Electronic Supplementary Information

A multifunctional persistent luminescent nanoprobe for imaging guided dual-stimulus responsive and triple-synergistic therapy of drug resistant tumor cells

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1. Materials and Chemicals

$Zn(NO_3)_2 \cdot 6H_2O$ (99%), Ga_2O_3 (99.99%), GeO_2 (99.999%), $Cr(NO_3)_3 \cdot 9H_2O$ (99.99%), $Eu(NO_3)_3 \cdot 6H_2O$ (99.9%), polyethyleneimine (PEI, MW~10000), N,N′-Dicyclohexylcarbodiimide (DCC) and N-Hydroxysuccinimide (NHS) were obtained from Aladdin (Shanghai, China). Dopamine hydrochloride was purchased from J&K Scientific Ltd. (Beijing, China). Folic acid (FA) was purchased from Yuanye Biotechnology (Shanghai, China). Dialysis bag was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China). Doxorubicin hydrochloride was obtained from Huafeng Lianbo Technology Co., Ltd. (Beijing, China). Roswell park memorial institute (RPMI)-1640 medium, 4′,6-diamidino-2-phenylindole (DAPI) and 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Dingguo Biotechnology Co. (Beijing, China). P-Glycoprotein (P-gp) siRNA was purchased from GenePharma Biotechnology (Shanghai, China). 6-Carboxyfluorescein (FAM) labeled P-gp siRNA, control siRNA, and all synthetic DNA strands were obtained from Shanghai Sangon Biotech (Shanghai, China). RNA simple Total RNA Kit for extracting total RNA from cells was obtained from Tiangen Biotech Co., Ltd. (Beijing, China). PrimeScript™ RT reagent Kit (Perfect Real Time) and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). ELISA Kit for P-gp quantification was obtained from Jianglai Biotechnology Ltd. (Shanghai, China). The MCF-7/ADR cells and 293T cells were purchased from Gefan Biotechnology (Shanghai, China).

2. Instrumentation

The transmission electron microscopic (TEM) images were obtained on a Tecnai G2 F20 transmission electron microscope (FEI, USA). X-ray diffraction (XRD) experiments were performed on a D2 PHASER diffractometer (Bruker AXS, Germany). Zeta potential was obtained on a multi-angle dynamic/static laser scattering instrument (Brookhaven, USA). All fluorescent spectra were recorded on F-4500 fluorescence spectrophotometer (Hitachi, Japan). The absorption spectra were obtained on a U-3900 spectrophotometer (Hitachi, Japan). The Fourier transform infrared (FT-IR) spectra were acquired from a Nicolet 6700 spectrometer (Thermo Fisher Scientific, USA). A1+ confocal laser scanning microscope (Nikon, Japan) was employed for cell imaging. All NIR persistence photographs were collected on the IVIS Imaging System (PerkinElmer, USA).The absorbance of 96-well microplate was recorded on Synergy 2 multi-mode microplate reader (BioTek, USA). The PCR analysis of cDNA was performed on LightCycler® 96 Real-Time PCR System (Roche, Switzerland).

3. Synthesis of hydroxylated persistent luminescent nanoparticles (PLNP-OH)

The persistent luminescence nanoparticles (PLNP) $Zn_{1.1}Ga_{1.8}Ge_{0.1}O_4$:0.5%$Cr^{3+}$,0.5%$Eu^{3+}$ was synthesized according to a previous method with slight modifications.[1] Briefly, 9.18 mL of Zn^{2+} (0.5 M), 15 mL of Ga^{3+} (0.5 M), 0.834 mL of Ge^{4+} (0.5M), 0.75 mL of Cr^{3+} (0.05 M) and 3.75 mL of Eu^{3+} (0.01 M) were mixed under vigorous stirring. The pH value of the mixture was adjusted to 8.0 and continuously stirred for 3 h. The solution was kept at 160 ℃ for 24 h for hydrothermal reaction. The resulting solution was centrifuged and the precipitate was collected and sintered in air at 900 ℃ for 4 h. The as-synthesized PLNP was wet-ground

for 30 min and then dispersed in 5 mM NaOH solution. After vigorous stirring for 24 h with subsequent centrifugation at 3500 rpm for 5 min, the resulting supernatant was collected and further centrifuged at 10000 rpm for 5 min to acquire PLNP-OH.

4. Synthesis of PEI-FA

FA (0.2 g) was dissolved in 175 mL of DMSO, then DCC (0.1 g) and NHS (0.18 g) were added to the solution with continuous stirring for 12 h. PEI (0.8 g) in 5 mL of DMSO was dropwise added to the previous mixture for continuous reaction for 24 h. Centrifugation and suction filtration were carried out to remove the insoluble substance. The PEI-FA was purified by dialysis (MW cutoff = 3000 Da) and lyophilization.

5. Preparation of PLNP-PDA (PP) and PLNP-PDA-PEI-FA (PPP-FA)

1 mL of PLNP-OH (1 mg/mL) in Tris-HCl buffer (pH 8.5) was respectively mixed with 10, 30, 50, 70, 90, 110 μL of dopamine (1 mg/mL) in Tris-HCl buffer (pH 8.5) under stirring for 5 h. The resulting solution was centrifuged at 10000 rpm for 5 min, and the resulting PP was re-suspended in 1 mL of Tris-HCl buffer (pH 8.5). Two more centrifugation-redispersion cycles were needed to acquire 1 mg/mL of PP in Tris-HCl buffer (pH 8.5).

1 mL of PP (1 mg/mL) in Tris-HCl buffer (pH 8.5) was mixed with 1 mL of PEI-FA (2 mg/mL) in Tris-HCl buffer (pH 8.5) and stirred for 24 h. The resulting solution was centrifuged at 10000 rpm for 5 min, the precipitate was collected and re-suspended in PBS (pH 7.4). Two more centrifugation-redispersion cycles were needed to acquire 1 mg/mL of PPP-FA in PBS (pH 7.4).

6. Loading with Doxorubicin (DOX)

0.5 mL of DOX (1 mg/mL) in ultrapure water was mixed with 1 mL of PPP-FA (1 mg/mL) in PBS (pH 7.4) and stirred at 37 ℃ for 12 h. The obtained solution was washed with ultrapure water for three times and re-suspended in 1 mL of PBS (pH 7.4) to obtain PPP-FA-DOX.

7. Release of DOX

PPP-FA-DOX (1 mg/mL, 1 mL) in PBS (pH 7.4) in quadruplicate was centrifuged at 10000 rpm for 5 min and the precipitate was re-dispersed in PBS buffer solution at pH 5.5, 5.5, 7.4 and 7.4, respectively. The solutions were shaken at 37 ℃. At a fixed time, one of the pH 5.5 solutions and one of the pH 7.4 solutions was irradiated with 808 nm laser for 10 min. Afterward, the four samples were centrifuged at 10000 rpm for 5 min and the supernatant were collected to determine the released DOX. 1 mL of the PBS buffer (pH 5.5 or 7.4) was added to re-suspend the precipitate.

8. Preparation of PPP-FA-DOX-(P-gp) siRNA

PPP-FA-DOX in PBS (pH 7.4) was mixed with P-gp siRNA (0.264 mg/mL, 1 μL) with a series of weight ratio of 0:1, 1:1, 2:1, 4:1, 8:1, 16:1, 32:1. The mixture was incubated at 37 ℃ for 30 min and P-gp siRNA was absorbed on PPP-FA-DOX through electrostatic interaction.

PPP-FA-DOX-(P-gp) siRNA was analyzed by gel electrophoresis. PPP-FA-DOX-(P-gp) siRNA (5 μL) was mixed with 6×loading buffer (1 μL) to run gel electrophoresis under voltage of 90 V and electric current of 90 mA for 30 min.

9. Cell culture and confocal laser scanning microscope (CLSM) imaging

293T cells and MCF-7/ADR cells were cultured in (RPMI)-1640 medium

(Hyclone) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were maintained in a 100% humidified atmosphere containing 5% CO_2 at 37 ℃.

For CLSM imaging, cells were planted on a 4-chamber glass bottom dish in 100 μL culture medium and incubated at 37 ℃ for 24 h. The cells were washed with Dulbecco's Phosphate Buffered Saline (D-PBS, pH 7.4) and the D-PBS was replaced with 100 μL pure (RPMI)-1640 medium containing 20 μg/mL PPP-FA, PPP-FA-DOX or PPP-FA-DOX-(p-pg) siRNA, or replaced with 100 μL pure (RPMI)-1640 medium containing 5 μg/mL free DOX or 0.63 μg/mL free p-pg siRNA with continuous incubation at 37 ℃ for 24 h. The cells were washed with D-PBS for three times and fixed with 4% formaldehyde at 37 ℃ for 15 min. The cells then were washed three times with D-PBS and stained with DAPI for 5min. After thorough rinsing with D-PBS, the cells were soaked in D-PBS for cell imaging with Nikon A1+ confocal laser scanning microscope.

10. Evaluation of silencing efficiency of MDR1 mRNA and P-gp

MCF-7/ADR cells were planted in a six-well plate at 1.5×10^5 cells/well in full (RPMI)-1640 medium and incubation at 37 ℃ for 24 h. One well of MCF-7/ADR cells were used as a negative control. For other wells, pure (RPMI)-1640 medium containing 100 nM of P-gp siRNA, 50 μg/mL of PPP-FA-(P-gp) siRNA (containing 100 nM siRNA) or 50 μg/mL of PPP-FA-(control) siRNA (containing 100 nM control siRNA) was added to replace the old medium, and the cells were incubated at 37 ℃ for 24 h. All wells of cells were replaced with fresh full (RPMI)-1640 medium and incubation for

another 24h.

Evaluation of MDR1 mRNA expression. Total cellular RNA was extracted from the previous four cells using RNAsimple Total RNA Kit according to the manufacturer´s protocol. The cDNAs were synthesized by PrimeScript™ RT reagent Kit (Perfect Real Time) according to its indicated protocol. QRT-PCR assay of cDNAs was carried out by SYBR® Premix Ex Taq ™II (Tli RNaseH Plus) on LightCycler® 96 Real-Time PCR System. The PCR procedure was initiated by 95 ℃ for 2 min, and followed by 50 cycles: 95 ℃ for 30 s and 58 ℃ for 90 s. The forward and reverse primer of MDR1 is 5′-ATATCAGCAGCCCACATCAT-3′ and 5′-GAAGCACTGGGATGTCCGGT-3′, respectively. The forward and reverse primer of β-actin is 5′-AAAGACCTGTACGCCAACACAGTGCTGTCTGG-3′ and 5′-CGTCATACTCCTGCTTGCTGATCCACATCTGC-3′, respectively. The relative expression levels of MDR1 mRNA were determined via normalization to the expression of an endogenous control β-actin and the $2^{-\Delta\Delta}$Ct method.

Evaluation of P-gp expression. The four cells were lysed by Triton X-100 cell lysis buffer and the P-gp expression levels were assayed by using ELISA Kit according to the manufacturer´s protocol.

11. Cytotoxicity Assay and Therapy Efficiency

Cytotoxicity assay. MCF-7/ADR cells were planted into a 96-well microplate at 10^4 cells/well in full (RPMI)-1640 medium. After incubation for 24 h, the cells were rinsed with D-PBS. Pure (RPMI)-1640 medium containing PPP-FA (40, 80, 120 μg/mL) were added to replace the old medium, then the cells were further incubated at 37 ℃ for 24

h. Then, MTT (0.5 μg/mL) in full medium were added to each well to replace the old medium, and incubation for 4 h. MTT in medium was discarded and DMSO (100 μL) was added to dissolve formazan through vibrating for 15 min. The absorbance of 490 nm of each well was acquired on microplate reader.

Therapy effect on MCF-7/ADR cells. MCF-7/ADR cells were planted into a 96-well microplate at 10^4 cells/well in full (RPMI)-1640 medium. After incubation for 24 h, the cells were rinsed with D-PBS (pH 7.4). Pure (RPMI)-1640 medium containing PPP-FA-DOX (40, 80, 120 μg/mL) or PPP-FA-DOX-(P-gp) siRNA (40, 80, 120 μg/mL) were added to replace the old medium. After incubation for 6 h, the PPP-FA-DOX and PPP-FA-DOX-(P-gp) siRNA treated cells were irradiated with or without 808 nm NIR laser irradiation (2 W/cm^2) for 10 min. The cells were further incubated at 37 ℃ for 24 h. Then, the cell viability was studied following the similar procedure mentioned in the cytotoxicity assay.

12. **In vivo imaging**

All animal experiments were conducted in compliance with the guidelines of Tianjin Committee of Use and Care of Laboratory Animals, and approved by the Animal Ethics Committee of Nankai University. MCF-7 cells (1×10^7 cells in 100 μL PBS) were subcutaneously injected into the selected position of the nude mice (4-5 weeks) to produce a tumor about 5 mm in diameter for optical imaging. After 254 nm UV light excitation for 10 min, 100 μL of PPP-FA-DOX (2 mg/mL) was intratumorally injected into the tumor-bearing mice. The tumor-bearing mice were anesthetized with intraperitoneally administered pentobarbital, excited with LED light (650 nm) for 5 min

and subsequently bioimaged at regular intervals. The fluorescence of DOX was acquired under excitation of 500 nm.

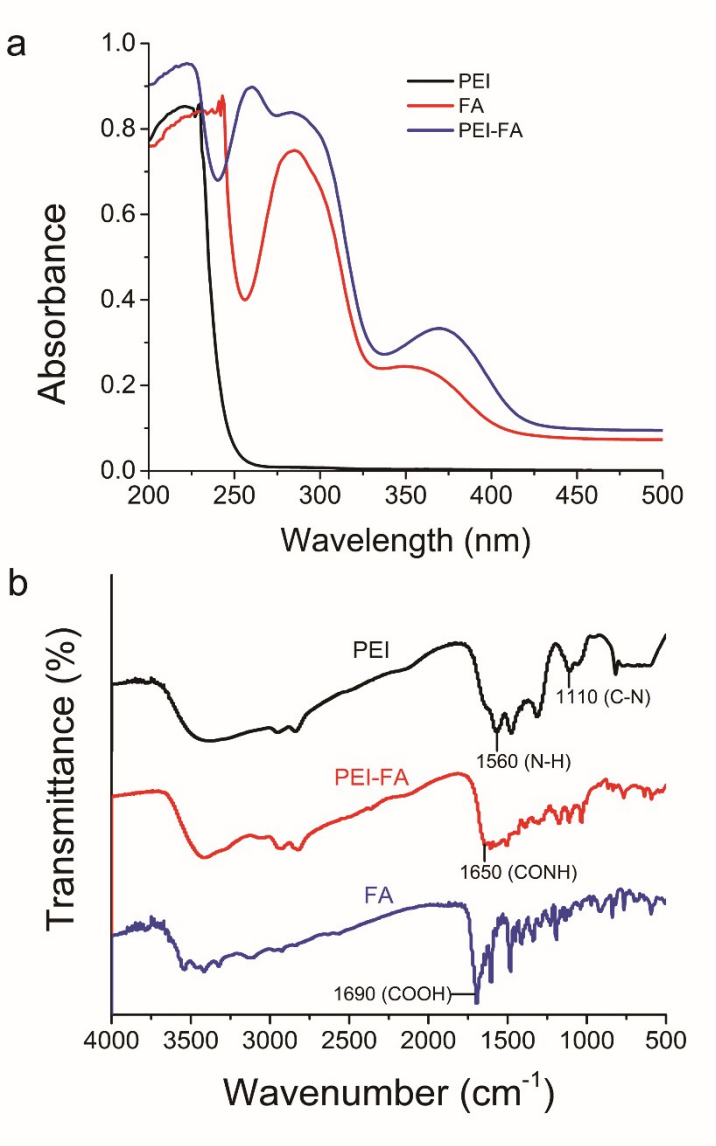

Fig. S1 (a) UV-vis absorption spectra of PEI, FA and PEI-FA. (b) FTIR spectra of PEI, PEI-FA and FA.

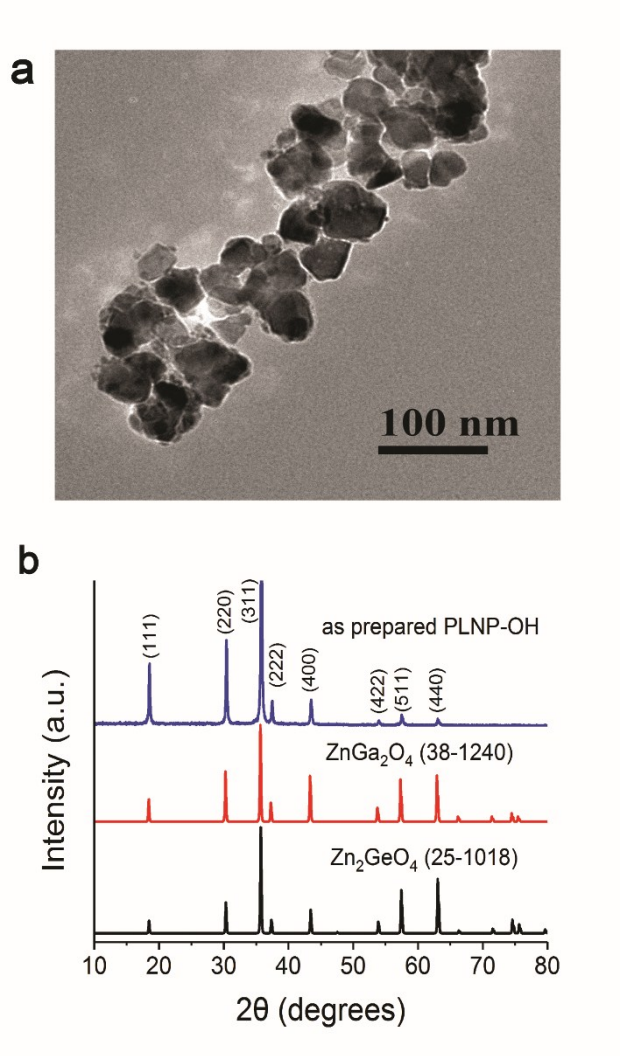

Fig. S2 (a) TEM image of the as-prepared PLNP-OH. (b) XRD patterns of the as-prepared PLNP-OH and the standard XRD patterns of $ZnGa_2O_4$ (JCPDS: 38-1240) and Zn_2GeO_4 (JCPDS: 25-1018).

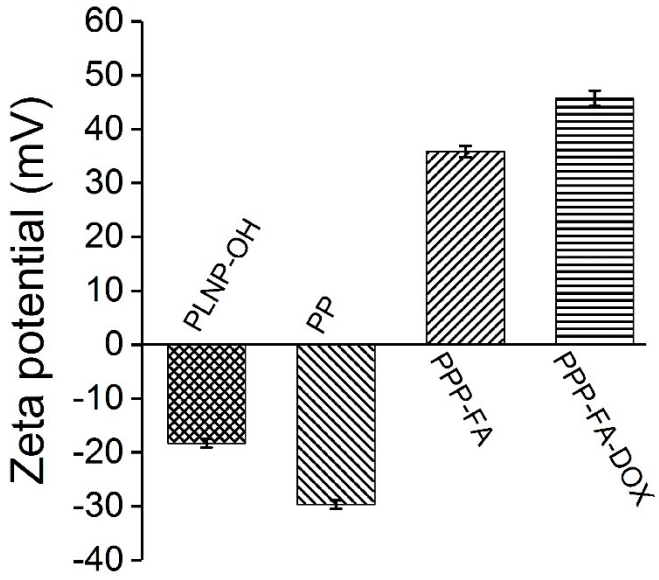

Fig. S3 Zeta potential of PLNP-OH, PP, PPP-FA and PPP-FA-DOX.

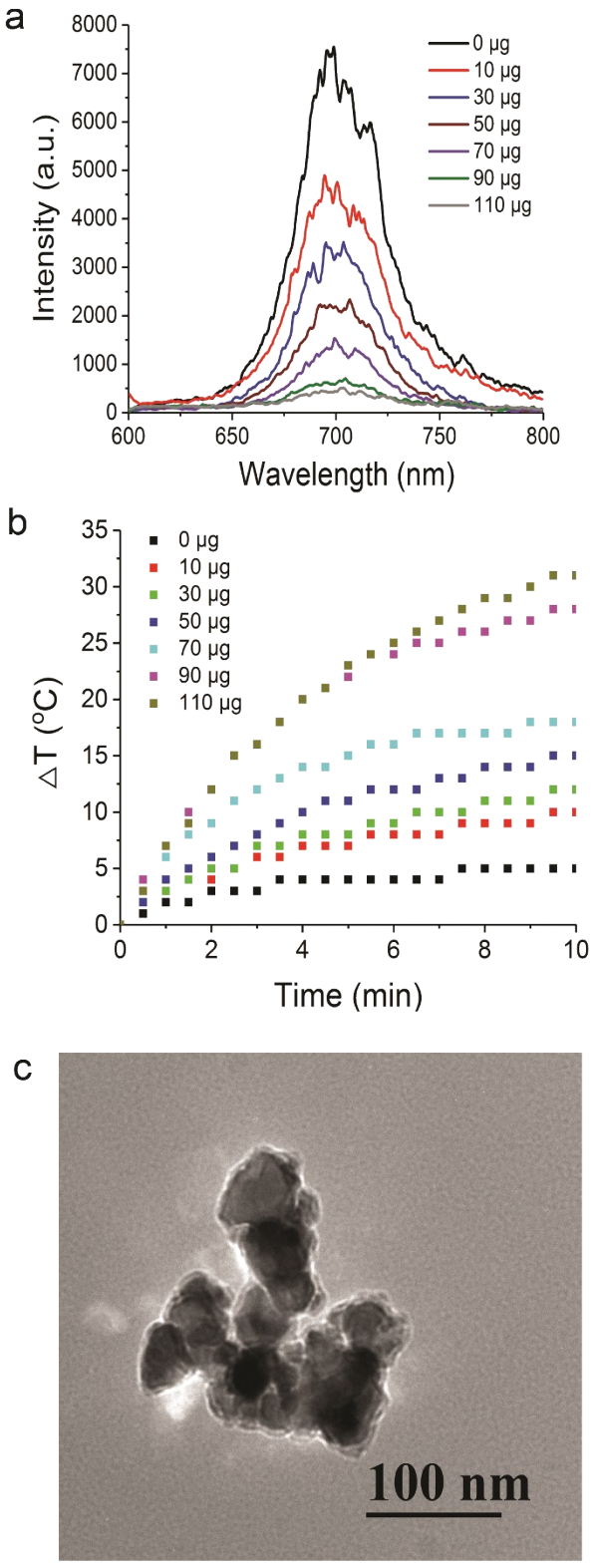

Fig. S4 (a) Emission spectra of PP. (b) Temperature change of PP under irradiation of 808 nm laser (2 W/cm^2) within 10 min. The PP result from the reaction of 1 mg of PLNP-OH with 0, 10, 30, 50, 70, 90, 110 μg of dopamine. (c) TEM image of PP.

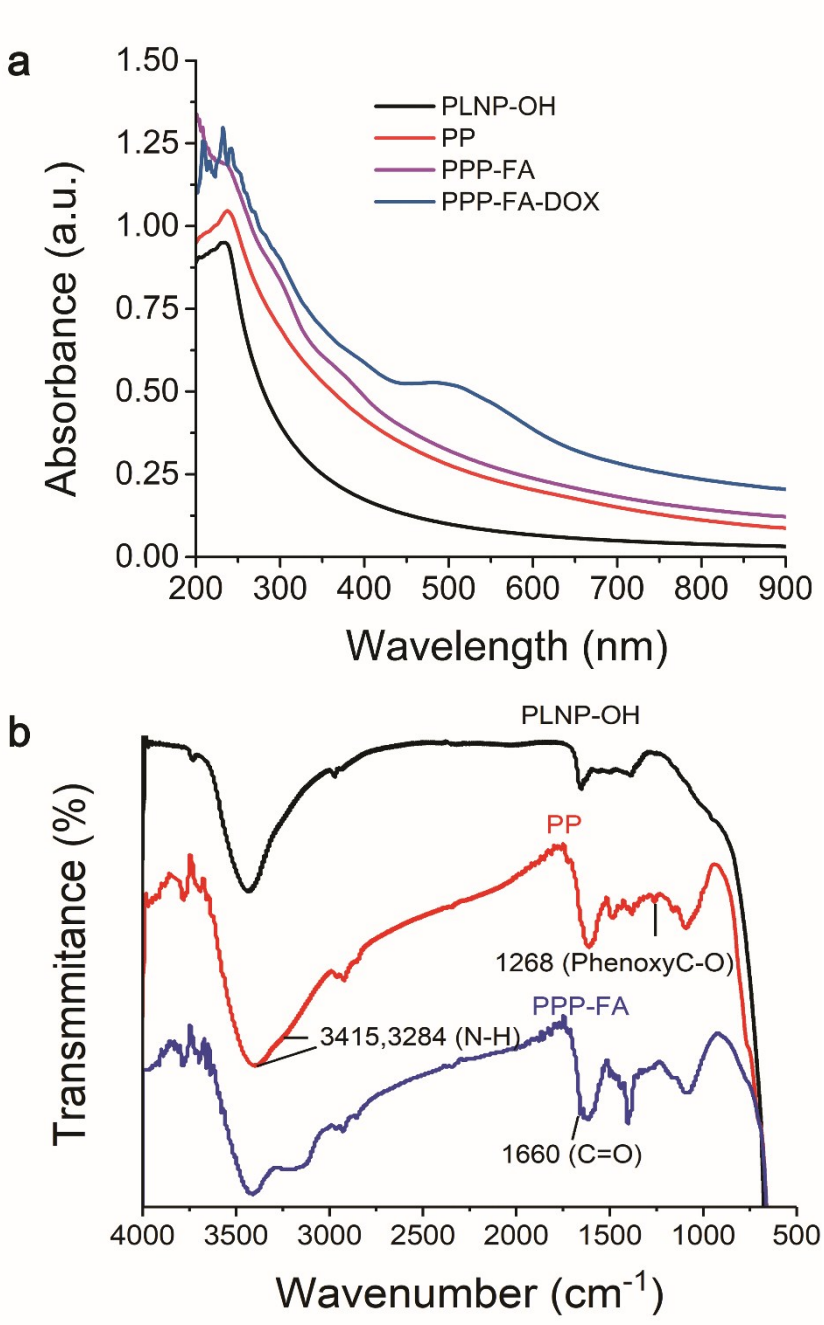

Fig. S5 (a) UV-Vis absorption spectra of PLNP-OH, PP, PPP-FA and PPP-FA-DOX. The high background in the absorption region of 800-900 nm results from the turbid solution; (b) FTIR spectra of PLNP-OH, PP and PPP-FA.

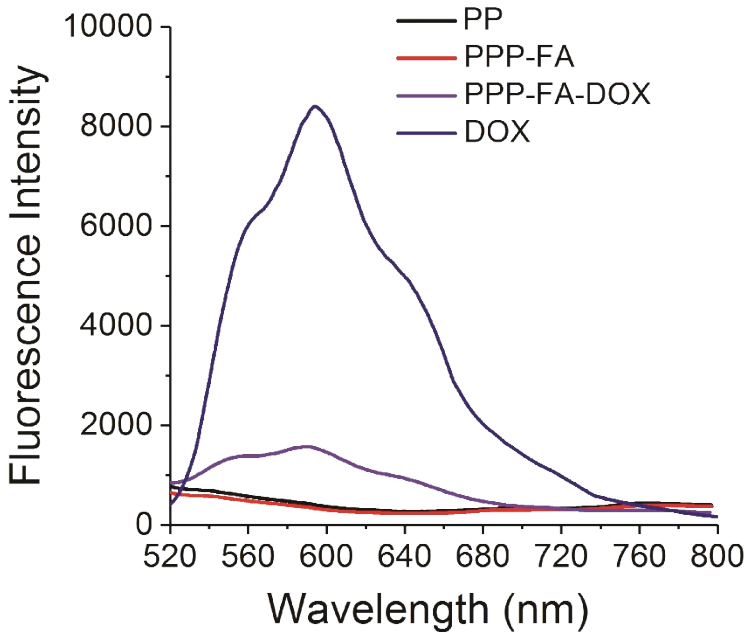

Fig. S6 Fluorescence spectra of PP, PPP-FA, PPP-FA-DOX, and DOX under excitation with 480 nm.

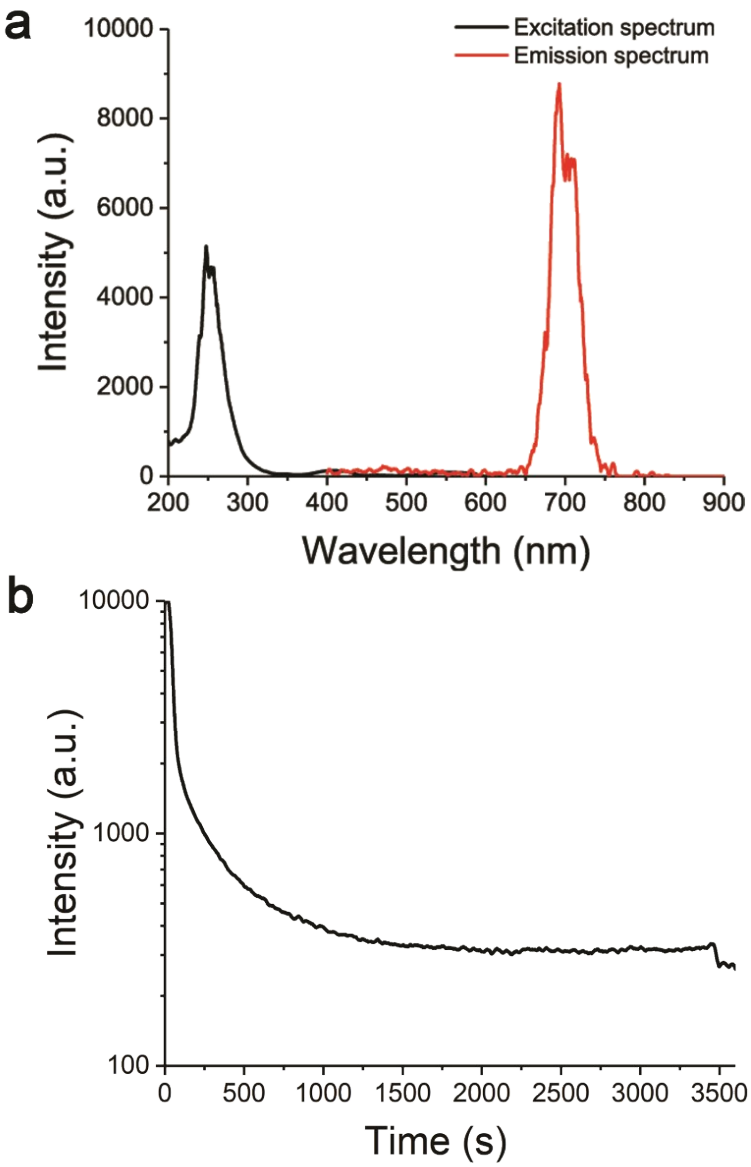

Fig. S7 (a) Excitation spectrum and emission spectrum of as-prepared PLNP-OH aqueous solution (1 mg/mL). (b) NIR luminescence decay curve of as-prepared PLNP-OH aqueous solution (1 mg/mL) monitored at 695 nm after irradiation with UV light for 5 min.

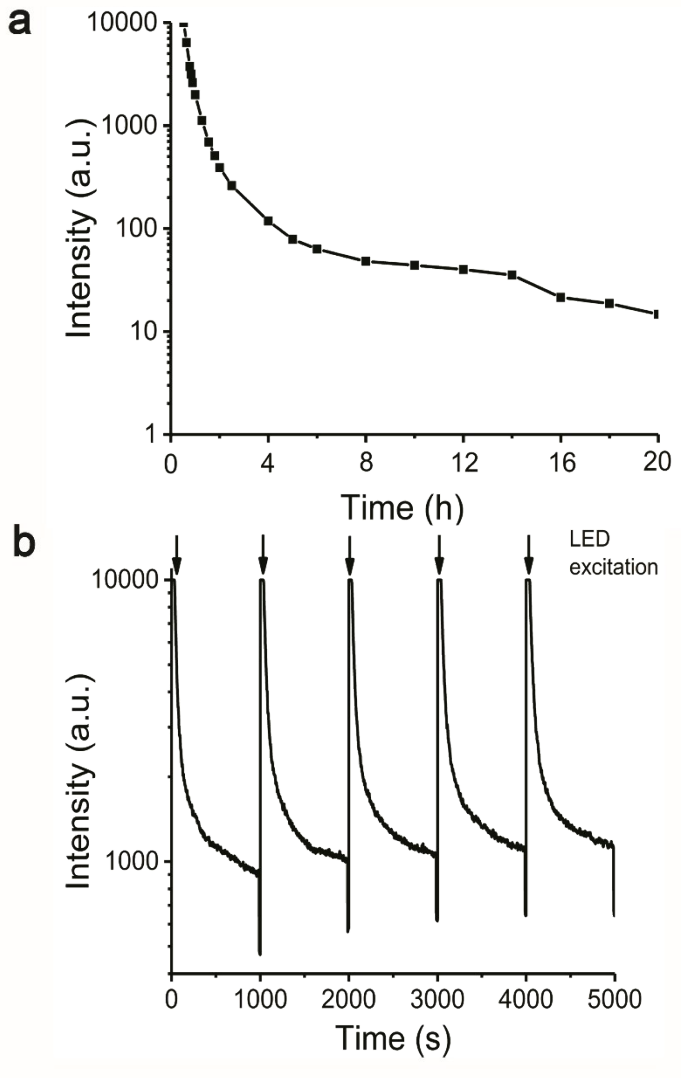

Fig. S8 (a) NIR luminescence decay curve of as-prepared PLNP-OH powder monitored at 695 nm after irradiation with UV light for 5 min. (b) NIR luminescence decay curve of PLNP-OH powder monitored at 695 nm reactivated with LED light every 1000 s.

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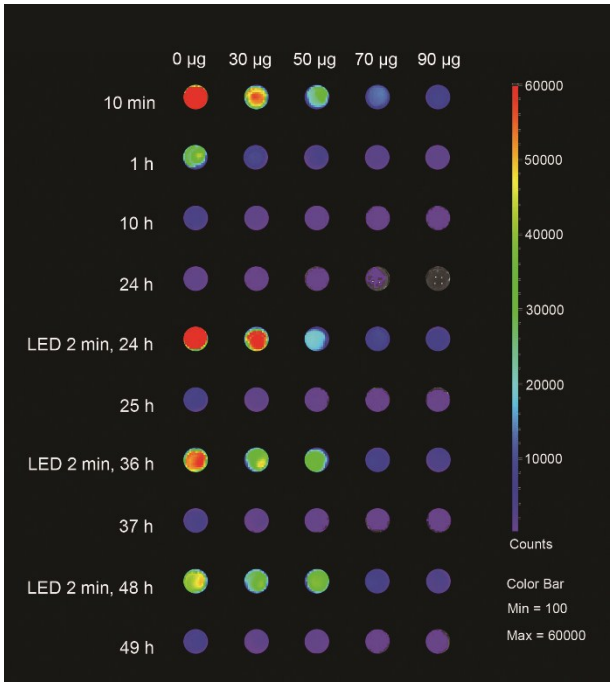

Fig. S9 NIR persistent luminescence and LED-reactivated persistent luminescence images of PP aqueous solution (1 mg/mL) resulting from 1 mg PLNP-OH with 0, 30, 50, 70, 90 μg of dopamine.

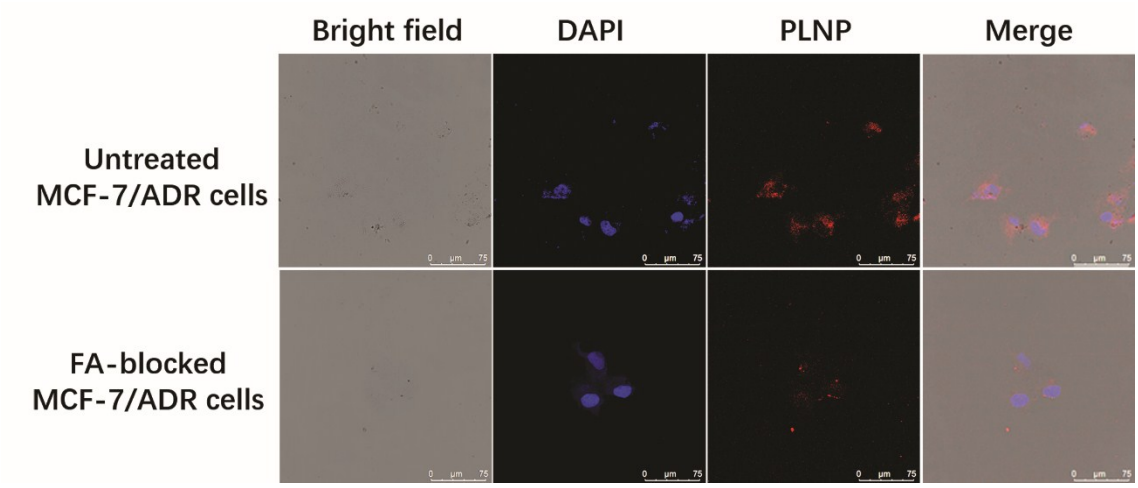

Fig. S10 CLSM images of untreated and FA-blocked MCF-7/ADR cells after incubation with PPP-FA. The fluorescence cell images were captured under the same conditions. Scale bar = 75 μm.

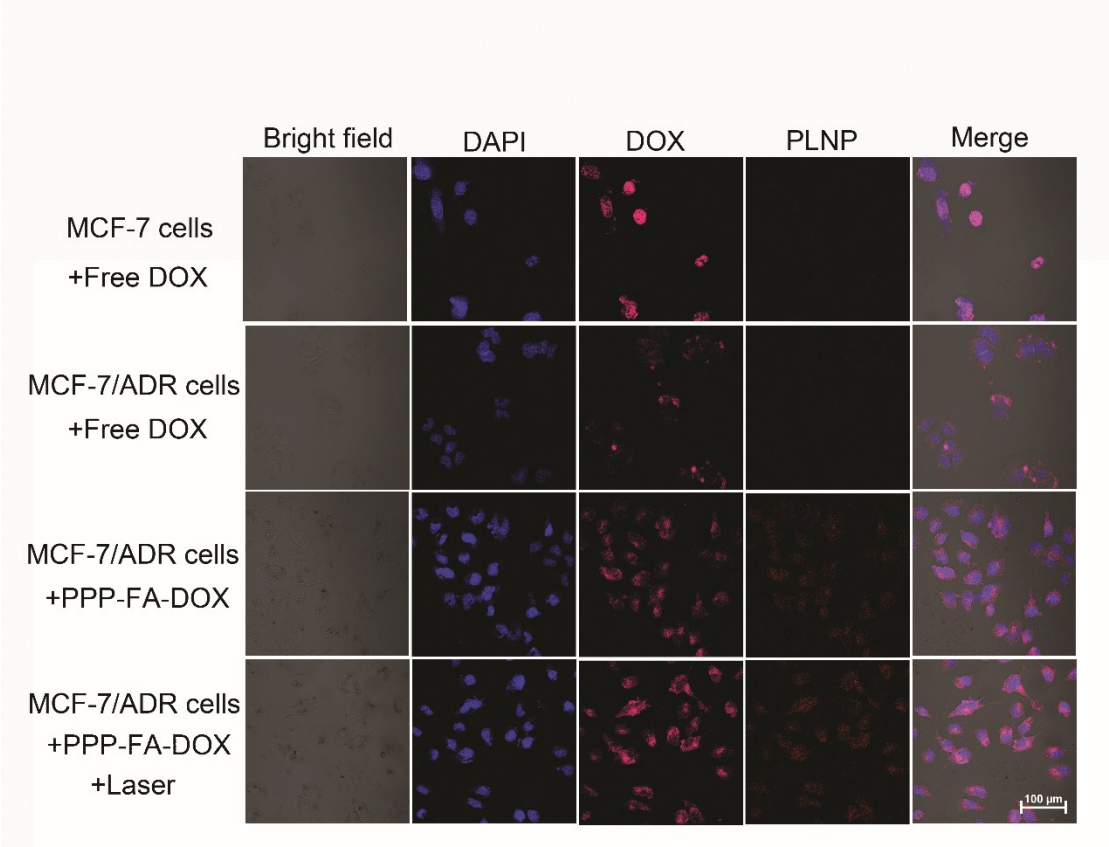

Fig. S11 CLSM images of MCF-7 and MCF-7/ADR cells after incubation with free DOX and PPP-FA-DOX, which was irradiated with 808 nm laser (2 W/cm^2, 10 min) or not. The fluorescence cell images were captured under the same conditions. Scale bar = 100 μm.

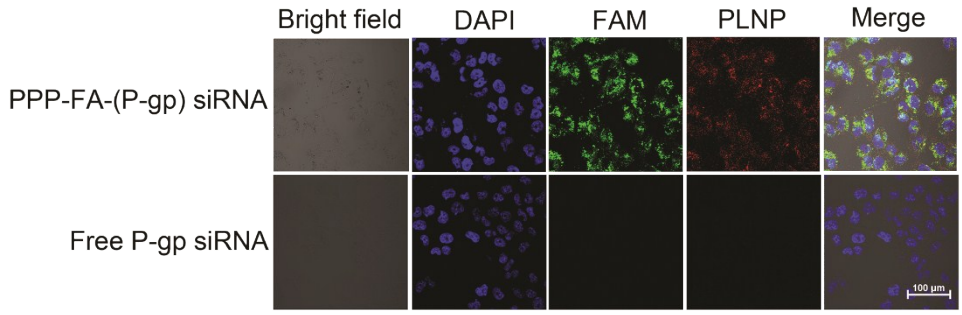

Fig. S12 CLSM images of MCF-7/ADR cells after incubation with PPP-FA-(P-gp) siRNA and free P-gp siRNA. The fluorescence cell images were captured under the same conditions. Scale bar = 100 μm.

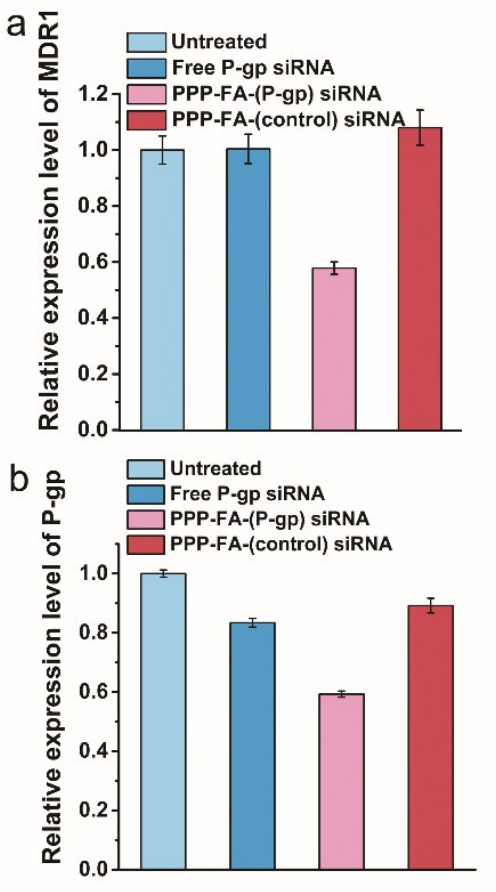

Fig. S13 Relative expression levels of MDR1 mRNA (a) and P-gp (b) in MCF-7/ADR cells untreated, incubated with free P-gp siRNA, PPP-FA-(P-gp) siRNA, and PPP-FA-(control) siRNA. The control siRNA is a negative control siRNA.

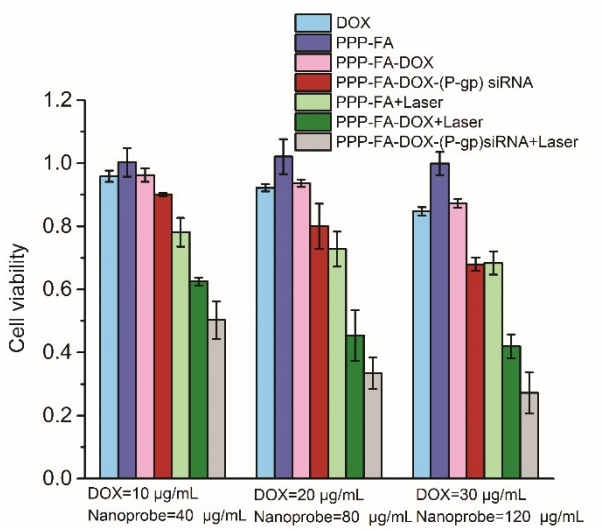

Fig. S14 Cell viability of MCF-7/ADR cells against different nanoprobes with various concentrations.

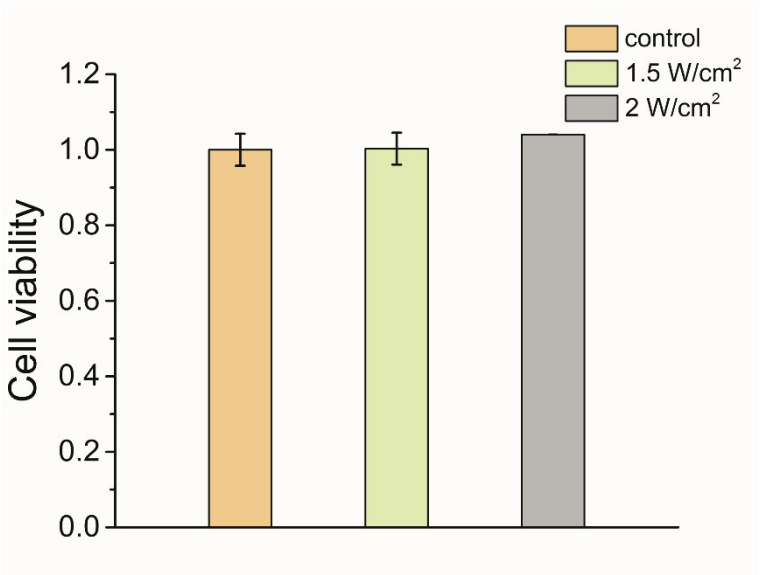

Fig. S15 Viability of MCF-7/ADR cells after irradiation with different 808 nm laser power density.

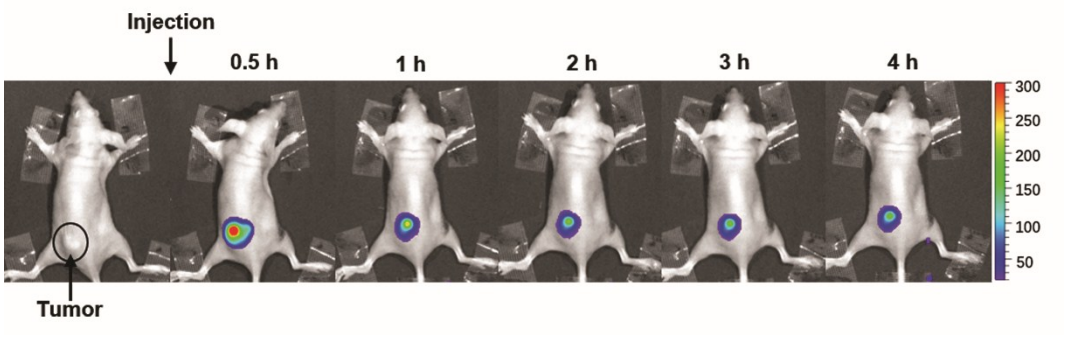

Fig. S16 In vivo luminescence images of MCF-7 tumor-bearing mice after intratumoral injection of PPP-FA-DOX. The nanoprobe was irradiated with a 254 nm UV light for 10 min before injection and irradiated with a 650 nm LED light for 5 min before each acquisition.

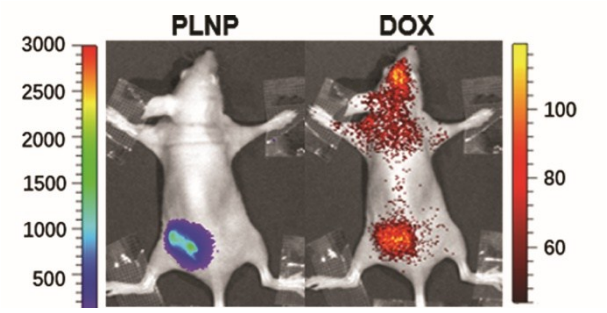

Fig. S17 In vivo imaging comparison of PLNP and DOX after intertumoral injection of PPP-FA-DOX.

References

1. S.-Q. Wu, C.-W. Chi, C.-X. Yang and X-P. Yan, *Anal. Chem.*, 2016, **88**, 4114.