Supporting Information

Ex Vivo Identification of the Circulating Tumor Cells in Peripheral Blood by Fluorometric "Turn on" Aptamer-Nanoparticles

Wenxi Xia[a], Xiaoyan Shangguan[a], Miao Li[a,b], Yang Wang[a], Dongmei Xi[a], Wen Sun[a], Jiangli Fan[a], Kun Shao*[,a] and Xiaojun Peng*[,a]

[a]State Key Laboratory of Fine Chemicals, Dalian University of Technology, 2 Linggong Road, Dalian 116024, PR China

[b]School of Biological Engineering, Dalian Polytechnic University, Ganjingzi District, Dalian 116034, PR China

Experimental Section

Materials

All reagents and solvents were reagent grade without specially purified and purchased from Sigma-Aldrich. Amino-PEG5000 thiols, azide-PEG5000 thiols and heterobifunctional linker succinimidyl- ([N-maleimidopropionamido]-diethyleneglycol) ester ($SM(EG)_2$) were purchased from Ponsure Biotech, Inc (Shanghai, China). Sterile defibrinated sheep blood and heparin sodium salt were obtained from Solarbio Science & Technology Co., Ltd (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermofisher. MCF-7 and 4T1 cells were obtained from Institute of Basic Medical Sciences (IBMS) of the Chinese Academy of Medical Sciences. The HBLV-mCherry-PURO was ordered from Hanbio Biotechnology Co., Ltd (Shanghai, China). Hypoxia-Inducible Factor-1 Alpha (HIF 1α) Human Recombinant protein (ab154478) was purchased from Abcam. Glucose Transporter GLUT1 Rabbit Monoclonal Antibody, Alex Fluor 488-

labeled Goat Anti-Rabbit lgG(H+L) and Alex Fluor 488-labeled Goat Anti-Mous lgG(H+L) were purchased from Beyotime. HIF 1α Mouse monoclonal were obtained from Abcam.

Balb-c mice (female, 15-20 g) were purchased from Dalian Medical University. All the animal procedures were carried out according to the Guidelines for Care and Use of Laboratory Animals of Dalian Medical University, and the experiment was approved by the Animal Protection and Use Committee of Dalian Medical University. All oligonucleotides were synthesized and HPLC-purfied by TaKaRa Biotechnology Co., Ltd (Dalian, China). DNA sequences were shown below:

(1) FAM-aptamer: 5′- FAM-

CGGATAACAACAAGTATGTGGAGGAACTGTGTGGTATCCG- 3′

(2) BHQ1-DNA: 5′- SH-CGGATACCACACAGT -BHQ1- 3′

All prepared glassware used for gold nanoparticle were steeped and cleaned with aqua regia (HCl (37 %)/HNO_3 (65 %) 3:1). All aqueous solutions used for nanoparticle were prepared by ultrapure deionized water. Mass spectrometric data was recorded on LTQ Orbit rap XL instruments. ^{1}H NMR spectra were performed on a Bruker Avance III 500 spectrometer. Single-photon confocal fluorescence imaging was observed using an Olympus FV3000-ASW multiphoton laser scanning confocal microscope.

Synthesis of 13 nm AuNPs

The synthesis of 13 nm AuNPs was referred to the previously published literature.[1] In brief, added 1 % sodium citrate to the boiling deionized water containing 0.01 % $HAuCl_4$, and the solution gradually turned red. After stirring for 15min, stopped the reaction and cooled to room temperature. The prepared 13 nm AuNPs was packed in a brown bottle for reservation.

Coupling of Aptamer-Oligonucleotides to PEG-Passivated AuNPs

a) Synthesis of PEG-passivated AuNPs

The synthesis procedure for PEG-passivated AuNPs was referred to the previous published literature.[2] The traditional passivation reactions were proceeded by adding the AuNPs to an aqueous solution containing sodium bicarbonate and a mixture of amino- and azide-PEG-thiols with the expected molar fraction of amino-terminated derivative ($0.09 < \chi_{NH2} < 0.33$). Stock solutions of thiols PEG was prepared in ethanol (5 mM) for reserve and stored at 4 °C.

b) Synthesis of functionalized AuNPs with the $SM(EG)_2$ linker

Stock solutions of freshly SM $(EG)_2$ linker was prepared in DMF (75 mM) for reserved and stored at 4 °C. The reaction process was add 5 mM linker to 30 mM PBS solution containing 2~6 nM AuNPs and shaken at 4 °C for 4 h. After the above reaction, the AuNPs were purified *via* ultrafiltration.

c) Preparation of double stranded DNA

First, the DNA was dissolved in STE Buffer (10 mM Tris pH 8.0, 50 mM NaCl, 1 mM EDTA) and the two strands were mixed with equal moles. Then, the DNA was kept at 94 °C for 5 min, and gradually cooled to r.t. Double stranded DNA stored at -20 °C after being separated.

d) Deprotection of the oligonucleotide 5′- thiol group

The 5′ -thiol group of oligonucleotide was deprotected by incubation with 100 equiv tris (2-carboxyethyl) phosphine hydrochloride (TCEP) in buffer for 2-3 h at room temperature. The thiol double stranded DNA could directly apply in the conjugation step with SM $(EG)_2$ functionalized AuNPs.

e) Oligonucleotide conjugation

The annealed and deprotected DNA double strands were added to the prepared SM $(EG)_2$ functionalized AuNPs and shaken for 12 h at 4 °C. The reaction volume ranged from 200~ 2000 μL in 20 mM PBS solution, where the concentration of AuNPs was 4~ 8 nM, NaCl was

200 mM and DNA double strands was 10 μM. After the above reaction finished, the AuNPs were purified and concentrated *via* ultrafiltration.

DNA-Gold Nanoparticle Characterization

Size/PDI and zeta potential of DHANs were analyzed by dynamic light scattering (ZS90, Malvern). The value of PDI were used to analysis size distribution. The morphological examination of nanoparticles were carried out using transmission electron microscope (TEM, HT7700 EXALENS).

Synthesis of DHA and DHA-PEG_{5000}-Thiols

DHA was synthesized as follow: potassium hydroxide (0.95 g, 17 mmol) was dissolved by adding deionized water (60 mL) in a round-bottomed flask under the nitrogen atmosphere. The above reaction mixture was added L-ascorbic acid (3 g, 17mmol) and 3-bromopropyne (3.03 mL, 34 mmol) in acetone (120 mL). The reaction was stirred at 40 °C for 48 h. The reaction system was extracted with ethyl acetate and washed with saturated sodium chloride, then dried of anhydrous sodium sulfate. The crude product was purified by silica gel column chromatography (DCM: acetone = 5:1) to obtain a white solid with the yield of 60%. ^{1}H NMR (500 MHz, DMSO-d_6) δ 5.10 (dd, J = 15.5, 2.1 Hz, 1H), 5.01 – 4.94 (m, 2H), 4.84 (t, J = 5.6 Hz, 1H), 4.79 (s, 1H), 3.69 (s, 1H), 3.65 (d, J = 6.6 Hz, 1H), 3.44 (d, J = 5.3 Hz, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.05, 148.96, 120.36, 79.10, 78.64, 74.49, 68.40, 61.68, 58.32. ESI-MS: *m/z* calcd for $C_9H_{10}O_6^-$ [2M-H]$^-$: 427.10, found: 427.09. Purified alkyne-DHA (2 equiv) was binding with azide-PEG_{5000}-thiols *via* Click-reaction under CuI (0.5 equiv) and DIPEA (1 equiv) catalyze. The target product was obtained by dialysis, then freeze-drying.

UV and Fluorescence Response of DNA or DNA-Gold Nanoparticle

UV spectra and fluorescence of associated with DNA were measured out using Nanodrop (Olympus). The experiment system was: 20 mM Tris-HCl, 20 mM NaCl, 2 mM $MgCl_2$, pH = 7.0. All oligonucleotide concentration was 2 μM. A stock solution of HIF 1α protein was

prepared by PBS buffer. HIF 1α and DNA double strands or DHANs were inbubated for 5 min and then tested immediately for preventing the degradation of HIF 1α *in vitro*.

***In Vitro* Cell Viability Assays**

MCF-7 cells and HepG2 cells were planted on 96-well plates (1×10^4 cells per well) and incubated at 37 °C for 24 h in DMEM. DHANs were added into each well and cultured for another 12 h with different concentrations. Then, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added into each well. The medium was removed out after 4 h incubation. The cytotoxicity was measured with a Bio-Rad microplate reader at the absorbance of 570 nm and 630 nm (n=6).

$$\textbf{Cell viability (\%)} = (OD_{PS} - OD_{blank\ control}/OD_{control} - OD_{blank\ control}) \times 100$$

Subcellular co-localization assay

4T1 cells were incubated on the 35 mm confocal dishes for 18 h. 4T1 cells were incubated with 200 μg/ml DHANs for 1 h, and then stained with LysoTracker Red for additional 10 min. The co-localization experiment was carried out on CLSM. The excitation wavelength for DHANs was 488 nm, and the emission wavelength was collected from 490-540 nm. The excitation wavelength for LysoTracker Red was 561 nm, and the emission wavelength was collected from 550-590 nm.

Investigation of Cellular Uptake and Internalization Mechanism

4T1 cells were planted in 35 mm glass bottom dishes at a density of 100000 cells/dish. After cultured at 37 °C under 5% CO_2 for 12 h, the cells grew to 60% -70% density. In order to mimic hypoxia tumor environment, cells were cultured in an incubator chamber (MIC-101, Billups-rothenberg) at 37 °C and 0.2% O_2 atmosphere for another 8 h. Furthermore, oxygen detector (Nuvair, O_2 Qucikstick) was used to monitor the oxygen concentration in the incubator chamber. In order to explore the mechanism of internalization, the cells were preincubated with

PBS (consist of 1% FBS) and DMEM under normoxia and hypoxia conditions, respectively. After preincubated for 30 min, DHANs (200 μg/ mL) were added to 4T1 cells at 37 °C for 0.5, 1, 2 and 3 h. In addition, to verify the effect of DHA on cell uptake, HANs (without DHA modification) also added to 4T1 cells under the equivalent conditions. Meanwhile, the cells were preincubated with PBS added 10 mM $_{D}$-glucose as GLUT1 transporter inhibitor. After incubation was finished, the medium was removed, and the cells were washed with PBS twice, then using FV3000 confocal laser scanning microscope to visualize.

Immunofluorescence and Flow Cytometry

Immunofluorescence: 4T1 cells are seeded in a 35 mm glass-bottom culture dishes and culture for 24 h at 37 °C in a 5% CO_2 humidified incubator. Then, the cells are fixed with 4% poly formaldehyde solution (4% PFA) for 15 min at room temperature, removed the PFA and washed twice with PBS. The cells are added 0.1% Triton X-100, cultured for 5 min and washed twice with PBS. Subsequently, the cells are blocked with 1% BSA for 30 min, and then incubate with specific concentration anti-HIF 1α and GLUT1 for 4 h at room temperature. After the incubation with antibody, the cells are washed with PBS to remove the extra antibody, and then added with Alexfluo488 lgG (H+L) for incubation 1 h. The immunofluorescence imaging is observed under CLSM.

Flow cytometry: 4T1 cells are seeded in a 6-Well cell culture plates and culture for 24 h at 37 °C in a 5% CO_2 humidified incubator. Then, the cells are digested, centrifuged and fixed with 4% poly formaldehyde solution (4% PFA) for 15 min at room temperature, removed the PFA and washed twice with PBS. The cells are added 0.1% Triton X-100, cultured for 5 min and washed twice with PBS. Subsequently, the cells are blocked with 1% BSA for 30 min, and then incubate with specific concentration anti-HIF 1α and GLUT1 overnight. After the incubation with antibody, the cells are washed with PBS to remove the extra antibody, and then

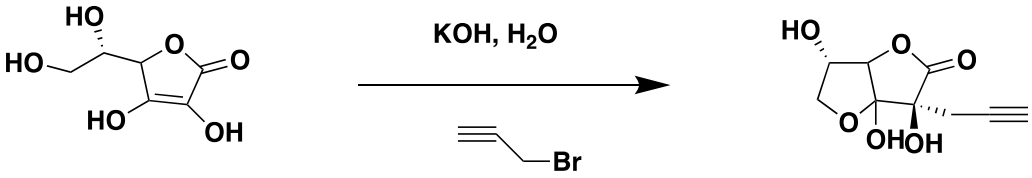
added with Alexfluo488 lgG (H+L) for incubation 1 h. The expression of enzyme is observed by flow cytometry.

Detection of Simulated CTC in Sheep Blood *in vitro*

Digested MCF-7 cells of different concentrations (50, 100, 500, 800, 1000, 3000, 7000 and 10000 cells/ mL) were plated onto multiwall glass slides obtained from Ibidi. After cultured at 37 °C under 5% CO_2 for 12 h, cells were transferred and cultured in an incubator chamber at 37 °C, 0.2% O_2 atmosphere for another 8 h. After cell incubation was finished, removed the medium, and added DHANs (200 μg/ mL) under the PBS medium. The cells were cultured at 37 °C for another 1 h, then using FV3000 confocal laser scanning microscope to visualize. The average fluorescence intensity was caculated by Cell Sens (a processing software from Olympus) based on randomly take 7 ROIs on the image. Taking more ROIs might reduce the biases.

Detection of Simulated CTC in Mice Blood *in vitro*

Blood was obtained from the vein of mice mimic CTC environment *in vitro*. Similar to the sheep blood experiments, digested 4T1 cells of different concentrations (50, 200, 500, 800, 1000 and 3000 cells/ mL) were plated onto multiwall glass slides. After cultured at 37 °C under 5% CO_2 for 12 h, cells were transferred and cultured in an incubator chamber at 37 °C, 0.2% O_2 atmosphere for another 8 h. After cell incubation was achieved, removed the medium, and added DHANs (200 μg/ mL) under the PBS medium. After the above experiment, cells were visualized under the FV3000 confocal laser scanning microscope.



Scheme S1. Synthetic route of DHA.

Table S1. Summary of Nanoparticle Properties

	Size (nm)	PDI	Zeta (mV)
AuNPs	22.90	0.240	-28.3
PEG-AuNPs	41.38	0.379	-17.2
PEG-SM(PG)2-AuNPs	43.38	0.328	-15.1
DHANs	65.50	0.401	-23.2

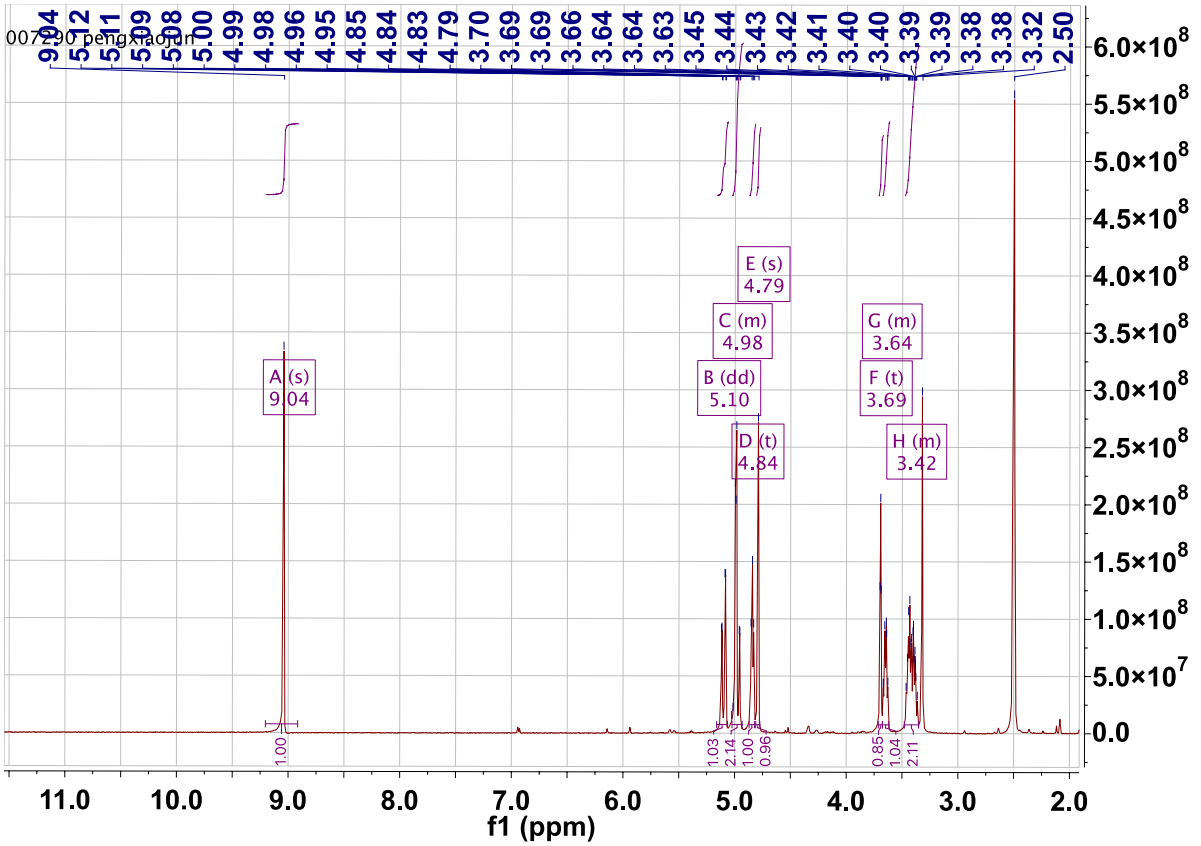


Figure S1. ^{1}H NMR spectra of DHA

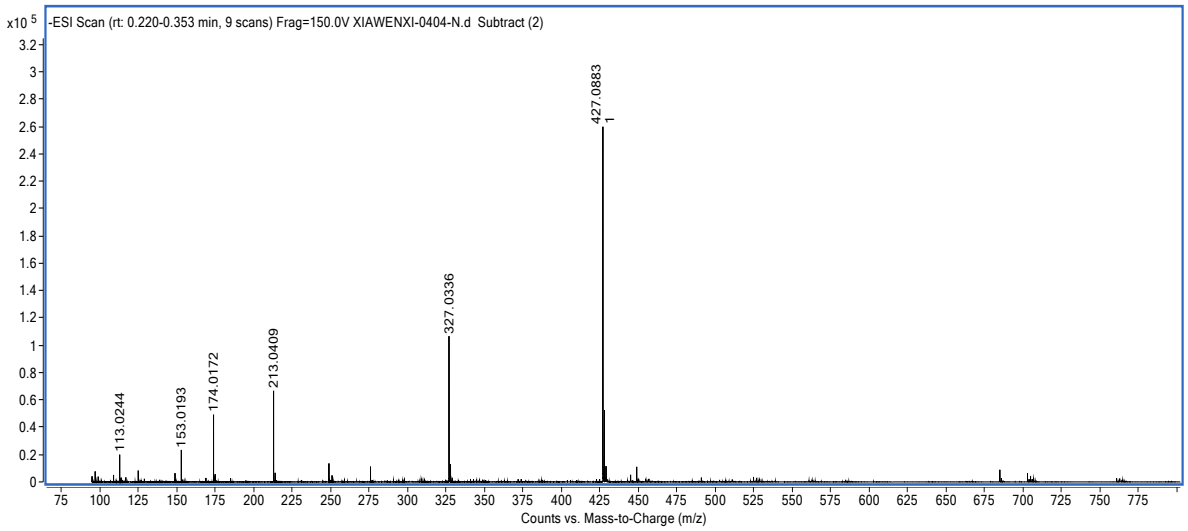


Figure S2. HRMS spectra of DHA.

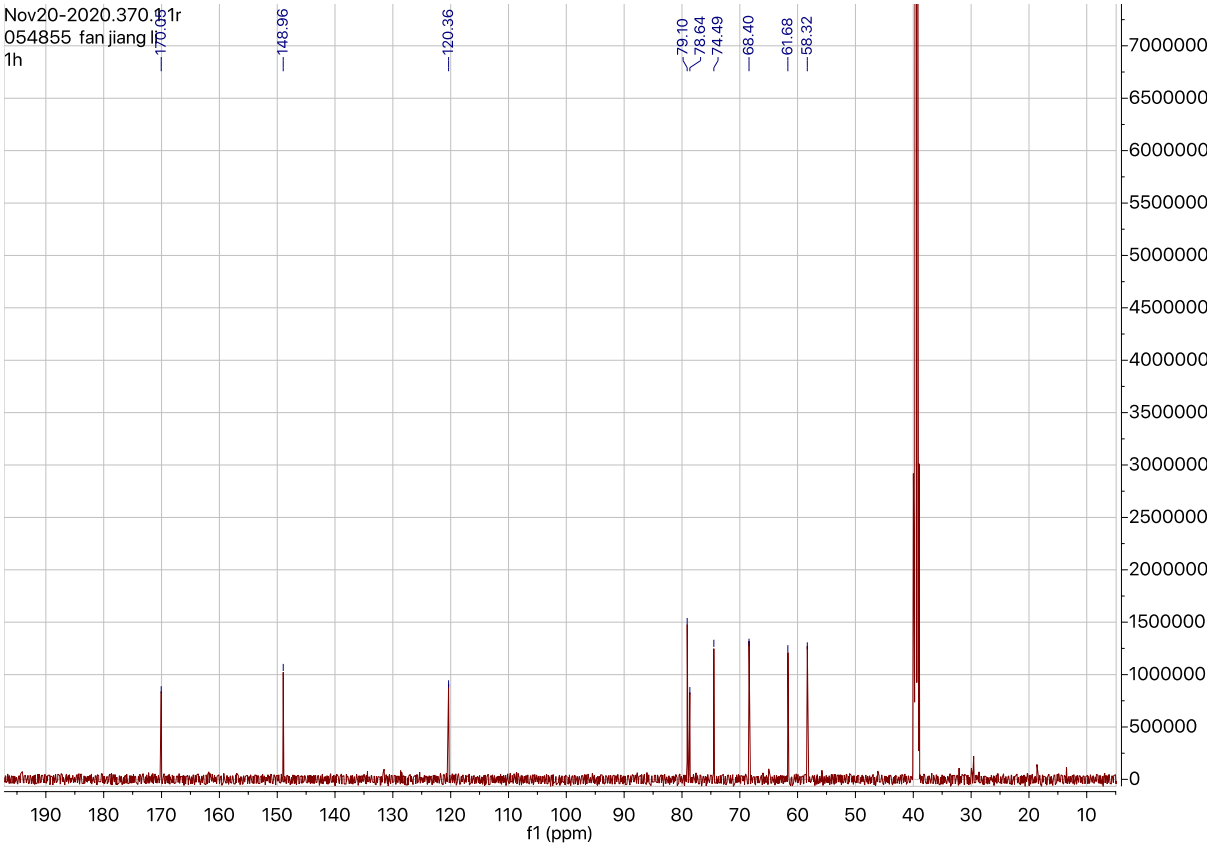


Figure S3. ^{13}C NMR spectra of DHA.

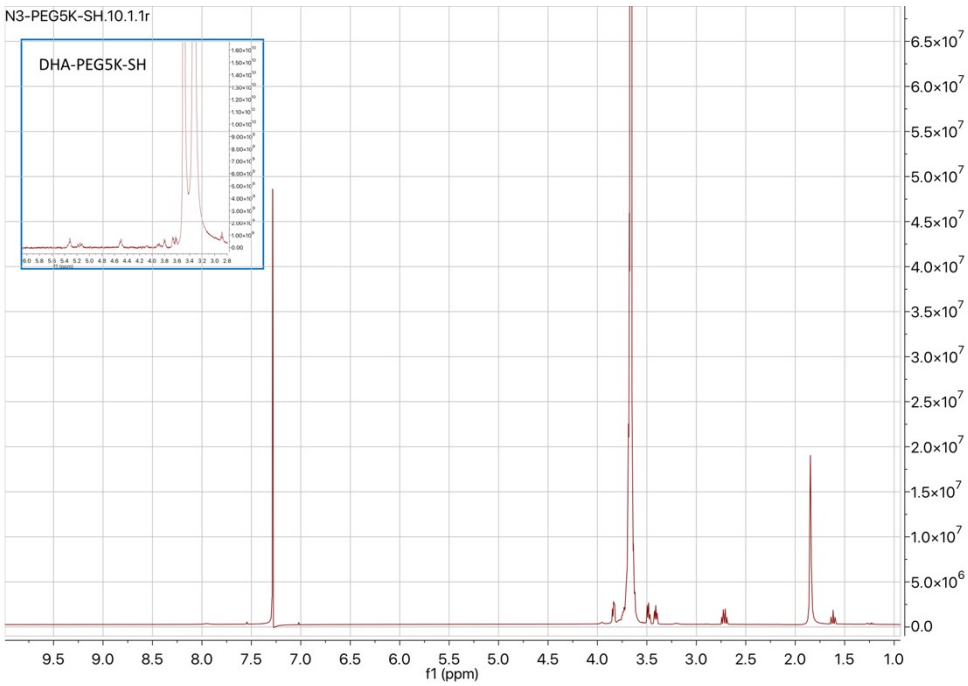


Figure S4. ^{1}H NMR spectra of DHA-PEG_{5000}-SH and N_3-PEG_{5000}-SH.

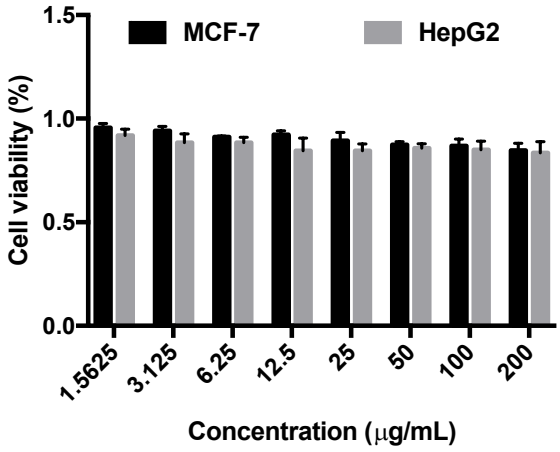


Figure S5. Cell viability of DNA-AuNPs in MCF-7 cells and HepG2 cells.

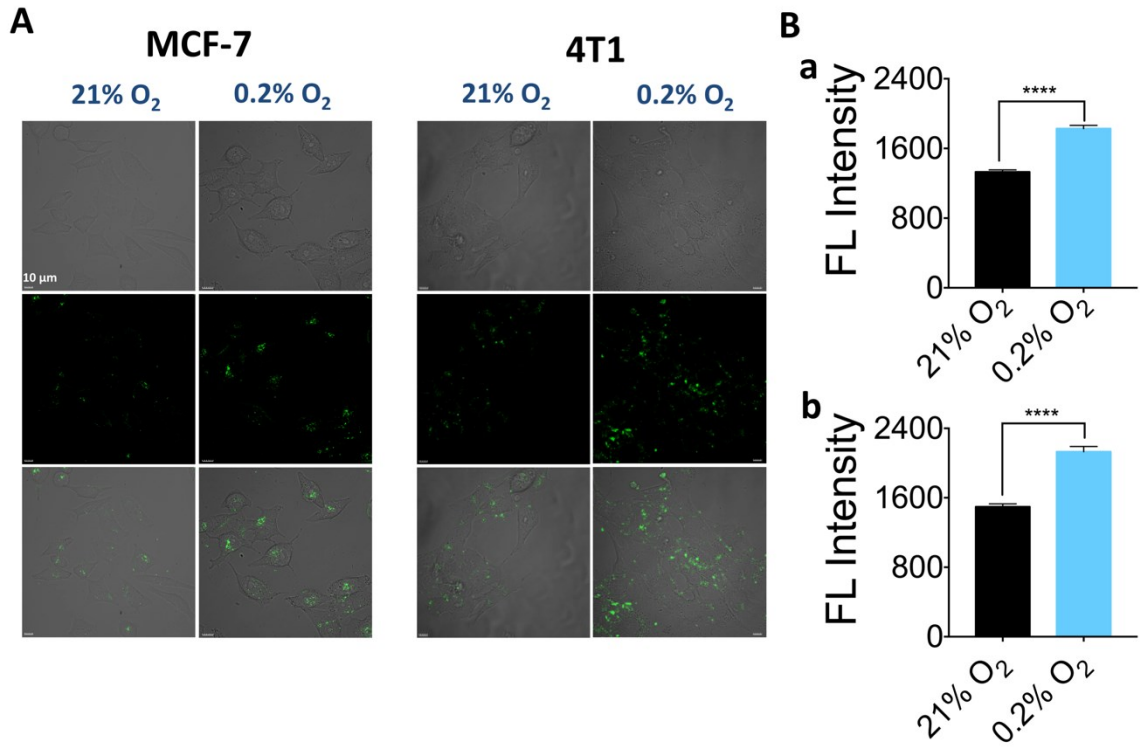


Figure S6. (A) Cellular uptake of DHANs (200 μg/mL) of MCF-7 cells and 4T1 cells in PBS medium under 21% or 0.2% O_2 conditions. DHANs: excitation wavelength = 488 nm, scan scope = 500-550 nm. Scale bar = 10 μm. (B) Quantitative analysis of average fluorescence intensity using the randomly selected 7 ROIs on each image of (a) MCF-7 cells and (b) 4T1 cells under normoxia and hypoxia environment. Date were assessed as mean ± SD *P < 0.05. **P < 0.01, ***P < 0.001, and ****P < 0.0001 determined by Student's *t* test.

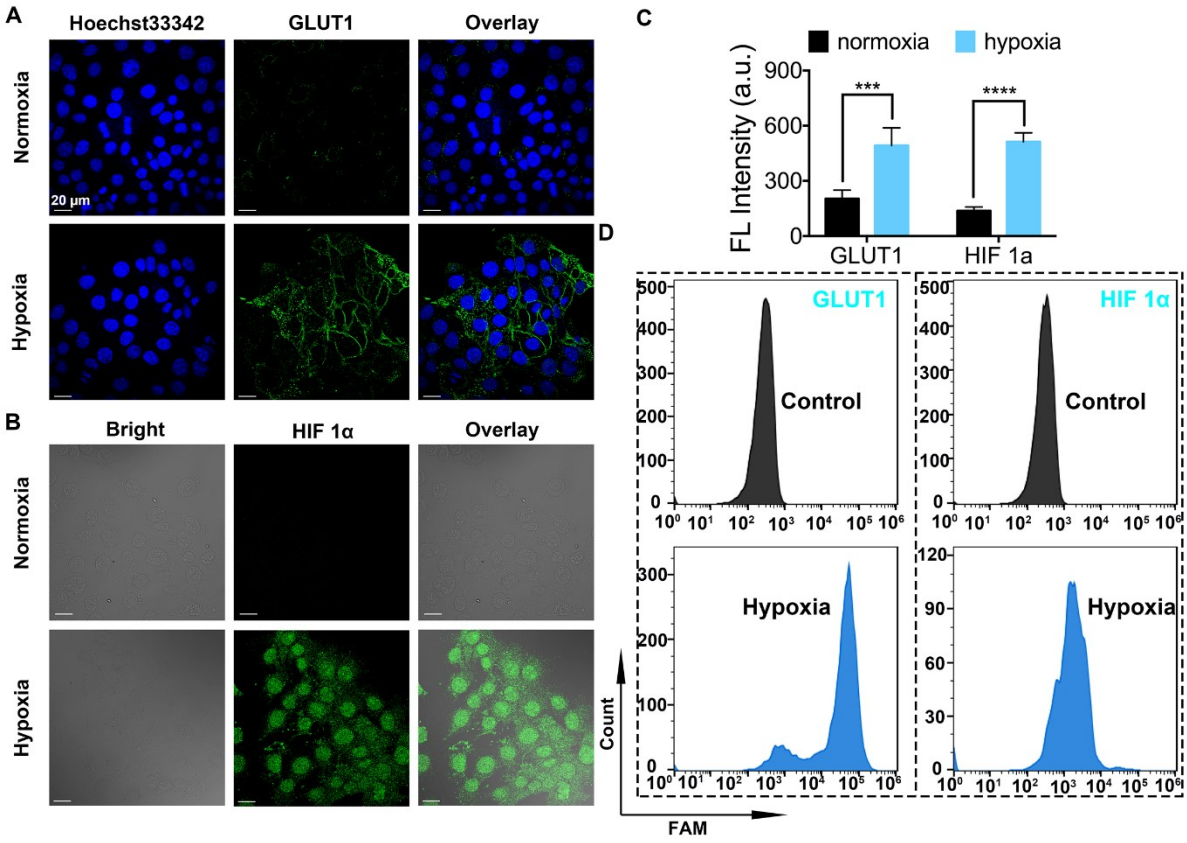


Figure S7. The expression of GLUT1 (A) and HIF 1α (B) in 4T1 cells under hypoxia and normoxia conditions by immunofluorescence. (C) The corresponding quantitative analysis of average fluorescence intensity using the randomly selected 7 ROI in each image under different conditions. (D) The expression of GLUT1 (A) and HIF 1α (B) in 4T1 cells under hypoxia conditions by flow cytometry.

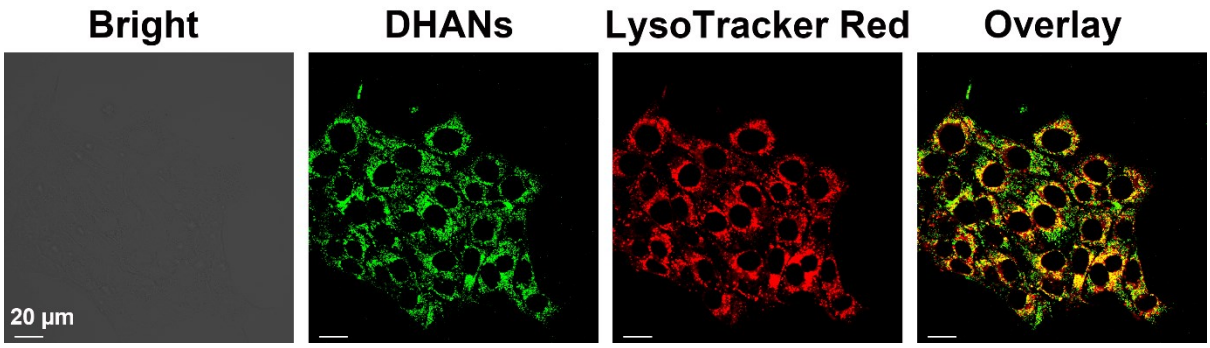


Figure S8. The co-localization of DHANs in 4T1 cells.

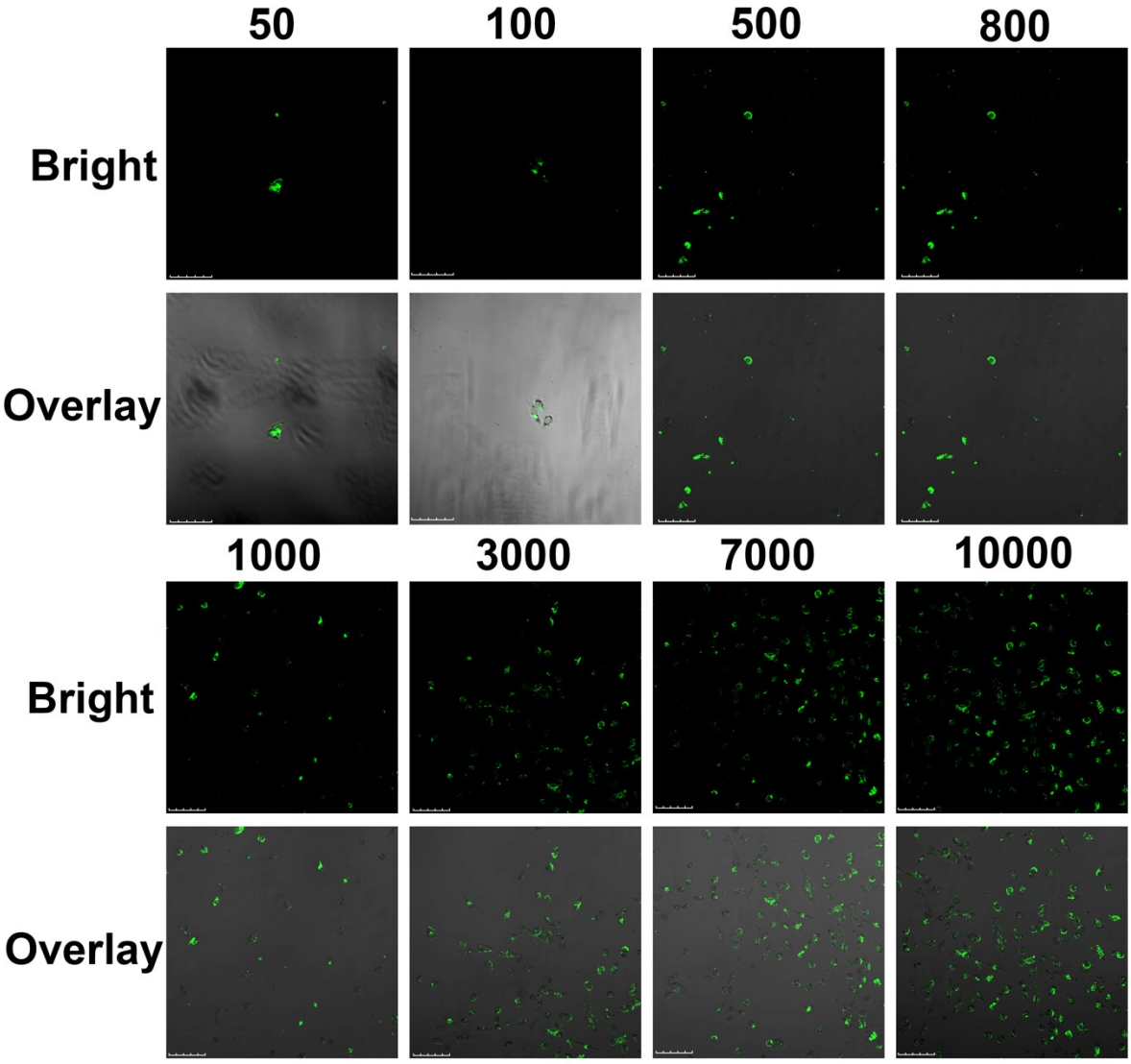


Figure S9. Fluorescence images of recognized spiked MCF-7 cells in whole sheep blood as mimic CTC samples. The FAM fluorescence represents MCF-7 cells. Excitation wavelength = 488 nm, scan scope = 500-550 nm. Scale bar = 100 μm.

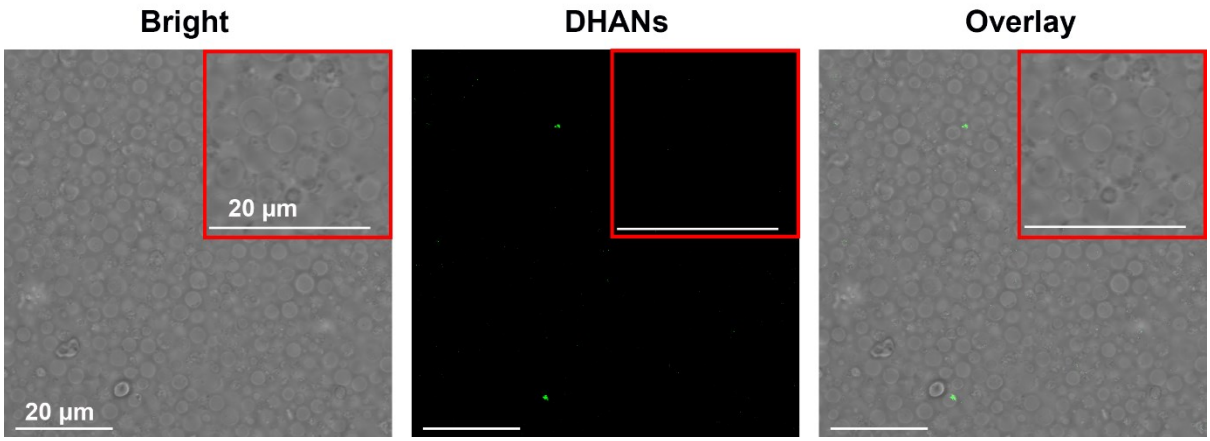


Figure S10. The CLSM imaging in the absence of added 4T1 cells.

References

1 L. Maus, O. Dick, H. Bading, J. P. Spatz and R. Fiammengo, *ACS Nano*, 2010, **4**, 6617–6628.

2 F. Degliangeli, P. Kshirsagar, V. Brunetti, P. P. Pompa and R. Fiammengo, *J. Am. Chem. Soc.*, 2014, **136**, 2264–2267.