Supporting Information

Multiple mRNAs controlled and heat-driven drug release from gold nanocages in targeted chemotherapy and photothermal therapy for tumor

Hao Zhang[a], Ze Gao[a], Xiaoxiao Li[a], Lu Li*[, a], Sujuan Ye*[, b] and Bo Tang*[, a]

[a.] *College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Provincial Key Laboratory of Clean Production of Fine Chemicals, Shandong Normal University, Jinan 250014, P. R. China.*

[b.] *Key Laboratory of Optic-electric Sensing and Analytical Chemistry for Life Science,MOE; State Key laboratory Base for Eco-chemical Engineering; College of Science and Technology, Qingdao 266042, P.R.China.*

E-mail: tangb@sdnu.edu.cn;
lilu5252@163.com;
Yesujuan2010@126.com.

Experimental Section

Materials and Reagents. Ethylene glycol (EG), polyvinylpyrrolidone (PVP, MW=55000), hydrochloric acid (HCl) and acetone were all obtained from Sinopharm Group. Chloroauric acid ($HAuCl_4 \cdot 3H_2O$), sodium hydro sulfide ($NaSH \cdot xH_2O$) and silver trifluoroacetate (CF_3COOAg) were all purchased from Aladdin of Shanghai. Sodium chloride (NaCl) was obtained from Shanghai McLean. RPMI-1640 liquid medium, 1×PBS buffer (sterile), 0.25% trypsin, and penicillin-streptomycin solution were purchased from HyClone. Fetal bovine serum (FBS) was purchased from Sijiqing. Doxorubicin hydrochloride (Dox) was purchased from Shanghai biological engineering co., LTD. 50 × TAE, PAGE pre-solution (40%), ammonium persulfate ($(NH_4)_2S_2O_8$), TEMED ($(CH_3)_2NCH_2CH_2N\text{-}(CH_3)_2$) were purchased from Beijing solebo company. The MCF-7/Hela cells used in this experiment were purchased from the tumor markers research center of the cancer hospital of the Chinese academy of medical scie nces in Shanghai, China. The DNA sequences were designed using the computer program NUPACK. The DNA used in the experiment was synthesized by bioengineering co., LTD. (Shanghai, China). All drugs are analytical grade and are not further purified.

H1: 5’-TTGGTGAAGCTAACGTTGAGG-BHQ-3’

H2: 5’-SH-GCTTTCACCAACCCTACCGCCAAAGACACCGTAAATATTCACCAA-Cy5-3’

H3: 5’-CCTCAACGGGCGGCGAGTGTCTTTGGCATACTTAGGAGCATAAGA-SH-3’

H4: 5’-TCTTATGCGGATAGTGAAAGC-3’

TK1: 5’-AAGTAATGCCAAAGACACTCGC-3’

GalNAc-T: 5’-GCTTTCACTATCCGCATAAGA-3’

C-myc: 5’-CCTCAACGTTAGCTTCACCAA-3’

H1': 5’-CGGAGGCCGCATCCCCGCGCGC-3’

H2': 5’-SH-GTTCTGCCAAAAGGCGAAGCGGTCAACCGCGATTCAAGGCCTCCG-3’

H3': 5’-GCGCGCGGCGCCACGTCGGTTGACCGCGAAACCGCCACGTGCGCT-SH-3’

H4': 5’-GGCGCACGACCACCGGCAGAAC-3’

Apparatus. The transmission electron microscopy (TEM) images were captured with a JEM 1200EX transmission electron microscope (JEOL, Japan). UV/Vis absorption spectra were obtained with a Cary 50 UV/Vis-NIR spectrophotometer (Varian, USA). Fluorescence imaging was performed using a Leica TCS SP8 inverted confocal microscope (Leica, Germany). Therapeutic effect evaluation was verified through flow cytometry (cytoflex).

Animals. Female adult Bal/c rats weighing 16-20 g (4-5 weeks of age) were housed in groups of three in controlled condition with access to food and water ad libitum. This study was performed in strict accordance with the NIH guidelines for the care and use of laboratory animals (NIH publication, 8th edition, 2011) and was approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU 2021049). All efforts were taken to minimize the animal numbers and their suffering.

Synthesis of Ag Nanocubes. The 100 mL round-bottomed flask was fixed in the heat-collecting magnetic agitator and adjusted the rotating speed at 300 r/min and the temperature as 150 °C. After the temperature is stable, 5 mL ethylene glycol was added into the round-bottom flask and preheated for 1 h with an inclined lid while stirring. Then, NaSH (60 μL; 3 mM in EG) was added into the round-bottomed flask quickly. Four minutes later, 0.5 mL HCl solution (3 mM in EG) and 1.25 mL PVP solution (20 mg/mL in EG) were added. After 4 min, 0.4 mL silver trifluoroacetate (0.4 mL, 282 mM in EG) was injected into the mixed solution quickly. The reaction lasted for 20

min, followed by removed the mixture in ice water for rapid cooling. The reaction solution was washed with acetone once, and then, the supernatant was removed after centrifugation. The resulting product was washed with ultrapure water three times for purifying. The end product was dispersed into 4 mL ultrapure water and kept it under airtight and dark conditions at 4 °C.[1]

Sythesis of Au Nanocages. The 50 mL round-bottom flask was connected with the spherical condenser tube and the complex was fixed on the intelligent magnetic agitator. 5 mL PVP solution (1 mg/mL in ultrapure water) was transferred into the round-bottomed flask, meanwhile, the temperature of the agitator was set as 190 °C and the magnetic mixture was turn on. 1.0 mL Ag nanocube solution was transferred into the flask and the mixture was heated to faint boiling lasting about 10 minutes. Chlorauric acid solution (200 μL chloroauric acid solution of 1% mass fraction was dissolved in 10 mL ultra-pure water) then pumped into the reaction solution at a rate of 45 mL/h until appropriate color was appeared. Heating for 30 min until the color of the solution was stable, the heating was turned off, and the temperature of the reaction mixture was reduced to room temperature at reflux. Sodium chloride was added to the reaction mixture until saturated and centrifuged at 9000 r/min for 30 min. The resulting precipitate was dispersed in ultra-pure water. Subsequently, the product was centrifuged at 10000 r/min for 30 min for three times. The product was dispersed in 2 mL deionized water, and stored it under 4 °C and avoiding light.

Polyacrylamide Gel Electrophoresis. The appropriate amount of DNA strands S1, S2, S3 and S4 were reacted at 37 °C for 3 h prior to use. 12.5% polyacrylamide gel was prepared with deionized water, 50× TAE, TEMED, 10% APS and 40% PAGE Pre-Solution. Then, the gel was put into two glass plate of the electrophoresis apparatus until it overflowed and the electrophoresis comb was put into the glass plate immediately for making lane. The gel was placed at room temperature about 3 h until the gel solidified. After the bomb was taken away, 1× TAE buffer was poured into the electrophoretic tank. After pre-electrophoresis, 10 μL DNA H1, H2, H3, H4 and their mixture were mixed with 1.5 μL 6× loading-buffer respectively and the mixtures were transferred into the lane. After the gel was running at 200 V for 6 min, the voltage was set as 125 V. Turn off the power until the yellow strip reaches 3/4 of the gel. All gel was stained with GelRed for 1 h and imaged.

Cell Culture. MCF-7 and Hela cells were employed in this experiment. The cells were grown in RPMI-1640 culture mediumat a density of 6×10^5 cells/dish in 25-cm^2 cell culture flasks, and cultured in medium (89%) with heat-inactivated bovine serum (10%), penicillin and streptomycin (1%) at 37 °C in a humidified atmosphere (5% CO_2 and 95% air).

Preparation of Drug-carrier Complex. 20 μL of 10^{-6} M H1, H2, H3 and H4 strands were added into an EP tube and shaken gently at 25 °C, and the I-shaped structure DNA was successfully synthesized. 200 μL of freshly prepared gold nanocages (AuNCs) were mixed equably with 1 mL of 10^{-7} M doxorubicin hydrochloride and shaken gently overnight. Subsequently, the prepared I-shaped DNAs were added and shaken overnight at 25 °C, and centrifuged at 10000 rpm for 20 min. The precipitate was dispersed into 20 μL of deionized water, and the drug-carrier complex was obtained.

Drug Loading, Release, Stability of AuNC/DNA@Dox Experiment. H1, H2 DNA strands (10^{-6} M) 20 μL were injected into centrifuge tube followed by the addition of 200 μL gold nanocages (dissolved in ultra-pure water) with continuous stirring for 16 h at 37 °C to obtain the resultant DNA-conjugated AuNCs. The purified DNA-conjugated AuNCs was incubated in the 1 mL 10^{-7} M

doxorubicin hydrochloride (Dox) solution over night and the mixture was centrifuged and washed with ultra-pure water to remove Dox molecules adsorbed physically on the surface of the AuNCs. Then 20 μL H3, H4 DNA strands (10^{-6} M) was mixed with the drug loaded DNA-conjugated AuNCs with continuous stirring for 16 h at 37 °C. The resulting solution was centrifuged at 10000 r/min for 20 min and the supernatant were removed to obtain AuNC/DNA@Dox. The mRNAs were divided into ten groups and treated as follows: added 20 μL PBS (10 mM, PH=7.4), 20 μL TK1 (10^{-6} M), 20 μL c-myc (10^{-6} M), 20 μL Gal (10^{-6} M), 20 μL TK1+c-myc+GalNAc-1 (10^{-6} M), 20 μL TK1+c-myc (10^{-6} M), 20 μL c-myc+GalNAc-1 (10^{-6} M), 20 μL TK1+ GalNAc-1 (10^{-6} M), 20 μL TK1+c-myc+GalNAc-1+NIR (10^{-6} M) and NIR. Appropriate amount of PBS solution was added to make the ten groups same volume. 50 μL $MgCl_2$ (0.5 mM) was added into the ten groups and the mixtures were vibrated for 24 h. The resulting samples were centrifuged at 10000 r/min for 20 min and the liquid supernate was used to be measured with fluorescence spectrophotometer to monitor Dox fluorescence intensity. Another three groups were exposed to 55 ℃, 85 ℃ and NIR light irradiation for six hours, respectively. Three removed to obtain AuNC/DNA@Dox. TK1+c-myc+GalNAc-1 (10^{-6} M) 20 μL were added in the resulting samples were centrifuged at 10000 r/min for 20 min and the liquid supernatant was used to be measured with fluorescence spectrophotometer to monitor Dox fluorescence intensity. Additionally, we controlled the temperature of AuNC@DNA complex solution by adjusting the laser power, and the solution temperature was constant at 85 ^{o}C and 55 ^{o}C, respectively. Then, we measured the fluorescence of DOX released by AuNC under different conditions with time.

DNA Grafting on The Surface of AuNPs. To evaluate DNA loading on the surface of AuNC, the amount of thiol-modified DNA loaded on the AuNC was determined through a reported protocol[3]. 20 μL DNA strands (100 nM, 500 nM, 1 μM, 10 μM) were injected into centrifuge tube followed by the addition of 200 μL gold nanocages (dissolved in ultra-pure water) with continuous stirring for 16 h at 37 °C to obtain the resultant DNA-conjugated AuNC. Generally, thiol-modified DNA was added to AuNC solutions and measured solution concentration by using NanoDrop One of Thermo. Then the mixture was stirred overnight at room temperature in the dark for ligand loading. Afterward, the loading DNA with thiol-modified were collected via centrifugation, and the DNA concentration of the supernatant solution was measured by using NanoDrop One of Thermo. In order to explore the DNA grafting effect of AuNC/DNA@Dox, the fluorescence intensities of free Dox in the supernatant after blocking at 596 nm under different encapsulation conditions were measured with a fluorescence spectrophotometer (Hitachi, Japan). All experiments were performed six times to check reproducibility. This results are listed in table S1, which are similar to those reported in the literature[4]. According to the following formula, the corresponding grafting efficiency (GE) were calculated.

$$GE\ (\%) = (C_{DNA}V_{DNA} - C_{free}V_{free}) / N \times 100\ \% \qquad (1)$$

C_{DNA} is the total concentration of DNA in solution, C_{free} is the concentration of DNA in supernate solution and N is the number of AuNC in the solution.

Drug Targeted Release Assay in cell. 100 μL of MCF-7, Hela and MCF-10A cells were inoculated in a cell culture dish and incubated in sterile incubator (37 °C, 5% CO_2) for 24 h. The drug carrier complexes were diluted into 2 mL of culture medium with 10% bovine serum and dropwise added into the culture dish. The cells were incubated for about 4 h, and the fluorescence imaging could be carried out.

MTT Experiment. The cytotoxicity of the AuNCs in MCF-7 cells was tested using the MTT assay.

100 μL of MCF-7 single-cell suspension was inoculated in a culture dish and incubated in sterile incubator for 16 h. The AuNCs were dispersed into 2 mL of culture medium and incubated with the cells for about 6, 12, 18, 24 h, respectively. And then, the culture medium was discarded and the cells were washed three times in the PBS buffer, and 50 μL of MTT solution was put in and continuously incubated for 4 h. Finally, 100 μL of dimethyl sulfoxide (DMSO) was added and shaken at low frequency for 10 min, and absorbance at 490 nm wavelength was measured.

Characterization of Silver Nanocubes (Ag Nanocubes) and Gold Nanocages (AuNCs). The synthesized Ag nanocubes and AuNCs were characterized by TEM and UV-visible spectra, exhibiting in Figure 4 in the following. We can see in Figure 4A, the Ag nanocubeshave sharp edges and angles with uniform size, and the side length is about 35 nm. Figure 4B depicts the UV-visible spectra curve with two distinct peaks at 355 nm (a shoulder peak) and 420 nm (the characteristic absorption peak), respectively. The synthesized AuNCs have a lighter color in the middle, which proves that they are hollow inside, and light transmittance on the walls attests to the hole walls. The spectra curve has a remarkable characteristic absorbance at 728 nm, which demonstrates the successful preparation of AuNCs.

Characterization of H-shaped DNA. To verify the hybridization of H1, H2, H3 and H4, the polyacrylamide gel electrophoresis (17.5% N-PAGE) was carried out, and the results were displayed in Figure 3. Lane a was DNA marker, lane b-e represented the strands H1-H4, and lane f represented the hybridized DNAs, which existed a new bright band, and thus demonstrating the successful synthesis of I-shaped structure DNAs.

UV-visible Spectra of the multifunctional drug delivery systems. In order to check the formation of the drug carrier complex, one terminal of strand H1 and H2 was labeled with BHQ and Cy5, respectively, and the UV-visible spectra of every material was measured through a Cary 50 UV/Vis-NIR spectrophotometer.

Drug Targeted Release Assay. The drug-carrier complexes were incubated in MCF-7,Hela and MCF-10A cells, and the dynamic release process of the AuNCs could be observed and imaged when TK1 mRNA, c-myc mRNA, and Gal mRNA were existed simultaneously. As is depicted in Figure 3, the fluorescence imaging was utilized to monitor the distribution of the AuNCs and the drug release due to the reaction between the sealing materials and mRNAs.

In Vitro Drug Loading, Release, Stability and Photothermal Performance of AuNCs/DNA@Dox. The AuNCs solution of 1mL loaded with Dox was heated for at least 30 minutes in a water bath of 80 ℃ and then centrifugedat 10000 rap/min for 10 minutes. The supernatant was removed and the precipitate was redispersed into 1mL ultrapure water. The fluorescence of the supernatant was excited at 490 nm and received at 590 nm (Hitachi, Japan). According to the linear relationship between Dox concentration and fluorescence intensity (Figure S2A and B) and the following formula, the corresponding encapsulation efficiency (EE) and drug loading efficiency (DL) were calculated.

$$EE(\%) = (M_{total} - M_{free}) / M_{total} \times 100\% \quad (2)$$

$$DL(\%) = (M_{total} - M_{free}) / M_{AuNCs} \times 100\% \quad (3)$$

The drug EE of AuNC+Dox and AuNC/DNA@Dox were 67.14 %, with the DL as 42.79 %, respectively. These results indicated that DNA nanolock had a good blocking effect, and Dox was encapsulated in AuNCs rather than adsorbed on the surface of AuNCs. According to the fluorescence linear calibration curve of Dox, the loading capacity of the Au nanocages was calculated to be 19.74 μg Dox per 1 mg AuNC. The detailed fluorescence information were

displayed in Figure S2.

Cell viability under infrared light irradiation. In order to verify the killing efficiency of AuNCs irradiated by infrared light, we incubated AuNCs into cells and irradiated them with infrared laser. Colorless trypsin was used to digest MCF-7 cells irradiated by infrared light for 0, 10, 20 and 40 minutes respectively. Then FITC-PI double staining kit was used to stain the cells and flow cytometry was used to detect the stained cells.

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 (Applied Biosystems, Foster City, CA). 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 c-myc forward, 5'-TCGGGTAGTGGAAAACCAGCAGCCT-3'; c-myc reverse, 5'-CCTCCTCGTCGCAGTAGAAATA-3'; TK1 forward, 5'-TATGCCAAAGACACTCGCTAC-3'; TK1 reverse, 5'-GCAGAACTCCACGAT-GTCAG-3'; GalNAc-T forward, 5'-CCAAGACCTTCCTCCGTTAT-3'; GalNAc-T reverse, 5'-AACCGTTGGGTAGAAGCG-3'; GAPDH forward, 5'-GGGAAACTGTGGCGTGAT-3'; GAPDH reverse, 5'-GAGTGGGTGTCGCTGTTGA-3'.

In Vivo Drug Release Behavior, Fluorescence Imaging in the NIR Region, and Tumour Imaging. For the in vivo drug release behavior of AuNC, the near infrared probe ICG loaded in AuNC was injected into 4T1-tumor-bearing mice. Under isoflurane anesthesia, in vivo NIR fluorescence imaging was performed using an IVIS Lumina II imaging system at 4 h postinjection. For in vivo fluorescence imaging of AuNC in the NIR region, an NIR fluorescence imaging system was applied. The excitation light was provided by a 780 nm diode laser with an 800 nm long-pass filter. After a single dose of 50.0 μmol/g AuNC was injected into the 4T1-tumor-bearing mice, NIR fluorescence imaging was performed. For in vivo tumour imaging, the AuNC locked by DNAs were injected into the 4T1-tumor-bearing mice. After 4 h later, we dissected the tumor from the mice. Then the tumour were observed using the Endra Nexus 128 system after near-infrared irradiation.

Simulated Heat Generation by Lasers at the Tumor site in vitro. We covered the mouse underarm skin on one side of the centrifuge tube, then irradiated the centrifuge tube with 808 nm laser and measured the heat change of the AuNC. The detailed information were displayed in Figure S7.

Supporting Figures:

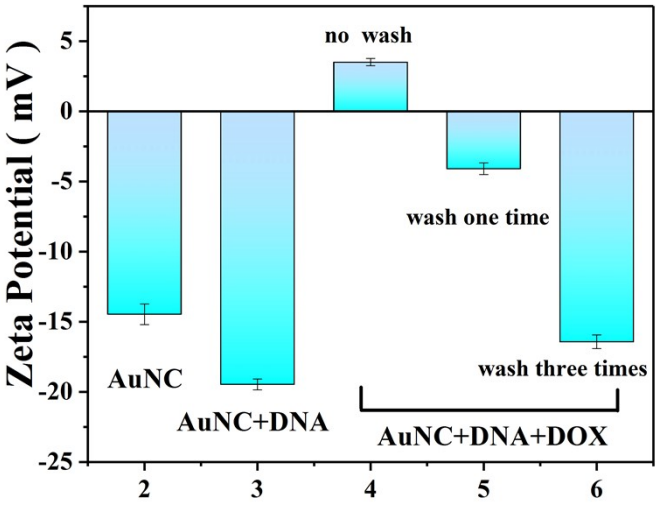

Fig.S1 Zeta potentials of AuNCs, AuNCs/DNA lock and AuNCs/DNA@Dox.

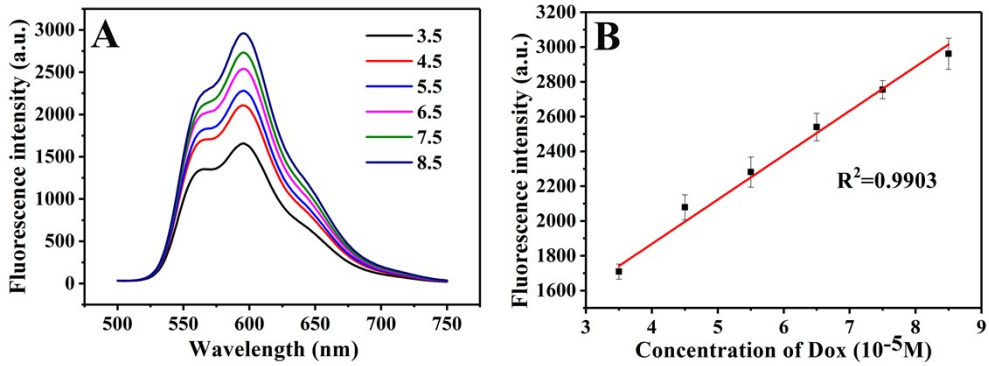

Fig.S2 (A) Fluorescence emission spectra of Dox at different concentrations (3.5, 4.5, 5.5, 6.5, 7.5, 8.5 μM) under 480 nm excitation and (B) the linear relationship between corresponding concentrations and fluorescence intensity.

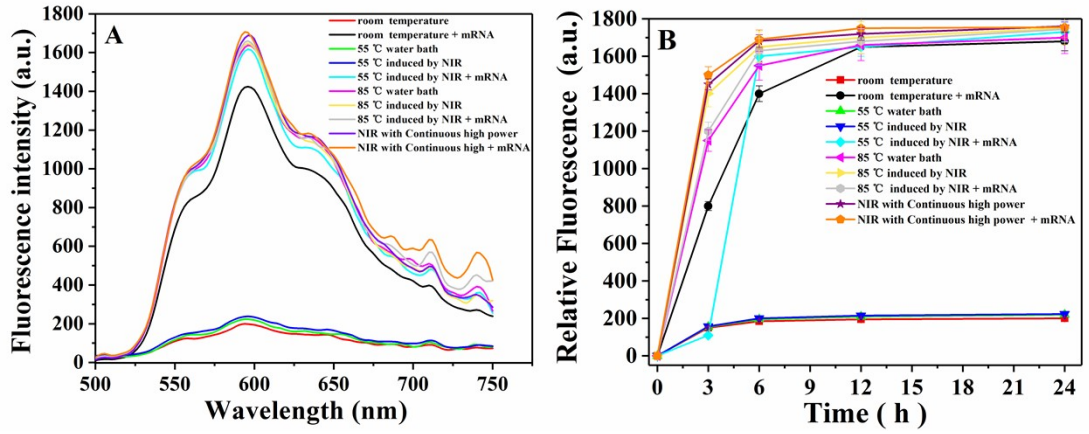

Fig.S3 The fluorescence intensity of DOX in the supernatant of DNA@AuNC at 6 h under different conditions (A) and the fluorescence intensity of DOX in the supernatant of DNA@AuNC with the different time under different conditions (B). There was no difference in the fluorescence intensity of released DOX induced by water bath or NIR irradiation, indicated that the temperature could be controlled stably by adjusting the laser power..

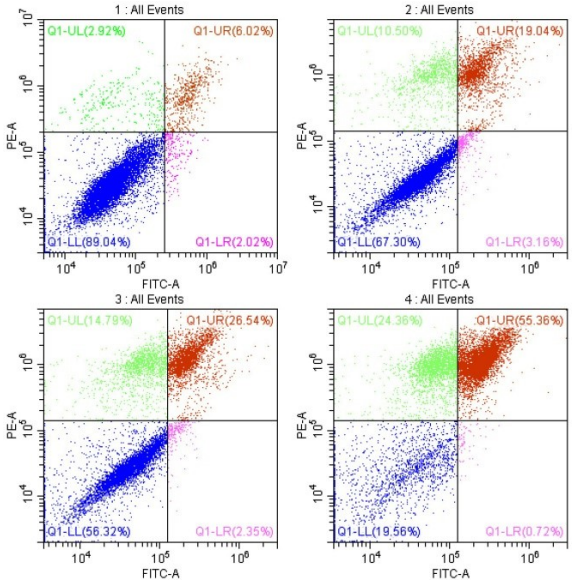

Fig.S4 Flow cytometry characterization of cell viability under infrared light irradiation. (1-4) represents the state of cells irradiated by infrared light for 0 s, 10 s, 2 minutes and 40 minutes, respectively.

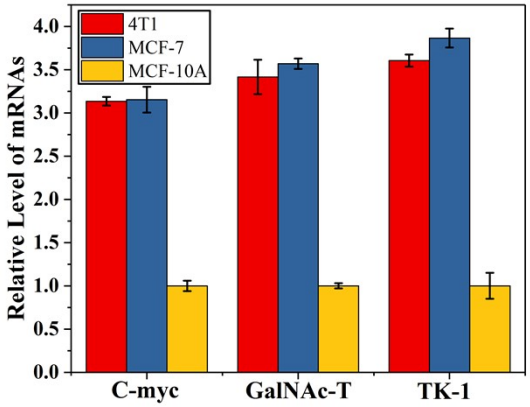

Fig.S5 Detection of the level of three tumor mRNAs by RT-PCR in cancer cells and their normal cells (MCF-10A, MCF-7 and 4T1).

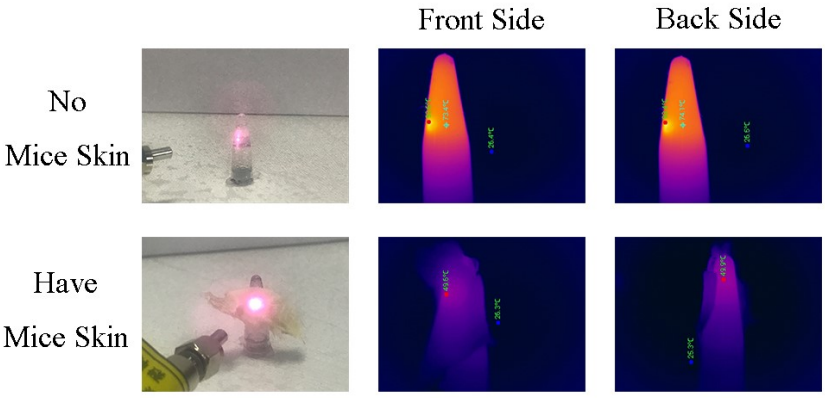

Fig.S6 simulated heat generation by lasers at the tumor site in vitro.

Table S1. The amount of RNA chains loaded on of AuNC.

DNA concentration	100 nM	500 nM	1 μM	10 μM
AuNC DNA grafting (counts)	26 ± 1	123 ± 1	128 ± 1	131 ± 1

Table S1 shows that the amount of DNA chains on AuNPs when DNA chains with different concentrations were combined with AuNC. Finally, 500 nM of DNA was chosen for AuNC, with 120 DNA chains on each AuNC.

References

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