Supplementary Material

The impact of light irradiation timing on the efficacy of nanoformula-based photo/chemo combination therapy

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Materials

Doxorubicin hydrochloride (DOX·HCl, 98%), pheophorbide A (PhA, >95%), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), Hoechst 33258 and LysoTracker Green DND-26 were purchased from J&K Scientific Ltd., Shanghai, China. Triethylamine (TEA), DMSO and ethanol were ordered from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. Deionized water (DI water) was obtained from a Milli-Q Biocel (Millipore Corporation, Breford, USA) water purification system. All reagents and solvents were of analytical grade.

Characterization

The fluorescence spectra were measured with a FluoroMax 4 (Horiba Jobin Yvon) Spectrofluorometer. UV-Vis absorption spectra were recorded on a PerkinElmer Lambda 750 UV/Vis/NIR Spectrophotometer. Scanning electron microscopy (SEM) images were observed on an FEI Quanta 200 FEG field emission scanning electron microscope. Transmission electron microscopy (TEM) images were observed using a Tecnai G220. DLS analysis measurements were recorded using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). In vitro fluorescence images of the cells were taken by a Leica laser scanning confocal microscope. In vivo fluorescence images of the mice were taken by a Maestro in vivo fluorescence imaging system (CRi, Inc.).

Cell culture

HeLa cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM). The culture medium contains 10% of fetal bovine serum (FBS) and antibiotics (50 U/mL penicillin

and 50 μg/mL streptomycin) at 37°C under a humidified atmosphere containing 5% CO_2.

Cell viability assay

The cytotoxicity experiments were carried out using MTT assay. HeLa cells (~5×10^3 cells/well) were seeded in a 96-well plate with 100 μL of complete DMEM medium and cultured for 24 h. The DOX-PhA NP (3.5:6.5) suspensions, DOX NP suspensions and PhA NP suspensions were diluted into different concentrations with PBS and added to the different groups. The cells were irradiated under 660 nm laser beam (50 mW/cm^2) for 5 min at different time points after incubation with NP suspensions of different concentrations, including 0.056, 0.113, 0.225, 0.45, 0.9, 1.8 and 3.6 μM. Then, the cells were incubated for another 24 h. All concentrations of DOX-PhA NP suspensions were marked by the concentration of DOX in them. In the groups of free PhA and free DOX, the concentrations were the same as those in the DOX-PhA NPs (0.056, 0.113, 0.225, 0.45, 0.9, 1.8 and 3.6 μM). The cells of the dark group were only given NPs (no irradiation). At the end of incubation, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well. After 5 h, the medium in each well was removed, and 150 μL of DMSO was added to dissolve the formed formazan crystals. Finally, the cell viabilities were determined by measuring absorbance. Cells without NPs were set as control for 100% viability. The value of IC50 (half maximal inhibitory concentration) of each group was also calculated.

A parallel experiment with laser power intensity of 100 mW/cm^2 was also performed.

Calcein AM/PI assay

HeLa cells (~1×10^5/plate) were seeded in 35 mm plates and cultured for 12 h. Then, the cells were incubated with DOX-PhA NPs (DOX: 0.023 mM, PhA: 0.043 mM) for different times (1 h, 2 h, 4 h). Then, cells were irradiated with a 660 nm (50 mW/cm2) laser for 5 min. The cells were stained with Calcein AM/PI for 15 min and then washed twice with PBS. The cells treated with PBS were taken as control. Finally, the cells were imaged by a confocal laser scanning microscope (CLSM).

Immunohistochemical analysis

Tumor tissues were excised from the mice at the end of treatment procedure and then fixed with 4% paraformaldehyde solution and embedded in paraffin for analysis. 8 μm paraffin tumor slices were prepared for immunohistochemical analysis. The sliced tumor tissues were stained by hematoxylin and eosin (H&E). Apoptotic cells were identified using a terminal transferase dUTP nick-end labeling (TUNEL) assay kit (Yeasen, Shanghai, China) according to the manufacturer's protocol. The images of H&E staining were obtained using a Nikon Eclipse 90i microscope. The nuclei were stained by hematoxylin as blue and the cytoplasm and muscle fiber were stained by eosin as red. The images of TUNEL staining were obtained by the CLSM.

Statistical analysis

The data are displayed as mean ± SEM. Statistical significance was analyzed by two-tailed Student's t test. *$p < 0.05$ and **$p < 0.01$ were considered significant.

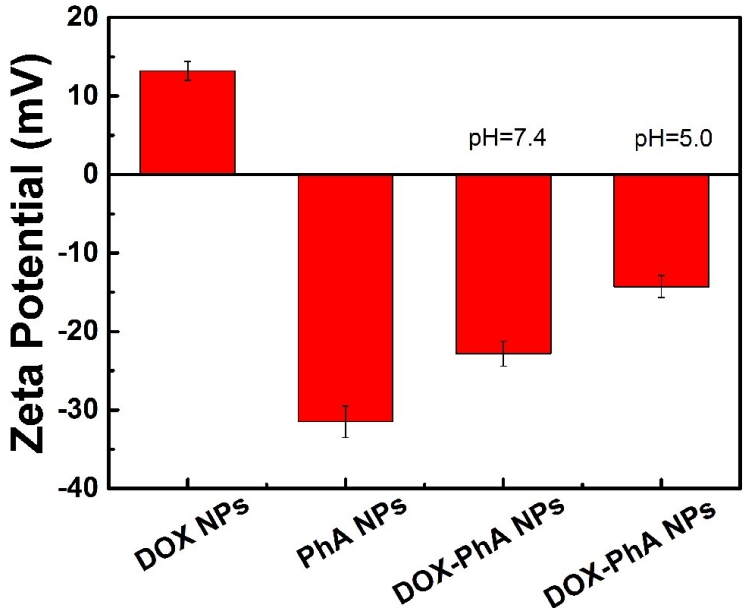

Figure S1. The Zeta potentials of pure DOX NPs in pH 7.4 buffer, pure PhA NPs in pH 7.4 buffer and DOX-PhA NPs (3.5:6.5) in pH 7.4 and 5.0 buffer, respectively. Error bars were based on standard error of mean (SEM) and n = 3.

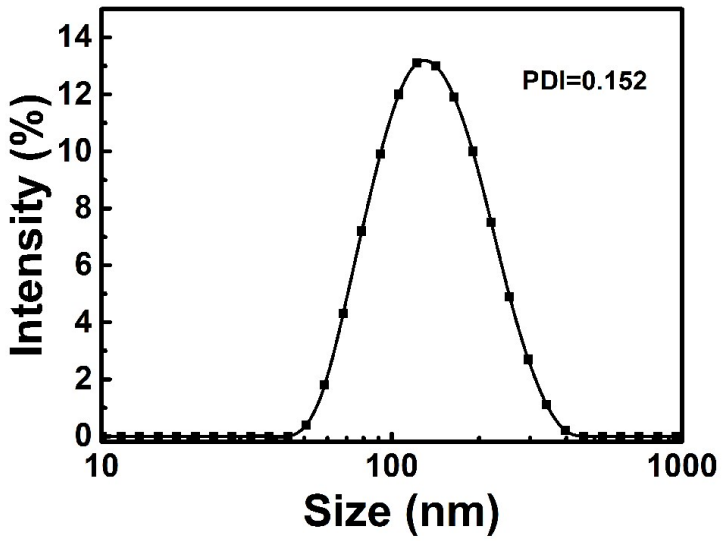

Figure S2. The size distribution of DOX-PhA NPs (3.5:6.5) presented by the intensity.

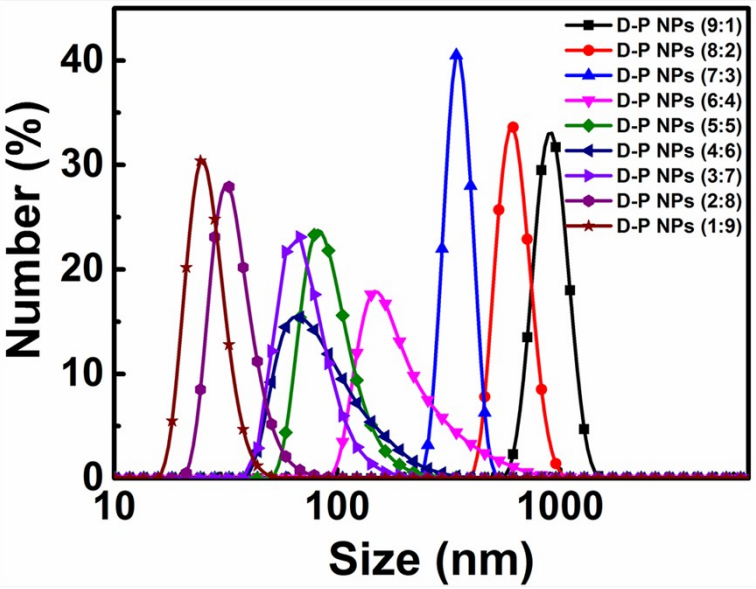

Figure S3. The diameters of the DOX-PhA NPs composed of different ratios (DOX:PhA=9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9) in deionized water.

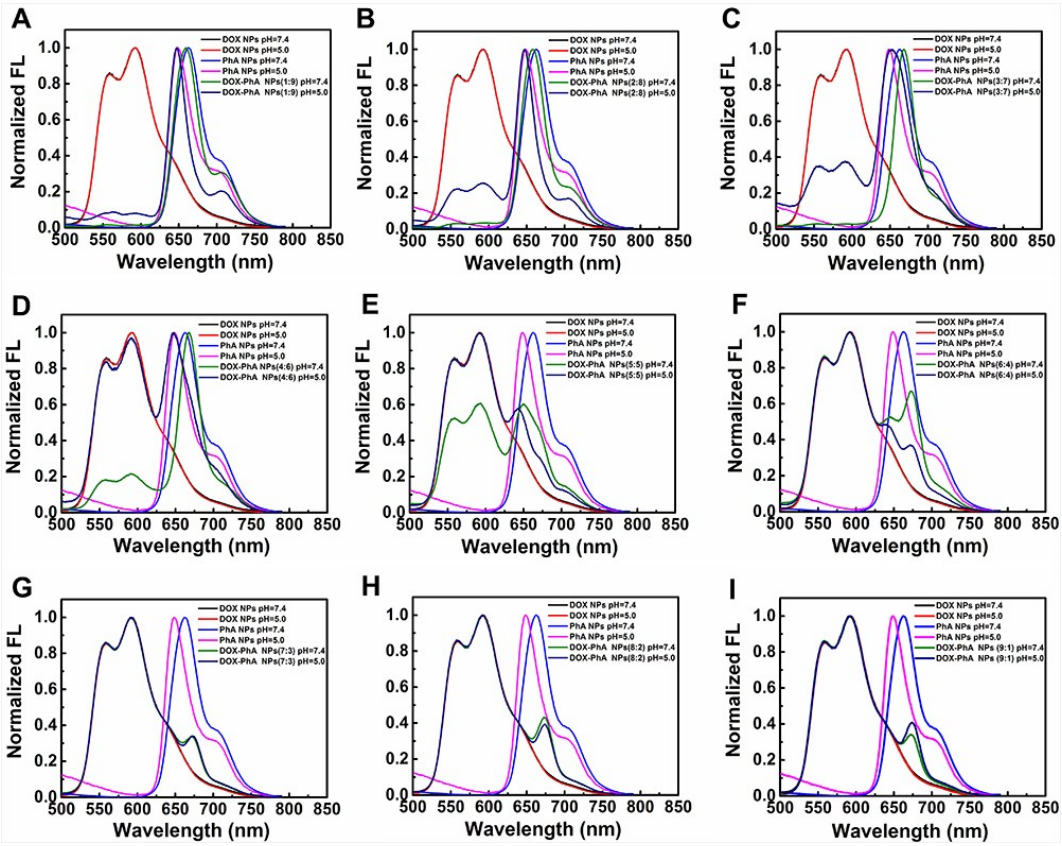

Figure S4. The fluorescence emissive spectrum of the DOX-PhA NPs composed of different ratios and the fluorescence emissive spectrum of DOX NPs and PhA NPs at two pH values. Ex = 405 nm. The ratios of DOX:PhA are **(A)** 1:9, **(B)** 2:8, **(C)** 3:7, **(D)** 4:6, **(E)** 5:5, **(F)** 6:4, **(G)** 7:3, **(H)** 8:2, **(I)** 9:1. In some ratios, the DOX-PhA NPs emission spectra vary significantly at different pH values (5.0 and 7.4). The emission enhancement at the DOX emission wavelength region at pH 5.0 could be used for monitoring the subcellular DOX release and distribution, which is a different fluorescence channel from that of PhA.

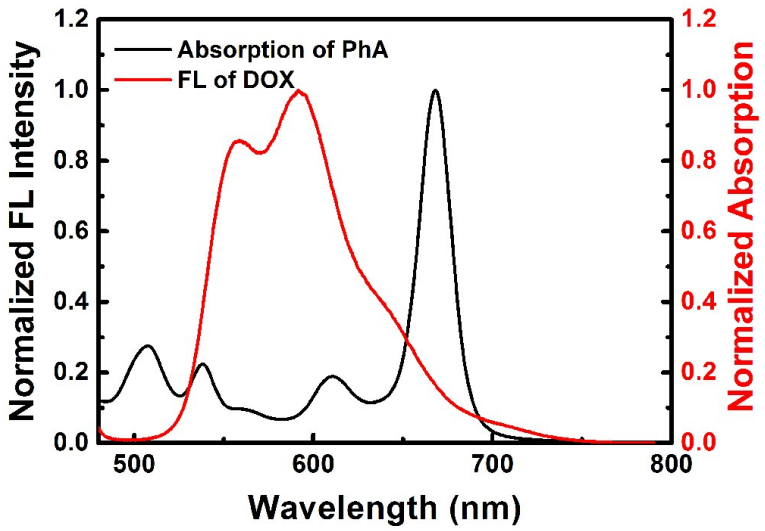

Figure S5. The normalized fluorescence emission spectrum of DOX and the normalized UV-vis absorption spectrum of PhA to indicate the wavelengths overlap for FRET.

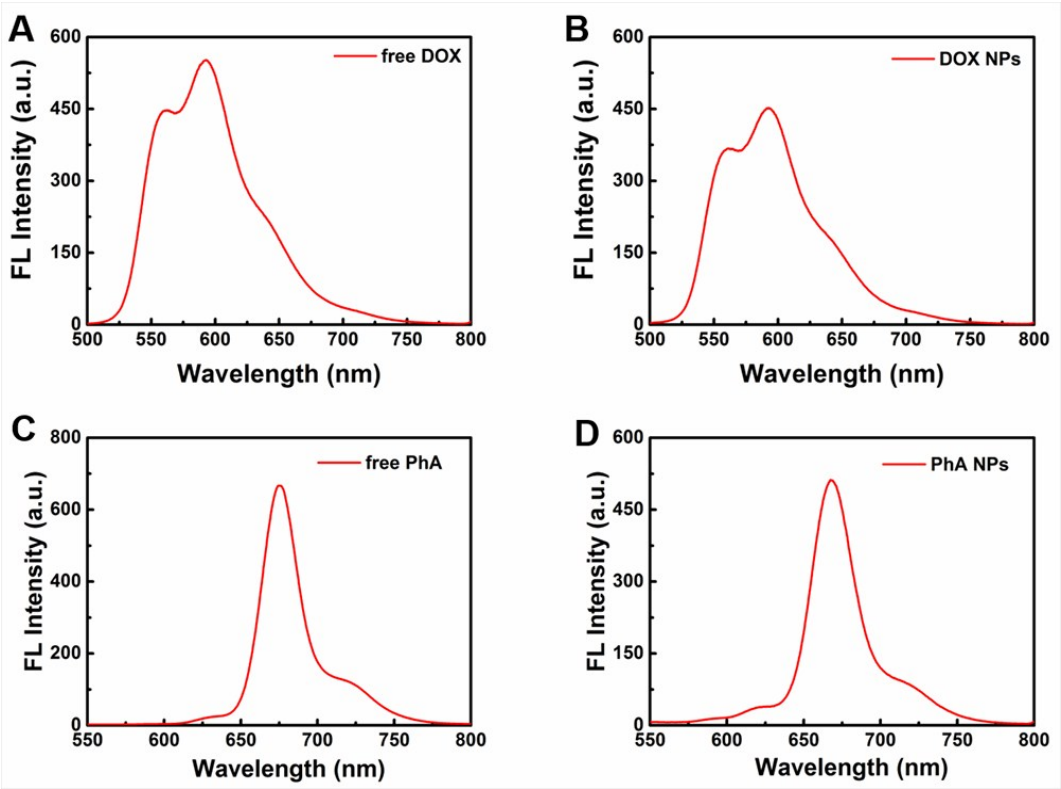

Figure S6. The fluorescent spectra of **(A)** free DOX, **(B)** DOX NPs, **(C)** free PhA and **(D)** PhA NPs. DOX NPs and PhA NPs were measured in the deionized water and free

DOX and free PhA were measured in DMSO.

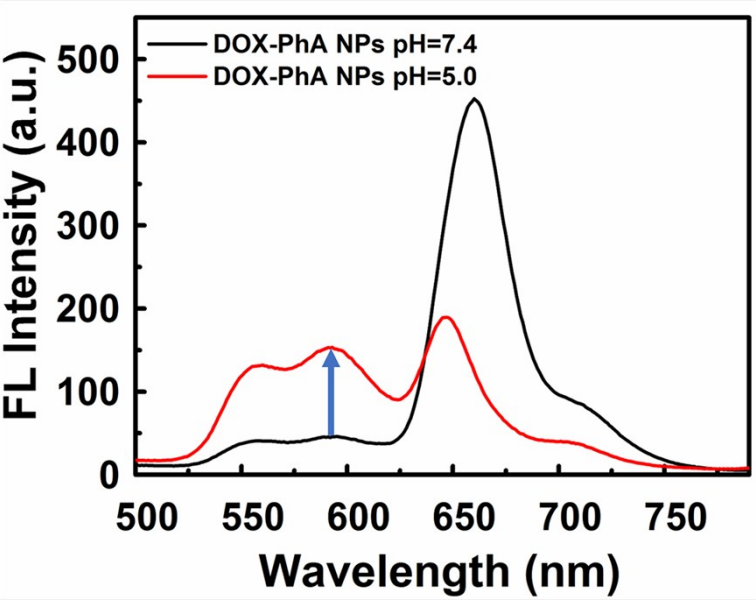

Figure S7. The DOX fluorescence increases ~ 3 times for DOX-PhA NPs at 599 nm when pH decreases from 7.4 to 5.0.

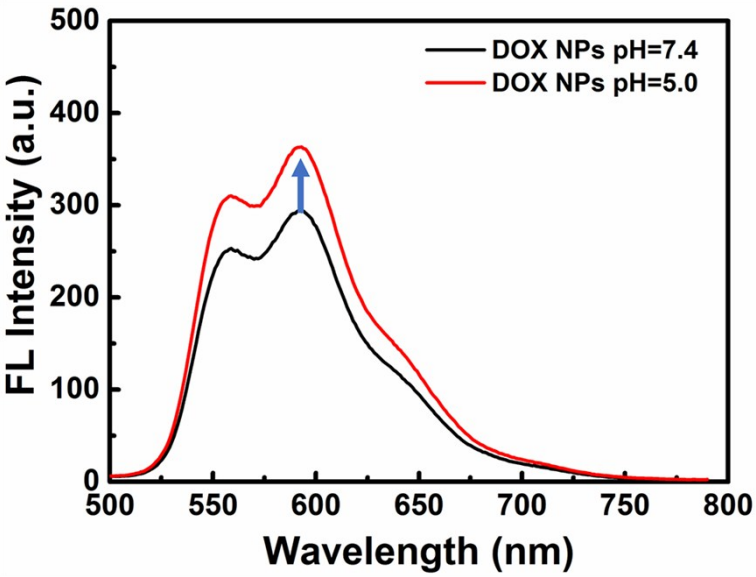

Figure S8. The DOX fluorescence increases ~ 1.24 times for pure DOX NPs at 599 nm when pH decreases from 7.4 to 5.0.

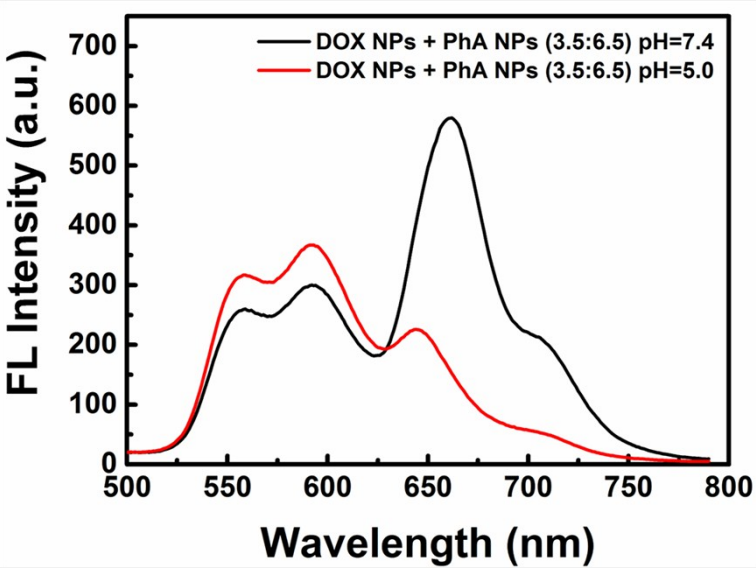

Figure S9. The fluorescent spectra of the mixture of DOX NPs and PhA NPs with the molar ratio of 3.5:6.5 under two pH values. No obvious FRET effect was observed.

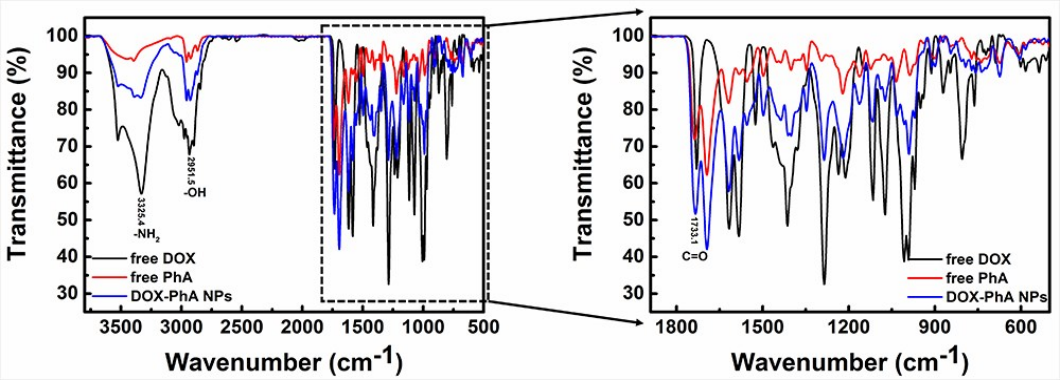

Figure S10. The infrared (IR) absorption spectra of free DOX, free PhA and DOX-PhA NPs. The wavenumber of 3325.4 cm^{-1} is the specific absorption peak of -NH_2 for DOX. The wavenumber of 2951.5 cm^{-1} is that of -OH and the wavenumber of 1733.1 cm^{-1} is that of C=O for PhA.

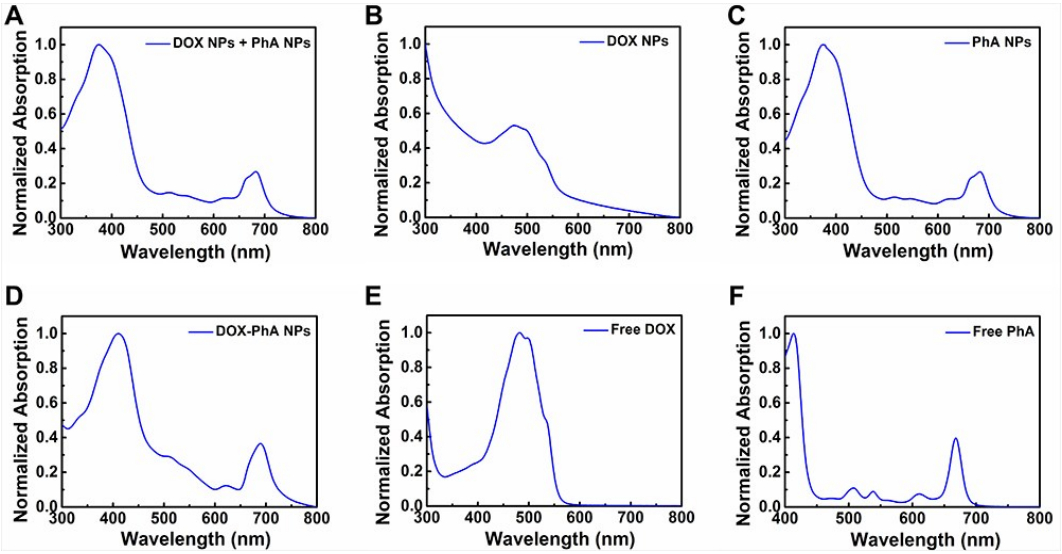

Figure S11. The UV-Vis absorption spectrum of different complex. **(A)** The mixture of DOX NPs and PhA NPs (DOX:PhA = 3.5:6.5) in deionized water. **(B)** DOX NPs in deionized water. **(C)** PhA NPs in deionized water. **(D)** DOX-PhA NPs (3.5:6.5) in deionized water. **(E)** Free DOX solution in DMSO. **(F)** Free PhA solution in DMSO.

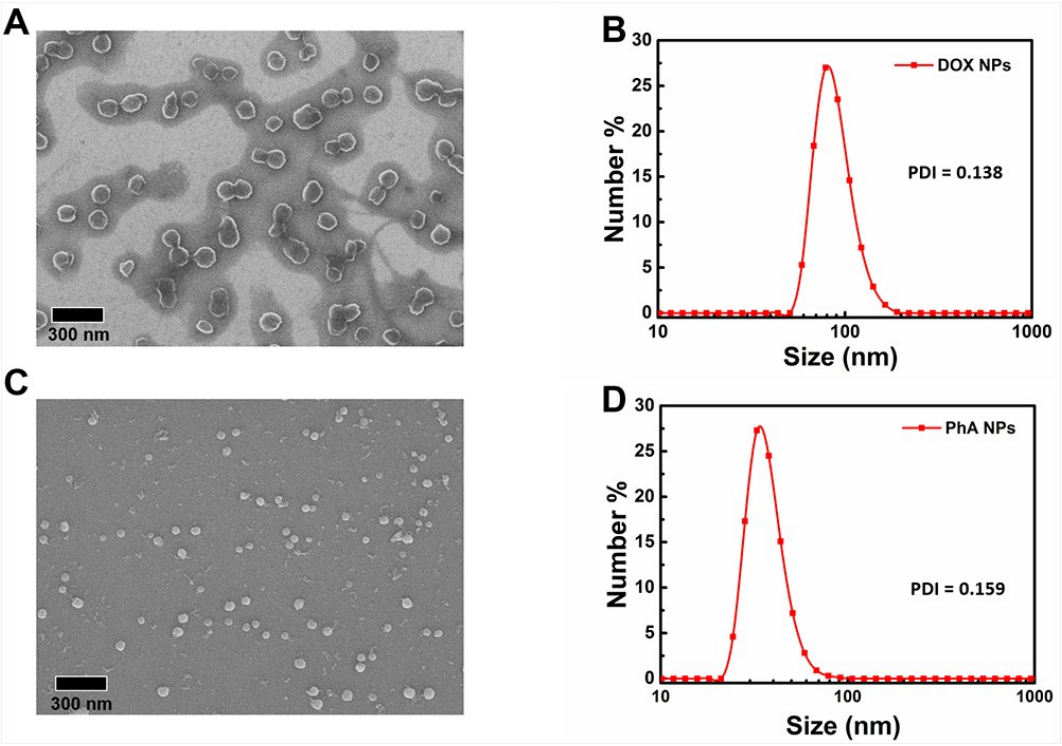

Figure S12. (A) The SEM images of DOX NPs. **(B)** The size distribution of DOX NPs

in deionized water measuring by DLS. The peak of size distribution of DOX NPs is about 100 nm. **(C)** The SEM images of PhA NPs. **(D)** The size distribution of PhA NPs in deionized water measuring by DLS. The peak of size distribution of PhA NPs is about 45 nm.

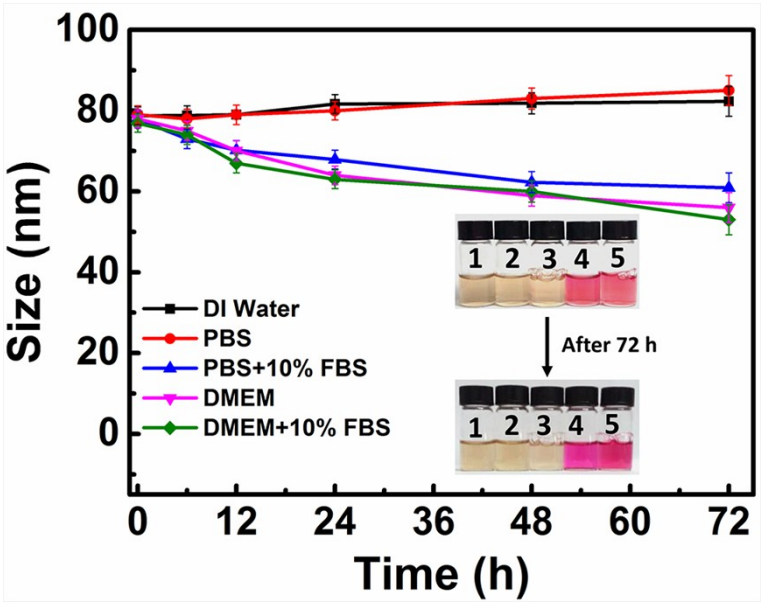

Figure S13. The diameters and the pictures of the DOX-PhA NPs (3.5:6.5) in different solutions over 72 h (1: DI (deionized) water; 2: PBS; 3: PBS + 10% FBS; 4: DMEM; 5: DMEN + 10% FBS). The diameters were measured by DLS. Error bars were based on standard error of mean (SEM) and n = 3.

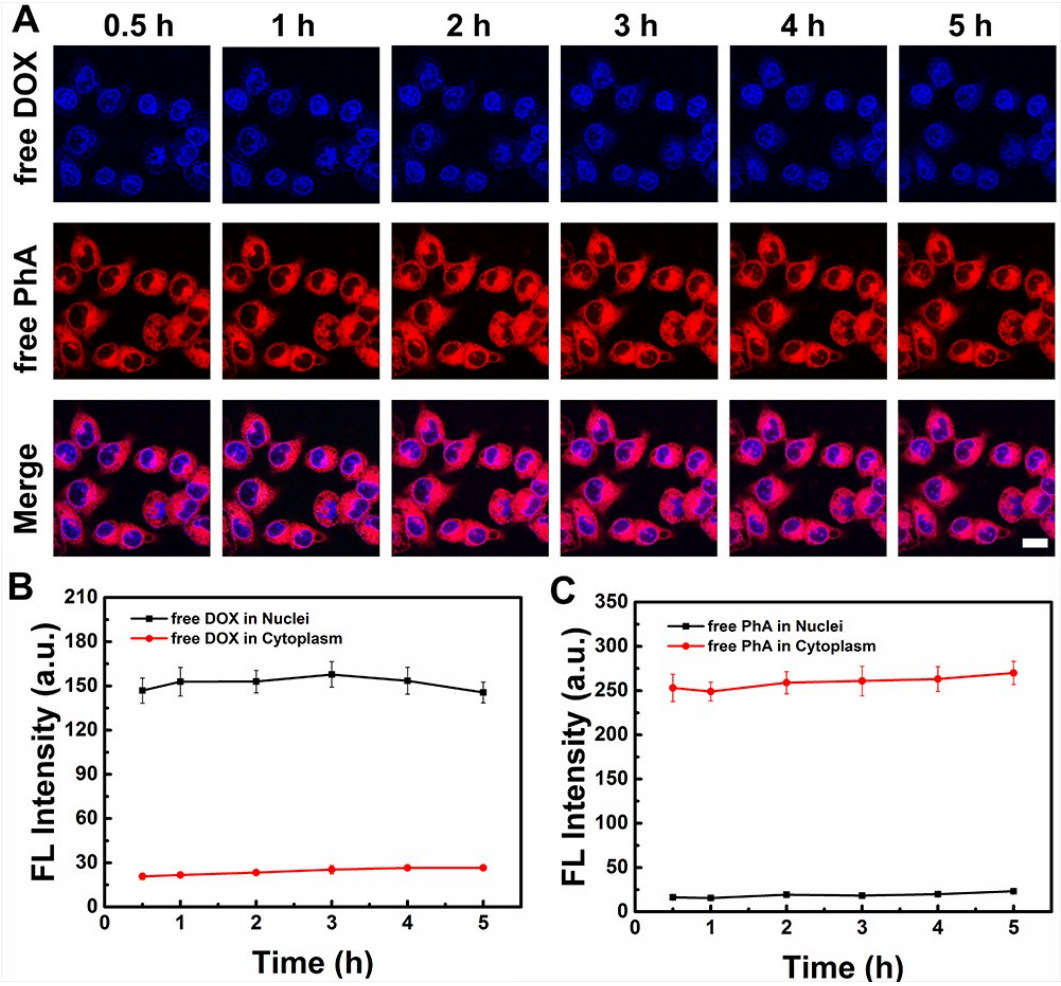

Figure S14. The in vitro real-time evaluation of the uptake kinetics of the mixture of free DOX (0.023 mM) and free PhA (0.043 mM). **(A)** The images were captured at the time points of 0.5 h, 1 h, 2 h, 3 h, 4 h and 5 h. The quantified fluorescence intensities of DOX **(B)** and PhA **(C)** in nuclei and cytoplasm over 5 h, respectively. Scale bar = 10 μm.

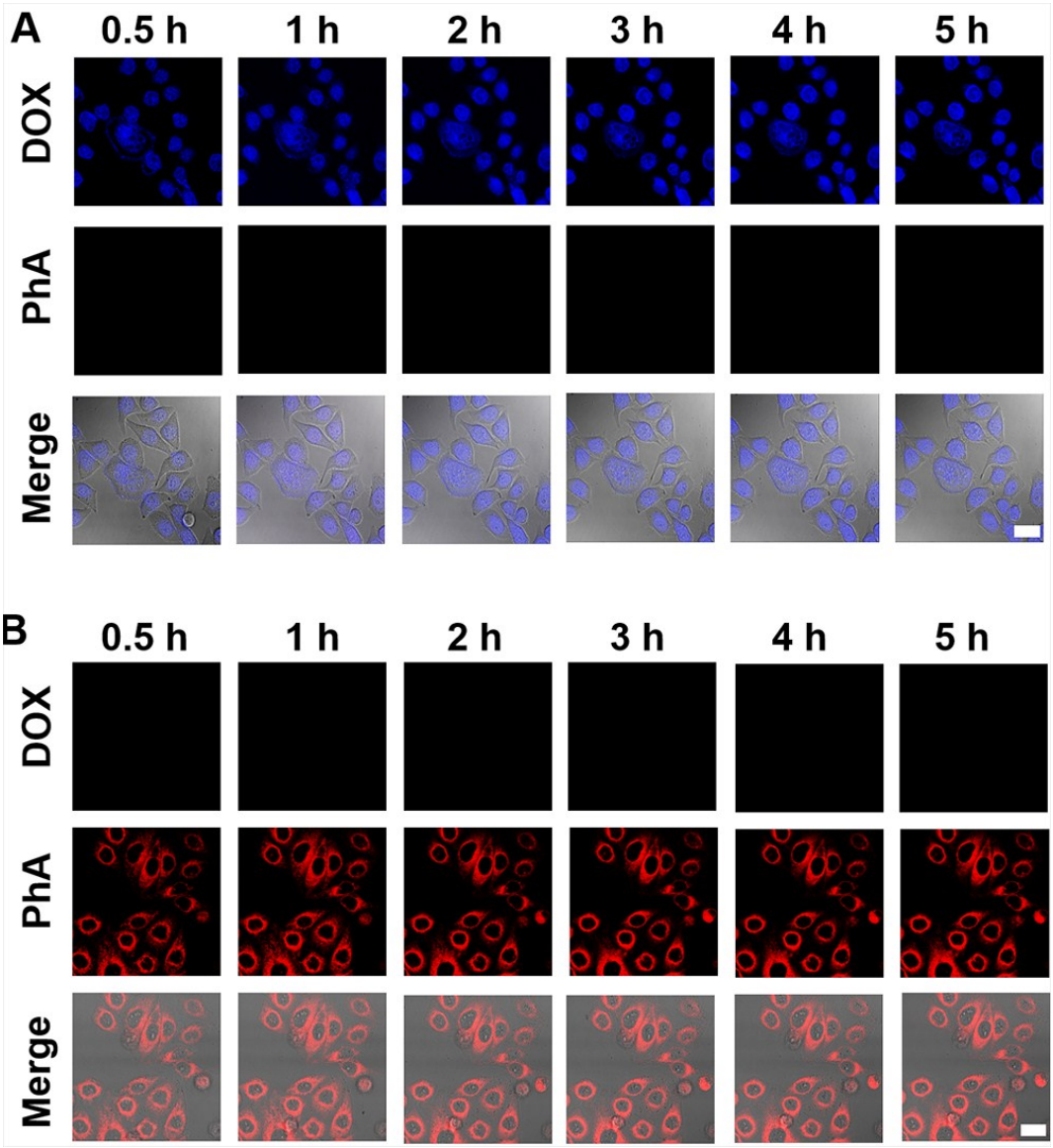

Figure S15. The CLSM images of HeLa cells by only treating **(A)** free DOX or **(B)** free PhA at both DOX and PhA imaging channels during 5 h. Scale bar = 20 μm. 405 nm wavelength laser was used to excite both DOX and PhA. The emission filters for DOX and PhA are 530-620 nm and 650-720 nm, respectively.

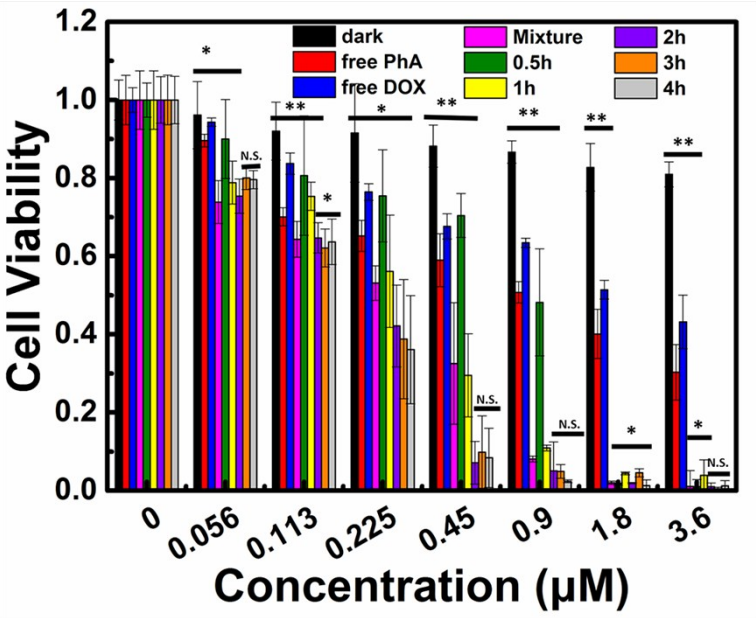

Figure S16. The cell viability of DOX-PhA NPs under the 660 nm laser (100 mW/cm^2, 5 min) after different incubation time. The group of dark, free DOX, free PhA and mixture (the mixture of free DOX and free PhA with the molar ratio of 3.5:6.5) were as controls. Error bars were based on standard error of mean (SEM) and n = 6. (N.S. no significance, *p < 0.05, **p < 0.01)

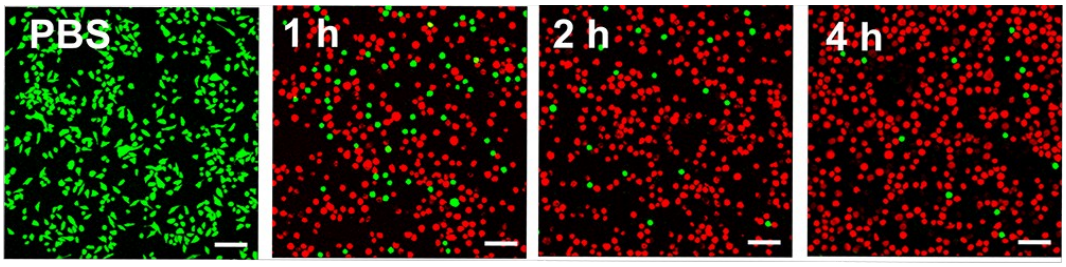

Figure S17. The Calcein AM/PI staining images of HeLa cells after different treatments. PBS: untreated cells; 1 h: irradiation applied after 1 h incubation with DOX-PhA NPs (3.5:6.5); 2 h: irradiation applied after 2 h incubation with DOX-PhA NPs (3.5:6.5); 4 h: irradiation applied after 4 h incubation with DOX-PhA NPs (3.5:6.5). DOX-PhA NPs suspensions contain 0.023 mM DOX and 0.043 mM PhA. Scale bar =

50 μm.

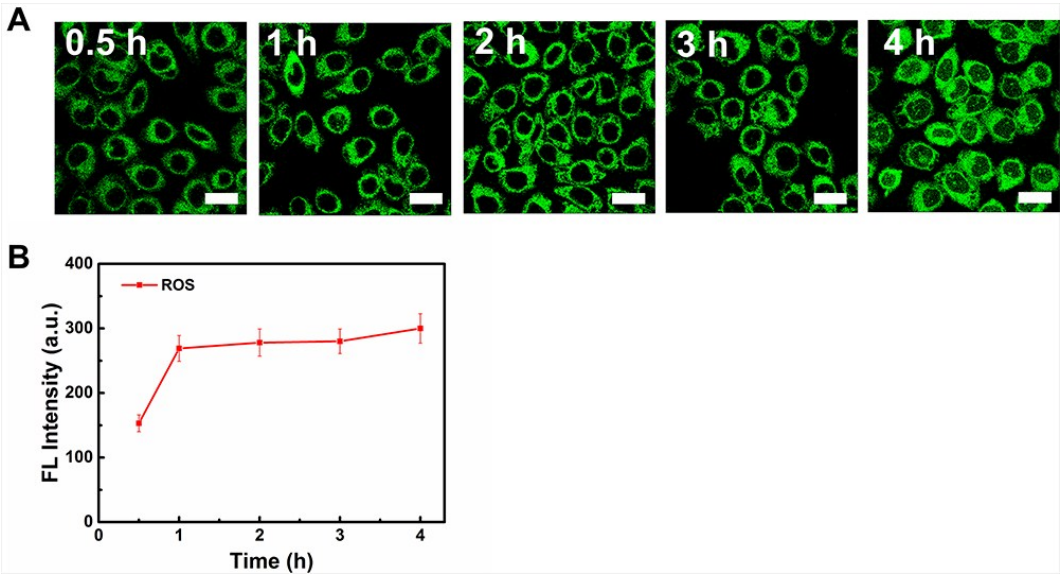

Figure S18. (A) The ROS generation of HeLa cells under 660 nm laser (50 mW/cm^2, 5 min) with different incubation time. The ROS generations were measured by using DCFH-DA and **(B)** the fluorescence intensities were measured by the CLSM software. Scale bar = 20 μm.

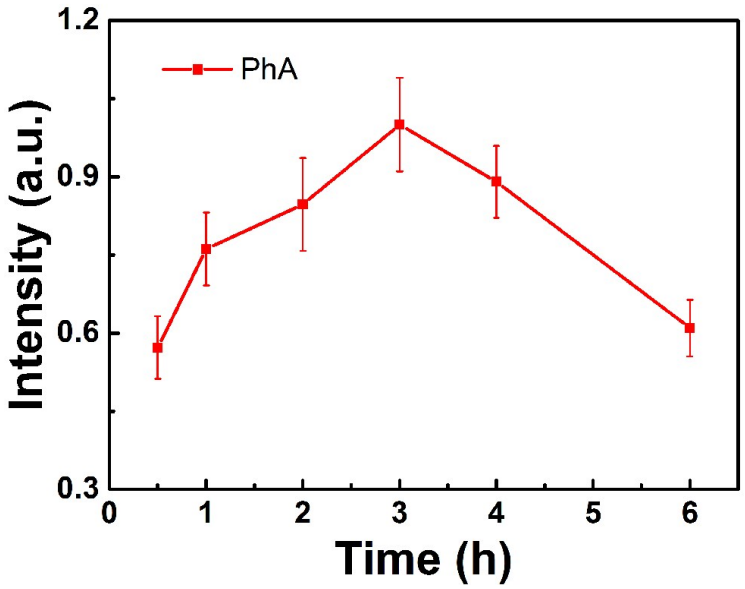

Figure S19. Tumor fluorescence intensity variation in PhA channel of HeLa tumor-

bearing mice over 6 h after intratumoral injection of DOX-PhA NPs (3.5:6.5). Error bars were based on standard error of mean (SEM) and n = 3.

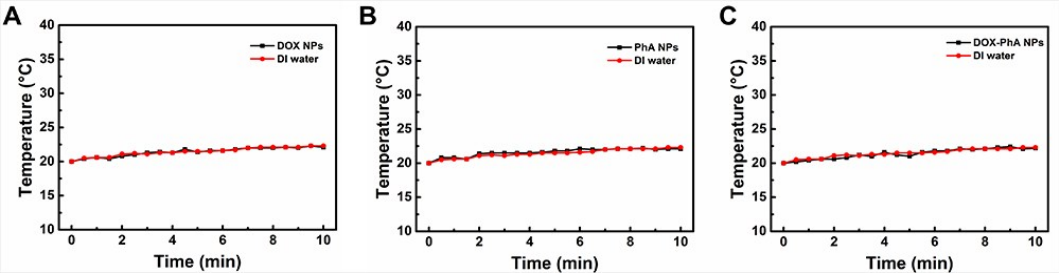

Figure S20. The temperature elevation curves of **(A)** DOX NPs (0.023 mM), **(B)** PhA NPs (0.043 mM) and **(C)** DOX-PhA NPs (3.5:6.5) under the 660 nm laser irradiation (100 mW/cm^2) within 10 min. DOX-PhA NPs suspensions contain 0.023 mM DOX and 0.043 mM PhA. DI water was used as control in each group.

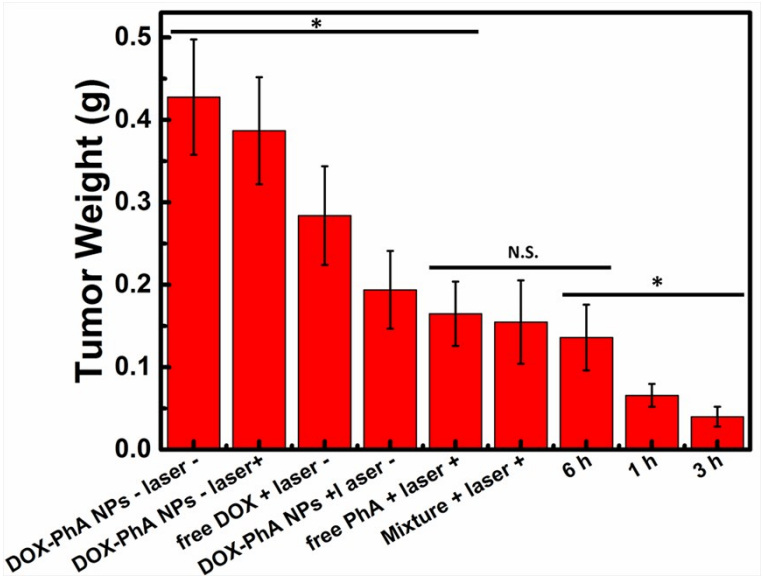

Figure S21. The tumor weight of different groups harvested at the end of anti-tumor combination therapy (21st day). Error bars were based on standard error of mean (SEM) and n = 6. (N.S. no significance, *p < 0.05, **p < 0.01)