Supporting Information

SiRNA-Templated 3D Framework Nucleic-Acids for Chemotactic Recognition, and Programmable and Visualized Precise Delivery for Synergistic Cancer Therapy

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Methods

Materials.

The DNA, mRNA and siRNA sequences were synthesized by Sangon Biological Co. Ltd (Shanghai, China). Adenosine 5'-triphosphate (ATP), Glutathione reduced (GSH), Oligomycin (OM), Dox, Dulbecco's modified Eagle's medium (DMEM), trypsin and phosphate buffered saline (PBS, pH 7.4, basic (1×)) were acquired from Sigma-Aldrich. Sepantronium Bromide (YM155), L-Buthionine Sulfoximine (BSO) and 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Aladdin (Shanghai, China). Fetal Bovine Serum (FBS) was obtained from Gibco. 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate (polyethylene glycol)-2000] (DSPE-PEG-FA) and cholesterol-catalase were purchased from Qiyue Biotech (Xian) Co.

Instruments.

The transmission electron microscopy (TEM) images were obtained with a FEI Talos F200s TEM (Thermo Fisher Scientific, USA). Ultraviolet-visible (UV-vis) spectra were acquired using a Shimadzu UV-2600 spectrophotometer (Shimadzu, Japan). The fluorescence spectra were recorded on an FS5 fluorescence spectrometer (Edinburgh, UK). The dynamic light scattering (DLS) and zeta potential data were conducted using the Nano-ZS Zetasizer ZEN3600 (Malvern, UK.). The liposome membrane solution was prepared using an Avanti mini extruder. The cell viability was measured by thiazolyl blue tetrazolium bromide (MTT) using a microplate reader at 490 nm on a microplate reader (BioTek, USA). Confocal fluorescence imaging of cells was performed on a Nikon A1R-si laser confocal laser scanning microscope (CLSM) (Nikon, Japan). Flow cytometric analysis was performed on CytoFLEX (Beckman, USA). Tissue slides images were captured using a Zeiss Axiovert 20 inverted fluorescence microscope (Carl Zeiss, Germany). In vivo imaging was performed on an IVIS Lumina III system (Caliper, USA).

Synthesis of siRNA-Templated 3D FNAs (ST-SFNA).

1) Preparation of ST-SFNA cores with Logic Gates.

Hybridization chain reaction (HCR) was employed to prepare the spherical 3D framework nucleic acids (FNAs). In the experiment, siRNA linkers with a linear structure triggered the linear polymerization (LP). Thus, with H1 (8 μL, 50 μM) and H2 (16 μL, 50 μM) as the monomers for 3 h of the polymerization, the linear polymer was obtained. After that, H3 (16 μL, 50 μM) were added for the reaction (2, 3 or 5 h), followed by another the reaction with H4 (8 μL, 50 μM) added (2, 3 or 5 h). Therefore, the branched chain polymerization (BCP) was accomplished to obtain FNAs. The different reaction time for BCP can result different size of the FNAs. Then, g-DNA was added and incubated for 2 h to stop the HCR and form ST-SFNA cores with controllable particle size. Finally, Doxorubicin solution (Dox: 2 μM) was incubated with the ST-SFNA cores for 12 h. The Dox-loaded ST-SFNA cores were purified with an ultra centrifugal filter (MWCO of 100kDa).

2) Tumor-Targeted ST-SFNA Preparation.

Liposomes were prepared using the thin-film hydration and extrusion technique. Stock solutions of the lipids were prepared in chloroform. The phospholipids 1, 2- dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate (polyethylene glycol)-2000] (DSPE-PEG-FA) were added to a round bottom flask in a molar ratio of 95:5. The solution was then evaporated using a rotavap to form a thin film of lipids. The products

were stored in a vacuum desiccator for 2 h to removal the trace chloroform solvent. The thin film was then rehydrated to 1 mL in 100 mM phosphate buffer (pH 7.4) and stirred continuously overnight to self-assemble the phospholipids into liposomes.

The resulting phospholipid membrane solution was then added into an equal volume of ST-SFNA cores and stirred continuously for 30 minutes. The mixture was then repetitively extruded 20 times through 400-, 200-, and 100-nm polycarbonate track etch porous membranes using an Avanti mini extruder. The resulting nanomaterials was incubated with cholesterol-conjugated catalase solution for 15 min to obtain ST-SFNAs.

Agarose gel electrophoresis.

Each DNA sample (5 μL) was mixed with 6x loading buffe (1 μL) and analyzed by 3 % agarose gel (weight / weight) at 100 V in 1×TAE buffer for 60 min. After electrophoresis, the gels were imaged with UV channel by ChampGel imaging system.

Sequential drug release and logic operation of ST-SFNAs.

ST-SFNA cores labeled with Cy5 and BHQ2 was incubated in PBS buffer (pH 7.4, supplemented with 5 mM Mg^{2+}) containing ATP (5 mM) and GSH (5 mM). Upon the addition of mRNAs and incubation at 37 ºC for 6 h, the emission spectra were measured (655-750 nm) with an excitation wavelength of 640 nm. To evaluate the release of the drug, the fluorescence of DOX (ex. 495 nm, em. 555 nm) were measured in the presence of 200 nM survivin DNA.

Catalytic Activity Assay.

Accordingly, the catalytic activity of the ST-SFNAs was evaluated using UV-vis spectrophotometer. Via the elimination of H_2O_2 substrate, the UV-vis absorbance decreased. The profile was carried out at different concentrations of catalase and H_2O_2. In the experiment, H_2O_2 was added into ST-SFNA solution in a quartz cuvette. The UV-vis absorbance of H_2O_2 at 240 nm was measured. Before tests, the solution was stirred to dislodge O_2 bubbles on the inner wall of the cuvette.

Motion in Solution.

The confocal laser scanning microscope (CLSM) was used to record the movement of the ST-SFNAs in XYT mode in real time. Different concentrations of H_2O_2 were added into 1 mL of ST-SFNAs, whose particle motion was recorded by time-lapse series scanning. The particle trajectory was analyzed using ImageJ software with manual tracking plugin and Chemotaxis and Migration Tool. The relationship between mean azimuth shift (MSD) and time was plotted to examine the motion of ST-SFNAs. The mean MSD of 15 nanoparticles indicated the directional motion of ST-SFNAs. MSD values were then calculated as a function of time interval (Δt). MSD was determined according to the equation $\langle MSD \rangle = 4 D\Delta t + v^2\Delta t^2$ with D being the diffusion coefficient and v, the speed of the nanomotors. The movement of the ST-SFNAs without fuel (controls) showed only a linear $\langle MSD \rangle = 4D\Delta t$ dependency, typical for a Brownian motion.

Cell Culture.

HeLa and HeLa-GFP cells were cultured in DMEM medium containing 10 % fetal bovine serum (FBS), 50 U / mL penicillin and 50 U / mL streptomycin in an atmosphere of 5 % CO_2 at 37 ºC.

Confocal Fluorescence Imaging.

Various cells (HeLa and HeLa-GFP) were seeded at an initial density of 2×10^4 cells/well in each confocal dish for 24 h. After incubation overnight, cells were washed three times with PBS, and ST-SFNAs were added at a concentration of 200 nM and incubated at 37 °C for different time (1 h, 2 h, 3 h, 4 h). Then the solutions were removed and the cells were carefully washed thrice with ice-cold PBS. Subsequently, the cells were incubated with fresh medium buffer containing 10 μg / mL nuclear stain (Hoechst 33342) for 20 min. Finally, all cellular fluorescent images were performed with a Nikon confocal laser scanning microscope, and the images were acquired using 40× objective lens.

Cell activity assessment.

The cytotoxicity of the ST-SFNA system was assessed with a standard MTT assay. 10 μL HeLa cells (5000 cells / well) were seeded to 96-well microplates and cultured for 24 h. Then the medium was removed and 200 μL new medium containing ST-SFNAs (50, 100, 150, 200 nM) was added to each well. After incubation for 48 h, 20 μL of MTT solution (5 mg / mL) was added into each well and incubated at 37 ºC for another 4 h. Finally, the supernatants were carefully removed and then 150 μL of DMSO was added to each well to dissolve the formazan crystals. Finally, the absorbance at 490 nm was measured and recorded using a microplate reader.

In order to evaluate the toxicity of the drugs, HeLa cells were seeded onto 96-well plates for 24 h and incubated with different solubility drugs, including YM155 (0, 1, 3, 5 nM) for 36 h, OM (0, 1, 5, 10 μM) and / or BSO (0, 50, 100, 200 μM) for 1 h. Then cells were treated with ST-SFNAs (200 nM) for 4 h. Next, the above procedures of adding MTT and DMSO were repeated for cell activity detection.

Logic computation of ST-SFNAs in live cells.

For logic computation, the high intracellular ATP, GSH and survivin levels in tumor cells were set as Input "1", while the depleted levels were set as Input "0". In this case, the cells were incubated with OM (10 μM) for 24 h, BSO (100 μM) for 24 h, and YM155 (1, 3, 5 nM) for 36 h, respectively. The group of HeLa cells without drug treatment was used as the control. After pretreatment, the logic devices of ST-SFNAs with "turn-on" signals were incubated with cells for logic computation.

Gene silencing of GFP in HeLa-GFP cells.

HeLa-GFP cells were seeded in 24-well plates at 2×10^4 cells per well and cultured overnight. After removing culture medium, 1 mL new medium containing ST-SFNAs-siGFP (50, 100, 200 nM) was added to each well. After 48 h treatment, the GFP expression in the control cells and experimental groups was assessed by confocal fluorescence imaging and flow cytometry analysis.

Cell Apoptosis Assay.

Cells were seeded in 6-well plates with a density of 1×10^5 cells per well for 24 h and then treated with ST-SFNAs for 12 h. The supernatant and the cell monolayer were collected, washed twice with cold PBS. Then, they were stained with Alexa Fluor 488 annexin V and Propidium Iodide using an Alexa Fluor 488 annexin V/Dead cell apoptosis kit according to the manufacturer's instructions. After staining, the percentage of apoptotic cells was examined with flow cytometry.

Tumor Sphere Culture and Observation.

The HeLa multicellular spheroids (MCSs) were established by a liquid overlay method. Briefly, a 96-well spheroid microplate was coated with 1.5 % agarose gel to prevent cell adhesion. Then, the cell suspensions (9×10^4 cells / 200 μL per well) were transferred into each well, gently agitated for 5 min, and maintained at 37 °C for 7 days. The uniform and complete spheroids were used for the penetration studies. The ST-SFNAs were incubated with the tumor spheroids at 37 °C for 4 h. Then, the medium was removed and the spheroids were gently washed with PBS three times. The fluorescent signal of Cy5 and Dox in spheroids was observed using the Z-stack images of the confocal microscope.

Tumor-bearing mice model.

All animal experiment protocols were reviewed and approved by the Animal Care and Use Committee of Institute of Beijing Normal University and complied with all relevant ethical regulations. The male BALB/c nude mice (18-20 g, 6-7 weeks) were purchased from Beijing Charles River Laboratory Animal Center and raised in a specific pathogen-free grade laboratory. The HeLa tumor-bearing mice model was established by subcutaneously inoculating about 5×10^6 HeLa cells in the right flank of BALB/c nude mice.

In vivo Imaging.

When the average tumor volume reached to 100-120 mm^3, the mice were intravenously injected with unmodified ST-SFNAs, ST-SFNAs-Cat, ST-SFNAs-FA and ST-SFNAs-(Cat+FA), at 30 nmol kg^{-1} dosage, respectively. Fluorescence imaging data were collected using the IVIS Lumina II in vivo imaging system at 6 h post-injection. The mice were euthanized and the main organs (liver, kidney, spleen, lung, and heart) of the mice were selected for ex vivo imaging by IVIS Lumina II in the vivo imaging system.

Tumor growth inhibition.

For in vivo antitumor study, the mice were randomly divided into four groups (three mice per groups) for the treatment to obtain the tumor volume of about 100-120 mm^3. 200 μL of therapeutic nanoagents (4 mg kg^{-1} Dox and / or 1 mg kg^{-1} siRNA) was injected into each nude mouse via tail vein every other day for 14 days and meanwhile tumor weight and size were monitored. Tumor size was measured by a caliper and tumor volume calculated according to the following formula:

$$\text{tumour volume}(mm^3) = \text{length}\times\text{width}^2 / 2$$

After 14^{th} therapy, tumors and major organs were sectioned for hematoxylin-eosin staining (H&E) analyses.

Video of ST-SFNA motion

Video of ST-SFNA motion in DI water (AVI, Video S1); Video of ST-SFNA motion in 3 mM H_2O_2 (AVI, Video S2); Video of ST-SFNA motion in 6 mM H_2O_2 (AVI, Video S3).

Supplementary Figures

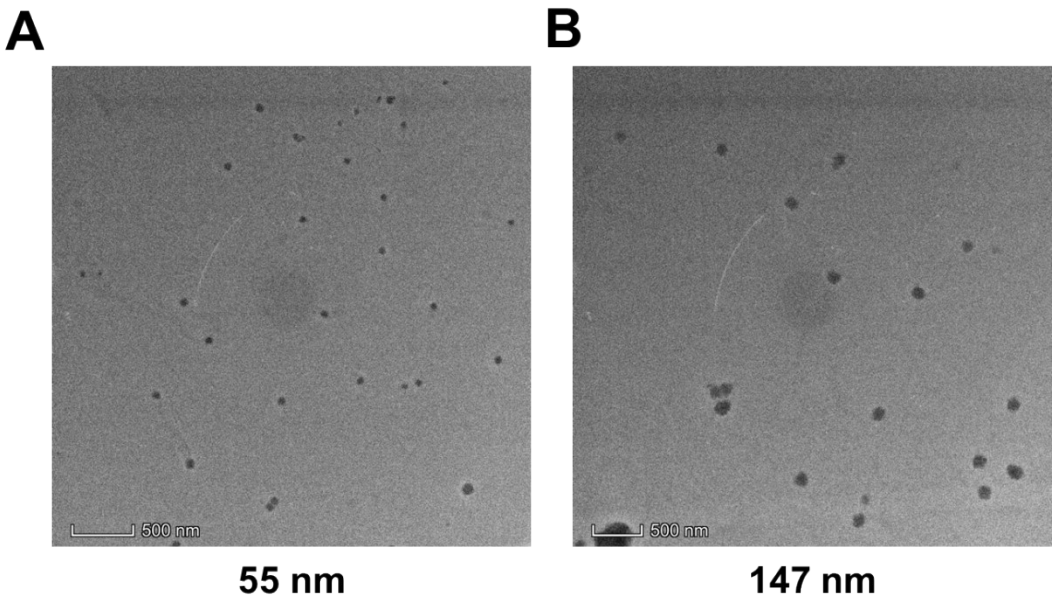

Figure S1. TEM characterization of ST-SFNA cores obtained by different times of branched chain polymerization after siRNA-templated linear polymerization (with H1 and H2 for 3 h). (A) Adding H3 for 2 h and H4 for 2 h of branched chain polymerization. (B) Adding H3 for 5 h and H4 for 5 h of branched chain polymerization.

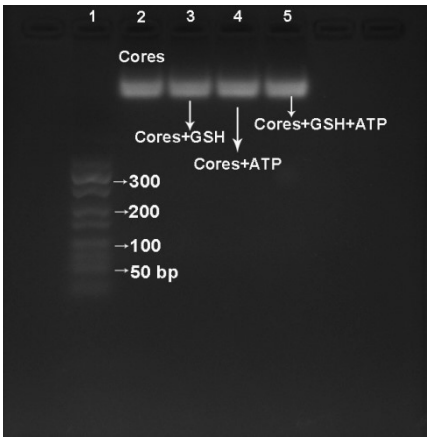

Figure S2. Agarose gel electrophoresis images: cascaded-logical responses of ST-SFNA cores. Lane 1: DNA marker; 2: ST-SFNA cores; 3: ST-SFNA cores with adding GSH; 4: ST-SFNA cores with adding ATP; 5: ST-SFNA cores with adding GSH and ATP.

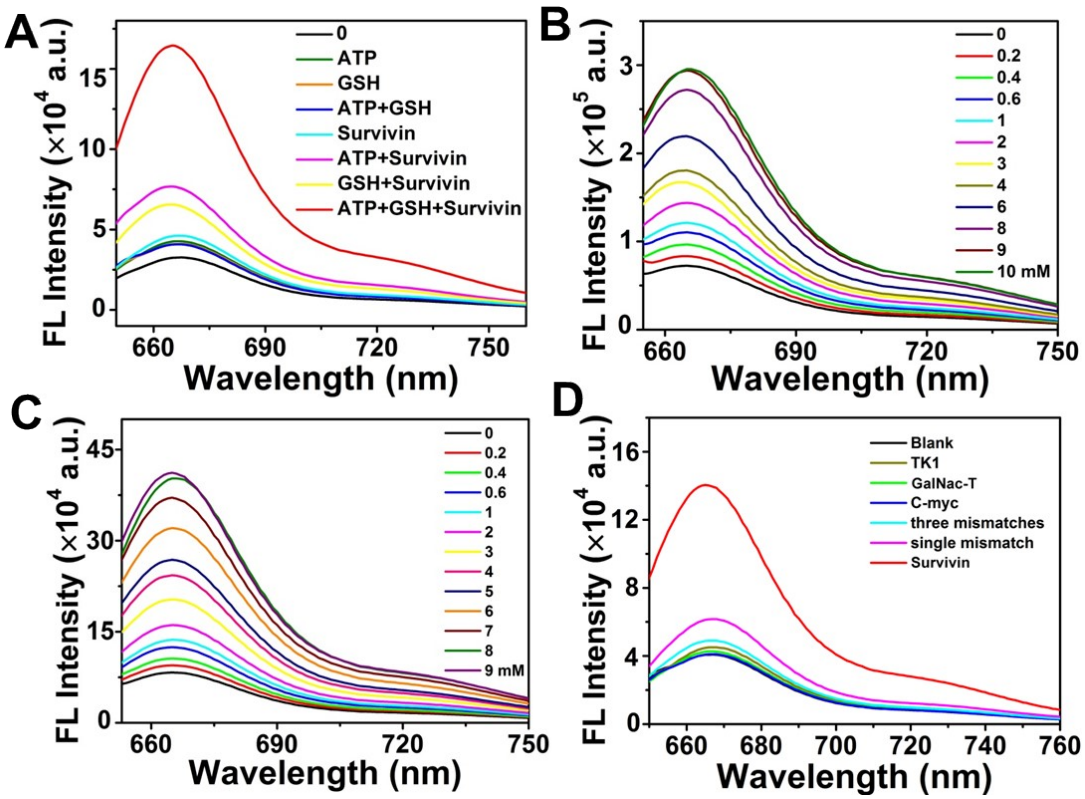

Figure S3. Original fluorescence spectra in Figure 2 of main text. (A) All input conditions corresponding to Figure 2B. (B) The ST-SFNA cores for ATP corresponding to Figure 2C. GSH: 5 mM, target DNA: 200 nM. (C) The ST-SFNA cores for GSH corresponding to Figure 2D. ATP: 5 mM, target DNA: 200 nM. (D) ST-SFNA cores selectivity for target DNA (survivin) against other counterparts, corresponding to Figure 2H.

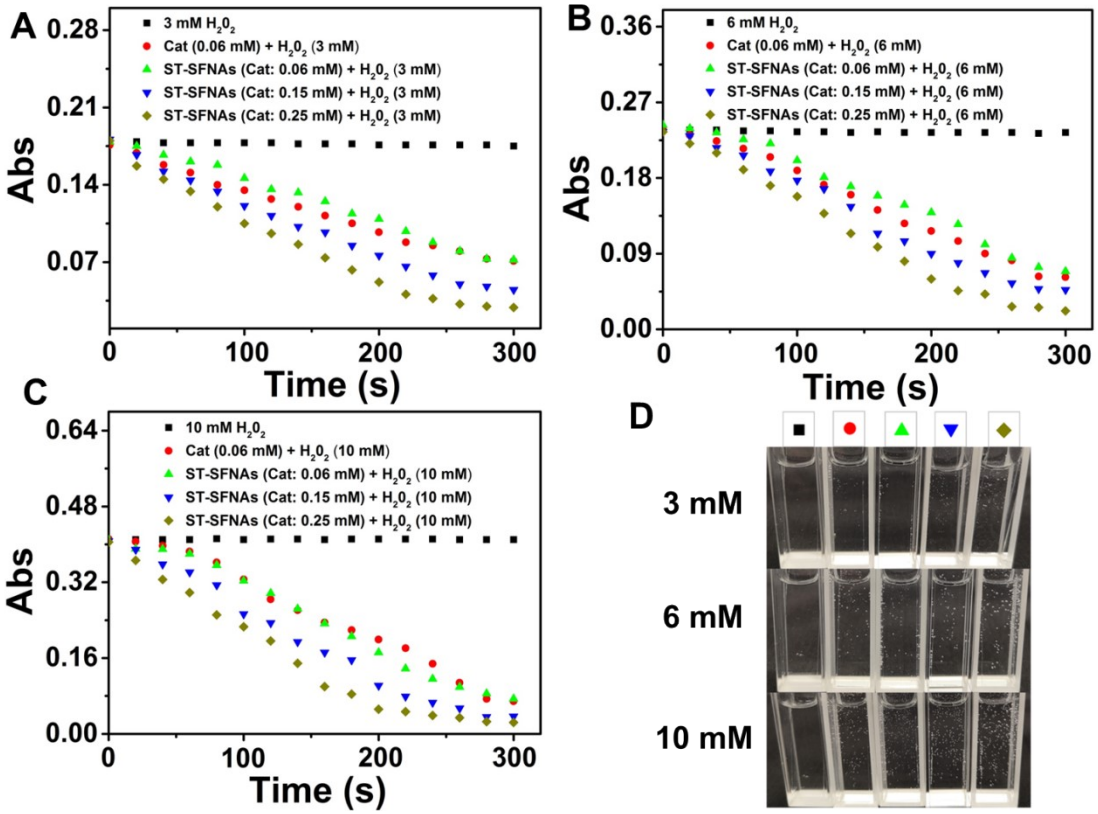

Figure S4. Evaluation of catalytic activity of ST-SFNAs at different concentrations of H_2O_2. (A) 3 mM, (B) 6 mM, (C) 10 mM. The absorbance of UV-vis spectra at 240 nm was recorded, corresponding to H_2O_2 substrate. (D) Optical photos during catalytic reactions.

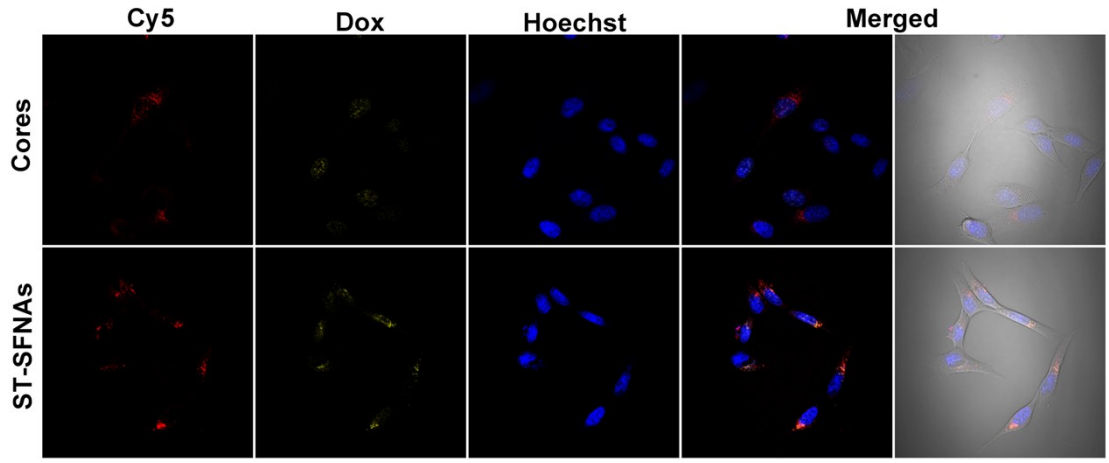

Figure S5. Confocal microscopy images of HeLa cells after the incubation with ST-SFNA cores and ST-SFNAs.

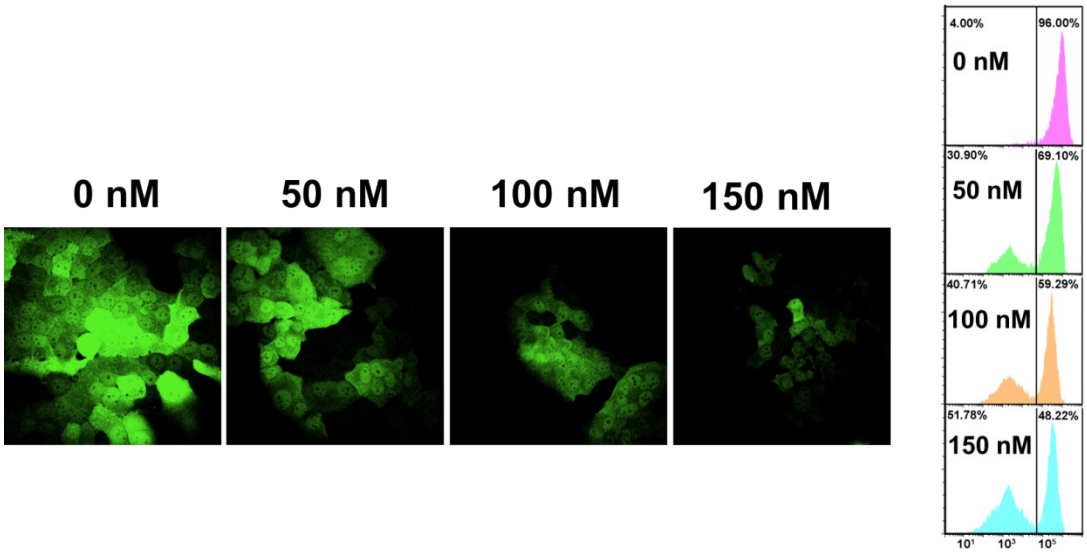

Figure S6. Fluorescence microscopy images and flow cytometric fluorescence intensity of the HeLa cells incubated by ST-SFNAs-siGFP at different doses.

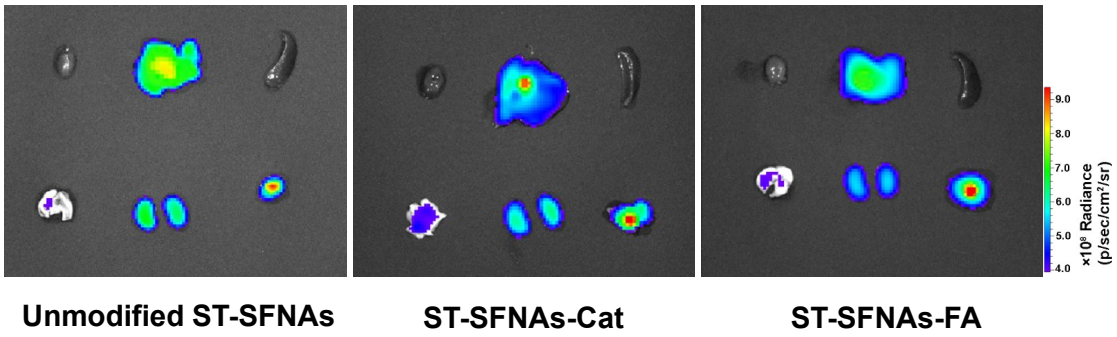

Figure S7. Fluorescent images of different tissues obtained from the mice, which treated with unmodified ST-SFNAs, ST-SFNAs-Cat, ST-SFNAs-FA.

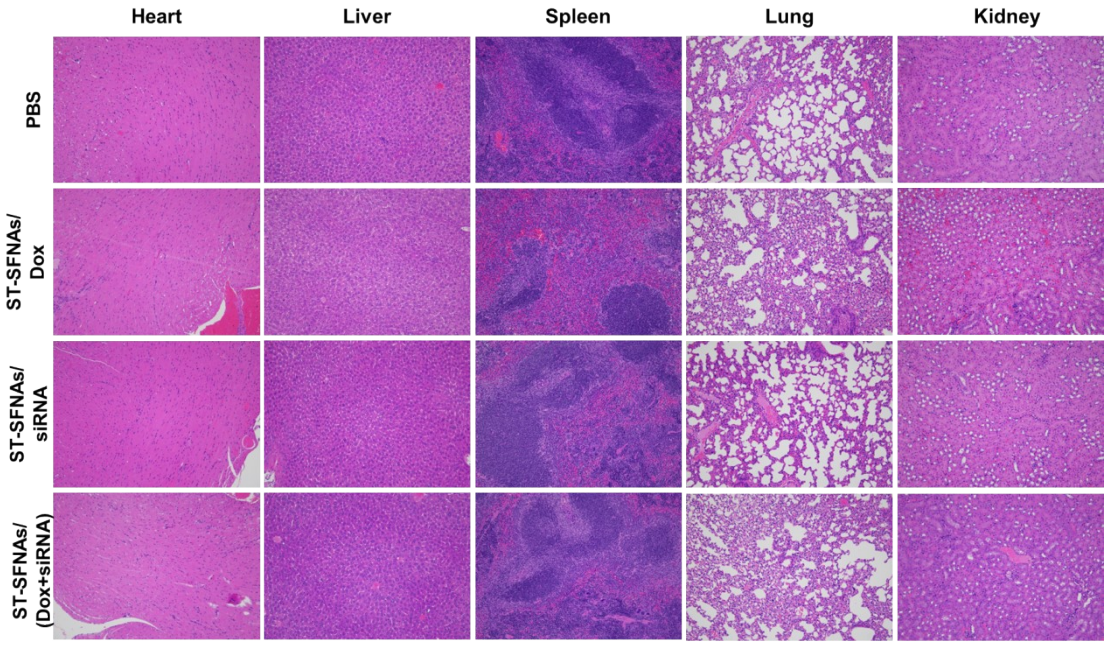

Figure S8. Images of heart, liver, spleen, lung, and kidney sections of HeLa tumor-bearing mice with indicated treatments after H&E staining.

Supplementary Table 1. DNA and RNA sequences used in this work.

Name	Sequence
H1	TTGAATGTAGAGATGCGGTGGTCCT/iCy5dT/GAGAAAGGGCTCTCAAGGACCACC GCAGTTTCC
H2	GCCCTTTC/iBHQ2dT/CAAGGACCACCGCAGGAAACTGCGGTGGTCCTTGAGACT TCACGGGTCTGCGGTGGTCCTTGAGA
H3	TTGAATGTAGAGATGCGGTGGTCCT/iCy5dT/GAGAAAGGGGCCTCTCAAGGACC ACCGCAGACCCG
H4	GGCCCCTTTC/iBHQ2dT/CAAGGACCACCGCACGGGTCTGCGGTGGTCCTTGAG AACCTTCCTCCGCAATACT
g-DNA	TGGAAGGAGGCGTTATGAGGGGGTCCATCTCTAC/iHS-SH/ATTCAATGGAAGGA GGCGTTATGAGGGGGTCCATCTCTAC/iHS-SH/ATTCAA
Survivin	CCCTTTCTCAAGGACCACCGCATCTCTACATTCAA
TK1	CAGTACAAGTGCCTGGTGATCAAGTATGCCAAAGA
GalNac-T	CTGCTTTCACTATCCGCATAAGACACCCGCCCAAC
C-myc	CCCCTCAACGTTAGCTTCACCAACAGGAACTATGA
Single mismatch	CCCTTTATCAAGGACCACCGCATCTCTACATTCAA
Three mismatches	CCCTTTATCAAGGACTACCGCATATCTACATTCAA
siPLK1 sense	UGAAGAAGAUCACCCUCCUUATT
siPLK1 antisense	UAAGGAGGGUGAUCUUCUUCATT-S-S-GGAAACTGCGGTGGTCCTTGAGA
siGFP sense	GGCUACGUCCAGGAGCGCATT
siGFP antisense	UGCGCUCCUGGACGUAGCCTT-S-S-GGAAACTGCGGTGGTCCTTGAGA