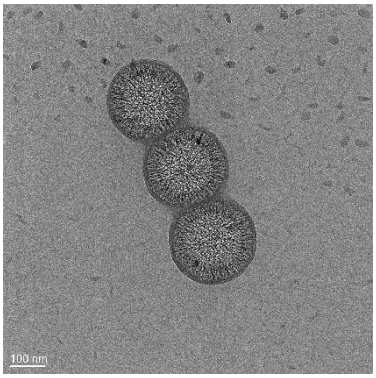

Fig. S5 TEM image of the HCNs/DOX/PEG/Apt-M.

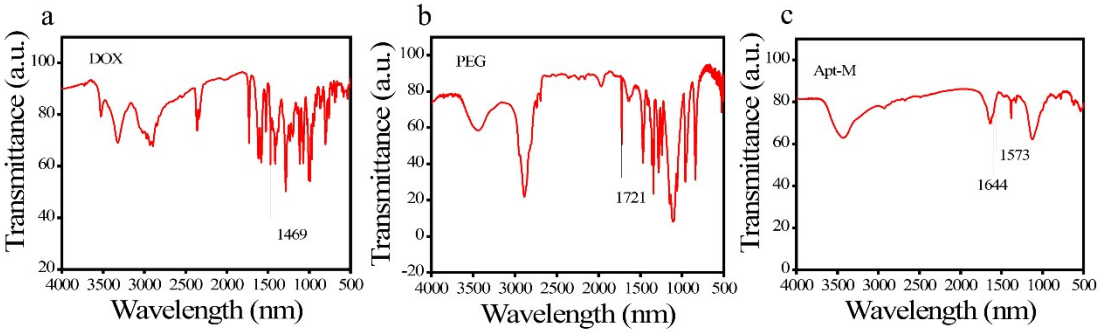

Fig. S6 FTIR spectra of (a) DOX, (b) PEG and (c) Apt-M.

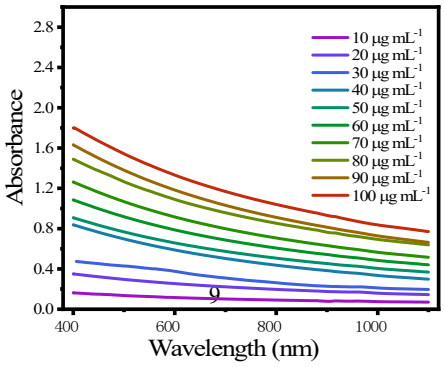

Fig. S7 UV-VIS spectra of HCNs/DOX/PEG/Apt-M at different concentrations.

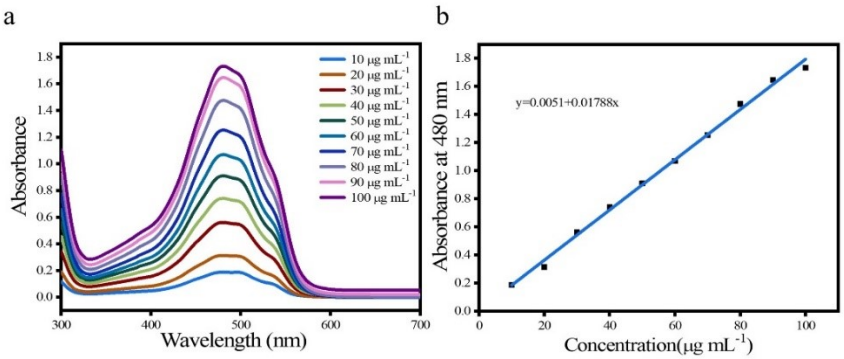

Fig. S8 (a) UV-VIS spectra of DOX with different concentrations. (b) Standard curve of DOX concentration and its corresponding absorbance value.

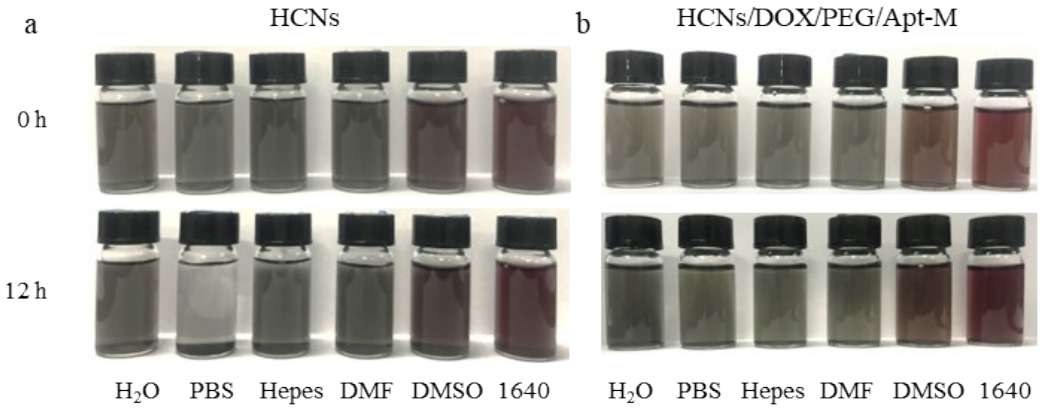

Fig. S9 Digital images of (a) HCNs and (b) HCNs/DOX/PEG/Apt-M dispersed in various solvents.

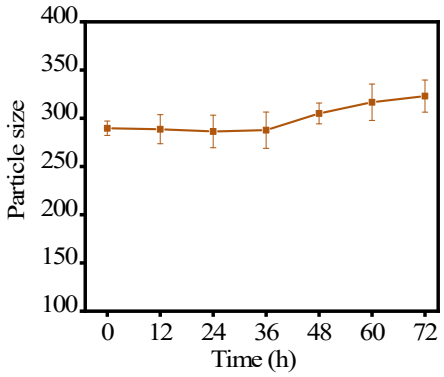

Fig. S10 Hydrodynamic diameter of HCNs/DOX/PEG/Apt-M.

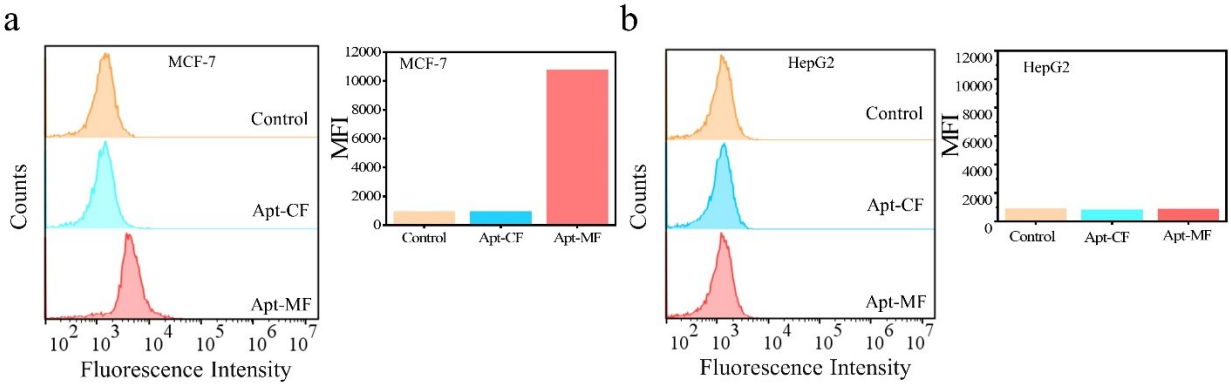

Fig. S11 Flow cytometry analysis and MFI values of (a) MCF-7 and (b) HepG2 cells after incubation with Apt-CF or Apt-MF.

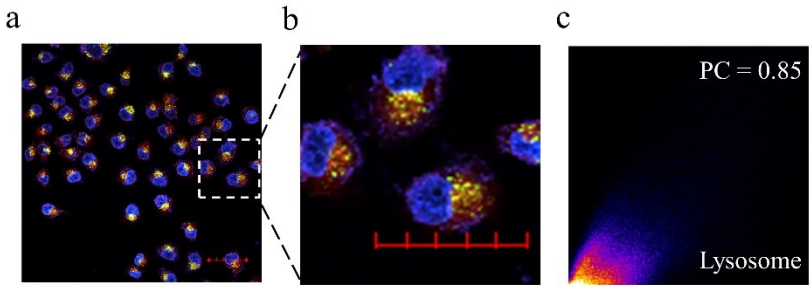

Fig. S12 (a) Colocalization experiments of MCF-7 cell co-stained with Hoechst 33342 and Lyso-Tracker Green. (b) a partial enlarged view of (a). (c) Colocalization analysis and Pearson correlation coefficient of MCF-7 cell. Scale bar: 50 μm.

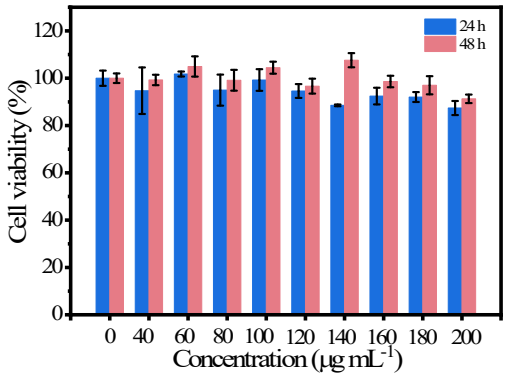

Fig. S13 Viability of HepG2 cell after incubation with HCNs/PEG/Apt-M of varied concentrations.

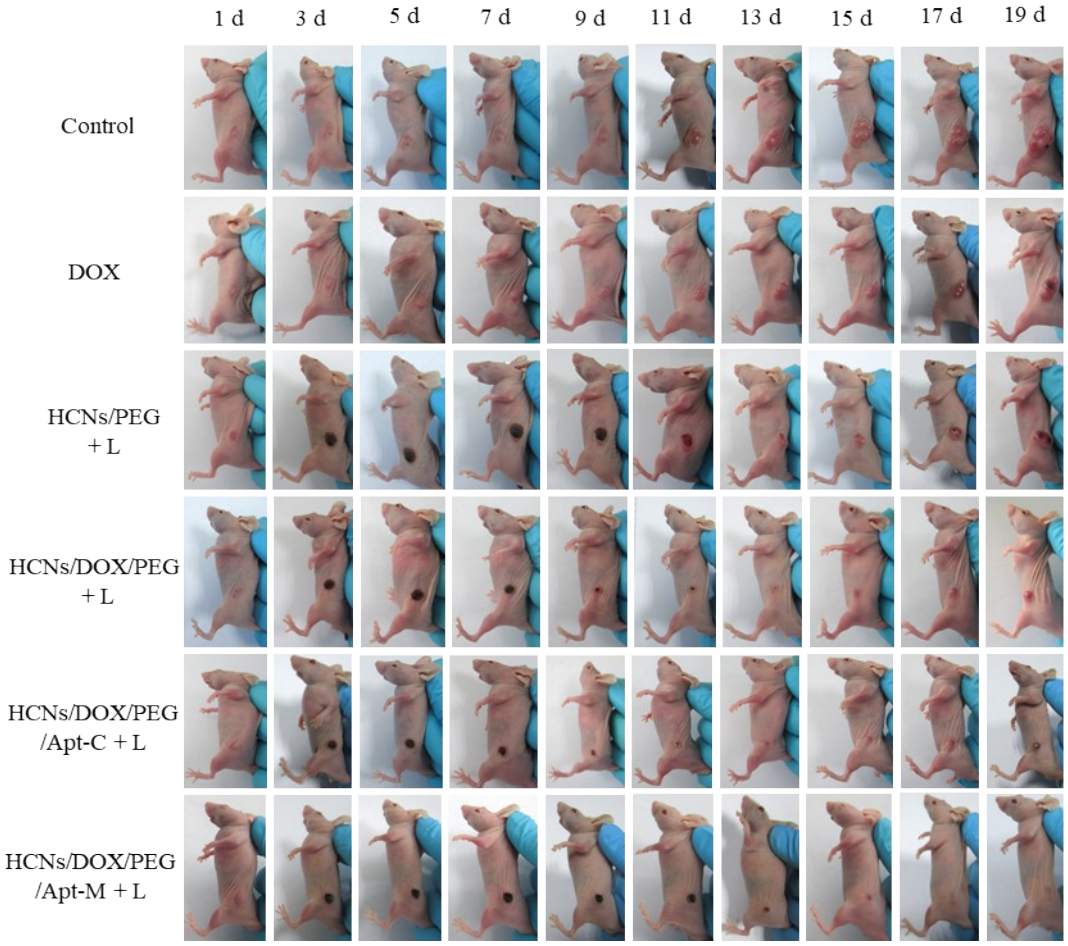

Fig. S14 Digital photographs of MCF-7 xenograft mice after different treatments in 19 days' period.

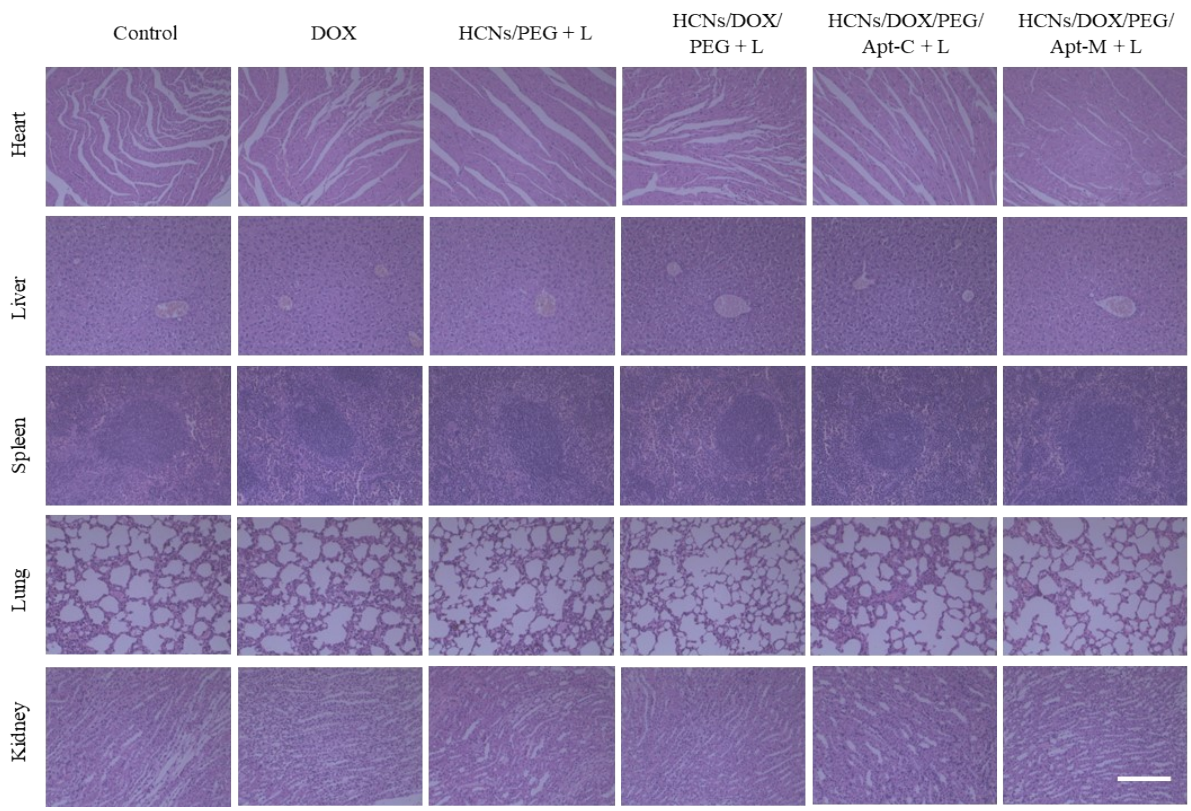

Fig. S15 Images of H&E stained tissue sections of major organs (heart, liver, spleen, lung and kidney) from mice with different treatments. Scale bar: 100 μm.