Electronic Supplementary Information (ESI)

Synergetic Effect Between Silicon Nanowires and Doxorubicin with Non-Toxic Doses Leads to High-Efficacy Destruction of Cancer Cells†

Binbin Chu‡, Fei Peng‡, Houyu Wang, Yuanyuan Su and Yao He*

Laboratory of Nanoscale Biochemical Analysis, Institute of Functional Nano & Soft Materials (FUNSOM), and Collaborative Innovation Center of Suzhou Nano Science and Technology (NANO-CIC), Soochow University, Suzhou, Jiangsu 215123, China

E-mail: yaohe@suda.edu.cn (Yao He)

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1. Experimental methods

1.1 Materials and devices

Hydrofluoric acid (≥40%), hydrogen peroxide (≥30%), silver nitrate (≥99.8%), and nitric acid (65-68%) are bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Silicon wafer (100, phosphate-doped (p-type)) is bought from Hefei Kejing Materials Technology Co., Ltd. (China). DOX is purchased from Huafeng United Technology CO., Ltd (Beijing, China). Milli-Q water (Millipore) is employed as the solvent for preparing solutions. The scanning electron microscopy (SEM) and transmission electronic microscopy (TEM)/high-resolution TEM images are captured by Philips CM 200 electron microscope and scanning electron microscopy (FEI Quanta 200F), respectively.

1.2 Synthesis and characterization of SiNWs

Free silicon nanowire (SiNW) arrays are produced through an HF-assisted etching method as described elsewhere.[1,2] In our experiment, Si wafer is firstly treated by ultrasonic treatment for 10 min in acetone solution, followed by washing with Milli-Q water for 3 times. Thereafter, the silicon wafer is immersed in a mixture solution (H_2SO_4 (98%) + H_2O_2 (30%), v/v =3:1) for half of an hour, following by washing Milli-Q water for three times. Afterward, the resultant Si wafer is treated with HF (5%) solution for 30 min, producing the hydrogen terminated Si wafer (H-Si wafer). The as-prepared H-Si wafer is immediately immersed in a mixture solution ($AgNO_3$+ HF (10%)) with slow stirring for 6 min to produce SiNW arrays on the surface of Si wafer. Ultrasonic treatment is then performed to detach the as-prepared SiNWs, which are collected for synergistic enhancement in cancer therapy in following experiments. SEM images show that the length of SiNWs are <1 μm.

1.3 Cell culture

MCF-7 cell line is cultured in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine (FBS) serum and 1% (v/v) penicillin-streptomycin solution. Meanwhile, the Human retinal epithelial cells (ARPE-19 cells) is cultured in F-12 medium supplemented with 10% (v/v) heat-inactivated fetal bovine (FBS) serum and 1% (v/v) penicillin-streptomycin solution. The cell line is incubated in a 5% CO_2 atmosphere at 37 ^oC and 100% humidity. For confocal imaging, FACS, and MTT assay, cells are cultured in 24-, 6-, and 96-well plates (Corning Incorporated, Corning, NY, US), respectively. Three independent assays are performed in triplicate for all measurements. Cells are treated with free DOX, free SiNWs, and SiNWs+DOX mixtures for different periods before biochemical analysis.

1.4 Lactate dehydrogenase (LDH) assay

In brief, MCF-7 cells were seeded in 96-well plates at ~1.5 ×10^4 cells per well overnight. Then, various concentrations (*e.g.*, 0-32 μg mL^{-1}) of SiNWs were introduced to cells for 24 h, with untreated cells (0 μg/mL) as control. The SiNWs-treated MCF-7 cells were firstly washed with PBS, and then treated with lysis buffer (10 μL) at 37 ^oC for 30 min. Afterward, the LDH working solution (100 μL) was added into each well for another 30 min at room temperature. The absorbance measurement was performed at 490 nm. Three independent assays are performed in triplicate for all measurements.

1.5 Cellular uptake experiment

MCF-7 cells are cultured on 24-well plates with cover slips at 1.2×10^5/well at 37^{o}C under 5% CO_2 for 24 h. Then the cells are cultured in RPMI-1640 medium, free DOX (0.64 μg mL^{-1}), free SiNWs (8 μg mL^{-1}), or SiNWs+ DOX mixtures (0.64 μg mL^{-1}, 8 μg mL^{-1}) at 37^{o}C for 24 h. Afterward, cells

are washed with PBS for three times to fully remove nonspecifically absorbed drug. To determine the intracellular localization, Hoechst 33258 is used for staining cells for 25 min. Thereafter, the treated cells are mounted on slides in fluoromount (Sigma, F4680) with cover slips. Cell images are performed using a laser-scanning confocal fluorescent microscope (Leica, TCS-SP5 Ⅱ) equipped with multi-line argon laser (458, 476, 488 and 514 nm) and diode laser (405 nm). Hoechst are excited by 3% power of diode laser ($\lambda_{excitation}$=405 nm) and DOX are excited by 20% power of argon laser ($\lambda_{excitation}$=488 nm). The emissions windows are set as the ranges of 420-480 or 560-620 nm for Hoechst 33258 or DOX, respectively. All images are captured under the same instrumental setting and processed with image analysis software.

1.6 Fluorescence activated cell sorting (FACS) analysis

Fluorescence activated cell sorting (FACS Calibur from Becton, Dickinson and Company) analysis is used to quantify the fluorescence from the MCF-7 cells treated with free DOX (0.64 μg mL^{-1}) or SiNWs+DOX mixtures (0.64 μg mL^{-1}, 8 μg mL^{-1}), similar to the manipulations for confocal imaging as mentioned above. The free DOX (0.64 μg mL^{-1}) or SiNWs+ DOX mixtures (0.64 μg mL^{-1}, 8 μg mL^{-1}) treated cells are then trypsinized and collected in Eppendorf tubes and washed with PBS. To determine the cellular fluorescence, the mixture is centrifuged at 1000 rpm for 3 min to dispose DMEM and PBS. The resultant cells finally are collected for FACS analysis. FlowJo software is used to analyze the data.

1.7 Cell cytoskeleton and formation

MCF-7 cells are cultured on 24-well plates with cover slips at 1.2×10^5/well at 37 ^oC under 5% CO_2 for 24 h. Then the cells are treated with RPMI-1640 medium, free DOX (0.64 μg mL^{-1}), free SiNWs (8 μg mL^{-1}), or SiNWs+DOX mixtures (0.64 μg mL^{-1}, 8 μg mL^{-1}) for 24 h. After treatment, cells are fixed with 4% paraformaldehyde-4% sucrose for 20 min and blocked with PBS containing 4% BSA for 40 min. The cytoskeleton is labeled with 300 nM FITC-labeled phalloidin (Sigma, USA) for 60 min, followed by washing with PBS containing 0.1% Tween20 for 3 times. Hoechst 33258 (3 mg/mL) is used for staining nuclei for 5 min. The resultant cells are finally observed by LSCM (Leica, Germany).

1.8 Cell cycle phase analysis

The cells were seeded with 2.4×10^5/well in 6-well plates. After incubated for 24 h, RPMI-1640 medium, free DOX (0.64 μg mL^{-1}), free SiNWs (8 μg mL^{-1}), or SiNWs+ DOX mixtures (0.64 μg mL^{-1}, 8 μg mL^{-1}) dissolved in culture medium are added to each well. After 6, 12, or 24-h incubation, cells were harvested, fixed in 70% ethanol and stored at -20 ^oC. Cells were then washed twice with ice cold PBS and incubated with RNase and DNA intercalating dye PI for 30 min in dark, and cell cycle phase analysis was performed using a BD FACS Calibur flow cytometer. FlowJo software is used to analyze the data.

1.9 Live-dead cells assay

The 3',6'-Di(O-acetyl)-4',5'-bis [N, N-bis(carboxymethyl) aminomethyl] fluorescein, tetraacetoxymethyl ester (Calcein-AM, Dojindo) and PI are used to labeled the live cells and dead cells in our experiments, respectively. In details, MCF-7 cells are seeded into 6-well cell-culture plate at 2.4×10^5/well, followed by incubation at 37 ^oC under 5% CO_2 for 24 h. Afterward, the cells are incubated with RPMI-1640 medium, free DOX (0.64 μg mL^{-1}), free SiNWs (8 μg mL^{-1}), or SiNWs+DOX mixtures (0.64 μg mL^{-1}, 8 μg mL^{-1}) for 24 h. Then all cells are collected, followed by washing with PBS for three times to fully remove residual RPMI-1640 medium and nonspecifically absorbed drug. And then, these collected cells are treated with the mixed

solution of calcein-AM (1 μM) and PI (1 mg mL^{-1}) for 15 min. The resultant cells are finally observed by laser scanning confocal microscope (Leica, Germany).

1.10 MTT assay

A standard colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) assay is carried out to determine cytotoxicity in our experiments. In details, MCF-7 cells are seeded into 96-well cell-culture plate at 1×10^4/well, followed by incubation at 37 ^oC under 5% CO_2 for 12 h. Afterward, the cells are incubated with serial concentrations of free DOX, free SiNWs, or SiNWs+DOX mixtures for 24 h. Then 20 μL stock MTT (5 mg mL^{-1}) is added to each well, and cells are cultured at 37 ^oC for 5 h. Acidified sodium dodecyl sulfate (SDS) is used to lyse the cells. The absorbance at 570 nm was tested by using the microplate reader (Bio-Rad 680, U.S.A.). Three independent assays are performed in triplicate for all measurements.

1.11 The fold increase (Fold) and combination index (CI) analysis

(1) Fold increase (Fold) analysis

The fold increase (Fold) in cell death of MCF-7 cells treated by the combination with SiNWs and DOX could be calculated through the equation (1) in previously reported work[3,4], as follows:

$$Fold = CD_E / (CD_{SiNWs} + CD_{DOX}) \quad \text{.....(1)}$$

In detail, CD_E represents the cell death of SiNWs+DOX-treated experimental groups, CD_{SiNWs} represents the cell death of free SiNWs-treated groups, and CD_{DOX} represents the cell death of free DOX-treated groups.

(2) Combination index (CI) analysis

Furthermore, if free SiNWs could be seen as a kind of anti-cancer drug to some extent, the combinatorial therapy indexes (CI) would be calculated according to the following equation:[4-7]

$$CI = (C_{A,x} / IC_{x,A}) + (C_{B,x} / IC_{x,B}) \quad \text{.....(2)}$$

In detail, $C_{A,x}$ and $C_{B,x}$ are the concentrations (μg mL^{-1}) of drug A (e.g., DOX in this paper) and drug B (e.g., SiNWs in this paper) used in combination to achieve x% inhibition of growth of the cells. $IC_{x,A}$ and $IC_{x,B}$ are the concentrations (μg mL^{-1}) for single agents to achieve the same inhibition of growth of the cells. According to Equation (2), CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively.[4-7]

2. Scanning electron microscopy (SEM) images of SiNWs

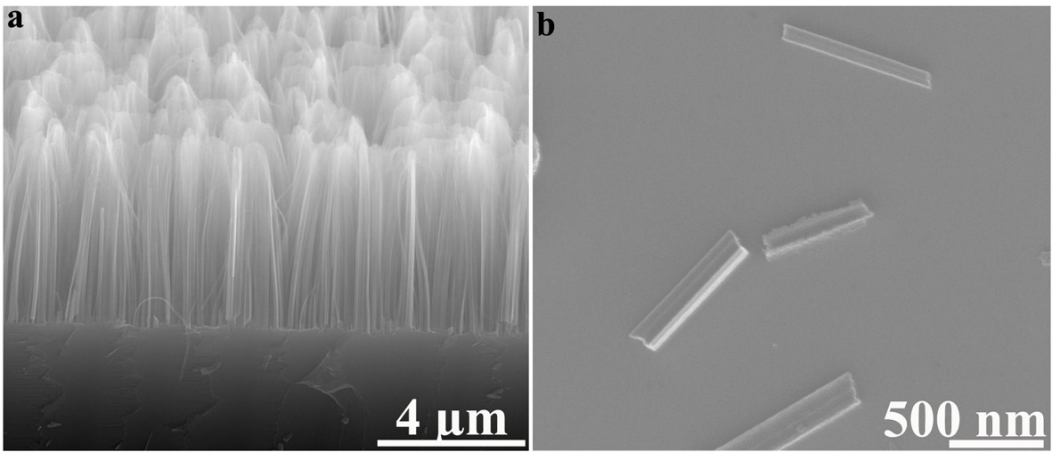

Figure S1. (a) SEM image of the as-prepared SiNWs arrays. (b) shows SEM image of the free-standing SiNWs detached from the surface of Si wafer by ultrasonic treatment.

3. Concentration determination of SiNWs

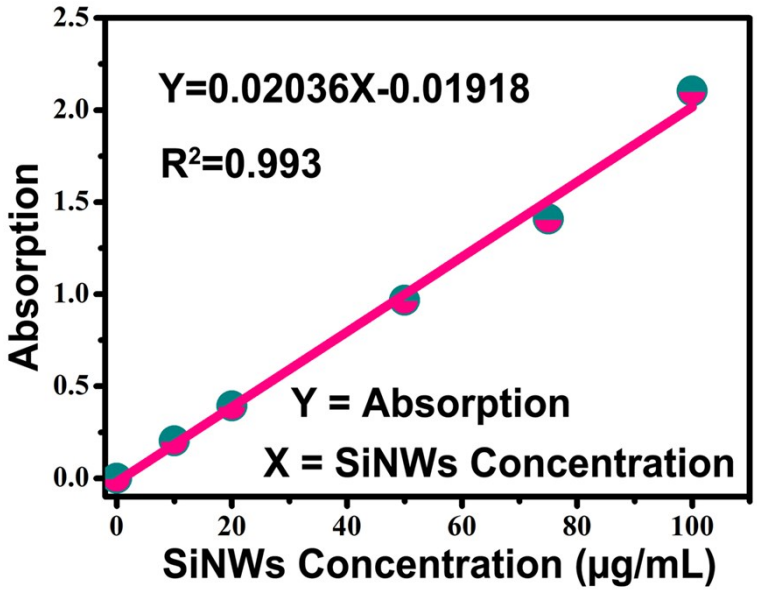

Figure S2. UV-vis-NIR spectrum of SiNWs aqueous solution versus different concentrations. Absorbance at 808 nm vs free SiNWs concentrations. Solid line is the linear fit using the analysis tool in Origin software with R^2= 0.993.

4. Cytotoxicity of SiNWs with different concentrations

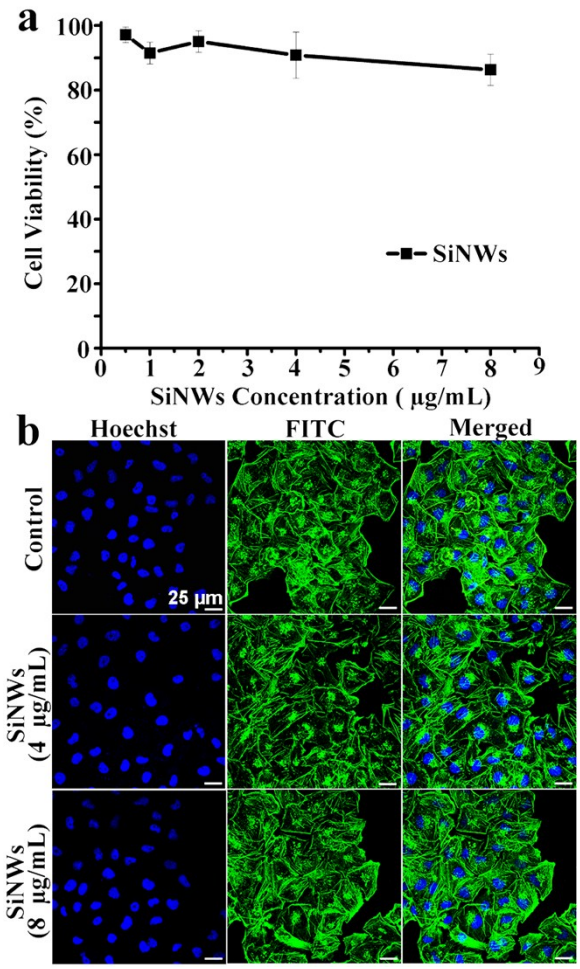

Figure S3. The cellular viability and confocal images of MCF-7 cells treated with SiNWs at different concentrations.[8] (a) The cellular viability of MCF-7 treated with SiNWs at different concentrations. After treated with SiNWs at 37 °C in the humidified atmosphere with 5% CO_2, the cell viability is calculated as a percentage from the viability of untreated cells. The results are means ± SD from three or five impendent experiments. (b) Confocal images of MCF-7 cells treated with free SiNWs at different concentrations (0, 4, and 8 μg mL^{-1}) for 24 h. The nucleus and actin are stained with Hoechst 33258 (blue) and FITC-phalloidin (green), respectively. Scale bars, 25 μm.

5. Mitochondria damage of MCF-7 cells treated by SiNWs+DOX

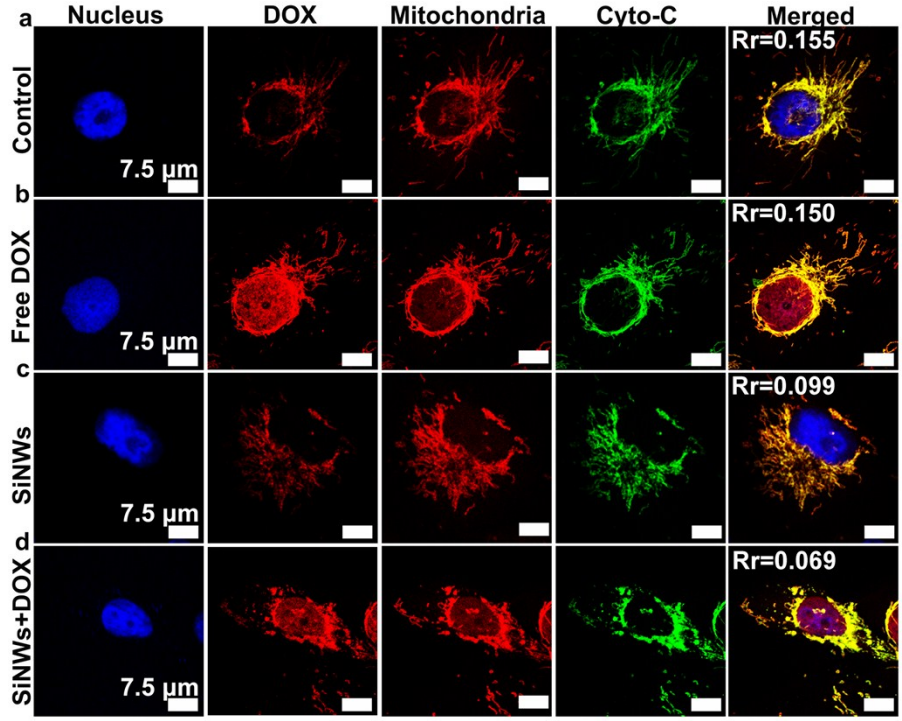

Figure S4. The destruction of mitochondria of MCF-7 cells. Confocal images on mitochondria of MCF-7 cells. Confocal images of MCF-7 cells treated with RPMI-1640 medium (a), free DOX (b), free SiNWs (c), or SiNWs+DOX (d) for 24 h. The mitochondria are stained by Mito-Tracker Red (red). The cytochrome C are stained by FITC (green) and the nucleus are stained by Hoechst 33258 (blue). Scale bars, 7.5 μm.

As shown in Fig. S4 (ESI†), the free DOX- and SiNWs+DOX-treated MCF-7 cells show distinct red signals (originated from DOX) in cellular nucleus, while no obvious red signals are observed in control group (i.e., cells cultured in RPMI-1640 medium) and free SiNWs-treated group. Moreover, Pearson's coefficient (Rr), which is calculated by the software of ImageJ (NIH Image: http;//rsbweb.nih.gov./ij/)[9], is further employed for the quantitative assessments of subcellular colocalization of mitochondria (red signals) and cytochrome C (Cyto-C, green signals). In detail, after treated by SiNWs+DOX, the Rr decreases from 0.155 to 0.069 (Fig. S4, ESI†), suggesting that cytochrome C is partially released from mitochondria to some extent.

6. Distribution of cell cycle

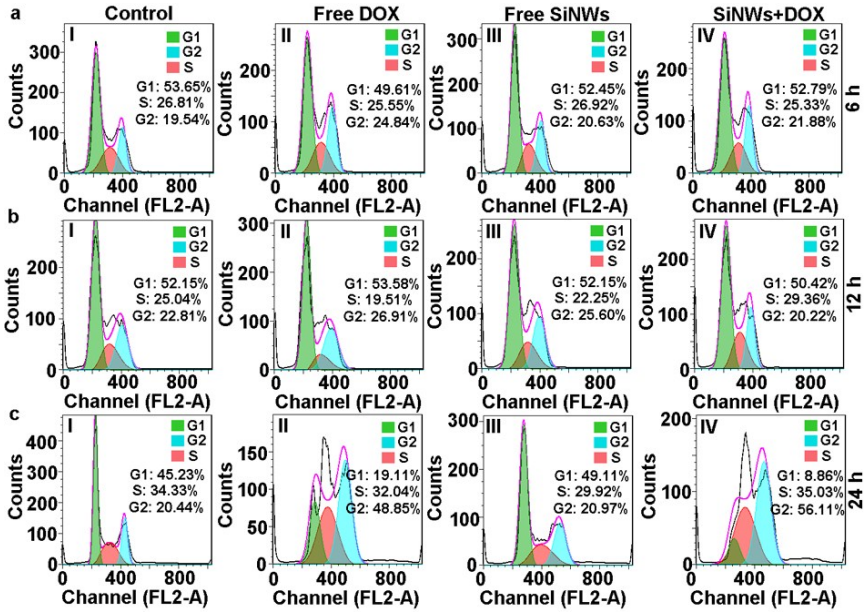

Figure S5. Cell cycle of MCF-7 cells. The distribution of cell cycle after treated with different concentrations of RPMI-1640 medium, free DOX, free SiNWs or SiNWs+DOX for 6 (a), 12 (b), and 24 (c) h. Green area, blue area, and red area represent G1 phase, G2 phase, and S phase respectively.

We assess the effects of free DOX, free SiNWs, and SiNWs+DOX mixtures system on MCF-7 cell cycle progression by the analysis of DNA content using flow cytometry. After treated by free DOX, free SiNWs, and SiNWs+DOX mixtures for 6 or 12 h, the cell cycle distribution of MCF-7 cells remains constant, suggesting no obvious influence of cell cycle for MCF-7 cells (Fig. S5a-b). Even after treated with free SiNWs for 24 h, the cell cycle distribution of MCF-7 cells is similar regardless of incubation time, indicating that the SiNWs produce negligible influence towards the cell cycle (Fig. S5c). Comparatively, free SiNWs and free DOX could cause a time-dependent arrest of MCF-7 cells in the G2 phase (Fig. 4 and Fig. S5c). In particular, the SiNWs+DOX mixtures could obviously induce the arrest of MCF-7 cells in the G2 phases and inhibit the growth by define and specific synergistic effect between SiNWs and DOX (Fig. S5c).

7. Confocal images of live-dead cells assay

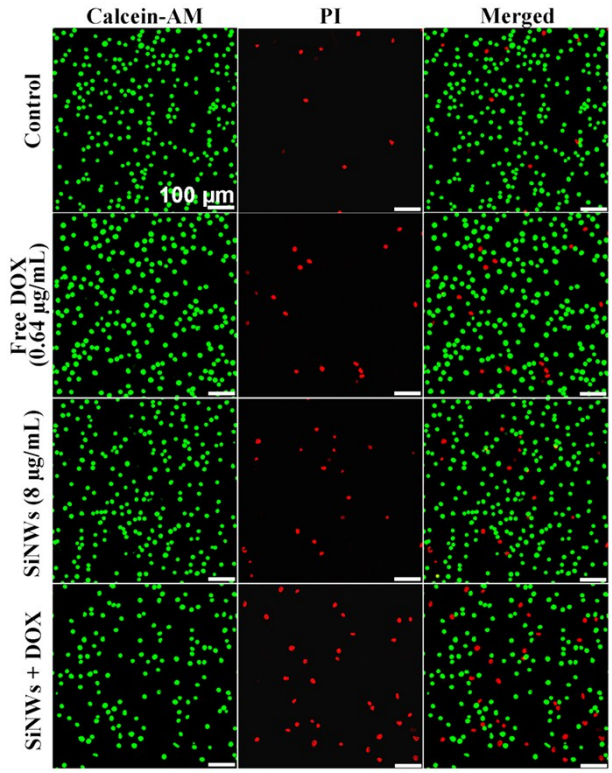

Figure S6. Live-dead cells staining assay of MCF-7 cells. Confocal images of MCF-7 cells treated with free DOX, free SiNWs and SiNWs+DOX for 24 h. The live cells and dead cells are stained with calcein-AM (green) and PI (red), respectively. Scale bars, 100 μm.

As displayed in Fig. S6, the ratio of live cells to dead cells decreases from 20 (control group) to 13.8 (free DOX-treated group), 10.8 (free SiNWs-treated group), or 4.5 (SiNWs+DOX-treated group) through different treatments for 24 h, suggesting obvious cell death of SiNWs+DOX-treated MCF-7 cells.

8. The fold increase (Fold) and combination index (CI) analysis

Table S1. Fold increase (Fold) in the cell death of MCF-7 cells treated by a combination with SiNWs and DOX.

Fold [a]	DOX (0.04 μg mL^{-1})	DOX (0.08 μg mL^{-1})	DOX (0.16 μg mL^{-1})	DOX (0.32 μg mL^{-1})	DOX (0.64 μg mL^{-1})
SiNWs (4 μg mL^{-1})	1.36	1.81	1.47	1.59	1.76
SiNWs (8 μg mL^{-1})	1.20	2.33	1.35	1.70	1.80

[a] Fold is calculated through Equation (1) in the experimental section of ESI† mentioned above.[3,4]

The value of Fold is listed in Table S1 (ESI†). Typically, the Fold value of SiNWs+DOX (SiNWs 4 μg mL^{-1}, DOX 0.32 μg mL^{-1}) is 1.59, which means that the combined effects of SiNWs+DOX is approximately 1.59-fold higher than the sum of individual effects of free SiNWs (4 μg mL^{-1}) and free DOX (DOX 0.32 μg mL^{-1}).[3,4] These results suggest good synergistic effects of SiNWs+DOX mixtures for destroying MCF-7 cells.

Table S2. Combination index (CI) calculated according to Equation (2).

Sample	CI [a]
SiNWs+DOX	0.5625

[a] CI represents the combination index of DOX and SiNWs for destroying MCF-7 cells, which is calculated through the Equation (2) in the experimental section of ESI†. Accordingly, CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively.[4-7]

The value of CI is listed in Table S2 (ESI†). Typically, the combination index (CI) for the combination of SiNWs and DOX works out to be ~0.56 (Table S2, which is calculated through Equation (2), ESI†), and as per the definition (CI < 1), this combination exhibits synergy in destroying MCF-7 cells.[4-7]

9. Cell viability of normal cells treated by SiNWs+DOX

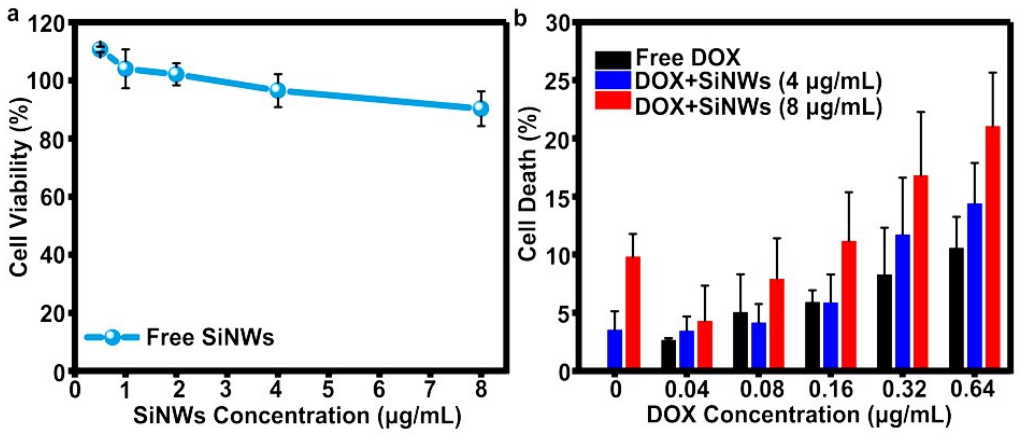

Figure S7. The cellular viability of ARPE-19 cells treated with free SiNWs or SiNWs+DOX mixtures. (a) The cellular viability of ARPE-19 cells treated by free SiNWs at different concentrations ranging from 0.5 to 8 μg mL^{-1}. (b) The cellular viability of ARPE-19 cells treated by SiNWs+DOX mixtures of various DOX concentrations (e.g., ~0-0.64 μg mL^{-1}). The results are means ± SD from three or five impendent experiments.

Fig. S7a shows that there are no significant decreases of metabolic activity of ARPE-19 cells treated by different concentrations (i.e., 0.5-8 μg mL^{-1}) of free SiNWs for 24 h, which is similar to the results of MCF-7 cell lines mentioned in Fig. S3. These results reveal that free SiNWs show feeble cytotoxicity to ARPE-19 cells. Moreover, compared with free DOX- or free SiNWs-treated groups, the SiNWs+DOX mixtures also show distinct cytotoxicity to normal cells (e.g., ARPE-19 cells, Fig. S7b). These results indicate no significant difference in cytotoxicity between cancer cell and normal cells treated with SiNWs+DOX.

10. Reference

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