Supporting Information

Photoacoustic and fluorescence dual-mode imaging of tumor-related mRNA with a covalent linkage-based DNA nanoprobe

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EXPERIMENTAL SECTION

Materials and Instruments. Carboxyl Graphene oxide (GO) was acquired from Nanjing XFNANO Materials Tech. Co., Ltd. (Nanjing, China). Glutathione were acquired from China National Pharmaceutical Group Corp. (Shanghai, China). 1-Ethyl-3-(3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Alfa Aesar Chemical Ltd. (Tianjin, China). Tamoxifen, β-Estradiol, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. Cell culture products were acquired from Hyclone. All of the DNA oligonucleotides were synthesized were synthesized and HPLC-purified by Sangon Biotechnology Co. Ltd. (Shanghai, China). Detailed DNA sequences and modifications are shown in Table S1. The human breast cancer cell line MCF-7, the normal immortalized human mammary epithelial cell line MCF-10A, human hepatocellular liver carcinoma cell line HepG2 and human hepatocyte cell line HL-7702 were obtained from Procell Life Science Co., Ltd.

High resolution transmission electron microscopys (HRTEM) was taken on a JEM-

2100 electron microscope. Atomic force microscopy (AFM) was carried out Dimension Icon (Bruker Inc.). Fluorescence spectra measurements were performed Fluorescence Spectrometer (Edinburgh, FLS-920). RT-PCR was taken on a LineGene 9620 (Bioer, Binjiang, China). MTT assay was measured on a microplate reader (Synergy 2, Biotek, USA). Fluorescence imaging in cells were performed with Leica TCS SP8 Confocal Laser Scanning Microscope. Flow cytometry was performed with ImageStreamX Mark Ⅱ Imaging Flow Cytometer (Merck Millipore, USA). Photoacoustic imaging was accomplished using an Endra Nexus 128 (Ann Arbor, Michigan).

Preparation of the nanoprobe. Firstly, EDC solution (1.5 mM) was added to 1 mL of carboxyl GO solution (PBS: 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) for 30 min at 0 °C to activate carboxylate groups. Then, 20 μL of DNA-NH_2 (25 μM) was added into the above mixture and stired overnight at room temperature. The resulting precipitates were centrifuged (10000 rpm, 5 min) and resuspended in 1 mL PBS solution. Subsequently, TK1 recognition segments (DNA-Cy5.5) were mixed with the above simple for 30 min at 37 °C in darkness. After the reaction, the product was collected and washed several times with PBS. To remove the physisorbed DNA-Cy5.5 on the GO surface, auxiliary DNA was further added to the above product solution for 30 min. Then, the nanoprobe was obtained by centrifuged (10000 rpm, 5 min) and washed several times with PBS. After resuspended in PBS, the nanoprobe was stored at 4 °C in darkness. For a comparison study, the control probe was synthesized under the same procedure but using the non recognition segment instead of TK1 recognition segment.

The response of the nanoprobe. For detection of TK1 mRNA, various concentrations of TK1 targets were added to the nanoprobe solution (25 μg/mL), and the fluorescence and photoacoustic measurements were carried out after 30 min. Unless otherwise noted, the fluorescence intensities were measured at $\lambda_{ex}/\lambda_{em}$ = 675/700 nm and the photoacoustic signals were exited at 690 nm. In addition, **t**he nanoprobe incubated with the target for different times were examined for studying the kinetics. GalNAc-T target (200 nM) and c-myc (200 nM) were also investigated to study the selectivity of the

nanoprobe.

The physisorption probe was prepared by directly adding the DNA-Cy5.5 into the GO solution. To optimize the concentration of GO, DNA-Cy5.5 were mixed with different concentrations of GO (4, 12, 16, 25, 36, 52, 70, 90 μg/mL) in PBS (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4).

For study the stability, the nanoprobe and physisorption probe were dispersed in Dulbecco's modified Eagles medium (DMEM), RPMI-1640 medium, DMEM + 10% fetal bovine serum (FBS), and RPMI-1640 medium + 10% FBS solutions. Furthermore, the fluorescence and photoacoustic intensities of the nanoprobe and physisorption probe in the presence of BSA (50 μg/mL) were studied, respectively.

To measure the nuclease stability, the nanoprobe in buffer solution (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) was divided into two groups. DNase I (2U/L) was added to one group. Another group without DNase I was used as the control. After the targets (200 nM) were added to the two groups for 0.5 h, the fluorescence and photoacoustic measurements were performed.

Cell Culture. MCF-7, MCF-10A and HepG2 were cultured in Dulbecco's modified Eagles medium (DMEM). HL-7702 cells were cultured in RPMI-1640 medium. All the culture medium were supplemented with 10% fetal bovine serum (FBS) and 100 U/mL of 1% antibiotics penicillin/streptomycin.

Cytotoxicity of the Nanoprobe. HepG2 and HL-7702 were chose to evaluate the cytotoxicity of the nanoprobe. After the cells seeded in a 96-well microplate, various concentrations of nanoprobe (25, 50, 75, 100, 125, 150 μg/mL) were dispersed in the culture medium. After incubation for 24 h, the culture medium was replaced and the cells were treated with the 150 μL of MTT solution (0.5 mg/mL). The MTT solution was removed after 4 h. Then, DMSO (150 μL) was added to each well, the absorbance was measured on RT 6000 microplate reader at 490 nm. To further investigate the cytotoxicity of the nanoprobe in vivo, the mice were intravenously injected with the nanoprobe solution (50 μL). Five major organs (heart, liver, spleen, lung, and kidney) were collected after 3 days for H&E staining experiments.

Confocal Fluorescence Image Assay and Flow Cytometry. For confocal

fluorescence imaging, MCF-7, MCF-10A, HepG2 and HL-7702 cells were incubated with nanoprobe (25 g/mL) for 6 h at 37 °C. After culture, the cells were washed three times with PBS and examined by confocal laser scanning microscopy (CLSM). Confocal fluorescence imaging was excited at 633 nm and collected at 650−750 nm. Prior to flow cytometry imaging, all the cells were cultured in cell dishes for 24 h. Then, 1 mL of the nanoprobe (25 μg/mL) in medium was added into cell dishes respectively. The culture medium was removed after 6 h. Then, all the cells were digested with 1 mL trypsin and washed several times with PBS. Finally, all the cells were analyzed on a flow cytometer.

Reverse transcription-PCR (RT-PCR). Total cellular RNA was extracted from the cell line using RNA simple Total RNA Kit (Tiangen) according to the manufacturer's instructions. cDNA was synthesized by using the FastQuant RT Kit (Tiangen). RT-PCR analysis was performed with SuperReal PreMix Plus (Tiangen) on LineGene 9620. Relative level of mRNA was calculated from the quantity of mRNA PCR products and the quantity of GAPDH PCR products.

Animal tumor xenograft models and photoacoustic imaging. All animal experiments were conducted in accorded with the Principles of Laboratory Animal Care (People's Republic of China). For tumor models establishment, 1×10^7 MCF-7 and HepG2 cells in PBS were injected subcutaneously into the flank of female nude mice. For the subcutaneous xenograft mouse models, 50 μL of nanoprobe (1 mg/mL) were intravenously injected into mice. The groups of mice injected with the same amount of control probe were chosen as negative controls. Photoacoustic imaging was performed at 8 h post-injection. For detecting the changes of the relative expression levels of mRNA *in vivo*, the mice were randomly divided into three groups. One groups was treated with of β-estradiol (2 mg/kg) and the other group was treated with tamoxifen (20 mg/kg). One group without treatment was served as control. The photoacoustic images of the tumor sites were captured at 690 nm at 8 h post-injection. Photoacoustic intensities were quantified using OsiriX imaging software (OsiriX Foundation, Switzerland).

Table

Table S1. DNA sequences employed in this work.

Oligonucleotide	**Sequence (5'-3')**
DNA-NH_2	GCCAAAGACACTCGCTAAGCTAAAA-NH_2
TK1 recognition segment (DNA-Cy5.5)	Cy5.5-GCGAGTGTCTTTGGCATACTTGATCA
non recognition segment	Cy5.5-GCGAGTGTCTTTGGCTATCAAGTACT
Auxiliary DNA segment	GCCAAAGACACTCGC
TK1 target	TGATCAAGTATGCCAAAGACACTCGC
GalNAc-T target	GCTTTCACTATCCGCATAAGA
c-myc target	CCTCAACGTTAGCTTCACCAA

Figures

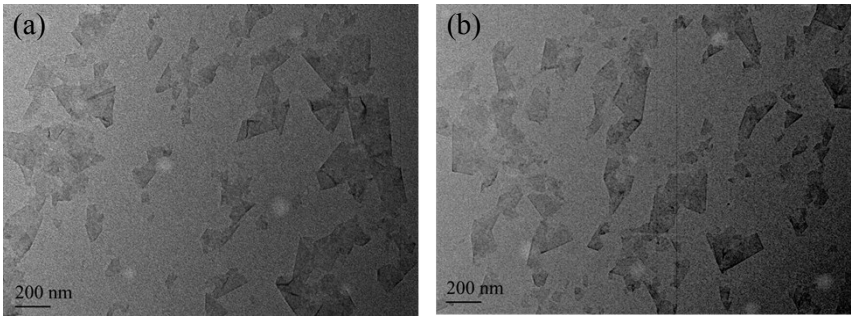

Fig. S1 HRTEM images of GO (a) and nanoprobe (b).

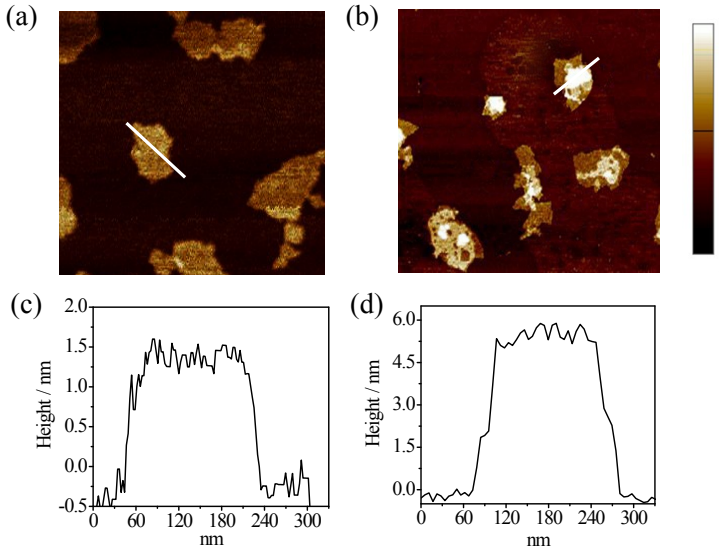

Fig. S2 AFM images of GO (a) and nanoprobe (b); (c) and (d) represent the height profiles of the regions labeled with the white line in (a) and (b), respectively.

Optimization of the concentration of auxiliary DNA

After DNA-NH_2 covalently linked with the carboxyl GO surface via the amido bond formation, TK1 recognition segment was added to allow hybridization. Since a portion of the recognition segment was inevitably adsorbed onto GO, various concentrations of the auxiliary DNA sequences that only hybridized with adsorption recognition probe were added to release the adsorption recognition segment from GO. When the amount of auxiliary DNA reached to 4 μM, the enhanced fluorescence intensity of the supernatant reached the maximum value (Fig. S3). Therefore, 4 μM of auxiliary DNA was used to fabricate the nanoprobe.

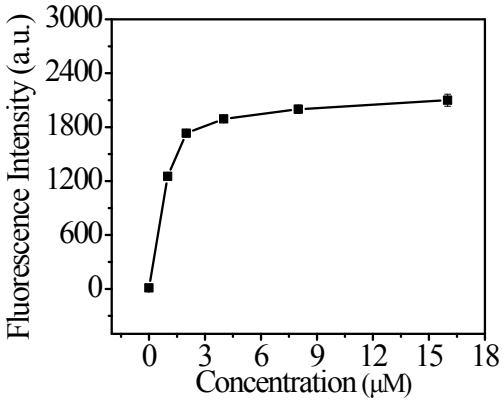

Fig. S3 Optimizing the concentration of auxiliary DNA segment. The excitation and emission wavelengths were 675 and 700 nm, respectively.

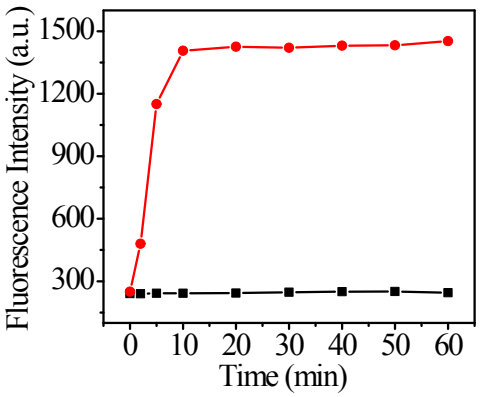

Fig. S4 Kinetic of the nanoprobe. The fluorescence intensity of the nanoprobe (black curve) and in the presence of target (red curve) for different times, the concentration of the target is 400 nM.

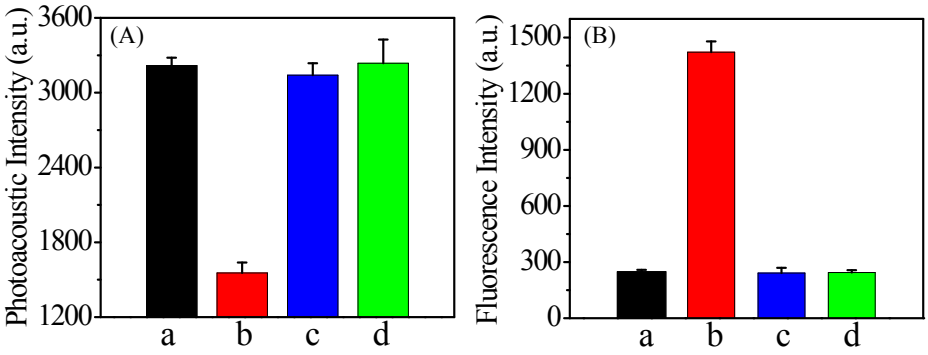

Fig. S5 The photoacoustic (A) and fluorescence intensity (B) of the nanoprobe in the presence of various interferents. (a) Control, (b) TK1 target, (c) GalNAc-T target, (d) c-myc target.

Preparation of the physisorption probe

The physisorption probe was prepared by directly adding DNA-Cy5.5 to the GO solution. The fluorescence intensity of DNA-Cy5.5 decreased gradually with the increasing concentration of GO. When the concentration of GO reached to 25 μg/mL, the fluorescence intensity of DNA-Cy5.5 was completely quenched (Fig. S6). Thus, 25 μg/mL was used to prepare GO-DNA-Cy5.5 complex.

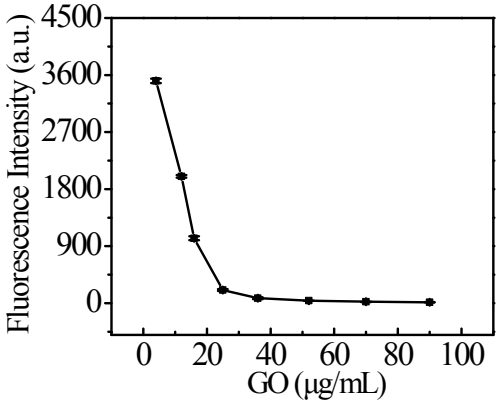

Fig. S6 Fluorescence quenching of the DNA-Cy5.5 at various concentrations of GO.

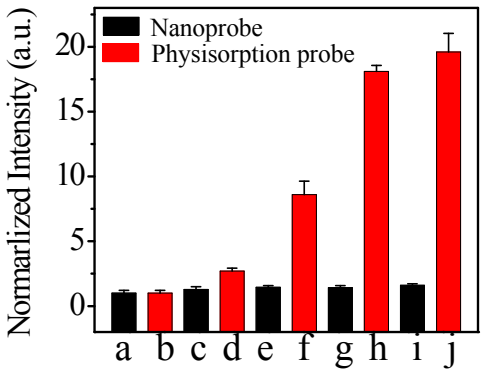

Fig. S7 The normalized intensity of the nanoprobe (black bars) and physisorption probe (red bars) under different conditions. (a, b) buffer solution, (c, d) Dulbecco's modified Eagles medium (DMEM), (e, f) RPMI-1640, (g, h) DMEM + 10% fetal bovine serum (FBS), and (i, j) RPMI-1640 + 10% FBS.

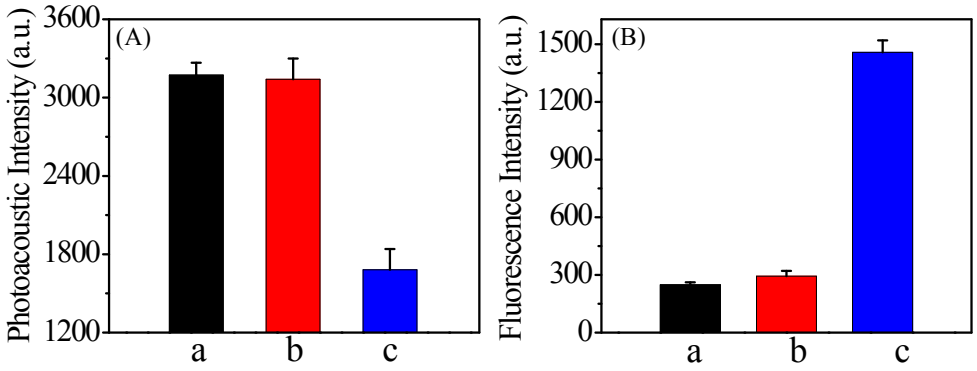

Fig. S8 Nuclease stability of the nanoprobe in the presence or absence of DNase I. The photoacoustic (A) and fluorescence intensity (B) of the nanoprobe (a) in response to DNase I (b) and corresponding target (c).

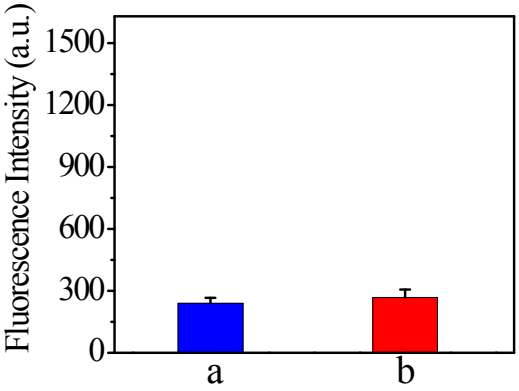

Fig. S9 The stability of the nanoprobe in the absence (a) or presence of (b) exonuclease III.

Cytotoxicity of the Nanoprobe in Living Cells and in Vivo

The cytotoxicity of the nanoprobe in living cells was studied and evaluated by MTT assay. HepG2 and HL-7702 cells were selected as the typical cancer and normal cells, rescpectively. Various concentrations of the nanoprobe were incubated with HepG2 and HL-7702 cells for 24 h. The nanoprobe showed almost no cytotoxicity or side effects in living cells under these conditions (Fig. S9), confirming that the nanoprobe was very suitable to detect intracellular mRNA.

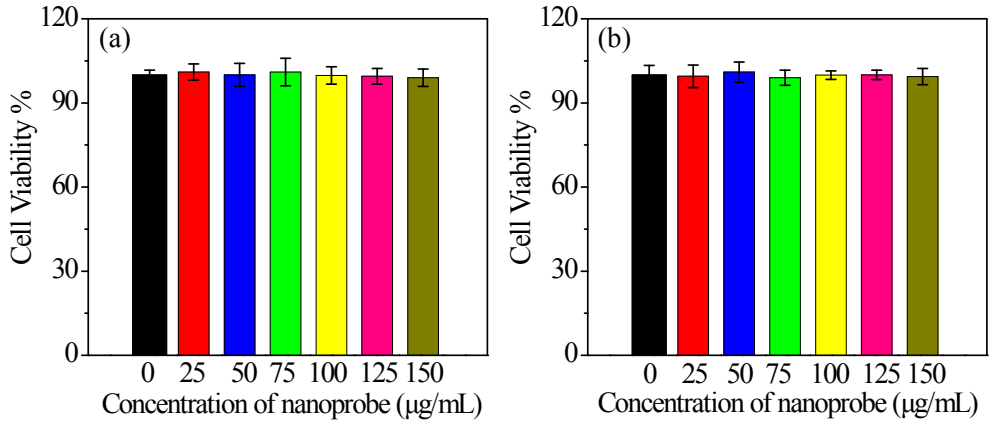

Fig. S10 Cell viability of HepG2 (a) and HL-7702 (b) cells. All cells were incubated with different concentrations of the nanoprobe for 24 h.

To further investigate the cytotoxicity of the nanoprobe in vivo, the histological effect of nanoprobe on five major organs (heart, liver, spleen, lung, and kidney) of the mice was studied. After the mice was intravenously injected with the nanoprobe for 48 h, the H&E staining experiments were performed. No histopathological abnormalities were found in these tissues (Fig. S10), implying that the nanoprobe displayed no cytotoxicity in vivo. All these results demonstrated that the nanoprobe was an approving candidate when applied in living cells and in vivo.

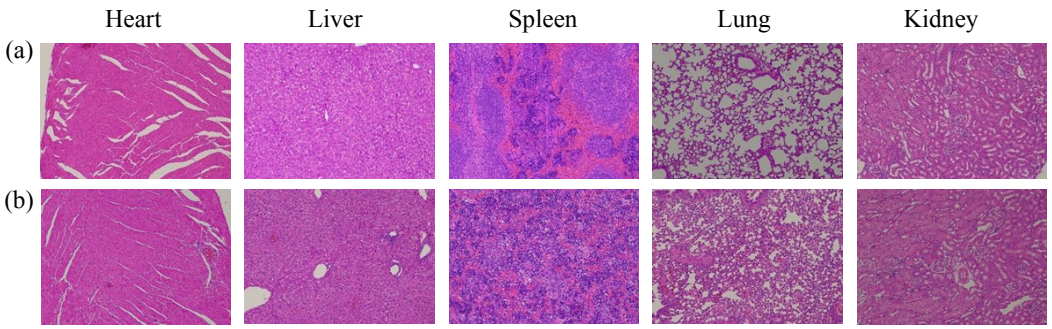

Fig. S11 H&E staining images of five major organs (heart, liver, spleen, lung, and kidney) in mice at control group (a) and nanoprobe treatment group (b).

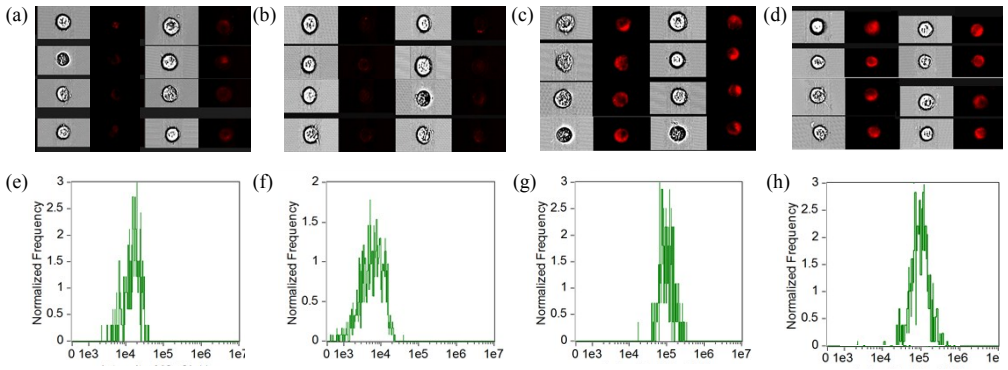

Fig. S12 Flow cytometry analysis. Cell images (top) and data (bottom) of MCF-10A (a, e), HL-7702 (b, f), MCF-7 (c, g) and HepG2 (d, h) cells incubated with nanoprobe, respectively.

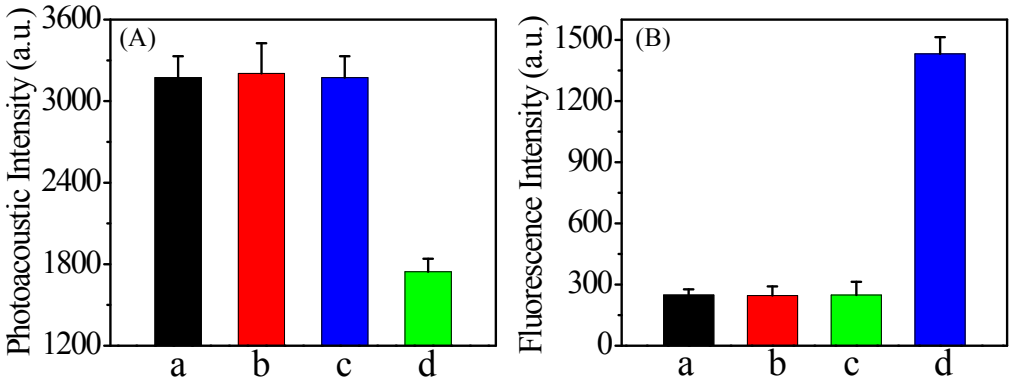

Fig. S13 The photoacoustic (A) and fluorescence intensity (B) when the nanoprobe and control probe were mixed with the DNA targets, respectively. The control probe treated without (a) and with (b) DNA targets, the nanoprobe treated without (c) and with (d) DNA targets.

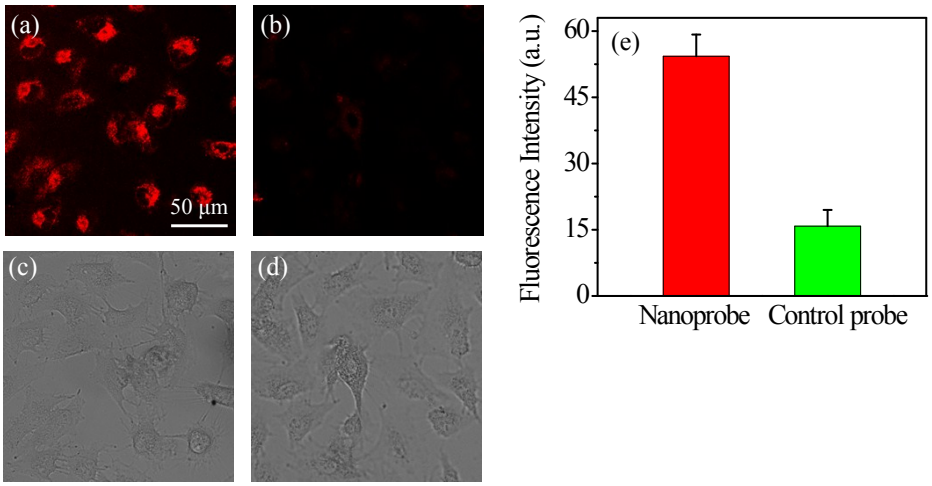

Fig. S14 Specificity of the nanoprobe in MCF-7 Cells. Fluorescence images of MCF-7 cells treated with the nanoprobe (a, c) and control probe (b, d). Images c and d were the bright-field images of a and b, respectively. (e) The fluorescence intensity of the cells in panels a and b. Scale bars are 50 μm.

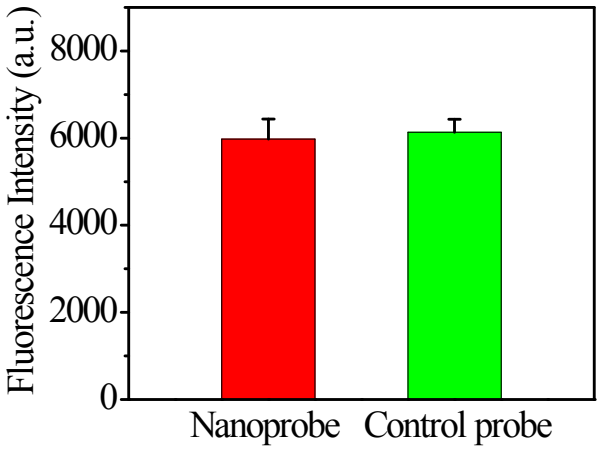

Fig. S15 The fluorescence intensity of the mice bearing HepG2 tumors treated with the nanoprobe and control probe via intravenous injection.

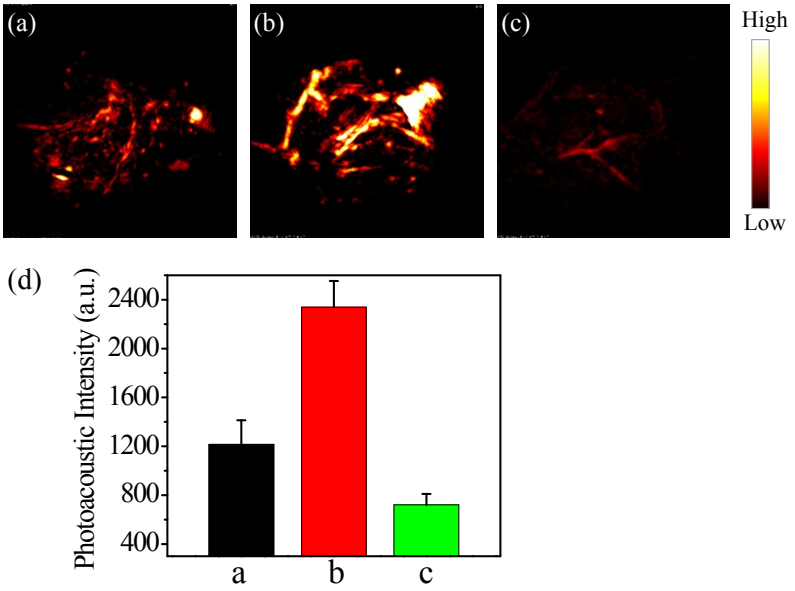

Fig. S16 Photoacoustic imaging of the different levels of TK1 mRNA in mice bearing MCF-7 tumors treated without (a), with tamoxifen (b) and β-estradiol (c). The treated and untreated mice were injected with the nanoprobe intravenously. (d) Quantification of photoacoustic intensity from images a, b and c. The excitation wavelength was 690 nm.