Supplementary Information

Multiplexed gene silencing in living cells and in vivo using a DNAzymes-CoOOH nanocomposite

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Experimental details

Materials. Cobaltous chloride hexahydrate ($CoCl_2 \cdot 6H_2O$) was purchased from Tianjin Guangfu Fine Chemical (Tianjin, China). N, N-Dimethylformamide (DMF), sodium hypochlorite (NaClO), sodium hydroxide (NaOH), sodium chloride (NaCl) and magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) were purchased from China National Pharmaceutical Group (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and L-glutathione reduced (GSH) were purchased from Sigma Aldrich (U. S.). 3-aminopropyltriethoxysilane (APTES) was purchased from Alfa Aesar (Tianjin, China). Deoxyribonuclease I (DNase I) was purchased from Solarbio (Beijing, China). All the reagents were analytical grade and used without further purification. Ultrapure water (18.2 MΩ·cm) was used throughout the experiments. DNA oligonucleotides were synthesized and purified by Takara Biotechnology (Dalian, China) and Sangon Biotechnology (Shanghai, China). The sequences of the DNA oligonucleotides are shown in Table S1. Anti-Thymidine Kinase 1 antibody, anti-Survivin antibody, anti-AKT1 antibody, anti-caspase-3 antibody and secondary antibody (Alexa Fluor 647) were purchased from Abcam (Germany). Beta Actin Polyclonal antibody was purchased from Proteintech (U. S.). The human breast cancer cell line MCF-7 was purchased from KeyGEN

Biotechnology (Nanjing, China), and the normal immortalized human mammary epithelial cells line MCF-10A were purchased from Shanghai BioLeaf Biotechnology (Shanghai, China).

Instruments. High resolution transmission electron microscopy (HRTEM) was carried out on a JEM-2100 electron microscope. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, China) with a combined glass-calomel electrode. Zeta potential was performed on a Malvern zeta sizer Nano-ZS90. UV-Vis absorption spectra were measured on a pharmaspec UV-1700 UV-visible spectrophotometer (Shimadzu, Japan). Fluorescence spectra were obtained with FLS-920 Edinburgh fluorescence spectrometer with a xenon lamp. The absorbance of formazan was measured with a microplate reader (RT 6000, Rayto, U. S.) in the MTT assay. RT-PCR was carried out with LineGene 9620 sequence detection system (Bioer, Hangzhou, China). Confocal fluorescence imaging studies were performed with TCS SP5 confocal laser scanning microscopy (Leica, Germany) and LSM 880 confocal laser scanning microscope (Zeiss, Germany) with an objective lens (×20). Nanodrop experiment was performed with a NanoDrop 2000 spectrophotometer (Thermo Scientific).

Synthesis of Amino-Functionalized Cobalt Oxyhydroxide Nanoflakes (COHN). Cobalt oxyhydroxide (CoOOH) were synthesized following previous method. [15] 100 μL of NaOH (0.8 M) and 100 μL of NaClO (0.1 M) were added to 100 μL of $CoCl_2$ (10 mM) solution, and then the mixture was sonicated for 1 min. After that, the solution was washed with water for three times and then dried in oven. The as-synthesized neutralized CoOOH (8 mg) nanosheets were dissolved in 4 mL anhydrous DMF, and 40 μL APTES was added to the mixture for 12 h at 80 °C under continuous mechanical stirring to initiate amino group. The product was washed sequentially with water and ethanol for four times, and finally dispersed in water and stored at room temperature. The amino groups were measured by ninhydrin: 1% of ninhydrin was added to 1 ml COHN or supernatant of COHN, and then brought to boiling for 30 min.

Preparation of DNAzyme-COHN Nanocomposite. The same molar ratios of three kinds of DNAzymes were mixed and added to COHN nanosheets in HEPES buffer

(20 mM, pH 7.2, containing 150 mM NaCl and 2 mM $MgCl_2$). The mixture was stirred at room temperature overnight and further purified by centrifugation at 13,000 rpm for 30 min. The product was dispersed in HEPES buffer, and finally saved in the fridge at 4 °C for the biological use.

***In Vitro* Cleavage Reactions.** Firstly, different concentration of nanocomposites with 10 μM AKT1 DNAzyme absorbed on the COHN (0 and 540 μg/mL) were prepared in Tris-HCl buffer (90 μL, 50 mM, pH 7.5, 150mM NaCl, 2mM $MgCl_2$, 0.01% SDS). Afterwards, GSH solution (5 mM) was added into the nanocomposites for 48h. Then the mixture was centrifuged to obtain the supernatant solution. After that, 2.5 μM RhB labeled AKT1 target was incubated with the supernatant solution at 37 ℃ for 1h. Finally, 44 μg/mL GO was added to the solution in Tris-HCl buffer (50 mM, pH 7.5, 150mM NaCl, 2mM $MgCl_2$, 20mM KCl), and the fluorescence intensities of RhB was measured.

Nuclease Stability of the Nanocomposite. Three groups of AKT1 (RhB)-COHN (10 μg/mL) were incubated at 37 °C. After allowing the samples to equilibrate for 10 min, with one group as control, the other two groups were added by DNase I (2 U/L) and RNase A (10 μg/mL) respectively. The fluorescence signals of the three groups were monitored for 6 h and collected at 1 h time interval during this period. Followed by adding GSH (10 mM) to each group, they were incubated for overnight at 37 °C, and the fluorescence intensities were obtained after the solution cooled down to room temperature. The experiment was repeated at least three times and the data are shown as mean ± SD.

Nanodrop Experiment. With 10 μg/mL of nanocomposite absorbed on different quantities of DNAzymes, the solution was mixed with GSH solution (1 mM) and then incubated under 37 °C for 48h. The resulting mixture was centrifuged at 13,000 rpm for 30 min and clear supernatant solution was collected. Nanodrop 2000 was applied to quantify the concentration of DNAzyme in the supernatant solution. Typically, the optical surfaces of the microvolume spectrophotometer sample retention system were cleaned by deionized water. Then the blank measurement is complete by dispensing 1

μL of buffer onto the lower optical surface. Followed with dispersion of 1 μL of the supernatant solution onto the lower optical pedestal, the software automatically calculates the nucleic acid concentration and purity ratios. Each experiment was repeated at least three times and the data are shown as the mean ± SD.

Cell Culture. MCF-7 cells and MCF-10A cells were incubated in Dulbecco's Modified Eagles medium (DMEM) media supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C in 5% CO_2 atmosphere.

Cellular Uptake of the Nanocomposites. The cellular uptake of the nanocomposites using the MCF-7 cells was studied, and inductively coupled plasma atomic emission spectroscopy (ICP-AES) was applied to analyzed cobalt contents in MCF-7 cells. Three groups of MCF-7 cells (seeded at 1×10^5/mL) were incubated with 10, 20, 40 μg/mL COHN for 6 h. Then the cells were washed with PBS buffer to remove the nanoparticles that were not uptaken into the cells. After that, 3 mL fresh DMEM culture medium was added, and the cells were further cultured for 48 h. At the end of incubation, the cells were washed with PBS buffer and collected in centrifuge tubes. For the next step, the cells were treated with nitric acid (2 mL, 15.2 mol/L), perchloric acid (1 mL, 12.38 mol/L) and hydrofluoric acid (2 mL, 33.3 mol/L), and then heated to dissolve the COHN. The samples were finally analyzed for total Co content by ICP-AES (Thermo, IRIS Advantage, 228.62 nm) and the measurement was repeated three times.

The Reduction of COHN in Living cells. ICP-AES experiments were conducted to investigate whether COHN can be reduced by GSH in living cells. Two groups of MCF-7 cells were incubated with PBS and 40 μg/mL COHN for 48 h, respectively, and another group of 40 μg/mL COHN was incubated in fresh culture medium for 48 h. Then unreacted COHN in cells lysate and fresh culture medium was removed by centrifugation. After that, the supernatant of cell lysate and fresh culture medium were treated with nitric acid (2 mL, 15.2 mol/L), perchloric acid (1 mL, 12.38 mol/L) and hydrofluoric acid (2 mL, 33.3 mol/L), followed by heating to dissolve the COHN. The samples were finally analyzed for total Co content.

Confocal Fluorescence Imaging. MCF-7 and MCF-10A cells were seeded in confocal dishes and incubated for 24 h. AKT1 (RhB) (0.9 μM) and AKT1-COHN (30 μg/mL) were incubated with MCF-7 and MCF-10A cells in DMEM respectively. After 6 h incubation, cells were washed twice with 1 mL of PBS and subjected to CLSM imaging.

Cell Proliferation Assays. (1) MCF-7 cells were seeded in a 96-well plate and further cultured for 24 h. After removing the culture medium, cells were incubated with different concentrations of control DNA (0.9 μM), control DNA-COHN (30 μg/mL), DNAzyme (0.9 μM), SUR-COHN (30 μg/mL), AKT1-COHN (30 μg/mL), TK1-COHN (30 μg/mL), and DNAzymes-COHN (30 μg/mL) nanosystem at 37 °C for 6 h. Then, the cells were replaced with 200 μL of fresh medium and further cultured for 48 h. The cells without any treatment as control group were incubated for 48 h at 37 °C. In addition, 200 μL MTT solutions (0.5 mg/mL in PBS) were added to each well and incubated for 4 h. The formazan crystals formed by viable cells were solubilized in 200 μL dimethylsulfoxide and then the absorbance value was measured at 490 nm with microplate reader. (2) To evaluate the cytotoxicity of different samples as described above for normal cells, MTT assays in MCF-10A cells with each sample were performed for 6 h at 37 °C, respectively. Then, the cells were replaced with 200 μL of fresh medium and further cultured for 48 h. After 48 h incubation, MTT assays were carried out as the same procedure above. (3) In order to evaluate the cytotoxicity of COHN for normal cells, MTT assays in MCF-10A cells with different COHN concentrations (0, 1, 5, 10, 20, and 40 μg/mL) were performed respectively. After 48 h incubation, MTT assays were carried out as the same procedure above.

Immunofluorescence Assays. MCF-7 cells were seeded in confocal dishes and incubated for 24 h. After removing the culture medium, cells were incubated with DNAzymes-COHN (30 μg/mL) at 37 °C. With 6 h more incubation, the culture medium was replaced by another 2 mL fresh medium and the cells were cultured for 48 h. Then, the cells were fixed in 4% paraformaldehyde for 10 min at room temperature. By twice washing with PBS buffer, the cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min. Primary antibodies (Caspase-3 antibody) were

diluted 50 times in PBS and used to stain cells for 1 h. The cells were then washed with PBS, incubated with secondary antibodies conjugated with Alexa Fluor 647 (1:200) for 1 h at room temperature. At last, the cells were treated with Hoechst 33342 to visualize the nuclei. Subsequently, the cells were washed with ice-cold PBS for three times and imaged under CLSM.

Gene Expression Analysis by RT–PCR. MCF-7 cells were applied to perform RT-PCR. The cells were divided into two groups: one with no treatment while the other was incubated with different concentrations of control DNA, control DNA-COHN, DNAzyme, and DNAzymes-COHN nanosystem at 37 °C for 6 h. Then RT-PCR was performed 48 h later. The following primers were used:

TK1, Forward 5'-TATGCCAAAGACACTCGCTAC-3'

Reverse 5'-GCAGAACTCCACGATGTCAG-3'

AKT1, Forward 5'-TCTATGGCGCTGAGATTGTG-3'

Reverse 5'-CTTAATGTGCCCGTCCTTGT-3'

SUR, Forward 5'-TCCACTGCCCCACTGAGAAC-3'

Reverse 5'-TGGCTCCCAGCCTTCCA-3'

GAPDH, Forward 5'-GGGAAACTGTGGCGTGAT-3'

Reverse 5'-GAGTGGGTGTCGCTGTTGA-3'

Western Blot Analysis. MCF-7 cells were seeded in a 6-well plate and incubated for 24 h. Then the cells were incubated with control DNA, control DNA-COHN, DNAzyme, and DNAzymes-COHN nanosystem at 37 °C for 6 h. After 48 h, cells were washed with PBS three times. Subsequent steps were performed at 4 °C. Cells were lysed in 150 μL of lysis buffer (Beyotime Biotechnology), followed by centrifugation at 14000 rpm for 5 min. The protein content of the supernatants was determined using Enhanced BCA Protein Assay Kit (Beyotime Biotechnology). Cellular proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking in 5% skim milk, membranes were incubated with the primary antibodies diluted 5000 times at 4 °C overnight, followed by a HRP-conjugated goat anti-rabbit immunoglobulin G (IgG) (Hangzhou HuaAn, China) diluted 2500 times for 1 h. After

that, the protein bands were performed using the enhanced chemiluminescence solution reaction.

Cell Migration (Wound-Healing) and Invasion Assay. MCF-7 cells were seeded in a 6-well plate and incubated for 24 h. Then the cells were incubated with SUR-COHN, AKT1-COHN, TK1-COHN, and DNAzymes-COHN nanocomposites at 37 °C. The cells were wounded by dragging a 200 μL pipette tip through the monolayer after 6 h. By washing with PBS, cellular debris was removed and images were taken at time 0, 12, 24 and 48 h post-wounding. Cell invasion assays were conducted using Matrigel-coated invasion chambers.

Animal Studies. All animal experiments were carried out and following the Principles of Laboratory Animal Care (People's Republic of China). Specific pathogen-free (SPF) female BALB/c nude mice were used in accordance with the guidelines of the principles of the Animal Investigation Committee approved by Biology Institute of Shandong Academy of Science, China. Murine tumor xenograft models were generated by the subcutaneous injection of 1×10^6 MCF-7 cells in PBS (150 μl) into the flank of female nude mice (4-6 weeks old, ~20 g).

For gene silencing in vivo, when the tumor volume reached 80-100 mm^3, the mice (n ≥5 per group) were injected with PBS (40 μL) only, control DNA-COHN (0.75 mg/mL, 40 μL), DNAzymes-COHN (0.75 mg/mL, 40 μL) three times at day 1, 3 and 5. The growing tumors were measured with calipers every two days and up to 14 days. The tumor volume was calculated using the equation volume = (length × $width^2$)/2 and the relative tumor volume = V/V_0 (V_0 was the original tumor volume). The weight was also monitored closely. For histological analysis, mice were euthanized at the end of the treatments. Tumors and major organs were harvested for routine staining with haematoxylin and eosin (H&E).

Supplementary Table:

Table S1. The sequence of the DNA oligonucleotides

Oligonucleotide	Sequence (from 5' to 3')
TK1 DNAzyme	CCTATGAGGCTAGCTACAACGAAGCCACG
SUR DNAzyme	GAAGAAAGGCTAGCTACAACGAACTGGGC
AKT1 DNAzyme	CCCGGTAGGCTAGCTACAACGAACCACGT
RhB-AKT1 DNAzyme	RhB-CCCGGTAGGCTAGCTACAACGAACCACGT
TK1 target	CGTGGCTrGrUCATAGG
SUR target	GCCCAGTrGrUTTCTTC
AKT1 target	ACGTGGTrGrUACCGGG
RhB-AKT1 target	RhB-ACGTGGTrGrUACCGGG
Control TK1 DNA	CCTATGAGGCTACCTACAACGAAGCCACG
Control SUR DNA	GAAGAAAGGCTACCTACAACGAACTGGGC
Control AKT1 DNA	CCCGGTAGGCTACCTACAACGAACCACGT

[a] Underlined letters represent the catalytic domain; [b] Letters in red represent the mismatched site.

Supplementary Figures:

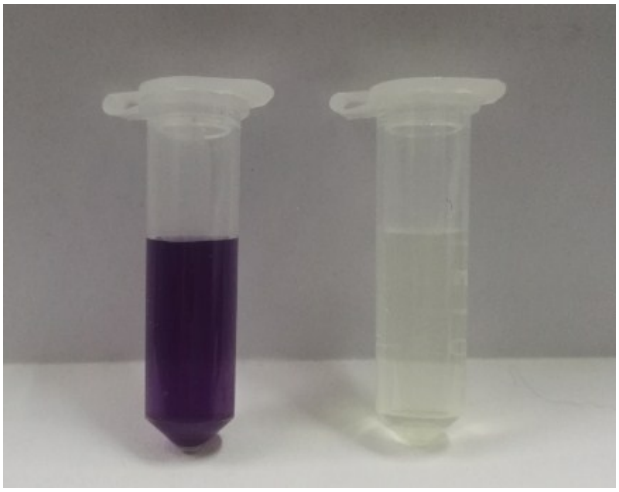

Fig. S1 Amino detection: COHN solution (left) and the supernatant of COHN solution (right) with ninhydrin.

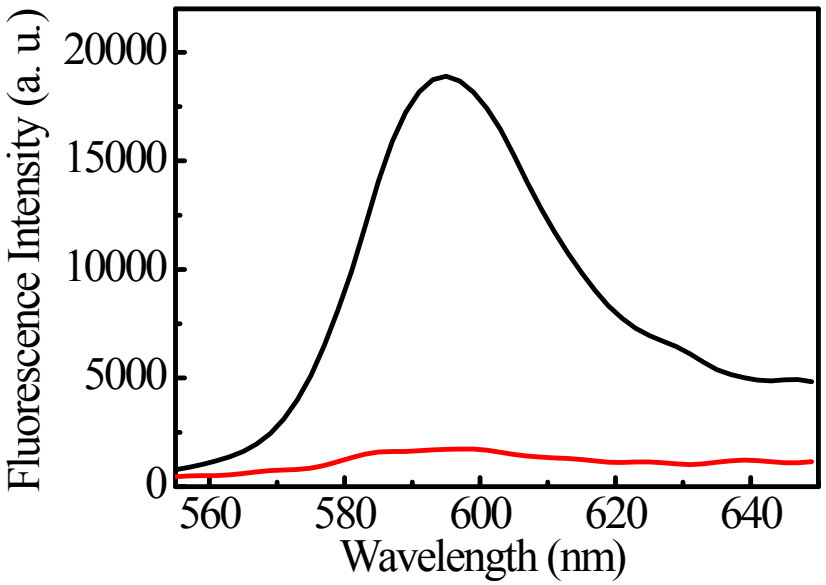

Fig. S2 Fluorescence spectra of the DNAzyme (black curve) and DNAzyme-COHN (red curve) in the presence of GSH, RhB labeled target and GO.

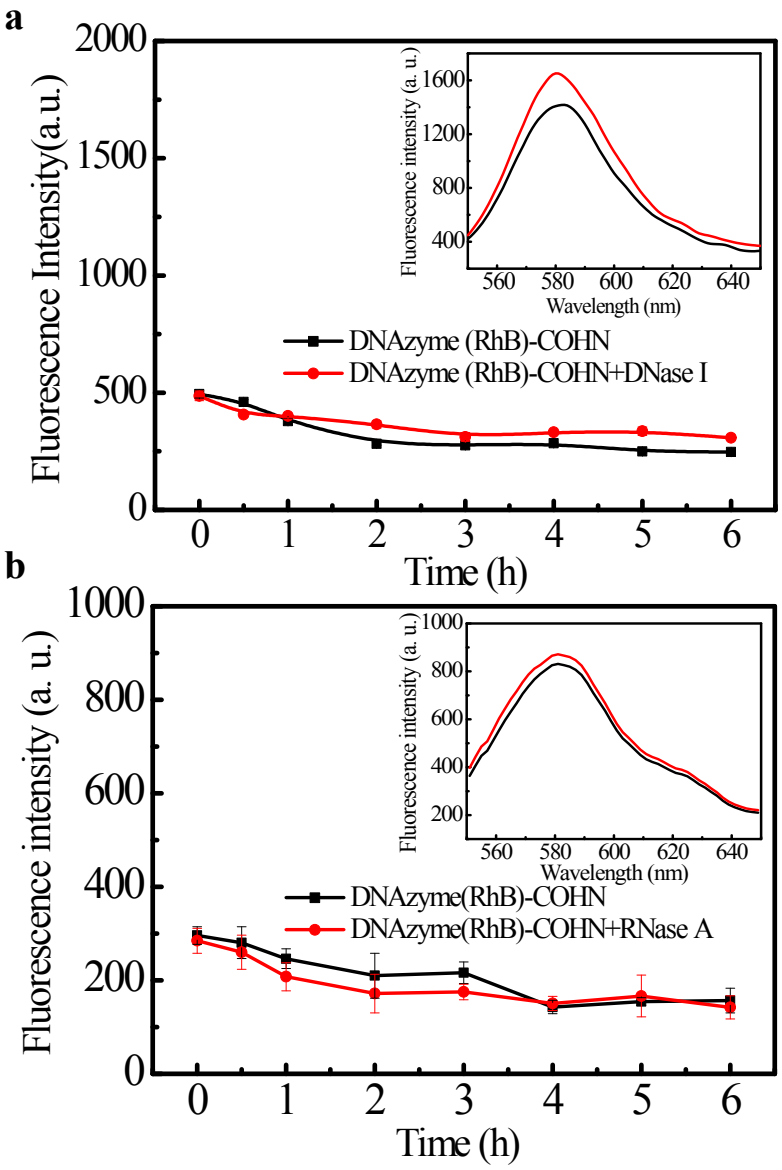

Fig S3 Nuclease stability of the AKT1 (RhB)-COHN nanocomposite for (a) DNase I or (b) RNase A. Fluorescence curve of the nanocomposite (10 μg/mL) without DNase I/RNase A (black) or in the presence of DNase I/RNase A (red) as a function of time. Inset: fluorescence spectra after adding GSH (10 mM) in the presence of DNase I/RNase A (red) and absence of DNase I/RNase A (black). The excitation wavelength was 532 nm.

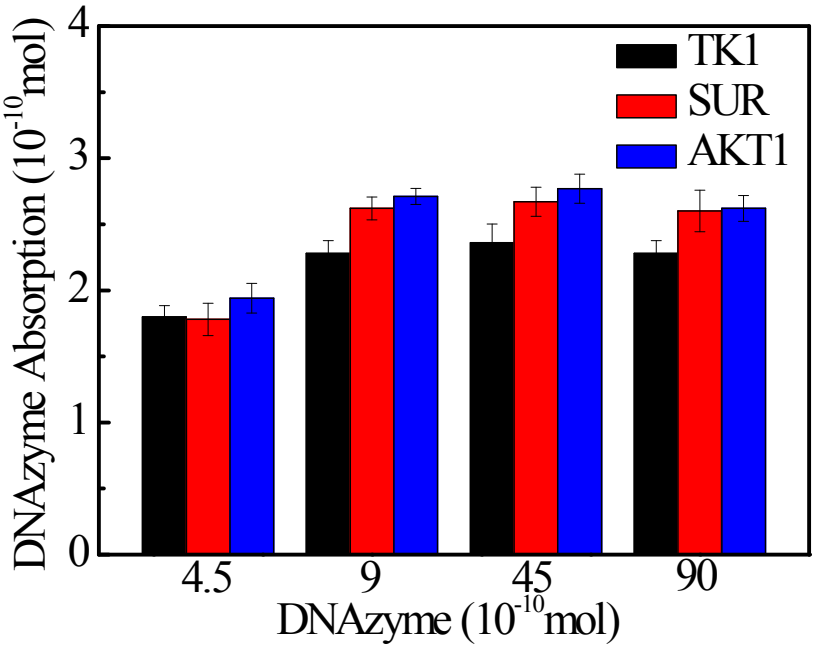

Fig S4 Nanodrop experiment for quantifing of the DNAzyme absorption on 10 μg/mL COHN with different concentration of DNAzyme.

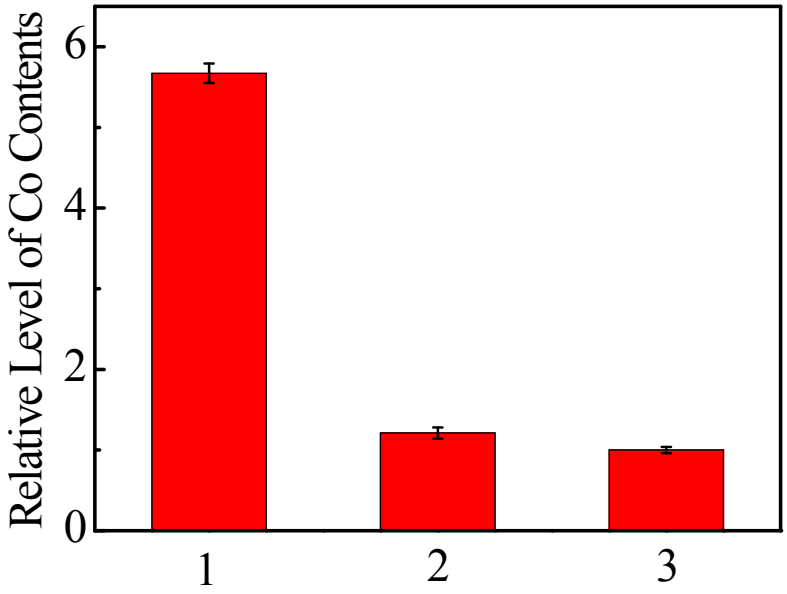

Fig. S5 ICP-AES analysis of Co content in 1: MCF-7 cells treated with COHN; 2: fresh culture medium treated with COHN; 3: MCF-7 cells treated with PBS.

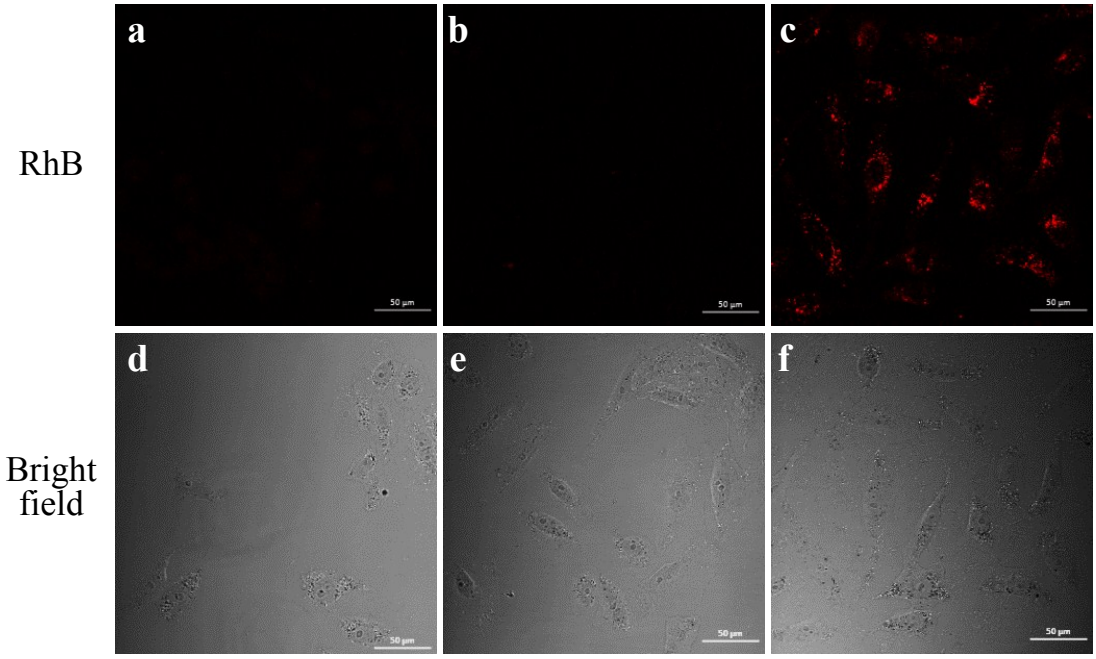

Fig. S6 Confocal fluorescence images of MCF-7 cells without treatment (left) and with treatment of AKT1 (RhB)-DNAzyme (middle) and AKT1 (RhB)-COHN nanocomposite (right). Scale bars are 50 μm.

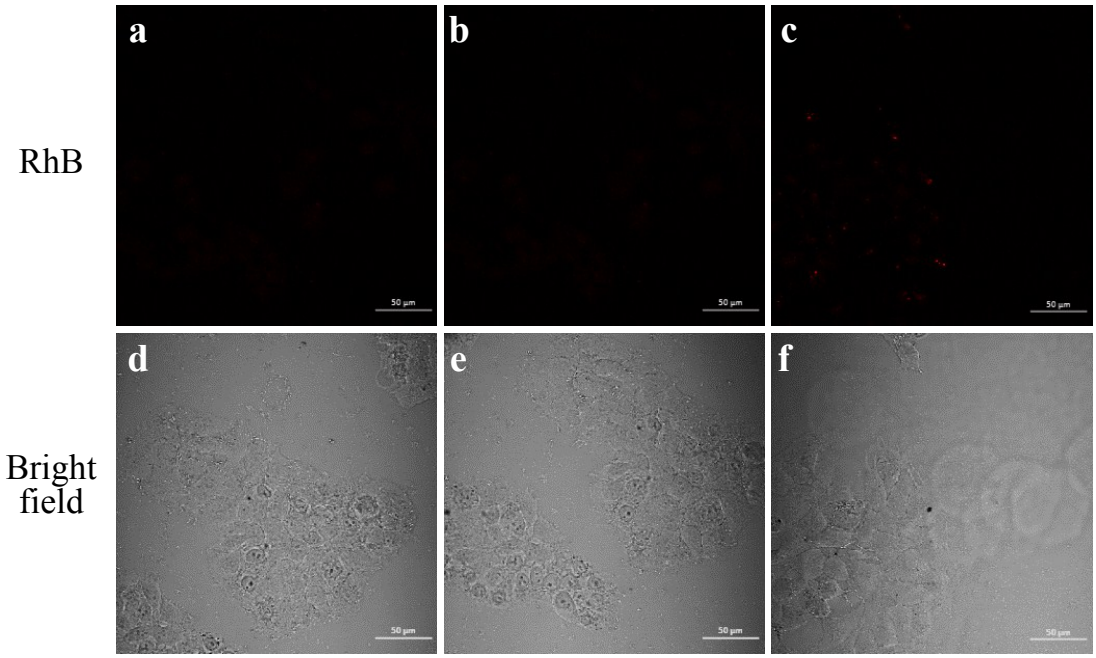

Fig. S7 Confocal fluorescence images of MCF-10A cells without treatment (left) and with treatment of DNAzyme (middle) and AKT1 (RhB)-COHN nanocomposite (right). Scale bars are 50 μm.

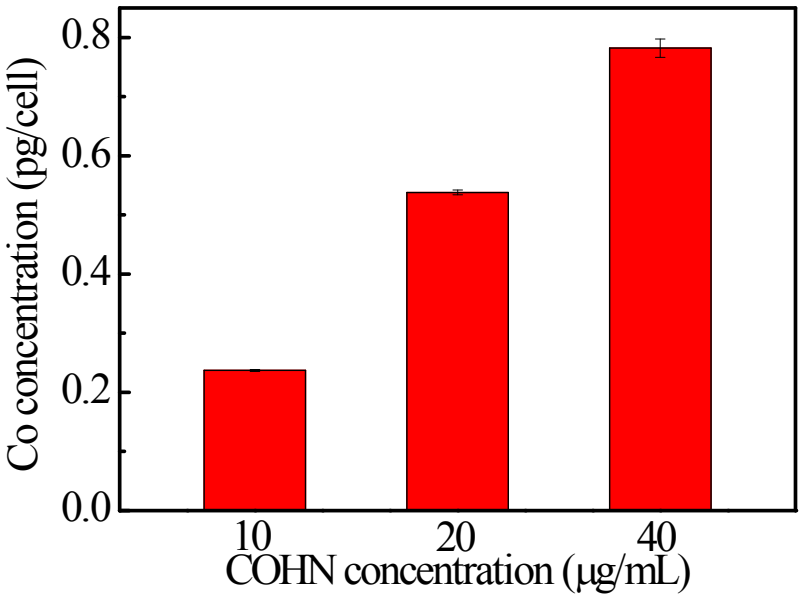

Fig. S8 ICP-AES analysis of Co content in the cells with different incubation concentrations.

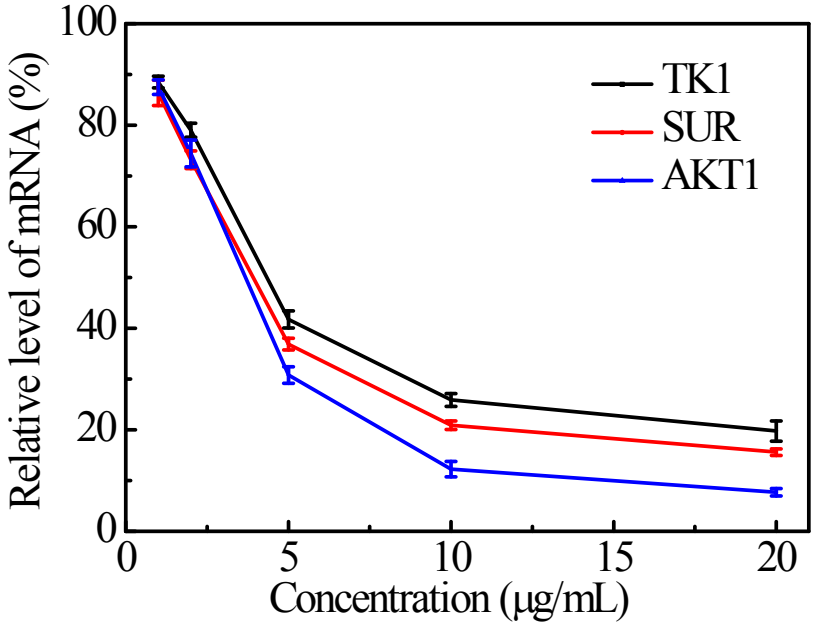

Fig. S9 TK1, SUR and AKT1 mRNAs expression in MCF-7 cells treated with different concentrations of nanocomposite for two days.

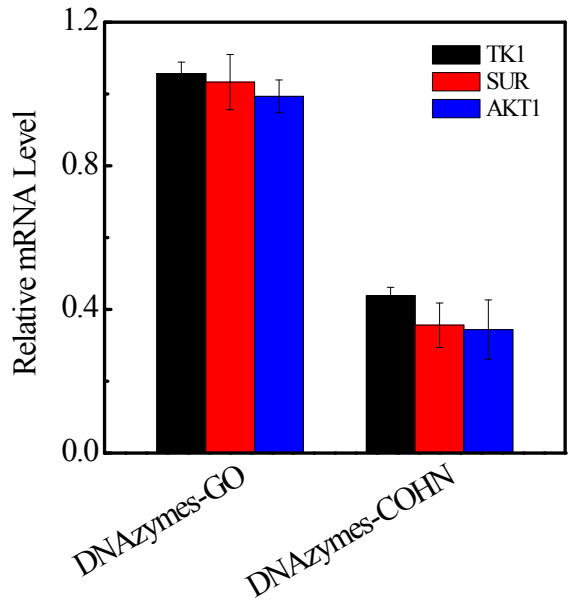

Fig. S10 TK1, SUR and AKT1 mRNAs expression in MCF-7 cells treated with DNAzymes-GO and DNAzymes-COHN for two days.

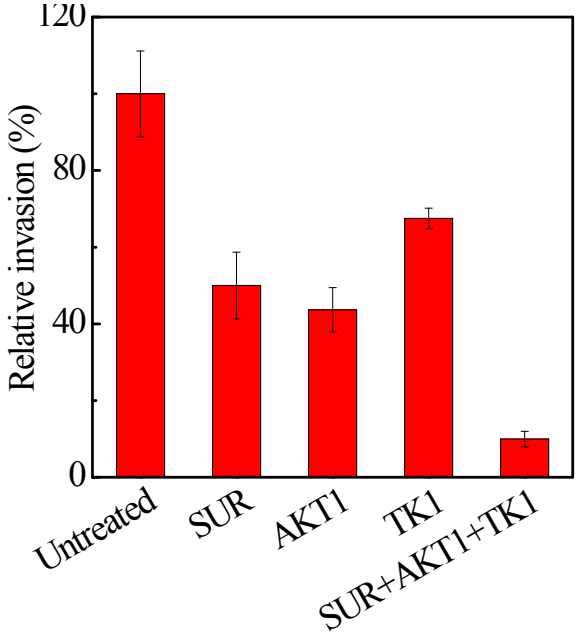

Fig. S11 Quantification of the chamber invasion assay to show relative invasion, and untreated groups were used as the control.

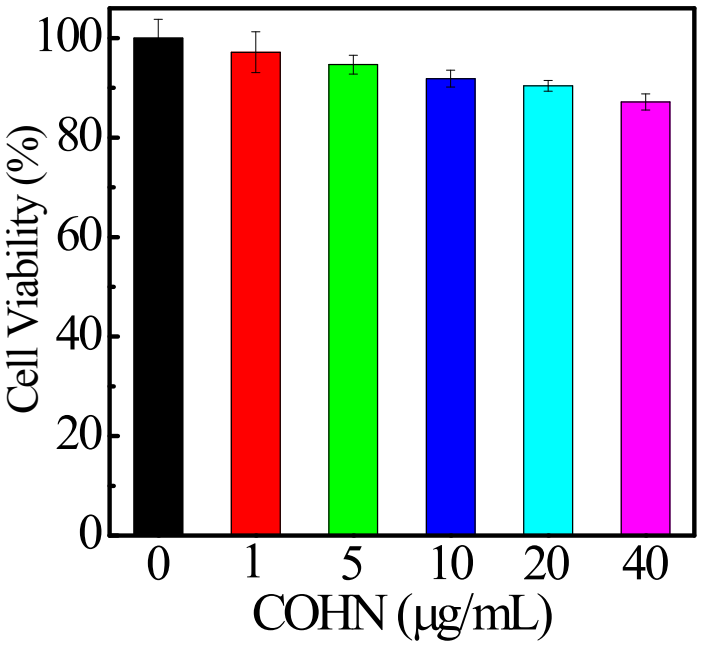

Fig. S12 Cytotoxicity of COHN incubated with MCF-10A cells for 48 h at different concentrations as indicated.

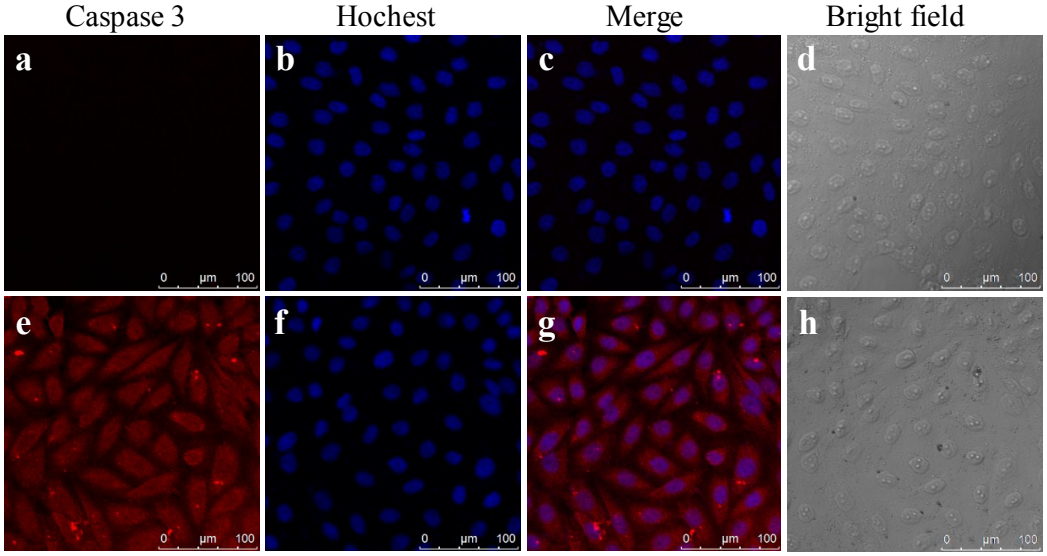

Fig. S13 Confocal microscope images of fluorescently stained active caspase-3 in MCF-7 cells. Active caspase-3 are immunostained with Alexa-647-labelled secondary antibodies (red) and nuclei are stained with Hochest 33342 (blue). MCF-7 cells without any treatment (top) and cells were treated with DNAzymes-COHN (bottom).

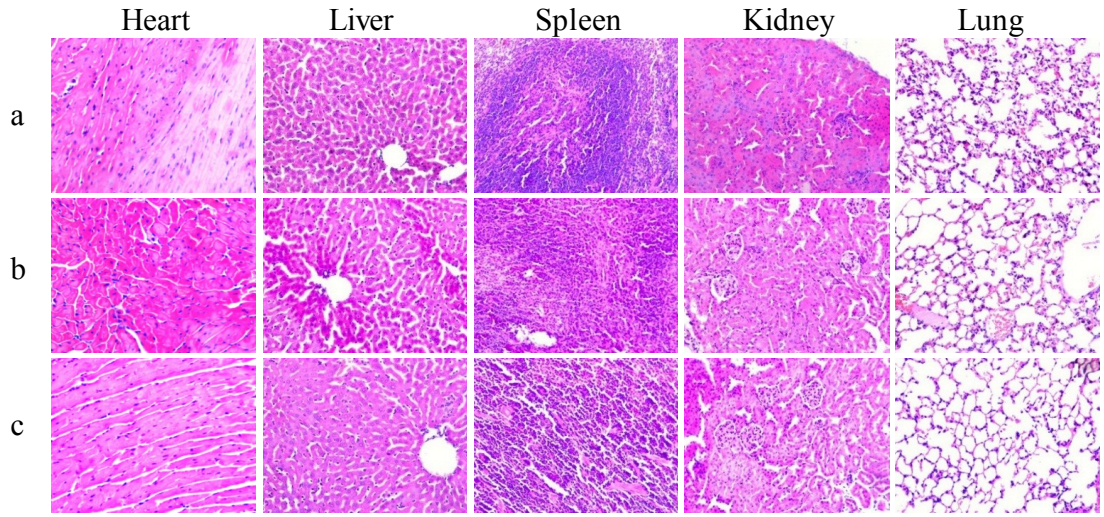

Fig. S14 H&E staining images of five major organs (heart, liver, spleen, kidney, and lung) in MCF-7 tumor-bearing mice at 7 day after different treatment groups: control (a), control DNA-COHN (b) or DNAzymes-COHN (c) at the tumor region.