Supplementary information

A tumour mRNA-triggered nanocarrier for multimodal cancer cell imaging and therapy

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EXPERIMENTAL DETAILS

Materials. DNA oligonucleotides were synthesized and purified by TAKARA Biotechnology Co., Ltd. (Dalian, China). Deoxyribonuclease I (DNase I) was purchased from Solarbio Science and Technology Co., Ltd. (Beijing, China); Trisodium citrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), Hydrogen tetrachloroaurate(III) ($HAuCl_4 \cdot 4H_2O$, 99.99%), $MgCl_2$ and KCl were purchased from China National Pharmaceutical Group Corporation (Shanghai, China). Bovine insulin, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Disodium of 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMD) and Doxorubicin (Dox) were purchased from Sigma Chemical Company; cell culture products, unless mentioned otherwise, were purchased from GIBCO. All the chemicals were of analytical grade and used without further purification. Sartorius ultrapure water (18.2 MΩ cm) was used throughout the experiments. The human breast cancer cell line MCF-7 was purchased from KeyGEN biotechnology Company (Nanjing, China), the normal immortalized human mammary

epithelial cell line MCF-10A was purchased from Shanghai Bioleaf Biotechnology Company (Shanghai, China), Human hepatocellular liver carcinoma cell line HepG2 and human hepatocyte cell line HL-7702 were obtained from the Committee on Type Culture Collection of the Chinese Academy of Sciences.

Instruments. Absorption spectra were measured on a pharmaspec UV-1700 UV-visible spectrophotometer (Shimadzu, Japan). Transmission electron microscopy (TEM) was carried out on a JEM-100CX II electron microscope. Near infrared (NIR) lasers with irradiation wavelength of 655 nm (MLL-III-655) were purchased from Changchun New Industries Optoelectronics Tech. Co., Ltd (Changchun, China). Fluorescence spectra were obtained with FLS-920 Edinburgh Fluorescence Spectrometer with a Xenon lamp and 1.0 cm quartz cells at the slits of 4.5/4.5 nm. All pH measurements were performed with a pH-3c digital pH-meter (Shanghai LeiCi Device Works, Shanghai, China) with a combined glass-calomel electrode. Absorbance was measured in a microplate reader (Synergy 2, Biotek, USA) in the MTT assay and $^1O_2$ activation experiment. RT-PCR was carried out with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). Confocal fluorescence imaging studies were performed with a TCS SP5 confocal laser scanning microscopy (Leica Co., Ltd. Germany) with an objective lens (×20).

Synthesis of oligonucleotides. All DNA oligonucleotides used to prepare and test mRNA target were synthesized and HPLC purified by TAKARA Biotechnology Co., Ltd. (Dalian, China). The sequences involved in this work are listed in Table S1. The thiolated oligonucleotides were reduced with Tris(2-carboxyethyl) phosphine hydrochloride (TCEP·HCl) before they were assembled on the surface of gold nanoparticles.

Preparation of gold nanoparticles. The gold nanoparticles (GNPs) of 13 nm were synthesized using the sodium citrate reduction method reported before[1]. All glassware was cleaned in aqua regia (HCl/HNO_3, 3:1), rinsed with H_2O, and oven-dried before the experiments. After that 100 mL of 0.01% $HAuCl_4$ was heated to boiling with vigorous stirring, and 2.0 mL trisodium citrate (1%) was added under stirring. The solution color turned from pale yellow to colorless and finally to burgundy. Boiling was continued for an additional 10 min. After the heating source was removed, the colloid was stirred until the solution cooled down to room temperature. Then it was filtered through a 0.45 μm Millipore membrane filter. Transmission electron microscopy (TEM) images indicated the particle sizes are 13±2 nm (100 particles sampled). The prepared GNPs were stored at 4 °C.

MB structure. The potential secondary structure of MB was predicted by using UNAfold on www.idtdna.com. It indicated that the "stem and loop" conformation has the binding sites of Dox.

Binding studies of Dox and MB. To confirm that the fluorescence of Dox could be quenched by MB, a fixed concentration of Dox (1 μM) was incubated with different concentrations of MB (0, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.75 μM) for 30 min, and the fluorescence spectra of Dox were measured with excitation and emission wavelength of 490 nm and 505-650 nm, respectively. Sequential decreases in the fluorescence intensity of Dox were observed with the increase of MB concentrations (Figure S1). The maximal quenching of the Dox fluorescence was observed when the MB concentration reached 0.5 μM, and the fluorescence intensity did not show obvious change with the further addition of MB (0.75 μM).

Preparation of the nanocarrier. GNPs (1 nM) were mixed with 100 nM MBs and shaken overnight. After 12 hours, SDS solution (0.1%) was added to the mixture and the final concentration of SDS was 0.01%. Phosphate buffer (0.1 M; pH = 7.4) was added to the mixture to achieve 0.01 M phosphate concentration and the NaCl concentration of the mixture was slowly increased to 0.1 M over an eight-hour period. Then the mixture was stayed at room temperature for 48 h. After that, the solution was centrifuged (13500 g, 30 min) and resuspended in phosphate buffered saline (PBS) for three times. Then the AuNP-MB conjugate (1 nM) was suspended in the Tris-HCl buffer (10 mM Tris·HCl, pH 8.0, 100 mM KCl and 1 mM $MgCl_2$). After addition of Dox (1 μM), the solution was mixed by vortexing overnight, and then was washed with the buffer for three times. Then the nanocarrier was sterilized using a 0.22 μm acetate syringe filter and resuspended in PBS with a concentration of 3 nM as stock solution stored at 4 °C. The concentration of Au NPs was determined by measuring their extinction at 524 nm ($\varepsilon = 2.7\times10^8$ L mol^{-1} cm^{-1}).

Quantitation of MB loaded on the nanocarrier. The amount of MB loaded on GNPs was quantitated according to the previous protocol[2]. Briefly, mercaptoethanol (ME) was added (final concentration 20 mM) to the nanocarrier solution (1 nM). After it was incubated overnight with shaking at room temperature, the MBs were released. Then the released MBs were separated via centrifugation and the fluorescence was measured. The fluorescence of Ce6 was excited at 406 nm and measured at 663 nm. The fluorescence of Dox was excited at 490 nm and measured at 590 nm. The fluorescence was converted to molar concentrations of the drugs by interpolation from a standard linear calibration curve that was prepared with known concentrations with identical buffer pH, ionic

strength and ME concentrations. Each GNP was calculated to carry about 48±4 MBs (based on the fluorescence of Dox) or 50±6 MBs (based on the fluorescence of Ce6).

Release of Dox and Ce6 of the nanocarrier with DNA target. The obtained nanocarrier was diluted to a concentration of 1 nM in buffer (10 mM PBS of pH 7.4, 10 mM NaCl, 10 mM $MgCl_2$) and treated with the complementary DNA target with an increasing concentration (0, 10, 20, 30, 40, 50, 90, 100, 120, 150, and 200 nM). After a 30 min incubation, the fluorescence spectra were obtained in the range from 505 to 650 nm by use of the maximal excitation wavelength at 490 nm (for Dox) and in the range from 630 to 700 nm by use of the maximal excitation wavelength at 406 nm (for Ce6).

Kinetic study. The nanocarrier solution was incubated without and with the perfectly matched DNA target (200 nM) at 37 ºC with different incubation time (0, 5, 10, 15, 20, 30, 40, 50, 60 min). The fluorescence intensities were measured with 490 nm and 406 nm excitation wavelengths for Dox and Ce6.

Specificity experiment. The complementary DNA target for the nanocarrier and other targets (TK1 single-base mismatched target, survivin target, GalNac-T target, c-myc target) were spiked in 1 mL hybridization buffer containing 1 nM nanocarrier, while the DNA targets were 200 nM. All experiments were repeated at least three times.

Stability of the nanocarrier. The fresh synthesized and purified nanocarrier (1 nM) was stored at room temperature, and the fluorescence intensity was measured with a 490 nm excitation wavelength at 0, 5, 10, 15, 20, 30 days, respectively. After that, the mercaptoethanol was added (final concentration was 20 mM) to the nanocarrier solution and the solution was incubated overnight with shaking. The fluorescence intensity of Dox was

measured with 406 nm excitation. The leakage percentage of nanocarrier was calculated as follows: (fluorescence intensity of each sample at different time)/(the final fluorescence intensity of the sample).

Nuclease assay. Two groups of nanocarrier were diluted to a concentration of 1 nM in buffer (10 mM PBS, pH 7.4, 2.5 mM $MgCl_2$, and 0.5 mM $CaCl_2$) and placed in a 96-well fluorescence microplate at 37 ºC. After allowing the samples to equilibrate (10 minutes), 1.3 μL of DNase I in assay buffer (2 U/L) was added to one group. The fluorescence of these samples was monitored for 60 min. Then 200 nM DNA targets were paralleled added into the two samples with incubation for 1 h at 37 ºC, the fluorescence was measured with 490 nm excitation and 590 nm emission wavelength (for Dox) or 406 nm excitation and 663 nm emission (for Ce6) after the solution was cooled to room temperature.

The $^1O_2$ activation experiment. Disodium of 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABMD) was employed to assess $^1O_2$ generation. ABMD molecules can react with $^1O_2$ to yield an endoperoxide, which caused a decrease in the intensity of ABMD absorption. The nanocarrier (1 nM) was incubated with the perfectly matched DNA target in PBS (pH at 7.4) for 1 h at 37 °C. Then ABMD (0.1 mM) was added into the above solution. The photo-oxidation of ABMD was monitored for 30 min under the irradiation with adiode laser at 655 nm and the UV-vis spectra were recorded every 5 min. The ability of $^1O_2$ generation of nanocarrier only or nanocarrier with target was then evaluated.

Cell culture. MCF-10A, MCF-7 and HepG2 cells were cultured in Dulbecco's modified Eagles medium (DMEM). HL-7702 cells were cultured in RPMI medium 1640. All cell lines were supplemented with 10% fetal bovine serum (except HL-7702, for which we used 20%

fetal bovine serum) and 100 U/ml 1% antibiotics penicillin/streptomycin and maintained at 37 °C in a 100% humidified atmosphere containing 5% CO_2 at 37 °C.

Confocal fluorescence imaging. In a comparative experiment of cancer cells and normal cells, all cells were plated on chamber slides for 24 h. Then, all of the cells were incubated with 1 nM nanocarrier for 4 h. After the incubation, the cells were washed with PBS (pH 7.4) before imaging. The cells were examined by confocal laser scanning microscopy (CLSM) with 488 nm excitation (for Dox) and 633 nm excitation (for Ce6).

RT-PCR. Total RNA from sorted cells was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA synthesis was performed using an iScript kit (Bio-Rad). RT-PCR was carried out with SYBR Green I (Qiagen) on an ABI PRISM 7000 sequence detection system. Relative level of tumor mRNA was calculated from the quantity of tumor mRNA PCR products and the quantity of GAPDH PCR products. The primers used in this experiment were TK1 forward, 5'-TATGCCAAAGACACTCGCTAC-3'; TK1 reverse, 5'-GCAGAACTCCACGATGTCAG-3'; GAPDH forward, 5'-GGGAAACTGTGGCGTGAT -3'; GAPDH reverse, 5'-GAGTGGGTGTCGCTGTTGA-3'.

Evaluation of the mRNA expression levels. The relative levels of TK1 mRNA expression in HepG2 cells were modulated by down-regulation with tamoxifen and up-regulation with β-Estradiol. The HepG2 cells were separated into three groups in parallel. One group was treated with tamoxifen (10^{-6} M) for 48 h to decrease the TK1 mRNA expression and another one was treated with β-Estradiol (10^{-8} M) for 48 h to increase the TK1 mRNA expression. The untreated one was used as control. Next, the nanocarrier (1 nM) was incubated with the

treated and untreated cells, respectively. Then the cells were examined by CLSM as mentioned above.

MTT assay. MCF-7 cells were cultured in 96-well microtiter plates and incubated at 37 °C in 5% CO_2 for 24 h. The MCF-7 cells were separated into five groups in parallel. Two groups were incubated with culture medium, another two groups were incubated with the nanocarrier (1 nM), the last one group was incubated with the GNP-MB conjugate (1 nM). After 12 h, the medium was removed, and three groups of cells with different treatment were irradiated with a 655 laser (0.25 W, 1cm*1cm) for 20 min. The cells were allowed to continue growth for 24 h. Next, 150 μL MTT solution (0.5 mg/ mL) was added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm with a RT 6000 microplate reader. Other three types of cells (MCF-10A, HepG2 and HL-7702) were then treated as mentioned above. All experiments were repeated at least three times.

References:

1. K. C. Grabar, R. G. Freeman, M. B. Hommer, M. J. Natan, *Anal. Chem.* 1995, **67**, 735.

2. L. M. Demers, C. A. Mirkin, R. C. Mucic, R. A. Reynolds, R. L. Letsinger, R. Elghanian, G. Viswanadham, *Anal. Chem.* 2000, **72**, 5535.

Supplementary table:

Table S1. DNA sequences employed in this work.

Oligonucleotide	Sequence
MB	5’-Ce6-ACGACGCCAGGGAGAACAGAAACCGTCGTAAAAAA -$(CH_2)_3$-SH-3’
TK1 perfectly matched target	5’-GTTTCTGTTCTCCCTGG-3’
TK1 single-base mismatched target	5’-GTTTCTGTGCTCCCTGG-3’
survivin target	5’-CAGCCCTTTCTCAA-3’
GalNAc-T target	5’-GCTTTCACTATCCGCATAAGA-3’
c-myc target	5’-CCTCAACGTTAGCTTCACCAA-3’

[a]Underlined letters represent the stem sequence; [b]Letters in red represent the mismatched site.

Supplementary figures:

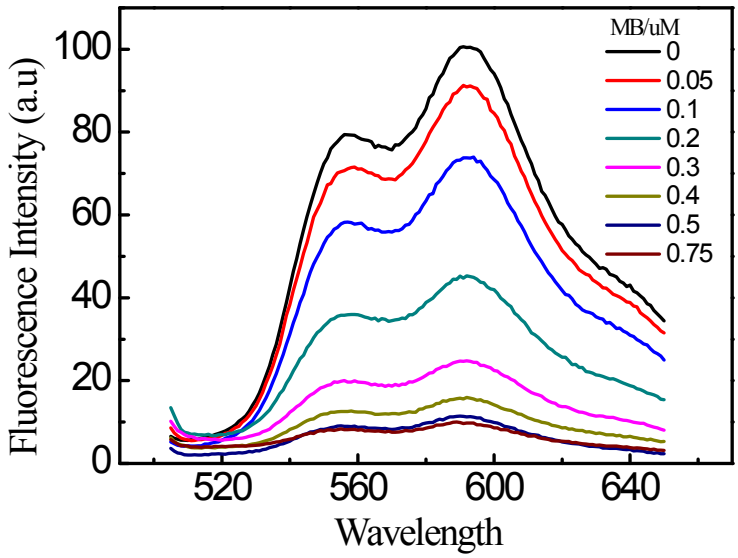
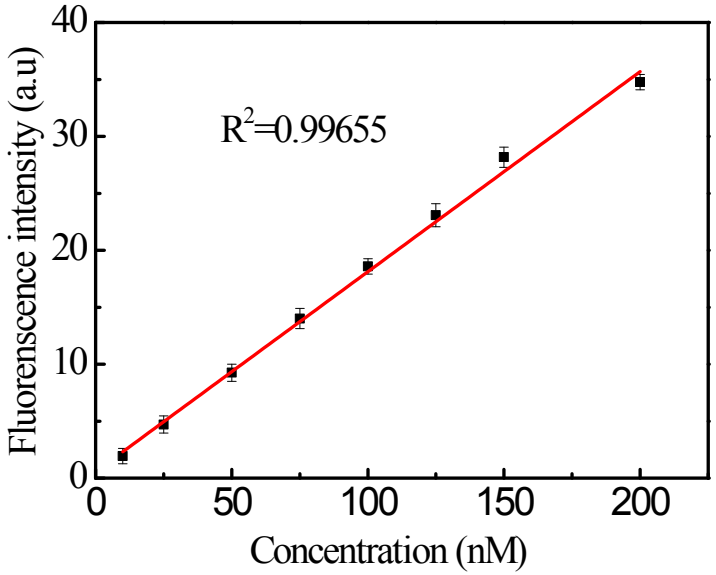


Fig. S1 Fluorescence spectra of Dox as a function of MB concentrations.

a



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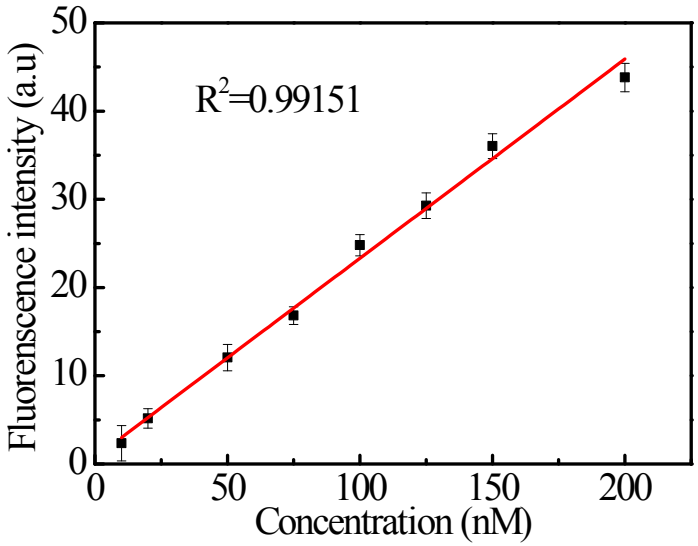


Fig. S2 Standard linear calibration curves of fluorescent dyes. a: Dox; b: Ce6.

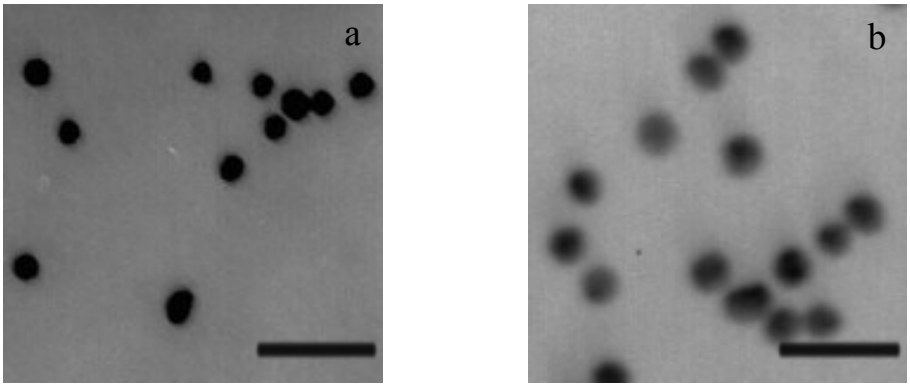


Fig. S3 TEM images of GNPs (a) and the nanocarrier (b). Scale bars are 50 nm

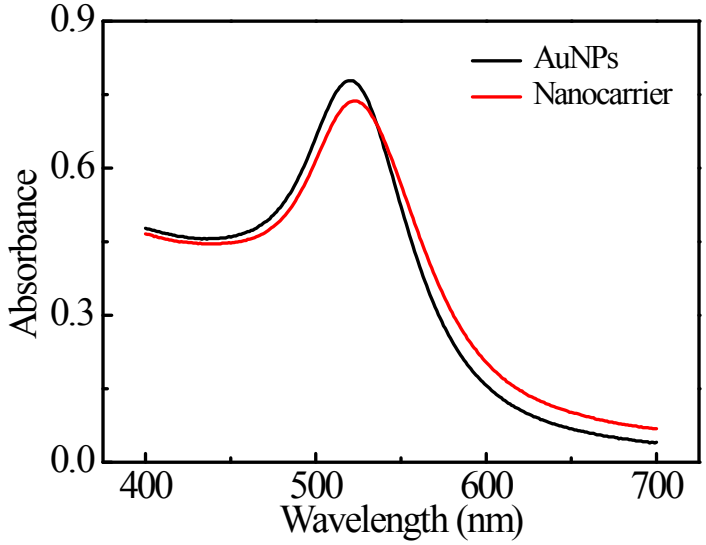
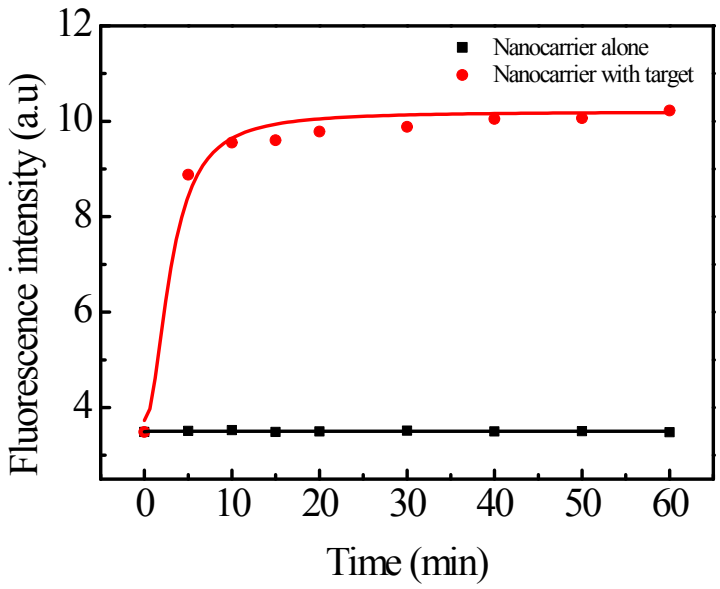


Fig. S4 UV-vis spectra for GNPs and nanocarrier.

a



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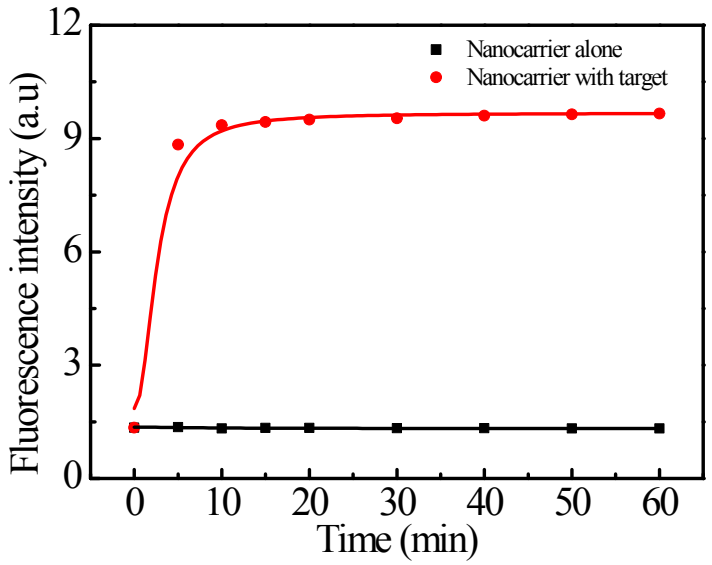
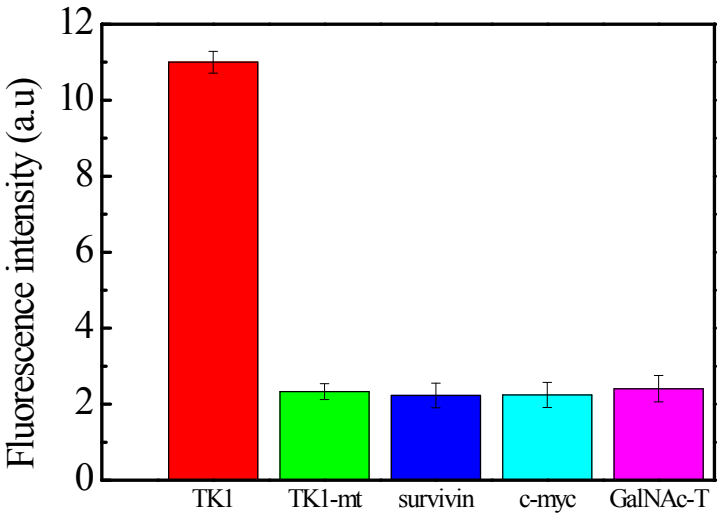


Fig. S5 Kinetic study of nanocarrier with perfectly matched DNA target (200 nM) and without perfectly matched DNA target. a: Dox; b: Ce6.

a



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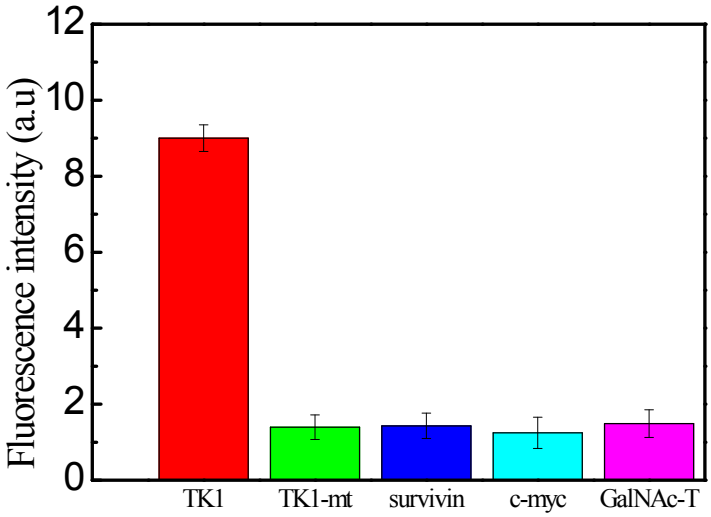

Fig. S6 Specificity of the nanocarrier over several DNA targets. The fluorescence intensity was measured when the nanocarrier was mixed with the perfectly matched target (red bar), single-base mismatched target (green bar) and other mRNA targets, respectively. a: Dox, b: Ce6.

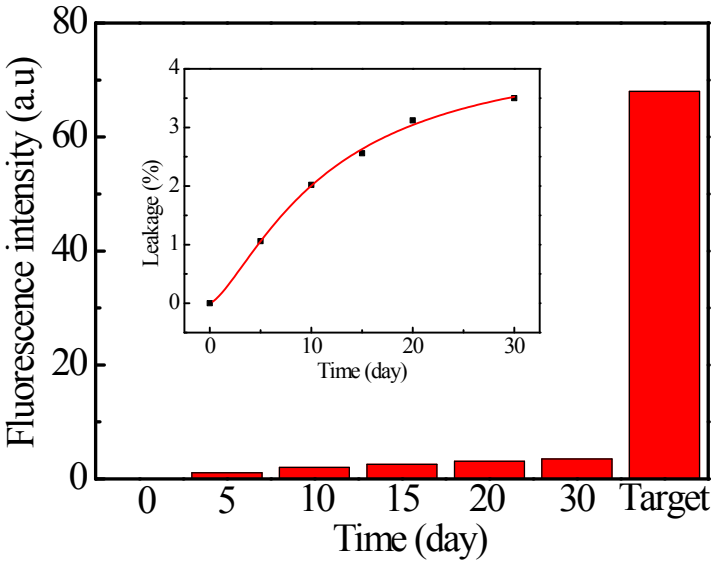

Fig. S7 Leakage percentage of nanocarrier over a time profile measured with 406 nm excitation wavelength at 0, 5, 10, 15, 20, 30 days, respectively.

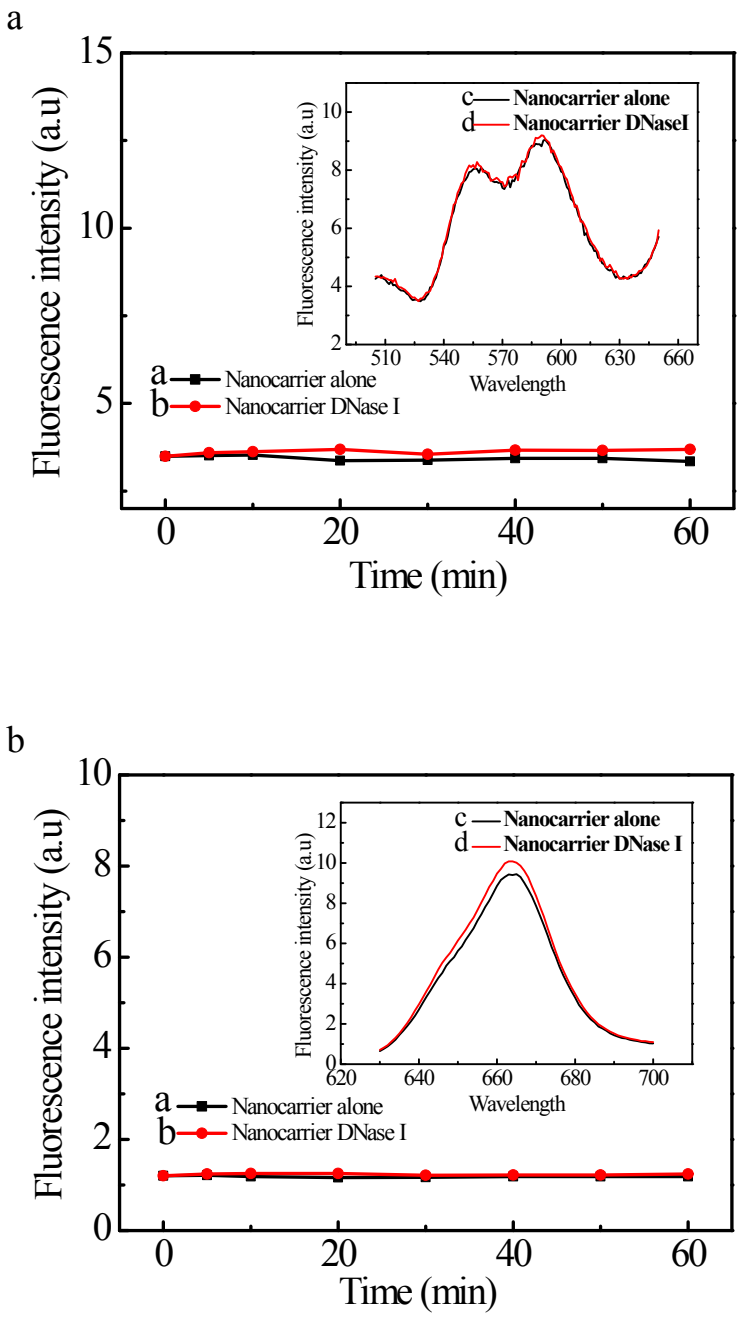

Fig. S8 The nuclease stability of the nanocarrier in the absence or presence of DNase I. Fluorescence curves of nanocarrier (1 nM) in PBS without DNase I (trace a), in the presence of DNase I (trace b). Inset: Fluorescence spectra of the two samples without (trace c) or with (trace d) DNase I after hybridization with 200 nM DNA target. a) Dox. b) Ce6.

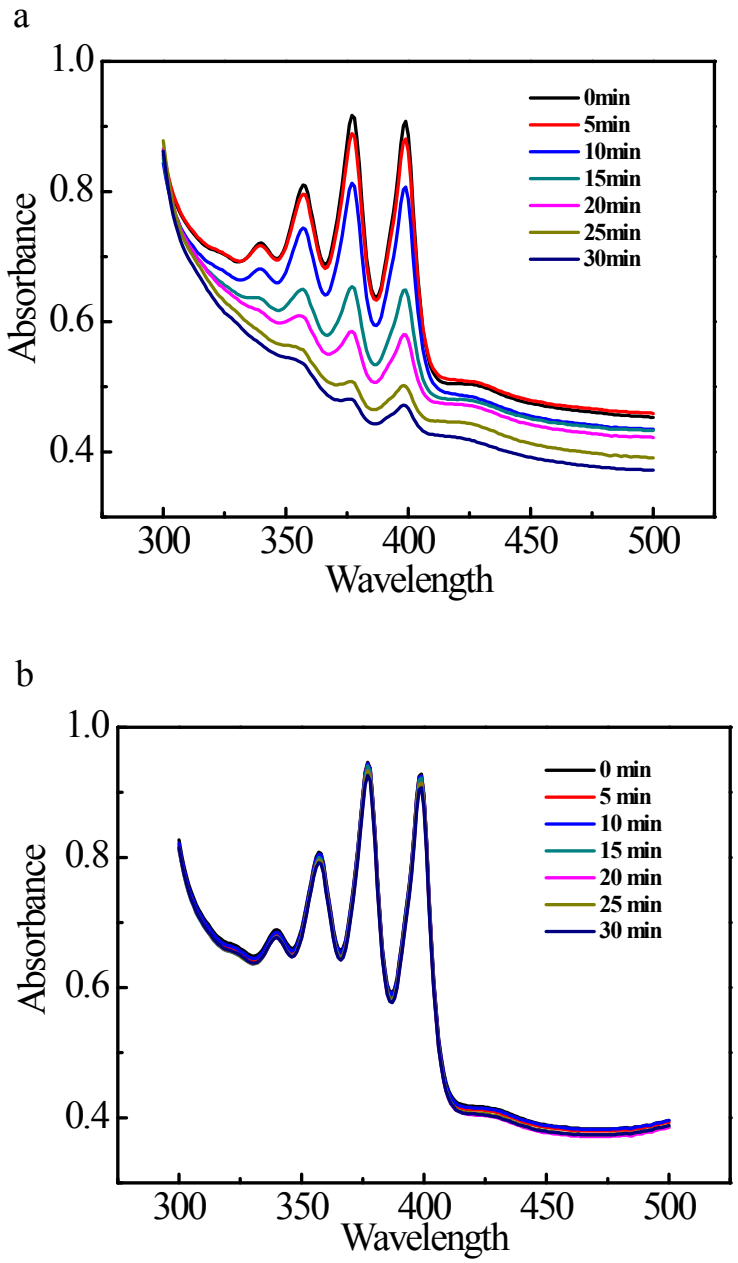

Fig. S9 The ABMD absorption spectra as a function of irradiation time in the presence of perfectly matched DNA target (200 nM) (a) or without perfectly matched DNA target (b).

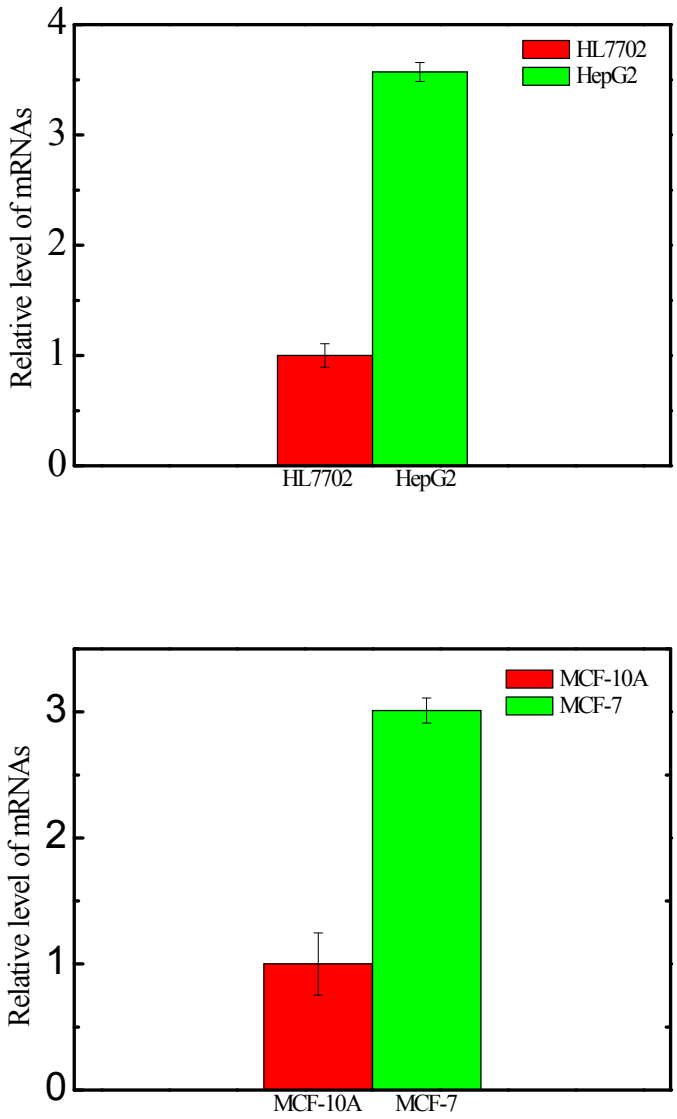

Fig. S10 Detection of the levels of TK mRNAs by RT-PCR in MCF-10A, MCF-7, HepG2 and HL7702 cells. The relative level of tumor mRNA was calculated from the quantity of tumor mRNA PCR products and the quantity of GAPDH PCR products and normalized to the expression level in normal cells.

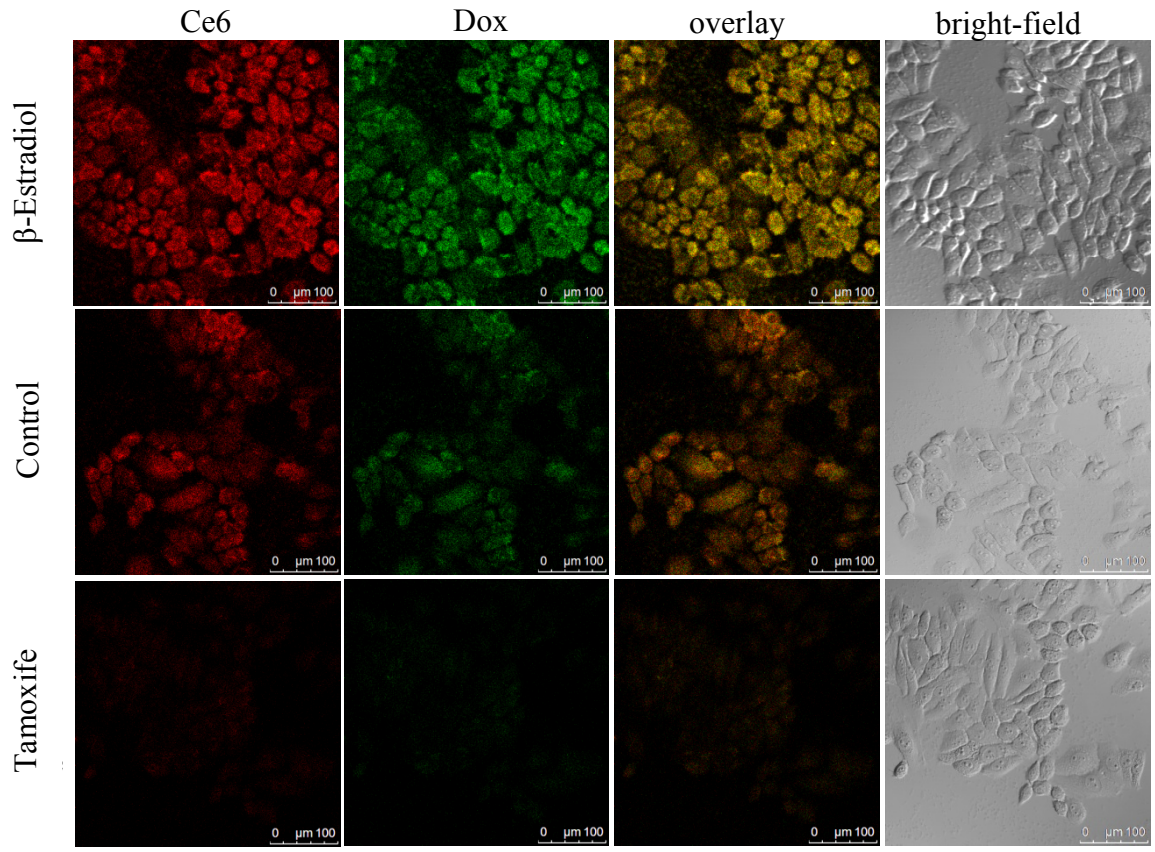

Fig. S11 Intracellular imaging of different levels of TK1 mRNA under CLSM. The treated and untreated HepG2 cells were incubated with the nanocarrier for 4 h at 37°C. The middle line was the control group, the first line was the β-estradiol-treated group and the bottom line was the tamoxifen-transduced group. Ce6 was excited by 633 nm laser and Dox was excited by 488 nm laser, respectively. Scale bars are 100 μm.