ADVANCED FUNCTIONAL MATERIALS

Supporting Information

for *Adv. Funct. Mater.*, DOI: 10.1002/adfm.201807772

Smart Supramolecular "Trojan Horse"-Inspired Nanogels for Realizing Light-Triggered Nuclear Drug Influx in Drug-Resistant Cancer Cells

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Materials and Methods

Materials. 3-[2-(2-Aminoethylamino)ethylamino]propyl-trimethoxysilane (AEEA) was purchased from Acros Organics (USA). Rose bengal (RB), genistein, and amiloride hydrochloride (amiloride) were obtained from Aladdin (China). Indocyine green (ICG) was bought from BBI Life Sciences (China). Methoxypoly(ethylene glycol)5k-*block*-poly(*L*-glutamic acid sodium salt)$_{200}$ with an average molecular weight of 31000 Da (abbreviated as PEG-PLE) was purchased from Alamanda Polymers (USA). Doxorubicin hydrochloride (Dox) was bought from Beijing Huafeng United Technology Co., Ltd (China). Singlet oxygen sensor green (SOSG) kit and sodium azide were purchased from Thermo Fisher Scientific (USA). Chlorpromazine hydrochloride (CPZ) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-*H*-tetrazolium bromide (MTT) were bought from Sigma Aldrich (USA). Propidium iodide (PI) was purchased from KeyGen Biotech (Nanjing, China). All solutions were prepared with deionized (DI) water (18.2 MΩ·cm) purified by a Milli-Q system (Millipore).

Characterization. Transmission electron microscopy (TEM) experiments were carried out on a JEM-2100 transmission electron microscope (JEOL). The hydrodynamic diameters and zeta potentials were measured on a zetasizer instrument (Nano ZS, Malvern). The fluorescence spectra excited at 511 and 552 nm were obtained by a Shimadzu RF-5301PC spectrofluorophotometer. The fluorescence spectra excited at 765 nm were collected using an F-4600 spectrofluorophotometer (Hitachi, Japan). The ultraviolet–visible–near infrared (UV–vis–NIR) absorption spectra were collected using a Shimadzu UV-3600 spectrophotometer. The mass spectra were collected on a Bruker UltrafleXtreme matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer using α-cyano-4-hydroxycinnamic acid as a matrix substance.

Near-Infrared Light-Activated Disassembly of SiPINGs and Dox@SiPINGs. The SiPING and Dox@SiPING suspensions were irradiated by an 808 nm laser (Nanjing Latron Laser Technology Co., Ltd.) with a power density of 0.5 W/cm^2 for 10 min. Then, the samples

were analyzed by UV–vis–NIR spectroscopy, and the corresponding TEM images were collected under the transmission electron microscope.

Temperature and Singlet Oxygen Measurement of SiPING Suspension after Irradiation. For the temperature measurements at room temperature or 37 ^{o}C, the SiPING suspension (ICG concentration: 1.25 or 2.50 μg/mL) was irradiated by an 808 nm laser (0.5 W/cm^2) for 10 min, and the temperature changes were monitored using an infrared thermal imaging camera (Ai50, Shanghai Infratest Electronics Co., Ltd., China). For the detection of singlet oxygen production, an SOSG kit was used. First, the SOSG reagent (1 μL, 5 mM) was added to 3 mL of SiPING suspension (ICG concentration: 1.25 or 2.50 μg/mL). Then, the samples were irradiated under an 808 nm laser (0.5 W/cm^2) for different time periods (0–15 min), and the fluorescence intensity change was recorded using a fluorescence spectrometer. The excitation and emission wavelengths were set as 504 and 525 nm, respectively.

Dox Release from Dox@SiPINGs with and without Irradiation. Two dialysis bags with 500 μL Dox@SiPING suspension (Dox concentration: 500 μg/mL) per bag were firstly prepared. Then, one bag was irradiated using an 808 nm laser (0.5 W/cm^2, 10 min), while the other was placed in the dark. After that, the two bags were placed in 4.5 mL DI water under stirring, and the dialysates were detected at different time points (0, 0.5, 1, 2, 4, 6, 8, or 12 h) using a fluorescence spectrometer to quantify the release of Dox. The excitation wavelength was 552 nm, and the emission was collected in the range of 570–700 nm.

Cell Culture. L02 (a normal human liver cell line), MCF-10A (a normal breast epithelial cell line), MCF-7 (a human breast cancer cell line), and multidrug-resistant MCF-7/ADR cells were used in this study. L02 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/mL of penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS). MCF-10A cells were cultured in endothelial cell medium (ECM) containing 5% FBS, 1× endothelial cell growth supplement (ECGS), 100 U/mL of penicillin, and 100 μg/mL streptomycin. MCF-7 cells and MCF-7/ADR were cultured in Roswell Park

Memorial Institute (RPMI) 1640 medium supplemented with 100 U/mL of penicillin, 100 μg/mL streptomycin, and 10% FBS. All cells were cultured in a humidified incubator at 37 ^{o}C and 5% CO_2.

Intracellular Uptake of Dox and Dox@SiPINGs. MCF-7 or MCF-7/ADR cells were seeded at a density of 5 × 10^3 cells/well in 96-well plates overnight and then incubated with Dox or Dox@SiPINGs (the Dox concentration was 0.5 μg/mL in MCF-7 cells and 5 μg/mL in MCF-7/ADR cells, respectively) for different time periods (0, 1, 3, 6, 12, or 24 h). Then, Hoechst 33342 was added and incubated with the cells for 10 min to stain the cell nuclei. Afterwards, the cells were washed with phosphate-buffered saline (PBS) solution and observed under a confocal microscope. Hoechst 33342 was excited at 405 nm and the emission was collected in the range of 410–470 nm. Free Dox and the Dox of Dox@SiPINGs were excited at 552 nm and the emissions were collected in the range of 555–650 nm. The OSiNDs channel of Dox@SiPINGs was excited at 488 nm and the emission was collected in the range of 495–540 nm. Finally, the intracellular fluorescence intensity was quantified using a flow cytometer (NovoCyte 2060, ACEA).

Cellular Uptake Pathways of SiPINGs, Dox, and Dox@SiPINGs. MCF-7/ADR cells were seeded at a density of 5 ×10^3 cells/well in a 96-well plate and incubated at 37 ^{o}C and 5% CO_2 for 24 h. Then, the cells were washed with PBS solution and pretreated with 50 μg/mL genistein, 5 μg/mL CPZ, 10 μg/mL amiloride, or 10 mM sodium azide, or cultured at 4 ^{o}C for 2 h in serum-free RPMI 1640 medium. Next, the medium was removed and complete RPMI 1640 containing Dox (5.0 μg/mL), SiPINGs (the OSiND concentration was 100 μg/mL), or Dox@SiPINGs (the Dox concentration was 5.0 μg/mL) with one of the above different inhibitors at 37 ^{o}C for another 2 h. The cells pretreated at 4 ^{o}C were incubated at 4 ^{o}C after the addition of complete RPMI 1640 with drugs. Finally, after washing with PBS solution, the cells were collected and analyzed by flow cytometry. Each group had three parallel samples.

Evaluation of the Integrity/Permeability of Nuclear Membranes of MCD-7/ADR Cells

via Flow Cytometry. MCF-7/ADR cells were cultured in 96-well plates (5×10^3 cells/well) overnight and then incubated with SiPINGs (the ICG concentration was 1.25 μg/mL) for 24 h. Afterwards, the cells were divided into two groups: irradiation group and non-irradiation group. The cells in the irradiation group were irradiated under an 808 nm laser (0.5 W/cm^2) for 10 min, while the cells in the non-irradiation group were placed in the dark. Then, fresh culture media containing 30 μM PI were added by replacing the old culture media. After incubation for 10 min, the integrity/permeability of nuclear membranes was analyzed by flow cytometry.

Intracellular Distribution of Dox@PSiNGs with and without Irradiation in MCF-7/ADR Cells and Cytotoxicity Evaluation of Dox@PSiNGs Using MCF-7/ADR Cells. MCF-7/ADR cells were seeded at a density of 5×10^3 cells/well in 96-well plates overnight. Then, to investigate the intracellular distribution of Dox@PSiNGs with and without irradiation, the cells were incubated with Dox@PSiNGs (the Dox concentration was 5.0 μg/mL) for 24 h. Next, the cells were divided into two groups: irradiation group and non-irradiation group. The cells in the irradiation group were irradiated under an 808 nm laser (0.5 W/cm^2, 10 min), while the cells in the non-irradiation group were placed in the dark. Then, Hoechst 33342 was added and incubated with the cells for 10 min to stain cell nuclei. After washing with PBS solution, the cells were observed under a confocal microscope. For the cytotoxicity evaluation of Dox@PSiNGs, the cells were cultured with different Dox concentrations of Dox@PSiNGs (the Dox concentration was 0, 1, 2, 5, 10, 20, or 50 μg/mL) for 24 h. Finally, the cell viability was evaluated using MTT assay.

Real-Time Cell Analysis (RTCA) Experiments. The real-time cytotoxicity of Dox and Dox@SiPINGs to L02 cells was evaluated using a real-time cell analyzer—the iCELLigence system (ACEA Biosciences Inc.). Briefly, L02 cells were seeded into the wells of E-Plate L8 at a density of 3.3×10^4 cells/mL in 450 μL culture medium. Next, different Dox concentrations of Dox or Dox@SiPINGs were added by replacing culture media when the cell

index value reached approximately 1. The real-time proliferation of cells was recorded based on an impedance-based system and finally presented as various curves with different growth rates.

In Vivo Experiments. Female BALB/c nude mice (18 ± 2 g) aged 4 weeks were purchased from Yangzhou University Medical Center (Yangzhou, China) and used under protocols approved by the Animal Care and Use Committee of Southeast University. To build the uterine cervical carcinoma (U14) xenograft tumor model, U14 cells (1×10^6) suspended in 100 μL of PBS were subcutaneously implanted into the back of each nude mouse. For the MCF-7/ADR xenograft tumor model, 1×10^8 MCF-7/ADR cells suspended in a mixture containing 50 μL PBS and 50 μL matrigel (BD Bioscience) were subcutaneously implanted into the back of each nude mouse. Then, when the U14 tumors reached approximately 50 mm^3 and MCF-7/ADR tumor reached around 20 mm^3 in volume, the mice were randomly divided into the following 6 groups at day 0: (1) Control, (2) Dox, (3) SiPINGs, (4) Dox@SiPINGs, (5) SiPINGs + irradiation (IR), and (6) Dox@SiPINGs + IR. Briefly, the mice in group 1 and 2 received tail vein injection with sterilized physiological saline (200 μL) and Dox solution (200 μL, 500 μg/mL), respectively. The mice in groups 3 and 5 were intravenously injected of SiPINGs (200 μL, the OSiND concentration was 1.25 mg/mL). The mice in groups 4 and 6 were intravenously injected with Dox@SiPINGs (200 μL, the Dox concentration was 500 μg/mL). Subsequently, a Perkin Elmer *in vivo* imaging system (IVIS Lumina XRMS Series III) was used to image the mice in groups 1–4 at different time points (0, 6, 12, 24, or 48 h) postinjection using the Dox channel with an excitation wavelength of 540 nm and an emission wavelength of 580 nm. Meanwhile, the mice in groups 5–6 were irradiated with an 808 nm laser (0.5 W/cm^2, 20 min) at 24 h postinjection. After the above treatments, the tumor volume and mouse weight were measured daily until day 14. The tumor volume was measured using a caliper and calculated as the volume = (tumor length) × (tumor width)^2/2. The relative tumor volume was calculated as V/V_0, where V_0 was the tumor volume

when the treatment was initiated, and *V* was the volume of the tumor measured daily after the treatment. Finally, after the completion of the entire experiment, all the mice were sacrificed and the tumor tissues were separated.

To evaluate the systemic toxicity of drugs, the mice injected with PBS (control) or drugs (Dox or Dox@SiPINGs) were sacrificed at day 14 postinjection. Then, the biocompatibility of the drugs was assessed by: (1) routine blood analysis using an automatic hematology analyzer (BC-2800Vet, Mindray, China), (2) examination of some organ function-correlated biomarkers such as the alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine (CRE) using a chemistry analyzer (Catalyst One, IDEXX), and (3) hematoxylin and eosin (H&E) staining of the organs including hearts, livers, spleens, lungs, and kidneys.

Statistical Analysis. All statistical results were presented as the mean ± standard deviation (SD). The differences between two groups were calculated using unpaired Student's *t*-test. For the in vitro experiments, the number of biological replicates was ≥ 5. For the in vivo experiments, the number of animals per group was ≥ 3. $P < 0.05$ was considered as statistically significant.

Supplementary Figures

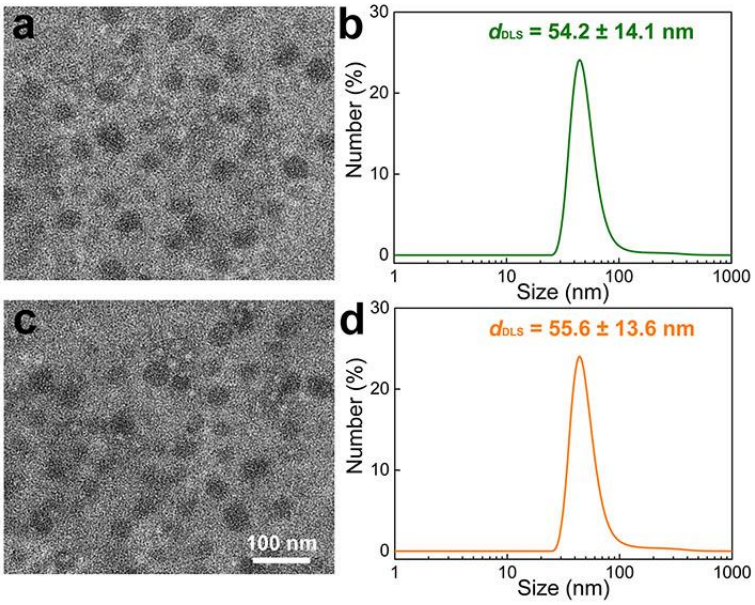

Figure S1. (a) TEM image and (b) dynamic light scattering (DLS) result of SiPINGs in water. (c) TEM image and (d) DLS result of Dox@SiPINGs in water.

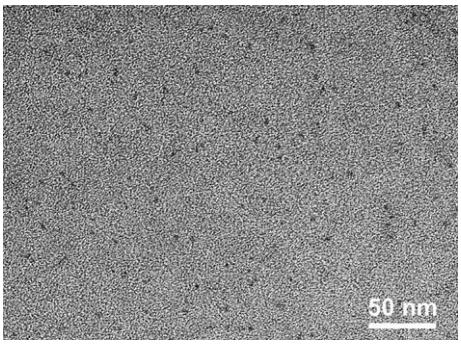

Figure S2. TEM image of OSiNDs in water.

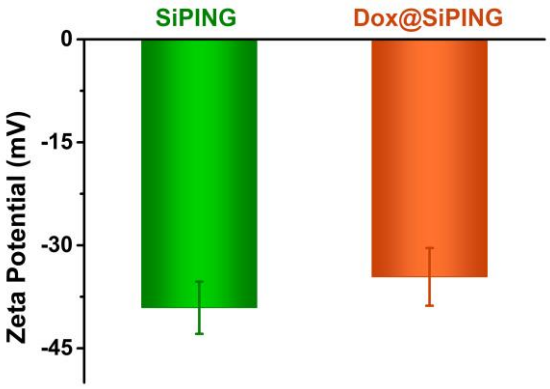

Figure S3. Zeta potentials of SiPINGs and Dox@SiPINGs in water.

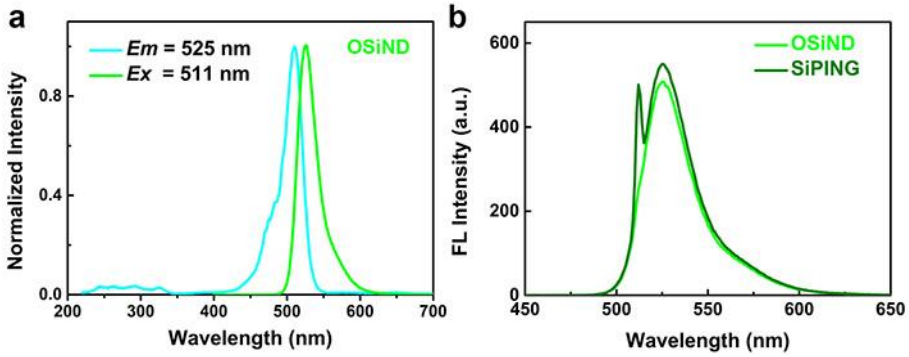

Figure S4. (a) Fluorescence excitation and emission spectra of OSiND suspension. (b) Fluorescence emission spectra of OSiND and SiPING suspensions with the same OSiND concentration of 50 μg/mL. The excitation wavelength was 511 nm.

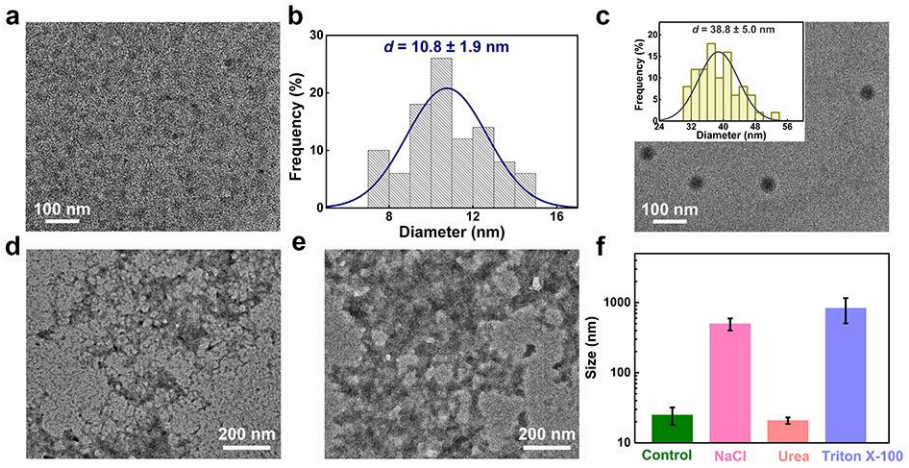

Figure S5. (a) TEM image and (b) corresponding size distribution histogram of the mixture of PEG-PLE, OSiNDs, and the degradation product of ICG (dICG). (c) TEM image and corresponding size distribution histogram (inset) of the mixture of PEG-PLE and OSiNDs. (d) TEM image of the mixture of PEG-PLE and dICG. (e) TEM image of the mixture of OSiNDs and dICG. The dICG was obtained from the ICG aqueous solution after laser irradiation (808 nm, 2 W/cm^2, 10 min). (f) Hydrodynamic sizes of the assembles formed by PEG-PLE, OSiNDs and dICG without (control) and with one of the three inhibitors (NaCl, urea, or Triton X-100; at a concentration of 200 mM).

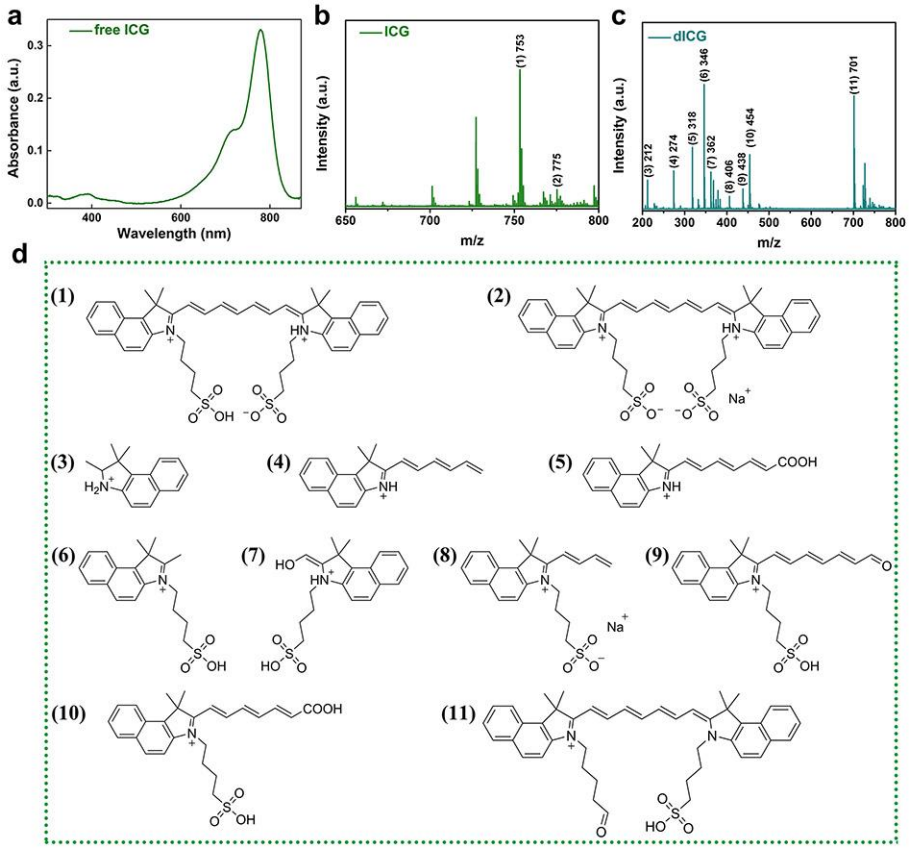

Figure S6. (a) Absorption spectrum of an ICG aqueous solution. MALDI-TOF mass spectra of (b) ICG and (c) dICG. (d) Molecular structures of the main products in (b) and (c).

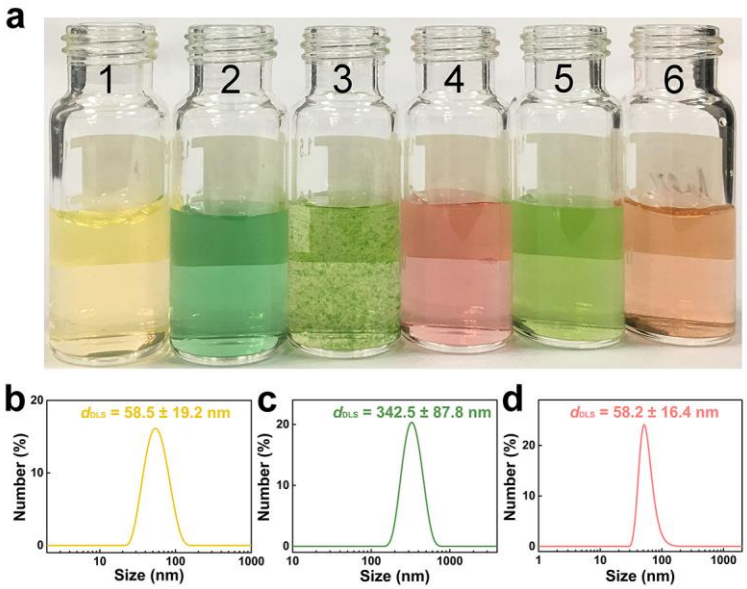

Figure S7. (a) Photographs of various mixtures of different reagents. 1: PEG-PLE and OSiNDs (1: 5, *w*/*w*, denoted as PSiNGs), 2: PEG-PLE and ICG (1: 0.5, *w*/*w*), 3: OSiNDs and ICG (2: 0.5, *w*/*w*), 4: PEG-PLE, OSiNDs, and Dox (1: 5: 2, *w*/*w*/*w*. denoted as Dox@PSiNGs), 5: SiPINGs, and 6: Dox@SiPINGs. (b–d) DLS results of PSiNGs, a mixture of PEG-PLE and ICG (2: 0.5, *w*/*w*), and Dox@PSiNGs.

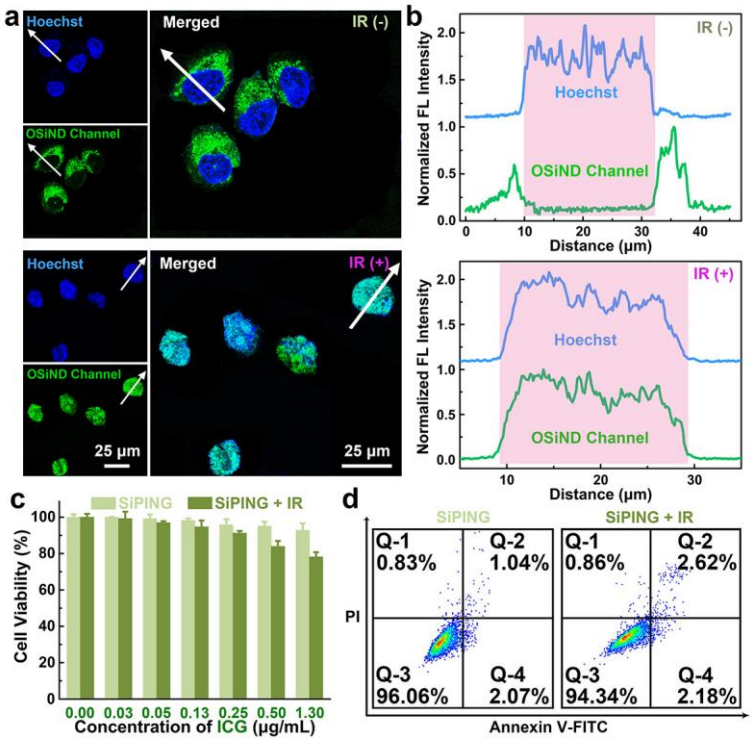

Figure S8. (a) Confocal fluorescence images of SiPINGs-treated MCF-7 cells before and after irradiation. The cell nuclei were stained with Hoechst 33342 (abbreviated as Hoechst). (b) Normalized line-scan FL intensity profiles of the marked positions (white arrows in (a)). (c) Viabilities of MCF-7 cells treated with different ICG concentrations of SiPINGs or SiPINGs + IR (808 nm, 0.5 W/cm^2, 10 min). (d) Flow cytometric results of MCF-7 cells treated with SiPINGs or SiPINGs + IR (808 nm, 0.5 W/cm^2, 10 min). The ICG concentration of SiPINGs was 0.13 μg/mL.

Table S1. Apoptosis assay results of MCF-7 cells without (control) or with the treatment of SiPINGs or SiPINGs + IR (808 nm, 0.5 W/cm^2, 10 min). The ICG concentration of SiPINGs was 0.13 μg/mL. (1), (2), and (3) listed in the table indicate the order number of the three parallel experiments for each treatment.

	Q-1 [%]	Q-2 [%]	Q-3 [%]	Q-4 [%]
SiPINGs (1)	0.83	1.04	96.06	2.07
SiPINGs (2)	0.90	2.61	95.50	0.99
SiPINGs (3)	0.87	2.33	96.62	1.18
SiPINGs + IR (1)	0.86	2.62	94.34	2.18
SiPINGs + IR (2)	0.74	0.84	96.86	1.55
SiPINGs + IR (3)	1.15	2.59	94.24	2.02

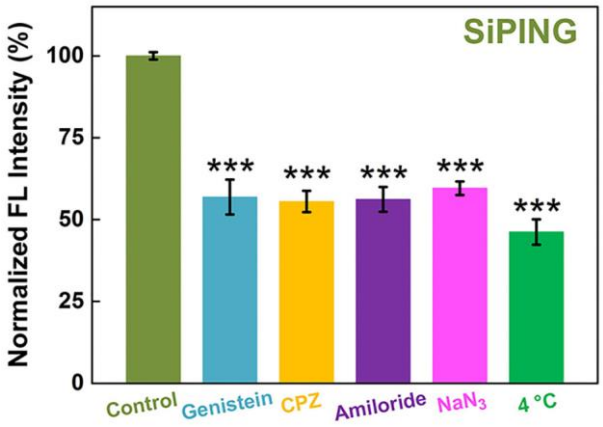

Figure S9. Flow cytometric results of MCF-7/ADR cells treated without inhibitors (control) and with genistein, CPZ, amiloride, sodium azide, and 4 ^{o}C incubation, respectively, followed by the addition of SiPINGs (the OSiND concentration was 100 μg/mL). ***P < 0.001 compared to the samples in the control group.

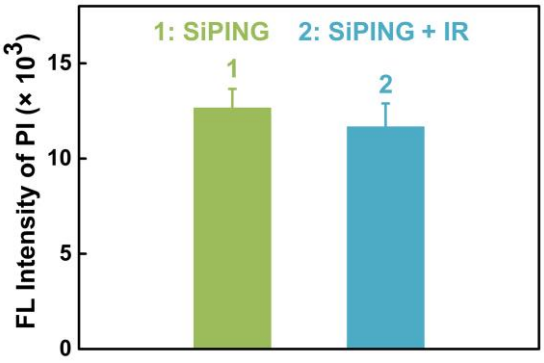

Figure S10. Evaluation of the nuclear membrane integrity/permeability of the SiPINGs- or "SiPINGs + IR"-treated MCF-7/ADR cells via flow cytometry. Before analysis, the cells were incubated with 30 μM PI for 10 min. The ICG concentration of SiPINGs was 1.25 μg/mL. IR: 808 nm, 0.5 W/cm^2, 10 min.

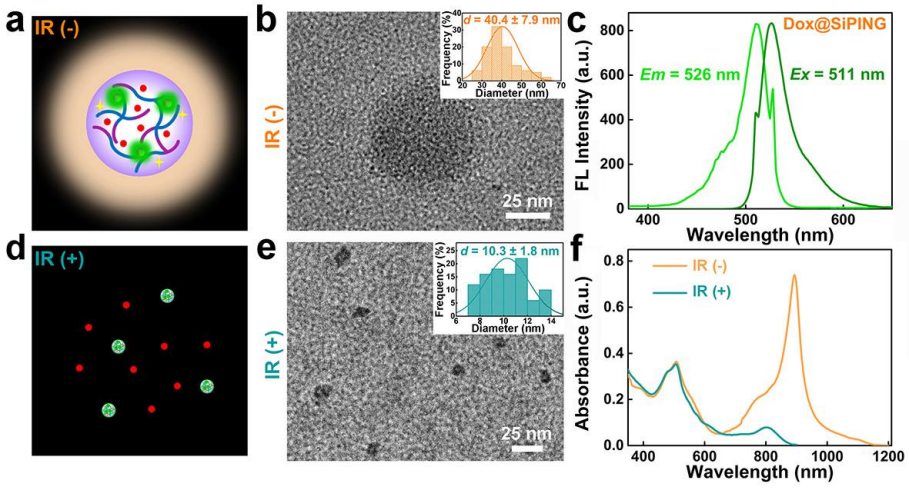

Figure S11. Characterization of Dox@SiPINGs before and after NIR light irradiation. (a) Schematic illustration, (b) TEM image (obtained at a high magnification) and the corresponding size distribution histogram (inset), and (c) fluorescence spectra of Dox@SiPING suspension without irradiation. (d) Schematic illustration and (e) TEM image (Inset: the corresponding size distribution histogram) of Dox@SiPINGs after irradiation. (f) Absorption spectra of Dox@SiPING suspension before and after irradiation. Irradiation condition: 808 nm, 0.5 W/cm^2, 10 min.

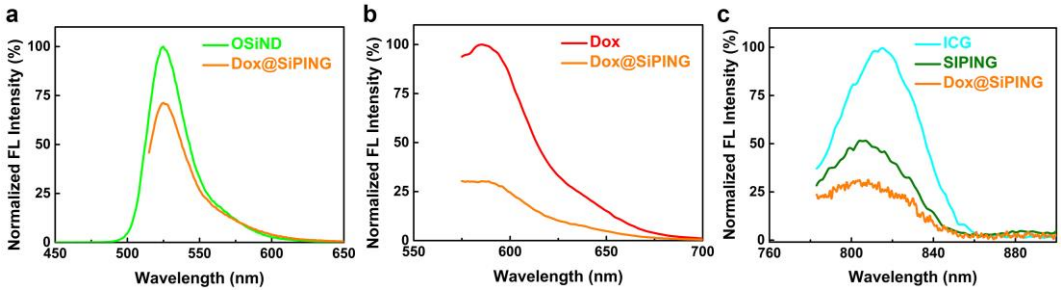

Figure S12. (a) Fluorescence emission spectra of OSiND and Dox@SiPING suspensions with the same OSiND concentration of 25 μg/mL. The excitation wavelength was 511 nm. (b) Fluorescence emission spectra of Dox solution and Dox@SiPING suspension with the same Dox concentration of 10 μg/mL. The excitation wavelength was 552 nm. (c) Fluorescence emission spectra of ICG solution, SiPING suspension, and Dox@SiPING suspension with the same ICG concentration of 25 μg/mL. The excitation wavelength was 765 nm.

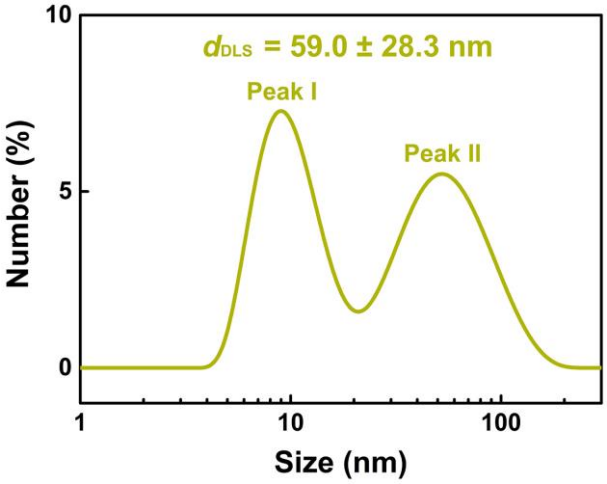

Figure S13. DLS result of Dox@SiPINGs in FBS-containing 1640 medium. Peak I (~10.4 nm) and Peak II (~59.0 nm) were assigned to FBS and Dox@SiPINGs, respectively.

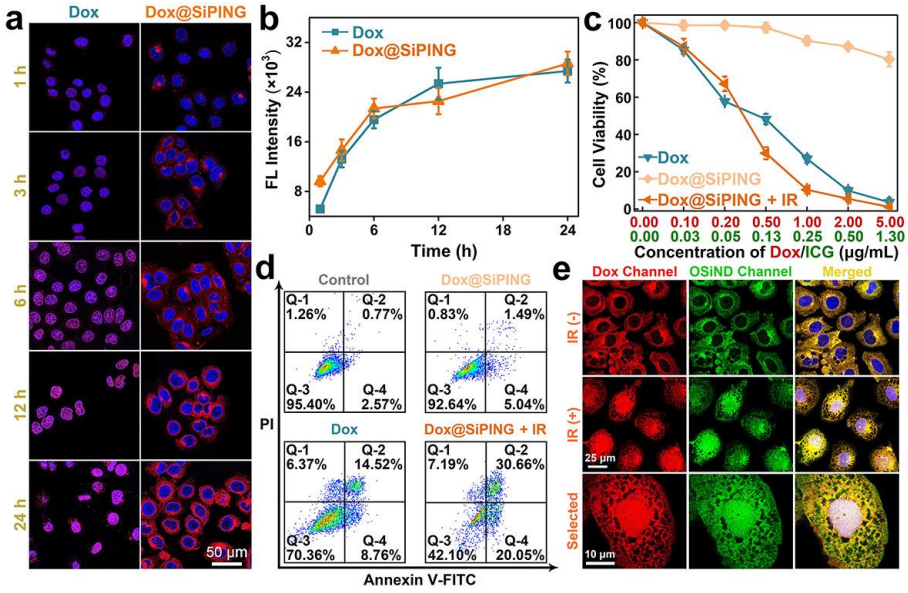

Figure S14. Near-infrared light-controllable Dox release of Dox@SiPINGs in MCF-7 cells. (a) Confocal fluorescence images showing the intracellular distribution and (b) the corresponding flow cytometry analysis results of Dox and Dox@SiPINGs in MCF-7 cells at different time points. (c) Cell viabilities of MCF-7 cells after various treatments. (d) Apoptosis assay results of MCF-7 cells without (control) and with the treatment of Dox, Dox@SiPINGs, or Dox@SiPINGs + IR (808 nm, 0.5 W/cm^2, 10 min). (e) Confocal fluorescence images of the intracellular distribution of Dox@SiPINGs in MCF-7 cells before and after irradiation (808 nm, 0.5 W/cm^2, 10 min). The yellow color indicates the co-localization of the green channel (from OSiNDs) and red channel (from Dox) of Dox@SiPINGs.

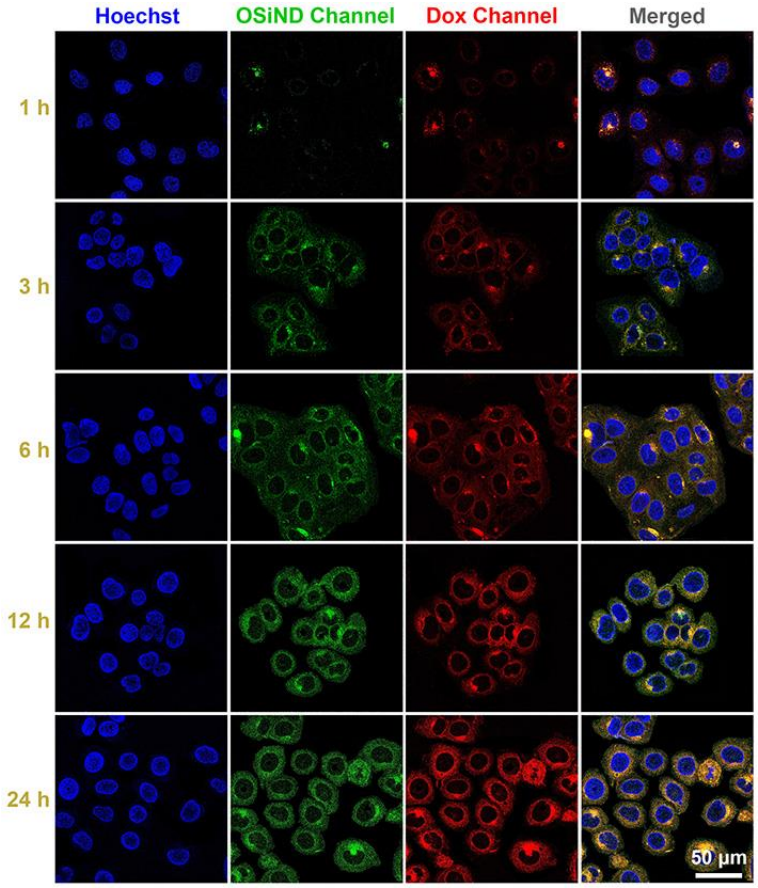

Figure S15. Confocal fluorescence images showing the intracellular distribution of Dox@SiPINGs in MCF-7 cells at different time points. The Dox concentration of Dox@SiPINGs was 0.5 μg/mL. Co-localization of the green color (from OSiNDs) and red color (from Dox) of Dox@SiPINGs produces yellow color in the merged images.

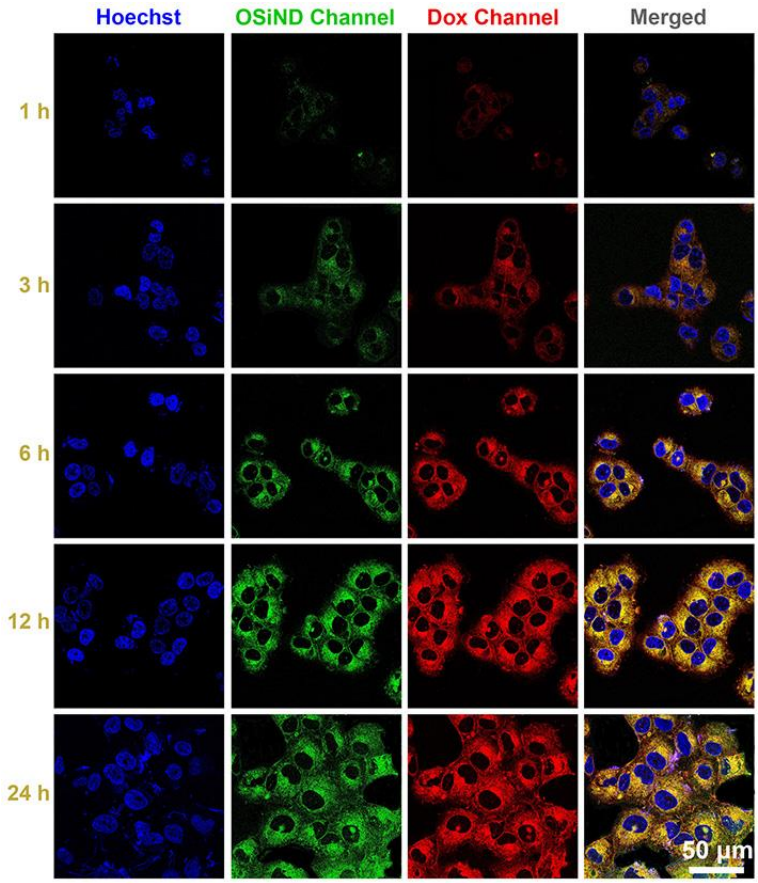

Figure S16. Confocal fluorescence images showing the intracellular distribution of Dox@SiPINGs in MCF-7/ADR cells at different time points. The Dox concentration of Dox@SiPINGs was 5.0 μg/mL. Co-localization of the green color (from OSiNDs) and red color (from Dox) of Dox@SiPINGs produces yellow color in the merged images.

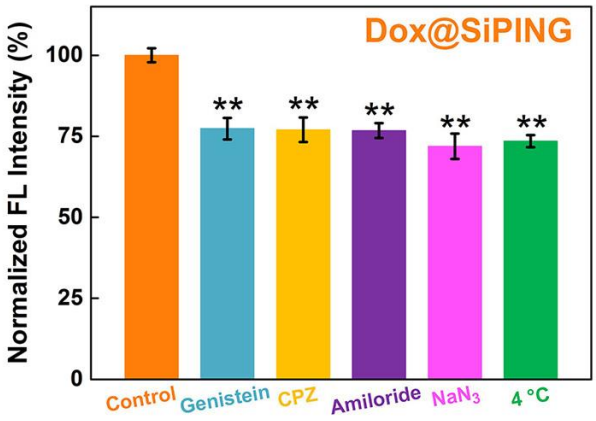

Figure S17. Flow cytometric results of MCF-7/ADR cells treated without inhibitors (control) and with genistein, CPZ, amiloride, sodium azide, and 4 ^{o}C incubation, respectively, followed by the addition of Dox@SiPINGs (the Dox concentration was 5.0 μg/mL). $^{**}P < 0.01$ compared to the samples in the control group.

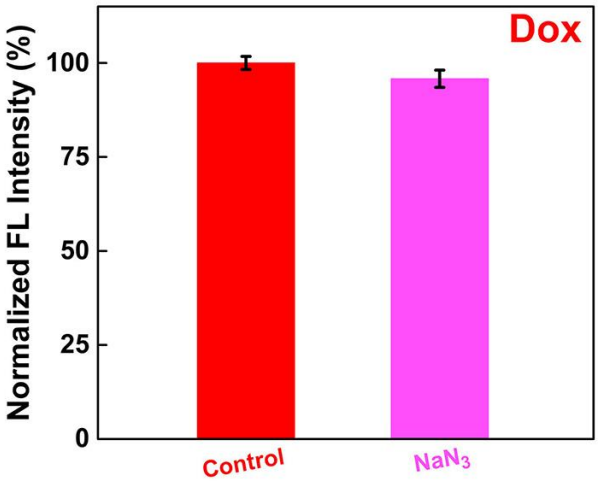

Figure S18. Flow cytometric results MCF-7/ADR cells treated without inhibitors and with the treatment of sodium azide (NaN_3) followed by the addition of Dox (5.0 μg/mL).

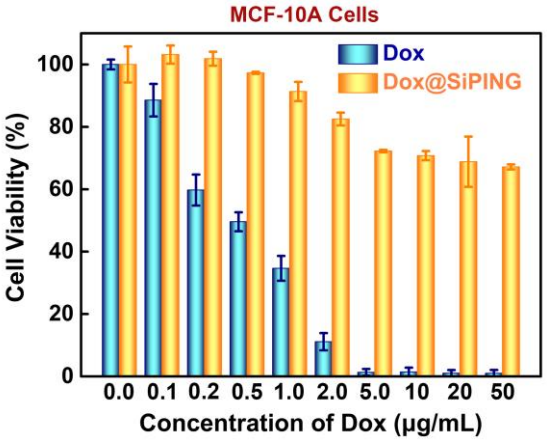

Figure S19. Cytotoxicity of Dox and Dox@SiPINGs to MCF-10A cells.

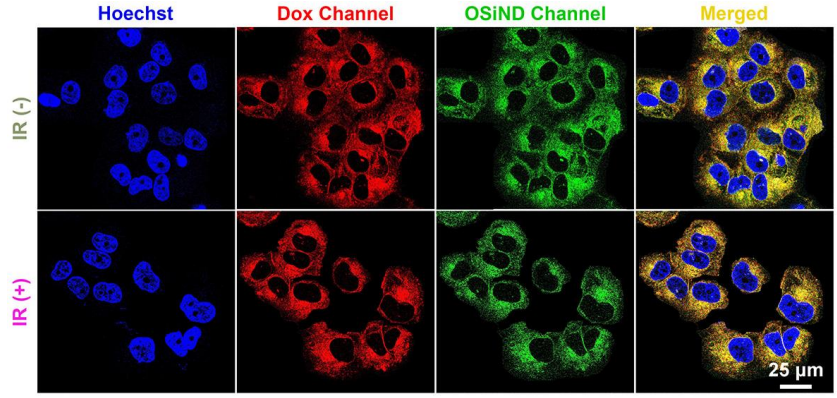

Figure S20. Confocal fluorescence images showing the intracellular distribution of Dox@PSiNGs in MCF-7/ADR cells before and after irradiation (808 nm, 0.5 W/cm^2, 10 min). The Dox concentration of Dox@PSiNGs was 5.0 μg/mL. Co-localization of the green color (from OSiNDs) and red color (from Dox) of Dox@PSiNGs produces the yellow color in the merged images.

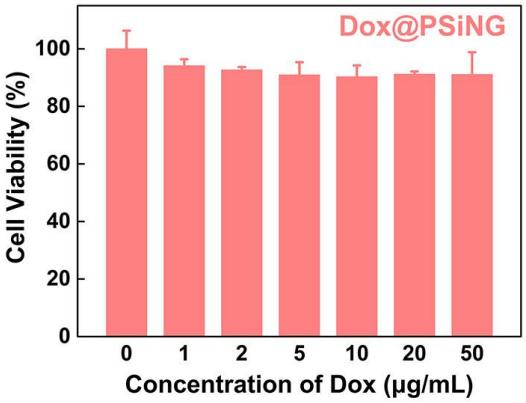

Figure S21. Cytotoxicity of Dox@PSiNGs to MCF-7/ADR cells.

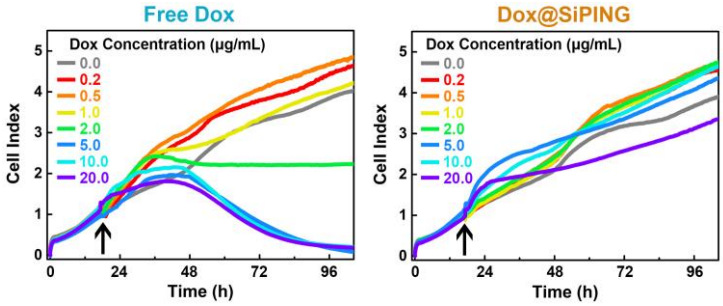

Figure S22. RTCA results of L02 cells after incubation with different Dox concentrations of Dox or Dox@SiPINGs. The arrows in the figures indicate the time of drug addition.

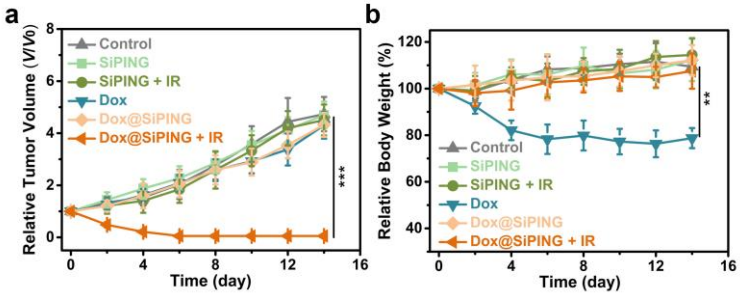

Figure S23. (a) Tumor growth curves and (b) body weight changes of the MCF-7/ADR tumor-bearing mice after various treatments. $^{**}P < 0.01$, $^{***}P < 0.001$.