SUPPORTING INFORMATION

Tumor-cell targeting polydiacetylene micelles encapsulated with an antitumor drug for the treatment of ovarian cancer†

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1. General information:

All reactions were carried out under an argon atmosphere using anhydrous freshly distilled solvents unless otherwise stated. All solvents were dried and distilled before use. Anhydrous dichloromethane (DCM) and dimethyl formamide (DMF) were distilled over $CaCl_2$ and CaH_2 respectively, and kept anhydrous with 4Å molecular sieves. The ultra-pure water was obtained by Millipore filtration system. Cy5.5 and 10, 12-tricosadiyonic acid were purchased from Energy Chemical Co., Ltd. (Shanghai); N, N-diisopropylcarbodiimide (DIC), N-Hydroxybenzotriazole (HOBt) and Fmoc-protected amino acids were purchased from GL Biochem (Shanghai) Ltd. All reagents and chemicals were AR grade and used without further purification.

The mass spectra of PA1 and PA2 were determined on an AB Sciex (MALDI-TOF) mass spectrometer. The analytical "High Performance Liquid Chromatography" (HPLC) was performed with the following parameters: RP-C_{18} HPLC column (10 μm particle size) and UV detector. The mobile phase was a gradient of 10-90% of methanol aqueous solution containing 0.5‰ trifluoroacetic acid at a total flow rate of 0.8 mL/min. The UV/Vis and fluorescence spectra were recorded with a Varian Cary 100 Conc UV-Visible Spectrometer and a Fluoromax-4 Spectrofluorometer (HORIBA Scientific), respectively. The pH was measured by a Mettler Toledo FE 20K pH meter.

2. Synthesis of peptides:

Synthesis of PA1 and PA2:

Peptides were prepared according to the reported general procedure. The Fmoc-Lys(Nph)-OH, a naphthalic acid fluorophore was attached to the side chain of lysine according to the literature.[1] Rink amide resin (390 mg, 0.64 mmol/g, 0.25 mmol, 1 equiv.) was weighed into a glass peptide synthesis vessel and allowed to swell in DMF (10 mL) for 2 h. Then, the Fmoc protection group was removed by treatment with piperidine (20%) in DMF (10 mL). After an intensive washing cycle with DMF (10 mL) and DCM (10 mL), the following amino acids including Fmoc-Lys(Nph)-OH, and diacetylene were attached: Fmoc-protected amino acid (0.75 mmol, 3.0 equiv), DIC (2.5 mmol, 10.0 equiv), HOBt (0.75 mmol, 3.0 equiv); 10, 12-tricosadiyonic acid (0.75 mmol, 3.0 equiv), DIC (2.5 mmol, 10.0 equiv), HOBt (0.75 mmol, 3.0 equiv). The final products were cleaved from the solid support by treatment with a cleavage cocktail of trifluoroacetic acid-triisopropylsilane-H_2O (95:2.5:2.5) for 2 h. After removing the solution, the white solid for PA1 and yellow solid for PA2 were precipitated with dry diethyl ether and centrifuged. The crude products were purified and isolated by preparative high performance liquid chromatography (HPLC) on a C-18 column. They were further characterized using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. PA1: A white solid (10 mg, purity HPLC: 99.0%). MALDI-TOF (m/z): calcd for 1157.72, found $[M+H]^+$ 1158.50. PA2: A yellow solid (12 mg, purity HPLC: 99.8%). MALDI-TOF (m/z): calcd for 1493.87, found $[M+H]^+$ 1494.85.

3. Fluorescence quantum yield measurements:

Relative fluorescence quantum yields were measured according to the literature.[2] The quantum yields of PDA were determined relative to fluorescent standard fluorescein (fluorescence quantum yield, 95%), with the possibility of correcting for differences between the refractive index of the reference n_R, and the sample solutions n_S, using the expression:

$$QY_S = QY_R \frac{I_S A_R n_S^2}{I_R A_S n_R^2}$$

Here the indices *S* and *R*, respectively, denote sample and reference. *I* represent the integrated emission intensities of standards and the samples, and *A* is the absorbance at the wavelength of excitation. *n* means the refractive index of the solvent that dissolve the compounds. Fluorescein in an aqueous 0.1 M NaOH solution was used as the external reference. The final value of quantum yield was obtained from an average of three measurements with different absorbance in the range between 0.02 and 0.05. The corresponding fluorescence spectra were measured using Fluoromax-4 Spectrofluorometer (HORIBA Scientific) at room temperature.

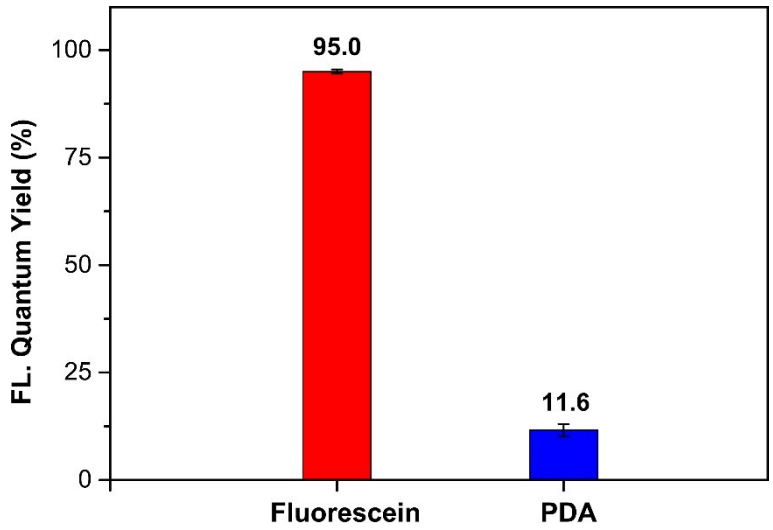

Figure S1. Fluorescence quantum yield of PDA relative to fluorescent standard fluorescein (fluorescence quantum yield, 95%).

4. Preparation of polydiacetylene (PDA) micelles:

The PDA, PDA-CPT and PDA-Cy5.5 micelles were prepared according to the reported general procedure.[3]

Preparation of PDA micelles:

A mixture of PA1 and PA2 (1:1 in molar ratio) was dissolved in ultrapure water and sonicated for 45 min, then transferred into a quartz cuvette and irradiated under UV light (254 nm, 8 mW/cm^2) for about 180 min. The solution was filtered through 0.45 μm membrane and freeze-dried to provide PDA micelles. The PDA micelles were dissolved in TBS (1mg/mL) and diluted to the desired

concentrations for tests.

Preparation of PDA-CPT micelles:

A mixed solution of PA1 and PA2 (1:1 in molar ratio) in ultrapure water was divided in two equal parts (solution A and B). Excess CPT (5 mg) was added to solution A, and the solution B was left without any additive. Two solutions/suspensions were sonicated for 45 min. The solutions/suspensions were then transferred into a quartz cuvette, submitted to UV light (254 nm, 8 mW/cm^2) irradiation for 180 min and filtered via 0.45 μm membranes. The loading of CPT in PDA micelles was quantified by freeze-drying the filtrates of solutions A and B and comparing the masses (M_A and M_B) of the obtained solid residues. The drug loading content (DLC, wt%) = (Weight of CPT loaded in micelles)/(Weight of CPT loaded micelles) × 100. The loading of CPT in PDA micelles was quantified by freeze-drying the filtrates of solutions A and B and comparing the masses (M_A and M_B) of the obtained solid residues：

$$L_{CPT} = \frac{M_A - M_B}{M_A} \times 100\%$$

Table S1. The results of preparation of CPT-loaded PDA micelles.

M_A(PDA-CPT)	M_B(PDA)	M_A-M_B	L_{CPT} (%)
5.3 mg	3.6 mg	1.7 mg	32.0 wt%

Preparation of PDA-Cy5.5 micelles:

A mixed solution of PA1 and PA2 (1:1 in molar ratio) was dissolved in ultrapure water and excess Cy5.5 (5 mg) was added to solution. After sonication for 45 min, the solution was transferred into a quartz cuvette using UV irradiation at 254 nm (8 mW/cm^2) for about 180 min and filtered through 0.45 μm membrane to remove insoluble Cy5.5. The solution was freeze-dried to provide PDA-Cy5.5 micelles.

5. UV/Vis and fluorescence experiments:

Absorption spectra were recorded using a Varian Cary 100 Conc UV-Visible Spectrometer and fluorescence spectra were measured with Fluoromax-4 Spectrofluorometer (HORIBA Scientific) at 25 °C. Samples were excited at 405 nm for PDA and PDA-CPT and 370 nm for CPT. The slit widths were set to 5 nm for excitation and emission. The data points were collected at 1.0 nm increments with a 0.1 s integration period. Samples were dissolved in TBS (50-mM Tris, pH 7.4, 25 °C).

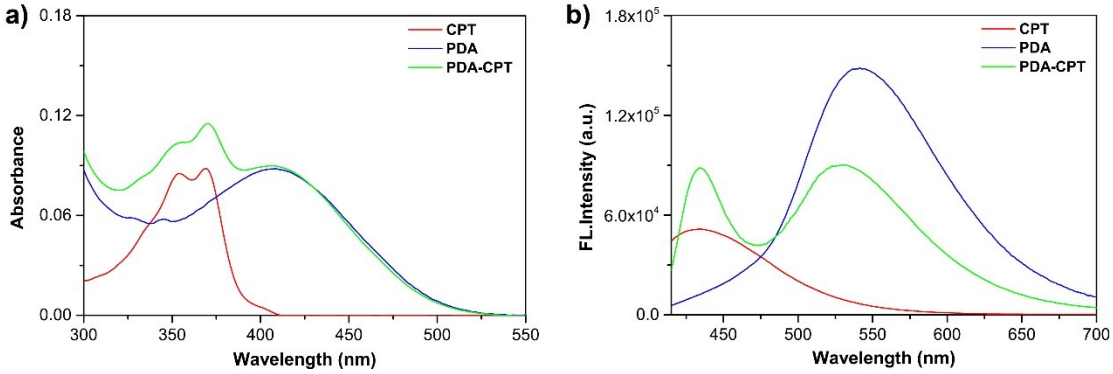

Figure S2. a) Absorption and b) fluorescence spectroscopy of CPT, PDA and PDA-CPT (10 µg/mL) in TBS.

6. Dynamic light scattering (DLS):

The hydrodynamic diameters of the PDA and PDA-CPT were measured using a dynamic light scattering spectrometer (DLS) on Nano-ZS (Zatasizer, Malvern) instrument. Samples of 10.0 µg/mL PDA and 10.0 µg/mL PDA-CPT in TBS (50-mM Tris, pH 7.4, 25 °C) in a total sample volume of 1.0 mL.

7. Transmission electron microscopy (TEM):

High-resolution images of PDA and PDA-CPT were acquired using TEM. Samples were prepared by placing a few droplets of 10.0 µg/mL PDA and 10.0 µg/mL PDA-CPT onto a carbon-coated grid with holes and stained with sodium phosphotungstate (2.0 wt% aqueous solution) and dried at room temperature. TEM characterization was performed using a JEM-2100 electron microscope (JEOL, Japan).

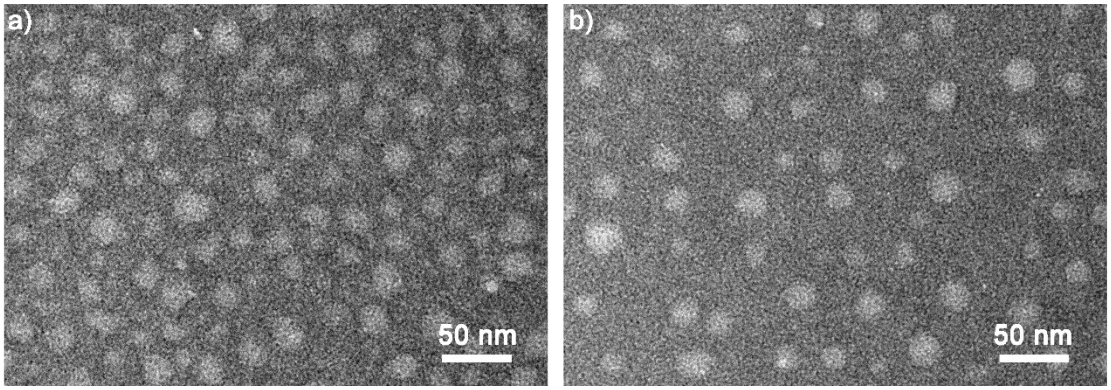

Figure S3. TEM images of a) PDA (10 µg/mL) and b) PDA-CPT (10 µg/mL) was stained with sodium phosphotungstate (2.0 wt% aqueous solution).

8. Zeta Potential measurement:

The zeta potential was measured with Nano-ZS (Zatasizer, Malvern) instrument. Samples were dissolved in TBS (50-mM Tris, 25 °C) at different pHs (4.5, 6.0, and 7.4). All data were corrected by subtracting the value tested for a blank scan of the buffer system at different pH (4.5, 6.0, and

7.4). Results were presented as mean ± SD (n=3).

9. *In vitro* drug release profiles:

The *in vitro* drug release profiles were acquired according to the literature.[4] The profiles for the *in vitro* release of CPT from PDA-CPT micelles were obtained by dialysis of the PDA-CPT micelles in TBS (50-mM Tris, 37 °C) at different pH (4.5, 6.0, 7.4). Drug release was carried out at 37 °C in a water bath with magnetic stirring (200 rpm) for 32 h. At defined intervals, 0.8 mL of external release buffer solution was collected and an equal volume of fresh release medium was replenished. The concentrations of the released CPT from the PDA-CPT micelles were analyzed using a standard working curve experimentally obtained by fluorescence spectroscopy at 435 nm. The experiments were carried out in triplicate and data were shown as mean ± standard deviation (SD). The drug release data were determined by three independent experiments. Cumulative drug release (%) = (the amount of drug released from micelles/the amount of drug loaded into micelles) × 100. The cumulative amount of CPT released from the PDA-CPT micelles was plotted as a function of time.

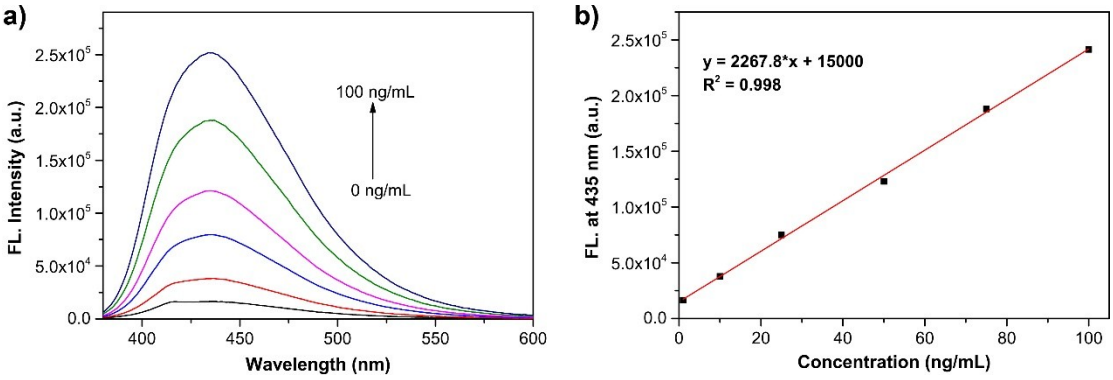

Figure S4. a) Fluorescence changes and b) the plot of the fluorescence intensity at 435 nm against CPT with different concentrations (0 – 100 ng/mL).

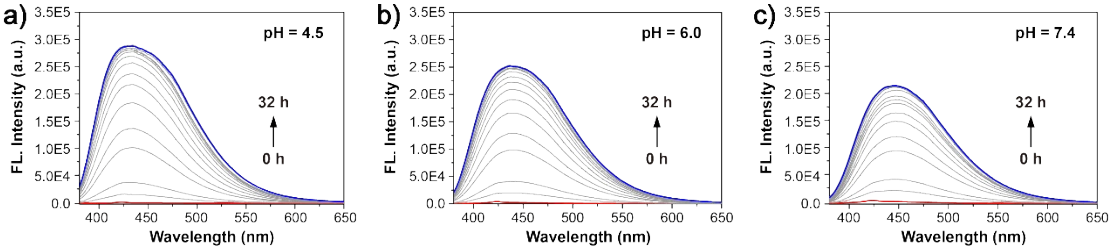

Figure S5. a, b, c) Fluorescence changes for CPT released from PDA-CPT micelles incubated in TBS (50-mM Tris, 37 °C) at different pHs (4.5, 6.0, 7.4).

10. Cell experiments:

Cell culture: SKOV-3 cells (human ovarian carcinoma epithelial cells) were purchased from Shanghai Bogoo Biotech Co., Ltd., China. SKOV-3 cells were cultured in McCoy's 5A (Gibco) at 37 °C under humidified conditions of 95% air and 5% CO_2. All media were supplemented with 10%

fetal bovine serum, 100 U penicillin and 0.1 mg of streptomycin (Gibco) per milliliter. The culture media were changed every two days to maintain exponential growth of the cells. Cells were passaged using 0.25% Trypsin/EDTA (Sigma) when they reached 80-90% confluence and seeded for the experiments.

Cytotoxicity Assay: The cytotoxicity was measured using a standard 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) assay with SKOV-3 cells. Cells growing in exponential growth phase were seeded into 96-well cell-culture plate at 1×10^4 cells/well. The cells were incubated overnight at 37 °C under humidified conditions of 95% air and 5% CO_2. CPT or PDA-CPT (100.0 μL/well) at concentrations of 2, 4, 8, 16, 32, 64 μg/mL in McCoy's 5A was added to the wells of the treatment group, whereas for final negative control group 100.0 μL McCoy's 5A culture media were added. The cells were incubated for 24 h at 37 °C under humidified conditions of 95% air and 5% CO_2. Subsequently, 10 μL of MTT solution (5 mg/mL in PBS) was added to the medium for additional 4 h incubation at 37 °C, allowing viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan, which precipitated as crystalline solids. After removal of the medium, formazan extraction was performed with 100 μL DMSO and its quantity was determined colorimetrically using a Multi-mode Plate Reader (BioTek, USA) at 490 nm (absorbance value). The following formula was used to calculate the viability of cell growth: Viability (%) = (mean absorbance value of the treatment group-blank/mean absorbance value of the control-blank) × 100. Furthermore, IC_{50} values were obtained by plotting the signal responses (OD_{490}) against the logarithm of analyst concentrations using Origin software (Origin 9.0). The 4-parameter logistic equation $y = A_2 + (A_1 - A_2) / [1 + (x/x_0)^p]$ was used for curve fitting in the whole concentration range, where A_1 is the maximum signal at no analyst, A_2 is the minimum signal at infinite concentration, p is the curve slope at the inflection point, and x_0 is the IC_{50} (analyst concentration causing a 50% inhibition of the maximum response, a measure of immunoassay detectability). [5]

Confocal microscopic imaging: SKOV-3 cells were seeded in a 35 mm petri dish with a glass cover slide and allowed to adhere overnight before treatment. The cells were incubated in McCoy's 5A at 37 °C under humidified conditions of 95% air and 5% CO_2. After washing the cells with PBS (pH 7.4, 1 mL × 1 times), the SKOV-3 cells were incubated with PDA-CPT (10 μg/mL) in McCoy's 5A for 1 h at 37 °C under humidified conditions of 95% air and 5% CO_2, respectively. For colocalization experiments, SKOV-3 cells were incubated with PDA-CPT (10 μg/mL) and Mito-Tracker Red (200 nM) or Lyso-Tracker Red (60 nM), respectively. Cells were pretreated with 10 μg/mL PDA-CPT for 60 min and Mito-Tracker Red for 30 min (or Lyso-Tracker Red for 60 min) at 37 °C. Cells imaging was then carried out after washing with PBS (pH 7.4, 1 mL × 5 times). Cell fluorescence images were obtained with a confocal laser scanning microscope (Nikon A1, Japan, 60×oil-immersion objective lens). Channel 1 for CPT: excitation: 405 nm, emission collected: 425-450 nm; Channel 2 for PDA: excitation: 405 nm, emission collected: 500-540 nm; Channel 3 for Mito-Tracker Red or Lyso-Tracker Red: excitation: 561 nm, emission collected: 580-610 nm.

11. *In vivo* experiments:

All animal handling was performed in accordance with Animal Research Committee guidelines of East China University of Science and Technology and conformed to the guide for the care and use of laboratory animals. SKOV-3 cells were washed with PBS (pH 7.4), and harvested using 0.25% Trypsin/EDTA (Sigma). After centrifugation, the harvested cells were then suspended in PBS (pH 7.4). Four-week-old (approximately 15 g) female BALB/c nude mice (Shanghai Slac Laboratory Animal Co. Ltd., China) were implanted subcutaneously on the right flank with 2 million SKOV-3 cells in 0.1 mL PBS (pH 7.4), and tumors developed within four weeks.

***In vivo* antitumor studies:** When the tumors reached a mean volume of 50 mm^3 after inoculation of SKOV-3 cells, the mice were randomly separated into three groups (n = 5 per group): (1) negative control (PBS); (2) CPT; (3) PDA-CPT. The mice received intravenous injections at 5 mg/kg in 0.1 mL PBS every 2 days for 16 days. During therapy, the tumor volumes and body weights were measured every two days. Length and width of tumors were measured individually using a Vernier caliper. Tumor volumes were calculated using the following formula: tumor volume = length × $width^2$ × 0.5. The mice were sacrificed 4 days after the treatments according to institutional guidelines. Tumors were resected, weighed, fixed in formalin and then embedded in paraffin. The therapeutic efficacy of the treatment was evaluated by the tumor-inhibition rate (TIR). This was calculated using the following equation: TIR = 100% × (mean tumor weight of control group - mean tumor weight of experimental group)/mean tumor weight of control group.

For staining of tissue slices: Freshly dissected heart, liver, kidneys, lungs, stomach, spleen and tumor from mice of three treatment groups were fixed and embedded in paraffin. After being cut into 4-μm slices, the sections were deparaffinized and stained with Hematoxylin and Eosin solution. Finally, the sections were dehydrated and mounted with Permount in a fume hood. The sections were scanned by a microscope (Nikon, Japan).

***In vivo* fluorescence imaging:** Two mice were imaged using an IVIS spectrum series *in vivo* imaging system (PerkinElmer). The fluorescence intensity from the tumor and other area was integrated. After mice were injected *via* the tail vein with Cy5.5 and PDA-Cy5.5 (with equivalent Cy5.5) in PBS (pH 7.4), they were placed in the IVIS spectrum series *in vivo* imaging system (PerkinElmer) and scanned to determine the NIR signal of tumors (excitation: 680 nm, emission collected: 720-740 nm). Mice were anesthetized by 2% isoflurane inhalation for 30 seconds in anaerobic box.

For *ex vivo* organ imaging: Two mice were euthanized by excess isoflurane inhalation and the organs were dissected after *in vivo* imaging experiments. The heart, liver, kidneys, lungs, stomach, spleen and tumor were obtained from mice at 24 h after injected with Cy5.5 and PDA-Cy5.5 (with equivalent Cy5.5). Fluorescence images of organs and tumors were obtained directly using an IVIS spectrum series *in vivo* imaging systems (PerkinElmer).

Statistical analysis: The statistical significance of treatments was assessed using the IBM SPSS Statistics 20. The statistical differences were determined by Student's t test. Values $p < 0.005$ indicate significant differences.

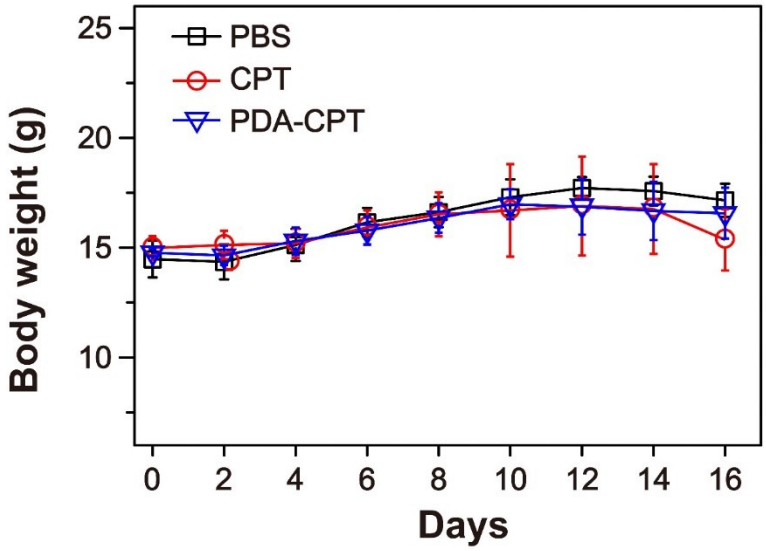

Figure S6. Changes of body weights of mice after intravenous injection of PBS, CPT and PDA-CPT at a dose of 5 mg/kg every two days by intravenous injection in SKOV-3 tumor-bearing mice.

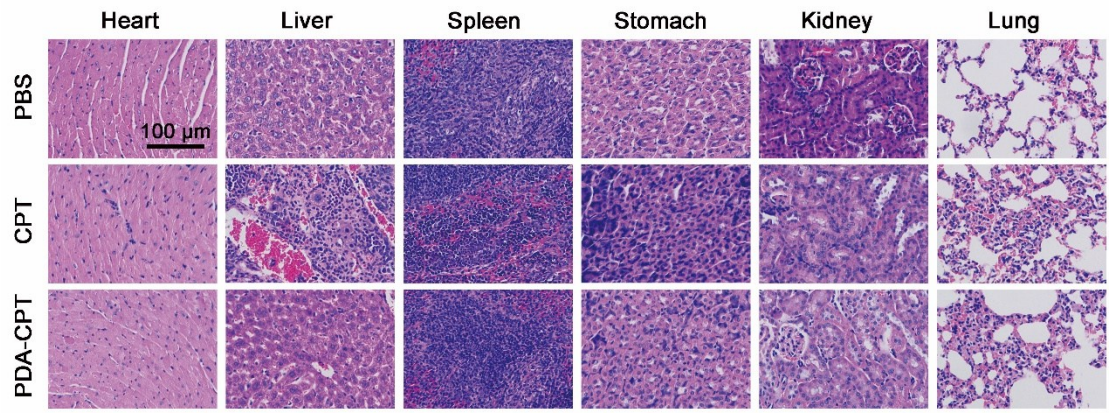

Figure S7. At the termination of the experiments, H&E staining of the paraffin sections of organs including heart, liver, spleen, stomach, kidneys, lungs from SKOV-3 xenograft tumor mice treated with PBS, CPT and PDA-CPT.

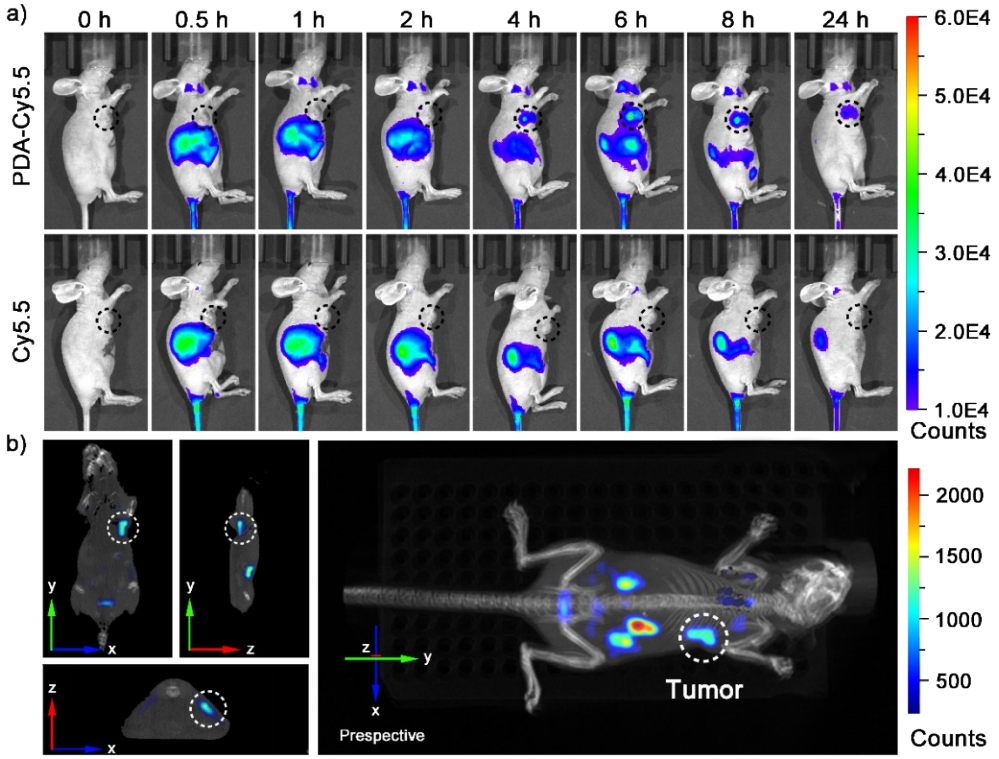

Fig. S**8**. a) *In vivo* NIR fluorescence imaging of a SKOV-3 xenograft tumor-bearing mouse intravenously injected with PDA-Cy5.5 or Cy5.5 over 0-24 h. All images were acquired under the same instrument conditions. b) Three-dimensional NIR image reconstruction of the xenograft tumor mouse 8 h after intravenously injected with PDA-Cy5.5 using an IVIS spectrum series in vivo imaging system. The circles indicate the tumor sites.

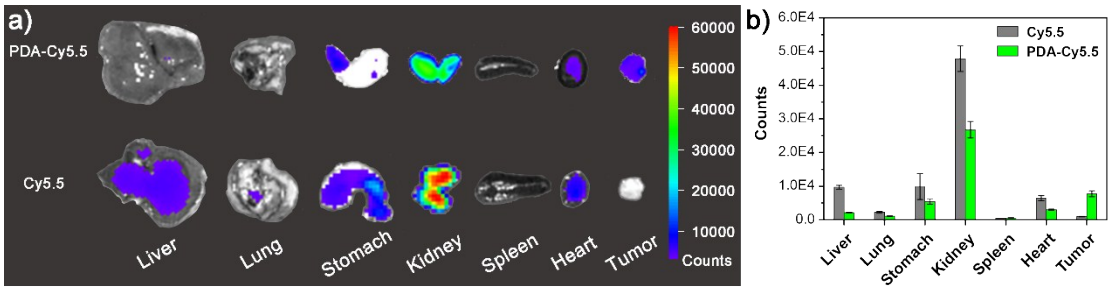

Figure S9. a) *Ex vivo* NIR fluorescence imaging of PDA-Cy5.5 or Cy5.5 distribution in isolated tumors and organs including liver, lungs, stomach, kidneys, spleen, heart at 24 h after intravenous injection. b) Quantitative analysis of relative organ accumulation in the xenograft tumor model system mice at 24 h after intravenously injected with PDA-Cy5.5 and Cy5.5. The error bars represent ± SD in the analysis of relative counts using the IVIS spectrum series *in vivo* imaging system.

References:

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