Supporting Information

of

A Logic-activated Nanoswitch for Killing Cancer Cells According to Assessment of Drug-Resistance

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Experimental Section

Chemicals

All the oligonucleotides used in this study were synthesized and HPLC-purified by Sangon Biotech (Shanghai, China). The sequences of oligonucleotides were listed in (**Table S1**). Ammonium persulfate, Tris, glacial acetic acid, copper sulfate pentahydrate, dimethyl sulfoxide, and spermine were purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Tetramethylammonium hydroxide (TMAH), manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), hydrogen peroxide aqueous solution (H_2O_2, 30wt%), methanol, glutathione (GSH), dimethyl sulfoxide (DMSO) and 3-(4,5- dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Doxorubicin Hydrochloride for Injection was purchased from Shenzhen Main Luck Pharmaceuticals Inc. (Shenzhen, China). Antibodies for P-gp (25081-1-AP; 1:1000), HMGA2 (20795-1-AP; 1:1000), β-actin (66009-1-Ig; 1:5000), and the HRP goat anti-rabbit IgG (SA00001-1; 1:6000) were obtained from Proteintech Co., Ltd (Wuhan, China). RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), trypsin, and fetal bovine serum (FBS) were obtained from Gibco Life Technologies Co., Ltd (Wuhan, China). Annexin V-FITC/propidium iodide (PI) cell apoptosis kit and Hoechst 33342 staining solution were obtained from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). All solutions were prepared using ultrapure water, which was prepared through a Millipore Milli-Q water purification system (Dubuque, USA), with an electrical resistance >18.3 MΩ. All the solutions and ultrapure water used were treated with DEPC and autoclaved to avoid degradation of RNA.

Apparatus

pH was measured by a San-Xin MP511 miniature pH meter (Shanghai, China). The fluorescent spectra were recorded on a PTI ASOC-10 fluorescence system (Photo Technology International, Birmingham, NJ, USA). Dynamic light scattering and Zeta potential were measured on Malvern Zetasizer Nano ZS90 (Malvern, United Kingdom). The morphology of the nanoparticles was imaged by Tecnai G2 F20S-TWIN (Hillsborough, Oregon, USA) transmission electron microscopy (TEM) and Bruker Dimension Icon (Bluker, Germany) atomic force microscopy (AFM). UV-Vis spectrophotometer (UV-2700; Shimadzu, Tokyo, Japan) was used to characterize the UV-Vis

absorption spectra. Cytotoxicity assays were performed on a Versa Max Microplate reader (Sunnyvale, CA, USA). The fluorescence imaging studies were carried out on an FV-3000 confocal laser scanning. Flow cytometric assay was carried out by A00-1-1102 flow cytometry (Beckman, USA).

Preparation of manganese dioxide nanosheets

The Manganese dioxide nanosheets were synthesized according to the previous reports.[1] Briefly, 20 mL of TMAH (0.6 M) and H_2O_2 (3 wt %) were mixed in an aqueous solution. Then 10 mL 0.3 M $MnCl_2$ was quickly added into the above solution within 15 s. The mixed solution immediately became a dark brown color and then was stirred vigorously for 24 h at room temperature. The prepared bulk MnO_2 was centrifugated at 2000 rpm for 20 min and washed with ultrapure water first and then methanol three times. Finally, the above-purified bulk MnO_2 was dried in a vacuum drying oven at 60 °C and kept at 4 °C for further experiments. Then, 10 mg MnO_2 was added in 20 mL ultrapure water and ultrasonicated for 12 h for further use.

Preparation and characterization of TNS and Con_L-TNS

10 μL of Anti-Pgp-E (1 μM) and 10 μL of let-7a mimic (1 μM) were first mixed in 80 μL of Tris-HAc buffer (10 mM, pH 6.5, containing 10 mM Tris, 10 mM $MgCl_2$, 1 mM spermine and 0.8 mM of $CuSO_4$). Then the mixture was heated to 95 °C for 5 min and rapidly cooled to 4 °C in the PCR Amplifier, followed by complete hybridization at 37 °C for 1.5 h at the speed of 400 rpm in an Eppendorf Thermomixer comfort to form TNS.

Con_L-TNS was prepared similarly as a control probe. 10 μL of Con_L-E (1 μM) and 10 μL of let-7a mimic (1 μM) were first mixed in 80 μL of Tris-HAc buffer. Then, the mixture was heated to 95 °C for 5 min and rapidly cooled to 4 °C in the PCR Amplifier, followed by complete hybridization at 37 °C for 1.5 h at 400 rpm to form Con_L-TNS.

15% native polyacrylamide gel electrophoresis (PAGE) was performed at 90 V in 1×TAMg buffer (20 mM Tris, 20 mM acetic acid, and 10 mM $MgCl_2$) to verify the formation of TNS (1 μM). Each sample solution was loaded into the gel and run on an FB-VE10-1 electrophoresis unit. After 120 min electrophoresis in an ice bath, the gel was stained with SuperGel Red for 30 min and then analyzed with Tanon 2500R (China).

For fluorescence analysis, the 5′ and 3′ ends of TNS were labeled with fluorescent dye Cy5 and Cy3, respectively. The fluorescence spectra of Cy5 were monitored by a Quanta Master fluorescence

spectrophotometer (Cy3: Ex=540 nm, Em=640-740 nm). The target-triggered disassembly of TNS was investigated after incubation with P-gp mRNA (molar ratio 1:1) at 37 °C for 2 h. The slit was set to be 10 nm for both the excitation and the emission.

Typically, 300 μL each sample (2 μM) was recorded by a MOS-500 circular dichroism (CD) spectrophotometer (Bio-Logic, France) in the range of 200~320 nm at room temperature. In each case, the background of the buffer solution was subtracted from the CD data and processed using Origin software.

Preparation and quantification of triplex and drugs loaded on the MnO_2@TNS/DOX

For TNS loading, the prepared 6.7 μL of MnO_2 sheets (0.25 mg/mL) were resuspended in DEPC-treated RNase water along with TNS (100 nM) for 30 min at room temperature allowing for sufficient drug adsorption to obtain the MnO_2@TNS at 16.8 μg/mL MnO_2 concentration. After 30 min incubation, the solution was centrifuged at 10000 rpm and the supernatant was collected for fluorescent spectral analysis.

For DOX loading, 3.5 μM DOX was added to the above solution MnO_2@TNS (16.8 μg/mL MnO_2 concentration) at 37 °C to obtain the MnO_2@TNS/DOX at 400 rpm. After 1 h incubation, the solution was centrifuged at 10000 rpm and the supernatant was collected for fluorescence analysis. For TNS and DOX co-loading, The MnO_2 sheet-to-TNS-DOX ratios (MnO_2@TNS/DOX at 16.8 μg/mL MnO_2 concentration consisting of 100 nM TNS, 3.5 μM DOX and 16.8 μg/mL MnO_2 sheets) were used for the following experiments.

Similarly, Con_L-MnO_2@TNS/DOX was prepared as a control probe.

Characterization of MnO_2@TNS/DOX

The encapsulation efficiency was determined by the fluorescence intensity of TNS and DOX from the MnO_2@TNS/DOX solution in buffer with the excitation wavelength of 540 (480) nm and the emission wavelength of 670 (600) nm for TNS (DOX). The particle size and zeta potential of the MnO_2@TNS/DOX were characterized by dynamic light scattering. The morphology of the MnO_2@TNS/DOX was examined by transmission electronic microscopy (TEM) and Atomic Force Microscope (AFM).

Investigation of the MnO_2@TNS/DOX stability

Drug stability is an essential factor that alters therapeutic efficiency. Thus, the stability of the prepared MnO_2@TNS/DOX was investigated by adding it to 10% fetal bovine serum and then kept

at 37 °C at different times. In addition, MnO_2 sheets were also investigated under the same conditions as controls.

Hemolysis assay

Mice whole blood was collected from the eyeballs, and ethylenediaminetetraacetic acid (EDTA, 1.5 mg/mL) was used as a stabilizer. The red blood cells (RBC) were collected by centrifugation at 1300 rpm for 5 min and washed 5 times with PBS, followed by resuspension in PBS (pH 7.4). Varying concentrations of MnO_2@TNS/DOX (serial dilution ranging from 8.4-33.6 μg/mL MnO_2 nanosheets) were added into the RBC-contained PBS buffer solutions and co-incubated at 37 °C for 1 h. All solutions were centrifuged to examine the hemolysis. For comparison, the RBC suspensions in PBS and DI water without MnO_2@TNS/DOX were used as control groups.

Drug release profile of MnO_2@TNS/DOX

The drug release studies of MnO_2@TNS/DOX were performed at 37 °C with or without GSH. For cumulative TNS release, after adding 1 mM GSH (pH 6.5) into the MnO_2@TNS/DOX at 16.8 μg/mL MnO_2 concentration solutions, the fluorescence intensity of Cy5 in these solutions was measured. (Cy3: Ex=540 nm, Em=560-740 nm).

For cumulative DOX release, after adding 1 mM GSH (pH 6.5) into the MnO_2@TNS/DOX at 16.8 μg/mL MnO_2 concentration solutions, the fluorescence intensity of DOX in these solutions was measured. (DOX: Ex=480 nm, Em=560-740 nm). The drug release studies were performed in triplicate for each of the samples.

Cell Culture

DOX drug-resistant MCF-7/ADR cells and non-drug-resistant MCF-7 cells were purchased from the cell bank of the Central Laboratory of Xiangya Hospital (Changsha, China). MCF-7 cells were cultured in a complete DMEM cell culture medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL of penicillin G sodium, and 100 mg/mL of streptomycin sulfate. MCF-7/ADR cells were cultured in the complete RPMI 1640 cell culture medium (Gibco) containing 1 μM DOX, 10% FBS, 100 $U \cdot mL^{-1}$ of penicillin G sodium, and 100 $mg \cdot mL^{-1}$ of streptomycin sulfate at 37 ^oC in a humidified incubator with 5% CO_2.

Confocal fluorescence imaging and analysis of cellular uptake of MnO_2@TNS/DOX

MCF-7, MCF-7/ADR cells were seeded in confocal dishes for 24 h at 37 °C under 5% CO_2. After removing the culture medium, the cells were incubated with MnO_2@TNS/DOX at 16.8 μg/mL

MnO_2 concentration in culture medium for 0 h, 2 h, 4 h, and 6 h. The cells were then washed with fresh PBS (1×, pH 7.4) three times to remove the remaining MnO_2@TNS/DOX. To assess the uptake of MnO_2@TNS/DOX nanoswitch, the cells were stained with Hoechst 33342 (5 μg/mL) for 10 min at room temperature before imaging. The cells were washed three times with fresh PBS buffer. Finally, the cells were performed using an FV3000-MPE confocal laser scanning microscopy system with 480 nm and 540 nm excitation light.

Cytotoxicity assay

Cytotoxicity experiments of the MnO_2@TNS/DOX nanoswitch were estimated using the MTT cell viability assay. MCF-7/ADR cells and MCF-7 cells were seeded (3×10^4 cells/well) separately into 96-well plates, respectively. The cells were incubated with different concentrations of different drug formulations (DOX, MnO_2@DOX, MnO_2@TNS, MnO_2@TNS/DOX, Con_L-MnO_2@TNS/DOX) for 4 h. Then the cell medium was then removed, the cells were washed 3 times with PBS and 200 μL of fresh cell medium was added. After 48 h incubation, 15 μL of MTT (5 mg/mL) solution was added into the cell medium to each well. After 4 h incubation, the cell viability was determined by measuring the absorbance at 490 nm, using a Synergy 2 Multi-Mode Microplate Reader (Bio-Tek, Winooski, VT). Experiments were repeated in triplicate, and the error bars represent the standard derivations.

Apoptosis mechanism assay

We investigated the MnO_2@TNS/DOX killing mechanism after treating cells with MnO_2@TNS/DOX and analyzed it by Annexin-FITC/PI two-color flow cytometry. MCF-7 and MCF-7/ADR cells were seeded in 6-well plate 5×10^5 cells/well for 24 h at 37 °C in a humidified incubator with 5% CO_2, respectively. The cells were incubated with MnO_2@TNS/DOX and Con_L-MnO_2@TNS/DOX at 25.1 μg/mL MnO_2 concentration for 48 h. Then the cells were washed three times with PBS, digested by trypsin, and collected in centrifuge tubes, followed by staining for 20 min with the Annexin V-FITC/PI cellular apoptosis fluorescent dye kit, according to the manufacturer's instructions. Finally, the cells were tested by flow cytometry.

Western blot assay

MCF-7 and MCF-7/MDR cells were treated with different nanoswitches at 25.1 μg/mL MnO_2 concentration and untreated cells were used as a negative control, respectively. The treated cells were lysed by RIPA buffer containing protease inhibitors to extract total proteins. The protein

concentrations were then determined with a bicinchoninic acid (BCA) protein assay kit (Invitrogen, Carlsbad, CA, USA). 30 μg of proteins were separated by 5-15% SDS-PACE and transferred to 0.22 μm nitrocellulose (NC) membranes (Millipore). Subsequently, the membranes were blocked with 5% blotting-grade milk and then incubated with primary and secondary antibodies successively. After incubating with antibodies, the protein bands were imaged by electrochemical luminescence (Invitrogen) and quantified by Image J software.

Supplementary Table

Table S1. All of the oligonucleotides and triple-helix nucleic acid switches used in this work.

Oligomer or Triple-helix nucleic acid switch		Sequence (5'-3')
TNS	Anti-Pgp-E	**CTA CTA CCT CA** *TCC ATC CCG ACC TCT TTT T* **ACT CCA TCA TC**
	let-7a mimic	**UGA GGU AGU AG** GUU GUA UAG UU
Con_L-TNS	Con_L-E	**CTA CTA CCT CA** *TTT ATG CCA TGC TCT ACGT* **ACT CCA TCA TC**
	let-7a mimic	**UGA GGU AGU AG** GUU GUA UAG UU

[a] In the above sequences, the loop of the triple-helix nucleic acid switch is shown underlined, and the stem of the triple-helix nucleic acid switch is displayed in italics.

[b] For Con_L-E, it is obtained by replacing the anti-Pgp sequence in Anti-Pgp-E with a random base composition, which can hybridize with let-7a mimic to form triplex stabilized Con_L-TNS.

Supplementary Figures

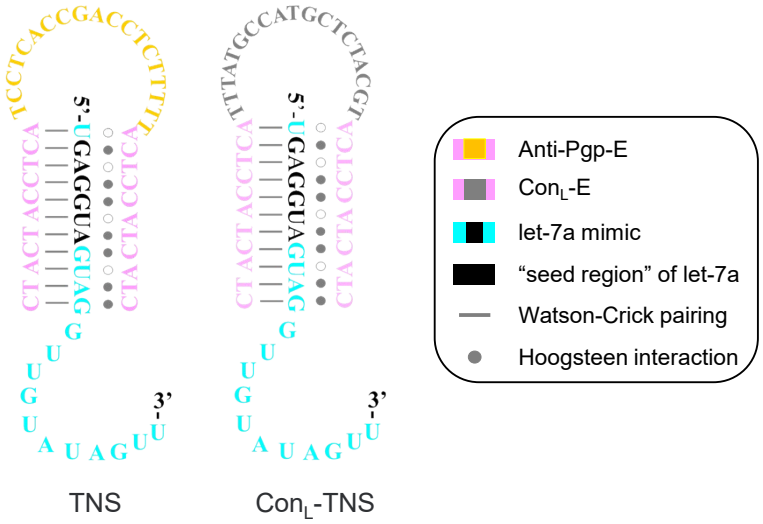

Figure S1. The sequence details of TNS, and Con$_L$-TNS with different triple-helix stems.

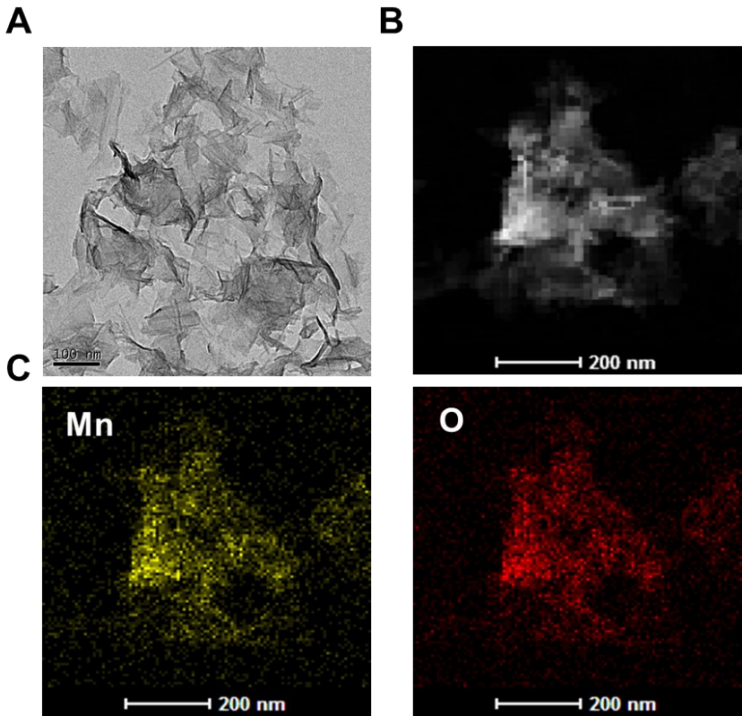

Figure S2. TEM images of the MnO_2 nanosheets (A-B) and elemental mapping of (C) Mn and O.

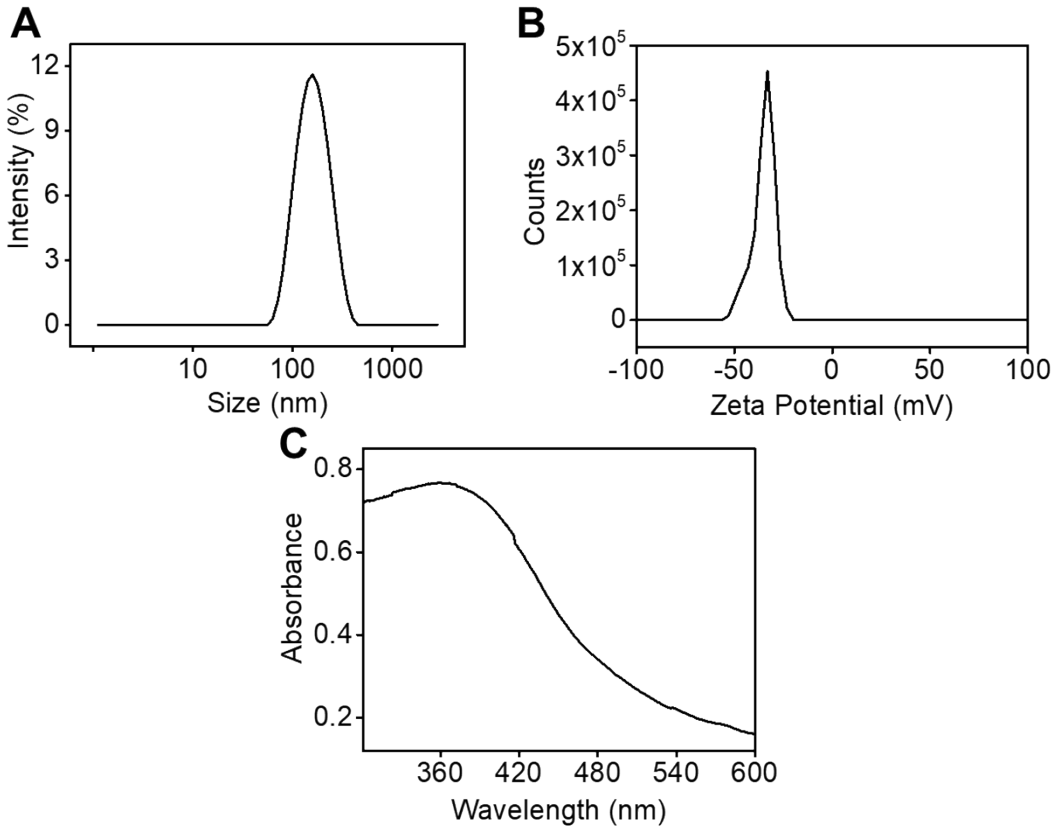

Figure S3. (A) Dynamic light scattering (DLS) (B) Zeta potential analysis and (C) UV-vis absorption spectrum of MnO_2 nanosheets.

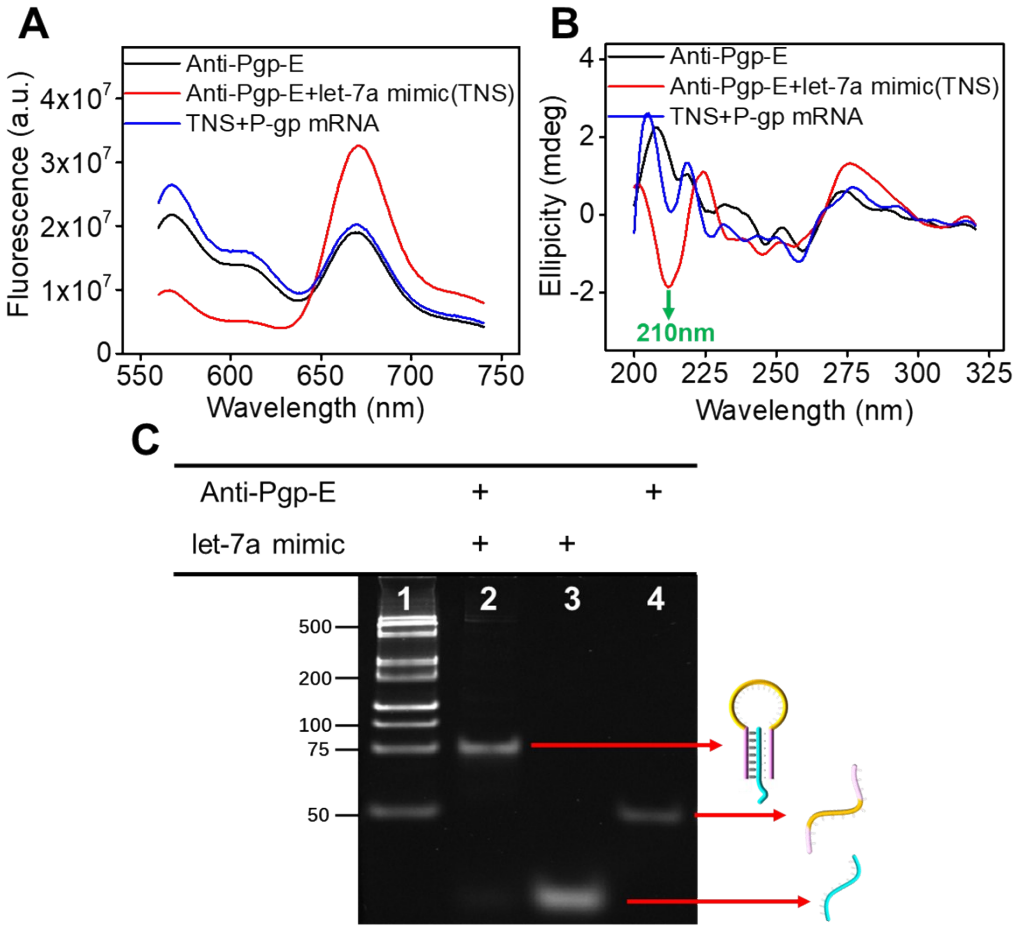

Figure S4. Validation of TNS's assembly and disassembly in response to P-gp mRNA. (A) Fluorescence behaviors of TNS during assembly and P-gp mRNA triggered disassembly. (B) Circular dichroism (CD) spectra of the assembly and disassembly of TNS. The CD spectral profile characterized by a negative band centered at ~210 nm, indicating the formation of triplex. (C) 15% PAGE analysis of the assembly and disassembly of TNS.

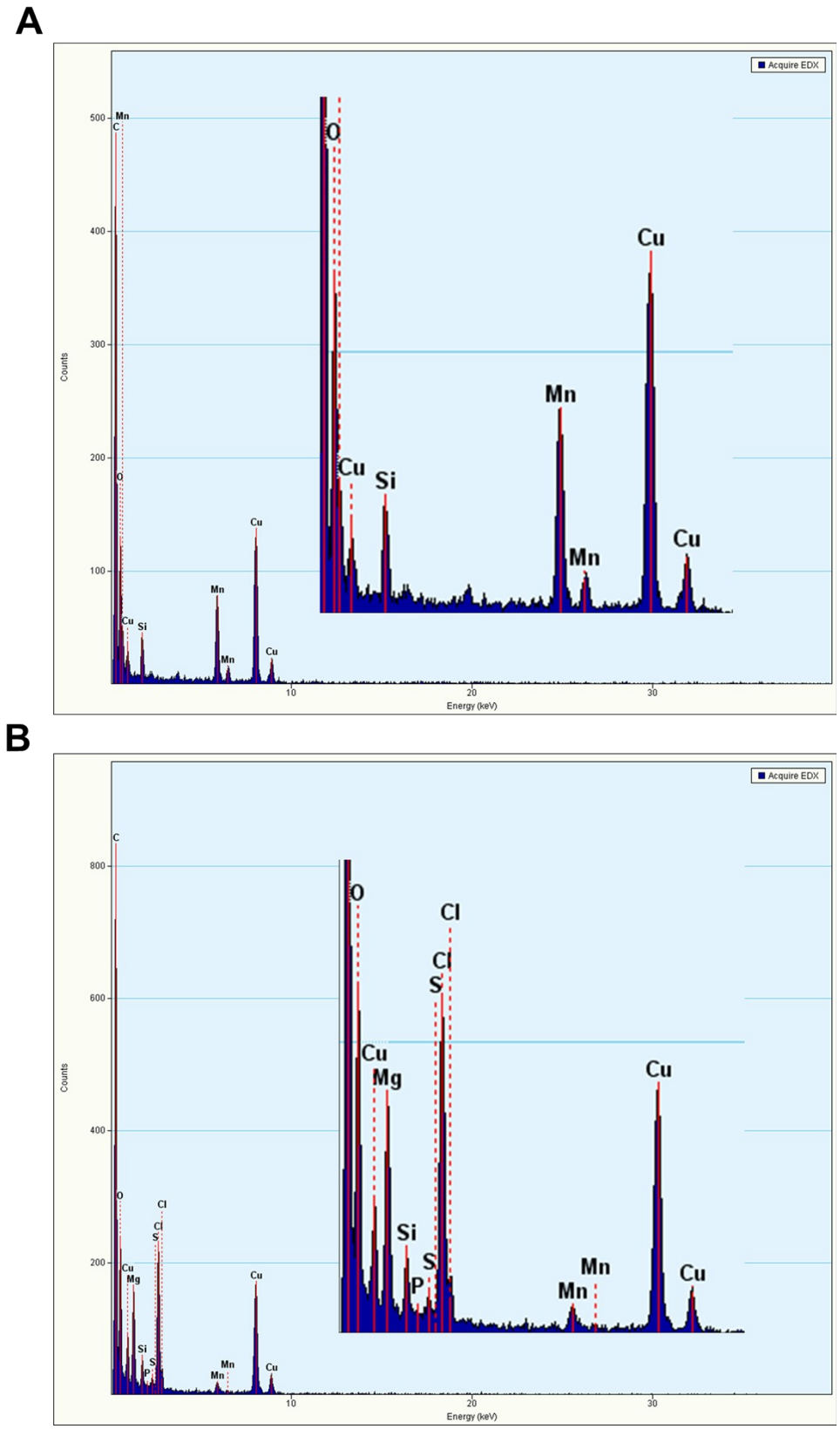

Figure S5. Integrated EDS drift corrected spectrum analysis of the (A) MnO_2 sheets and (B) MnO_2@TNS/DOX. The presence of both Mn and O peaks was observed in the MnO_2 sheets. The presence of both Mn, O, P, S and Cl peaks was observed in the MnO_2@TNS/DOX.

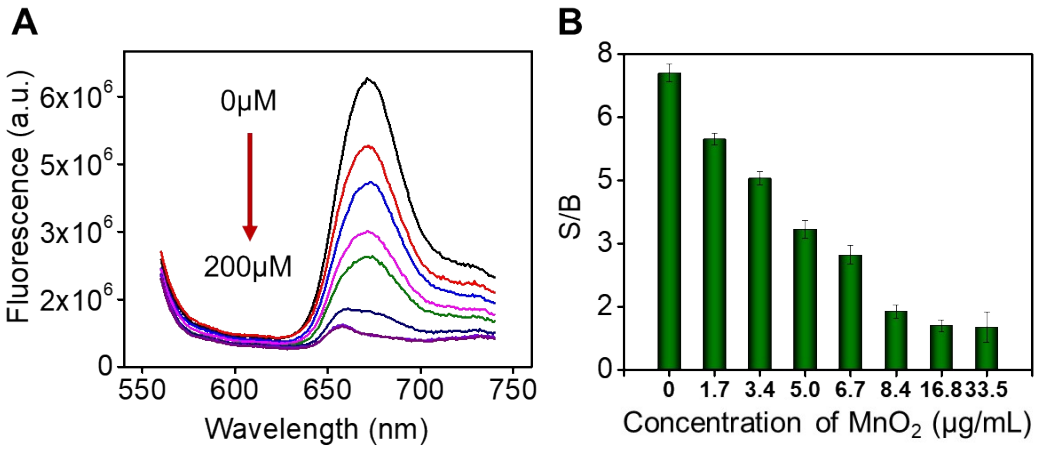

Figure S6. (A) Fluorescence changes and (B) S/B of TNS after incubation with the MnO_2 sheets of different concentrations (0 ~ 33.5 μg/mL) for 30 min away from light at room temperature.

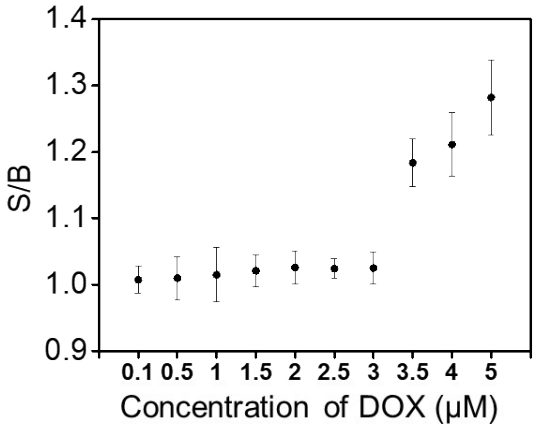

Figure S7. The fluorescence S/B of MnO_2@TNS at 16.8 μg/mL MnO_2 concentration with different concentrations DOX.

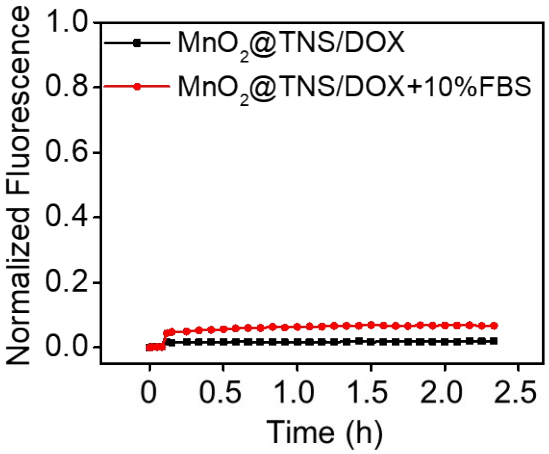

Figure S8. The stability of MnO_2@TNS/DOX (25.1 μg/mL MnO_2) in 10% FBS serum.

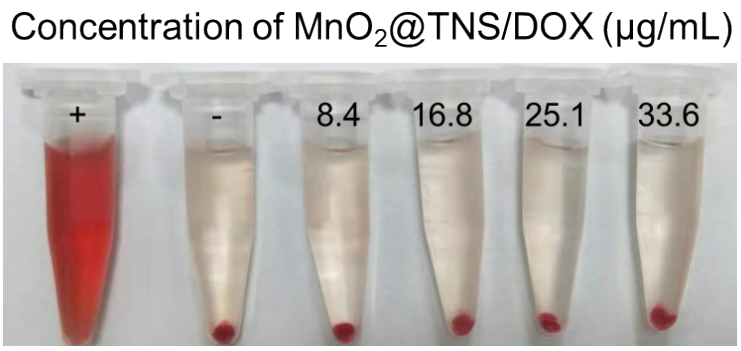

Figure S9. Hemolytic tests of MnO_2@TNS/DOX with different MnO_2 concentrations. Ultrapure water and PBS were used as positive and negative controls.

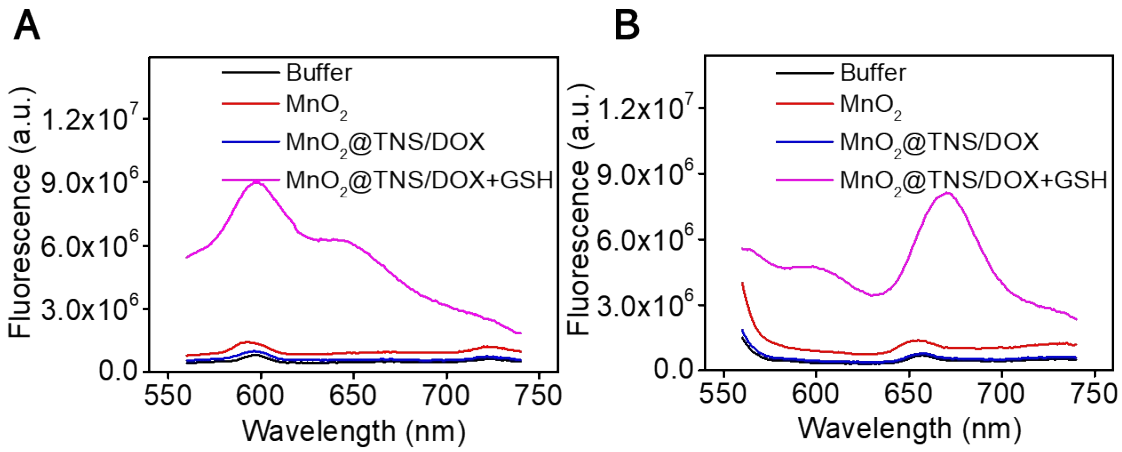

Figure S10. Fluorescence spectra of buffer, MnO_2, MnO_2@TNS/DOX (16.8 μg/mL MnO_2) without and with the addition of GSH (A) Ex= 480 nm (B) Ex= 540 nm.

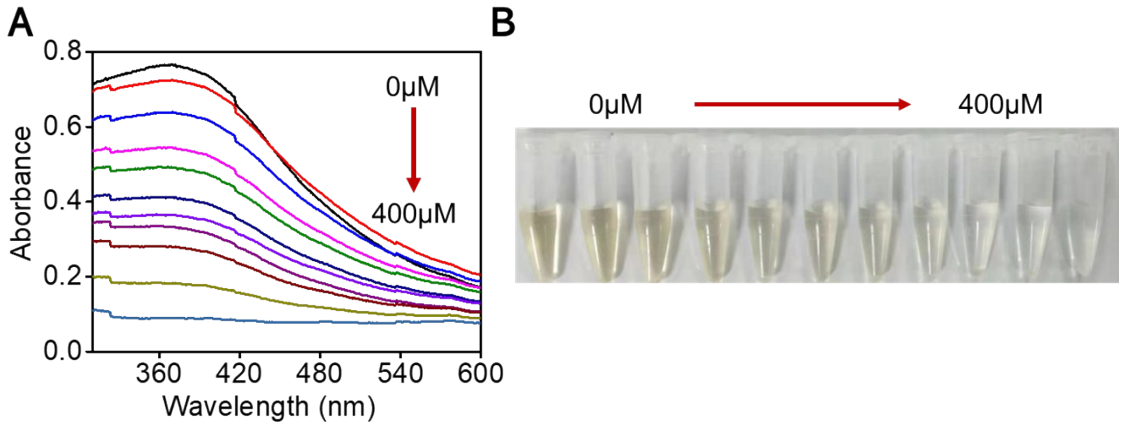

Figure S11. (A) UV-vis absorption spectra and (B) photograph of MnO_2 (16.8 μg/mL MnO_2) after reaction for 5 min at 37℃ in the presence of GSH with different concentrations.

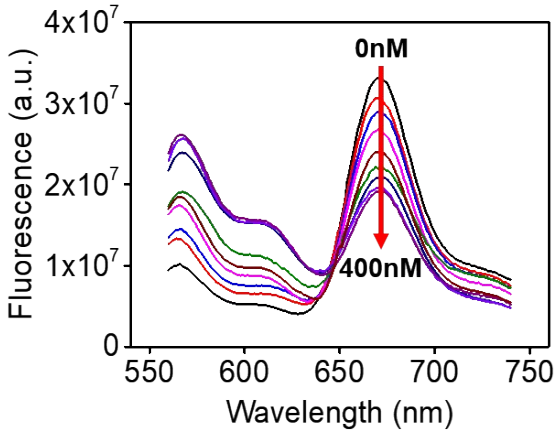

Figure S12. Fluorescence recovery of TNS in response to P-gp mRNA with varying concentrations (0, 3.0,10.0, 20.0, 35.0, 50.0, 80.0, 100.0, 400.0 nM).

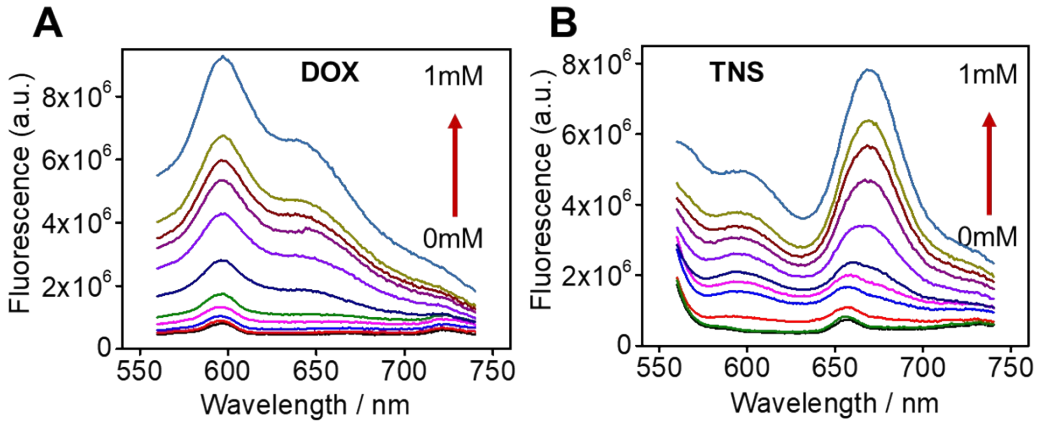

Figure S13. Fluorescence spectra of (A) DOX and (B) TNS release from MnO_2@TNS/DOX at 16.8 μg/mL MnO_2 concentration with different concentrations of GSH (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 mM) at 37 °C in buffer.

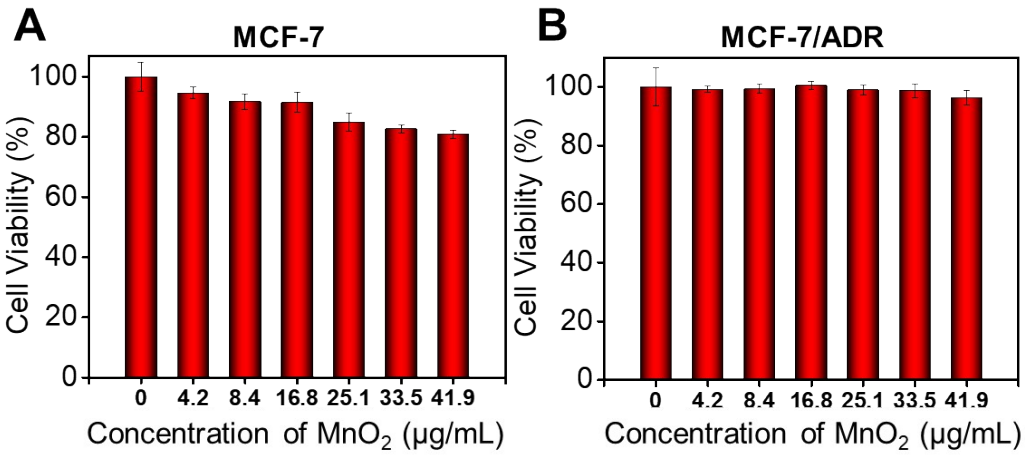

Figure S14. Cytotoxicity of MnO_2 nanosheets against (A) MCF-7 and (B) MCF-7/ADR cells for 48 h.

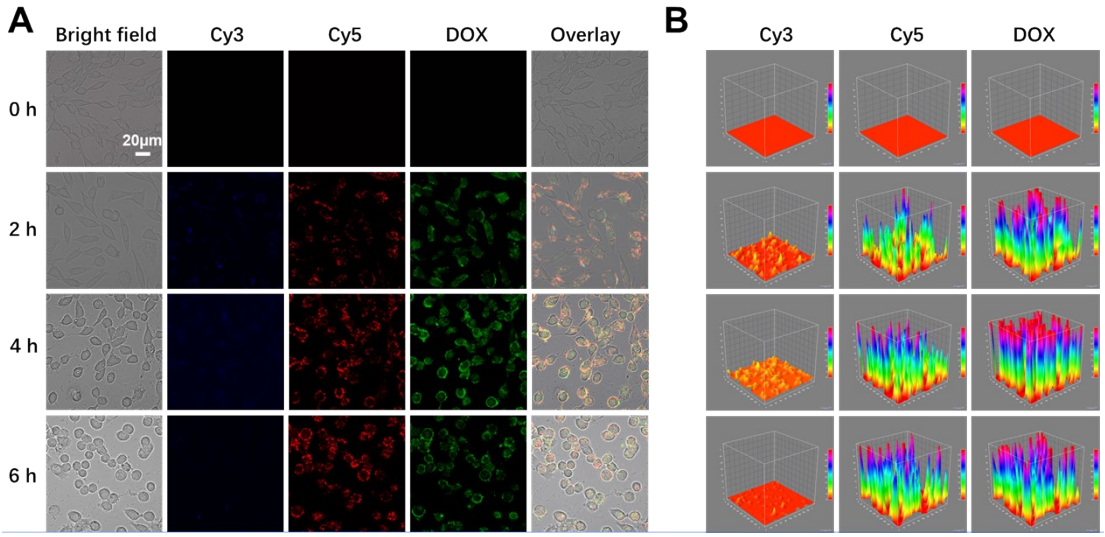

Figure S15. Confocal imaging of MCF-7 cells after incubation with MnO_2@TNS/DOX at different times. (B) Quantitative analysis of fluorescence intensity at the corresponding time. The concentration of MnO_2 nanosheets = 16.8 μg/mL. Size: 20 μm.

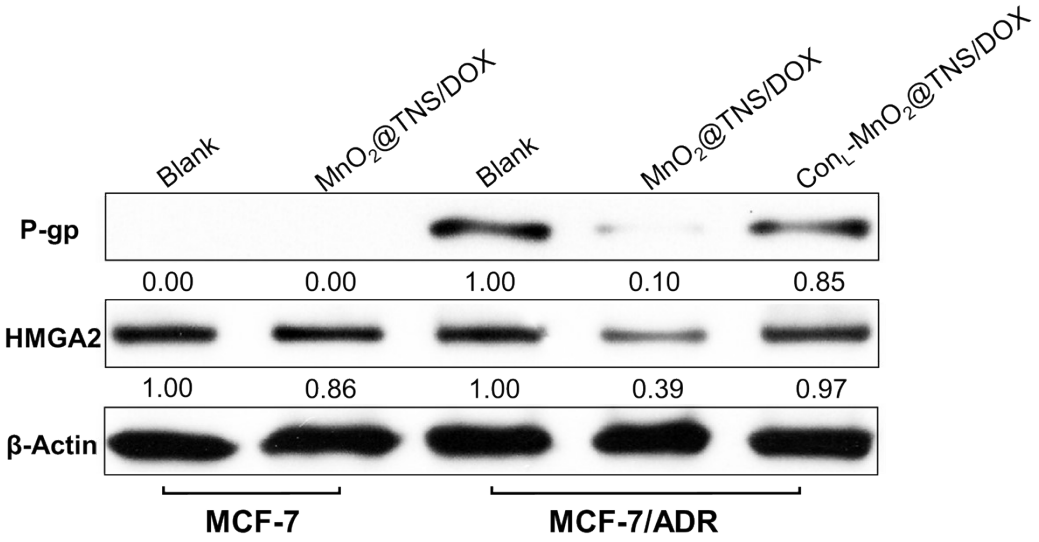

Figure S16. Western blot image of P-gp and HMGA2 protein expression in MCF-7 and MCF-7/ADR cells treated with different nanoswitches at 25.1 μg/mL MnO_2 concentration for 48 h. The relative intensity ratios of P-gp/β-Actin and HMGA2/β-Actin normalized to MCF-7/ADR are marked at the bottom.

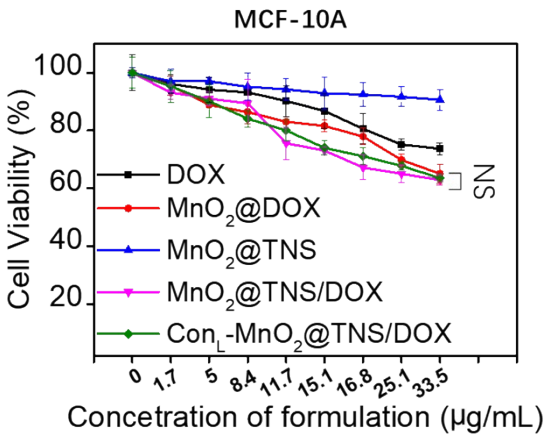

Figure S17. Cell viability of MCF-10A cells after incubation with different nanoswitches with different concentrations for 48 h. Statistical significance: **P < 0.01.

Reference

[1] L.L. Peng, X. Peng, B.R. Liu, C.Z. Wu, Y. Xie, G.H. Yu, Ultrathin two-dimensional MnO_2/graphene hybrid nanostructures for high-performance, flexible planar supercapacitors, Nano Letters., 13(2013): 2151-2157. https://doi.org/10.1021/nl400600x