Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary information

Tumor identification via in vivo portable Raman detection of sialic acid with dual gold nanoprobe system

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Experimental section

Materials and reagents. Chloroauric acid ($HAuCl_4 \bullet 4H_2O$) was obtained from Shanghai Chemical Reagent Company (Shanghai, China). Trisodium citrate was obtained from Sinopharm Chemical Reagent Co., Ltd. (China). N-hydroxysuccinimide (NHS), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC•HCl) and 3,3'-diethylthia tricarbocyanine iodide (DTTCI) were purchased from Aladdin Reagent Co., Ltd. (China). N-Acetylneuraminic acid was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (China). 3-Aminophenylboronic acid (3-APBA) was obtained from Sigma-Aldrich (USA). Thiol PEG 2K succinimidyl carboxymethyl ester (HS-PEG-NHS) and Thiol PEG 5K acid (HS-PEG-COOH) were purchased from Shanghai To Yong Bio (China). Phosphate buffered saline (PBS), Fetal bovine serum (FBS), Roswell Park Memorial Institute-1640 Medium (RPMI-1640), CCK8 assay kit, trypsin, and phosphate buffered saline (PBS) (pH 7.4, containing 136.7 mM NaCl, 2.7 mM KCl, 8.72 mM Na_2HPO_4, 1.41 mM KH_2PO_4, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) were purchased from KeyGen Biotech (China). Fluorescein *Sambucus Nigra* agglutinin (FSNA) was purchased from Vectorlabs (USA). All these reagents were used as received without further purification. All aqueous solutions were prepared using ultrapure water (≥18 MΩ, Milli-Q, Millipore).

Apparatus. The transmission electron microscopic (TEM) images were obtained on a JEM-2100 transmission electron microscope (JEOL Ltd., Japan). The scanning electron microscope (SEM) images were obtained on a JSM-7800F scanning electron microscope (JEOL Ltd., Japan). Dynamic light scattering (DLS) was performed on a 90 Plus/ BI-MAS equipment (Brook haven, USA). The UV-vis absorption spectra were recorded using a micro UV-vis spectrophotometer (Nanodrop-2000C, Nanodrop, USA). Fluorescence spectra were recorded on an F-7000 fluorescence spectrophotometer (Hitachi, Japan). Zeta potential analysis was performed on a Zetasizer (Nano-Z, Malvern, UK). Confocal fluorescence imaging of cells was performed on a SP8 STED 3X confocal laser scanning microscope (CLSM) (Leica, Germany). The cell concentration was determined using a Countess II FL automated cell counter (Life Technologies, USA). CCK8 assays were detected on a Varioskan Flash spectral scanning multimode reader (Thermo Fisher Scientific, USA). Raman images were collected on a Renishaw inVia confocal Raman microscope (Renishaw, UK) using 633 nm excitation. Raman spectra was collected on a Portman785 (Oceanhood, China) portable Raman detector using 785 nm excitation. The atomic absorption spectra (AAS) were performed on an Analytik Jena novAA350/ZEEnit650p atomic absorption spectrometer (Analytik Jena, Germany).

Synthesis of AuNP probes. Two sizes of gold nanoparticles (AuNPs) were prepared with different ratios of $HAuCl_4$ to trisodium citrate.[S1] The 10-nm AuNPs (Au10) were synthesized by adding 7.5 mL trisodium citrate (1%, w.t.) to 300 mL boiling $HAuCl_4$ solution (0.01%, w.t.), while 40-nm AuNPs (Au40) were synthesized by adding 3 mL to 300 mL boiling $HAuCl_4$ solution (0.01%, w.t.). After 15 min of boiling, the mixtures were cooled down and stored at 4 ^oC. Prior to use, the initial citrate-particle solution was removed by discard of the supernatants after centrifugation under 12000 rpm for Au10 or 6000 rpm for Au40. The AuNPs were resuspended in DI water and centrifugated again to further clean the minimal solution left over the particle pellet by discard of the supernatants, which was regarded as a "wash". The concentrations of AuNPs were determined using UV-vis absorption spectrometry.[S2]

To synthesize Au10-DTTC/PEG-PBA, HS-PEG-NHS (1mM, 5 μL) and DTTCI (1 mM, 20 μL) were added to 1 mL Au10 solution (10 nM) with slight shaking at room temperature for one night. After washed with water by centrifugation at 12000 rpm for 10 min twice, 3-APBA (1mM, 10μL) were added into the

obtained solution and shaked at room temperature for 12 h. The obtained Au10-DTTC/PEG-PBA were washed with PBS by centrifugation at 10000 rpm for 15 min twice and resuspended to 1 mL in PBS. The Au40-DTTC/PEG-PBA was obtained with similar procedure by replacing Au10 with Au40.

To synthesize Au40-PEG-SA, HS-PEG-COOH (1mM, 5 μL) was added to 1 mL Au40 solution (1 nM) and stirred at room temperature for 10 min. After washed with PBS by centrifugation at 6000 rpm for 10 min twice, the resuspended solution in 1 mL PBS was firstly incubated with EDC (3mg·mL^{-1}) and NHS (3mg·mL^{-1}) at room temperature for 1 h, which were subsequently added with SA (1mM, 10 μL) incubated for 12 h at room temperature. The obtained Au40-PEG-SA was washed with PBS by centrifugation at 6000 rpm for 10 min twice and resuspended to 1 mL PBS.

Cell culture. 4T1 and MCF-10A cells were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 μg·mL^{-1}), and streptomycin (100 μg·mL^{-1}) at 37 °C in a humidified atmosphere containing 5% CO_2, respectively.

Raman imaging of SAs on cell surface. 4T1 or MCF-10A cells seeded on confocal dishes were firstly incubated with 120 μL Au10-DTTC/PEG-PBA (10 nM) at 37 °C for 1 h. After washed with PBS twice, the cells were subsequently incubated with 120 μL Au40-PEG-SA (1 nM) for another 1 h at 37 °C. The obtained cells were washed with PBS twice and subjected to Raman imaging.

The Raman imaging acquisition mode was static scan type at a center wavenumber of 1000 cm^{-1} with 1-s exposure time, 1-time accumulation and 100% laser power, and 5 μm × 5 μm step. The Raman images of cells were generated using signal at point review mode at 1129 cm^{-1} by a WiRE 3.4 software using black to red color scale. The average Raman intensity of cells was obtained from statistics mean value by dividing the total red channel value with the total cell membrane length within the chosen Raman image area using Photoshop CS6 software.

SEM analysis of cells. The 4T1 cells were seeded in a 35-mm ITO slide. After undergoing adherent growth and the subsequent processing steps, cells were washed three times with PBS, and dealt with 4% glutaraldehyde fixation, gradient dehydration, and natural drying before subjecting to SEM imaging.

Animal experiments. All animal experiments were performed in strict accordance with the Laboratory Animal Management of Jiangsu Province for the care and use of laboratory animals (License No. SYXK 2019-0056) and was approved by the Department of Science and Technology of Jiangsu Province (Nanjing, China). The subcutaneous tumor xenograft models were prepared by inoculating 4T1 mouse breast cancer cells (1 × 10^6 cells/mouse) into the hind leg of the nude mice (female, 6-8 weeks).

After the tumor volume risen to 1 cm^3, 100 μL Au10-DTTC/PEG-PBA (10 nM) or Au10-DTTC/PEG (10 nM) were firstly subcutaneous injected at the tumor periphery of the mice. After a certain time, 100 μL Au40-PEG-SA (1 nM) or Au40-PEG (1 nM) were subsequently injected at different locations with distance of 2 cm from the tumor center. The portable Raman measurements were performed with a Portman785 system. A semiconductor diode near-infrared laser was used as the excitation source (wavelength: 785 nm, intensity: 30 mW, cumulative time: 10 s). Raman spectra were collected at the skin in tumor region.

At 4 h or 24 h after injections, the mice were euthanized to collect 0.1g of major organs (including heart, liver, spleen, kidney, and lung), which were digested in HNO_3 at 80 °C overnight, and the obtained yellow powders were dissolved in 3% HNO_3 for AAS analysis.

At 7 days after injections, the mice were euthanized to collect major organs (including heart, liver, spleen, kidney, and lung), which were harvested for routine staining with hematoxylin and eosin for histological observations.

Blood samples (approximately 250 μL/sample) were collected via orbital puncture from each mouse. For hematology, whole blood was collected in a potassium EDTA collection tube. The stabilized blood

with EDTA was centrifuged at 3500 rpm for 15 min and the serum was removed. Subsequently, the red blood cells (RBCs) were washed 5 times with isotonic PBS and dispersed in 5 times the volume of blood in isotonic PBS. 30 μL RBCs solutions were respectively added with 120 μL ultrapure water (positive), PBS (negative), Au10-DTTC/PEG-PBA (10 nM), Au40-PEG-SA (1 nM), combinations of Au10-DTTC/PEG-PBA (60 μL, 20 nM) and Au40-PEG-SA (60 μL, 2 nM). The mixtures were incubated in a 37 ^{o}C water bath for 3 h and centrifuged at 3500 rpm for 15 min. The hemoiysis rate was determined by (Abs_{test} - $Abs_{negative}$ / $Abs_{positive}$ - $Abs_{negative}$) × 100% through the absorbances (Abs) values of each sample at 570 nm.

Supplementary Figures

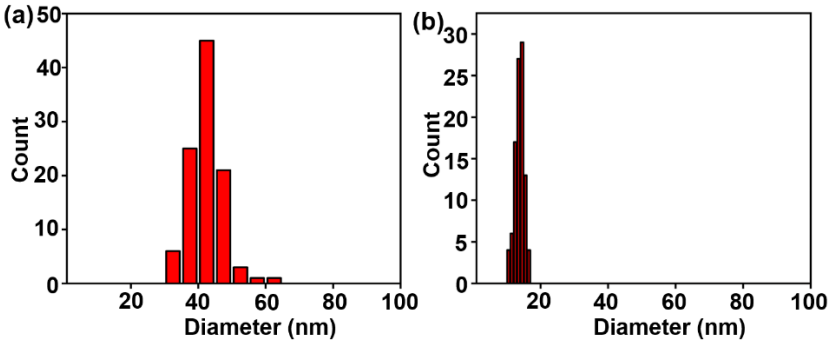

Fig. S1 Counts of the size distributions of (a) Au40-PEG-SA and (b) Au10-DTTC/PEG-PBA from TEM images in Fig. 1 using ImageJ software.

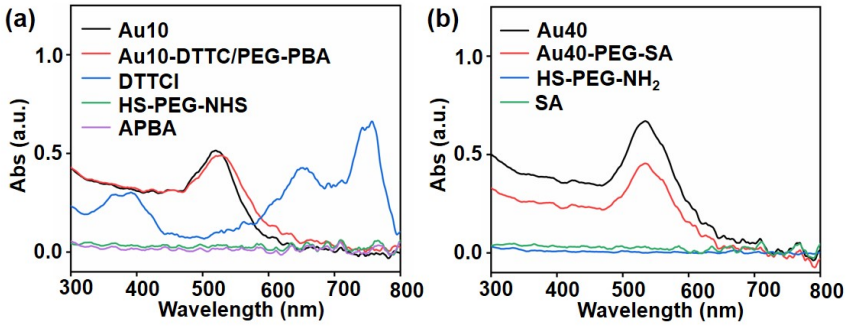

Fig. S2 UV spectra of (a) 5 nM Au10, 4 nM Au10-DTTC/PEG-PBA, 0.2 nM DTTCI, 0.5 nM HS-PEG-NHS, 0.5 nM APBA, and (b) 0.07 nM Au40, 0.05 nM Au40-PEG-SA, 0.5 nM HS-PEG-NH_2, 0.5 nM SA.

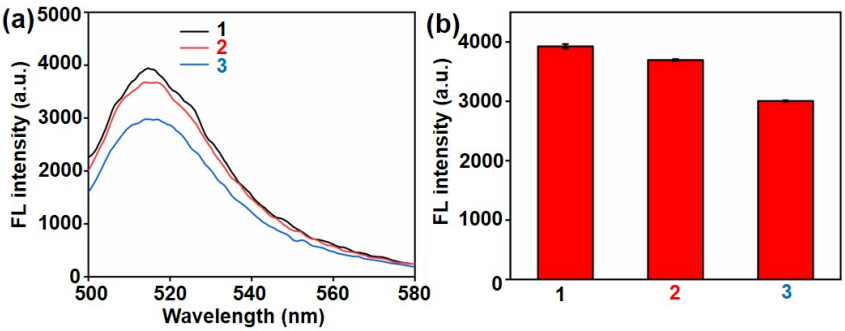

Fig. S3 (a) Fluorescence spectra of 5 μg/ml FSNA solutions (1), the supernatants of 5 μg/ml FSNA incubated with 0.2 nM Au40-PEG (2) or 0.2 nM Au40-PEG-SA (3). (b) Histograms of the fluorescence intensity at 518 nm from (a). Error bars represent ± S.D. (n=3).

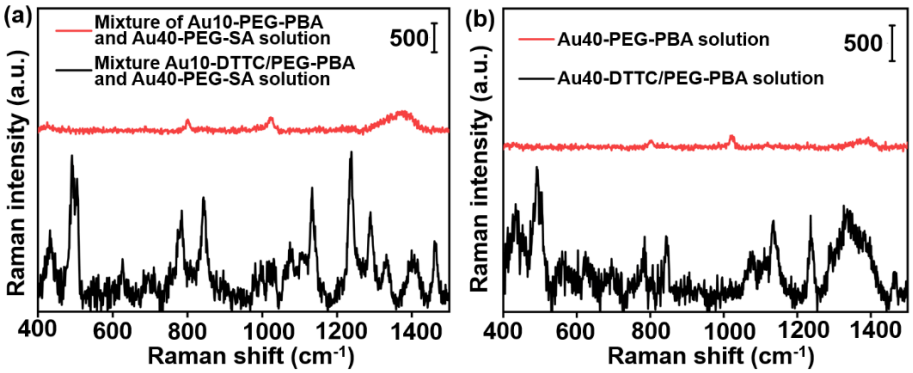

Fig. S4 Raman spectra of (a) the mixture of Au10-PEG-PBA with Au40-PEG-SA, and Au10-DTTC/PEG-PBA with Au40-PEG-SA solutions, and (b) Au40-DTTC/PEG-PBA and Au40-PEG-PBA solutions (wavelength: 785 nm, intensity: 30 mW, cumulative time: 1 s)

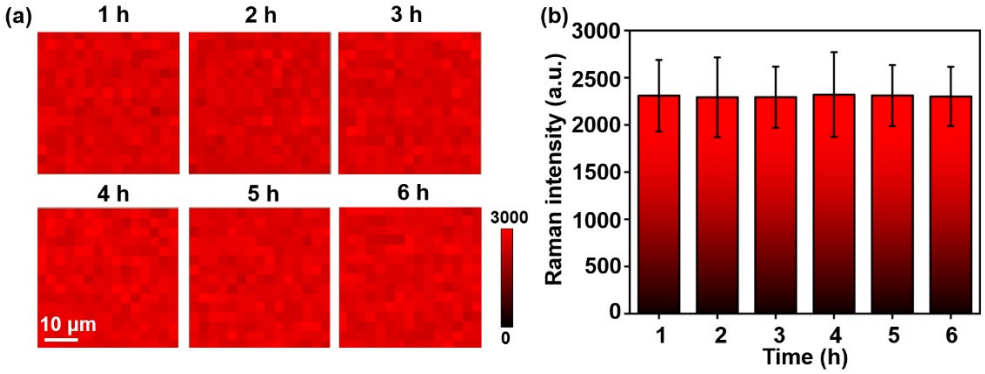

Fig. S5 (a) Raman imaging of the mixture solutionS of 10 nM Au10-DTTC/PEG-PBA and 2 nM Au40-PEG-SA for 1 - 6 h. All images have same scale. (b) Statistical Raman intensities from (a).

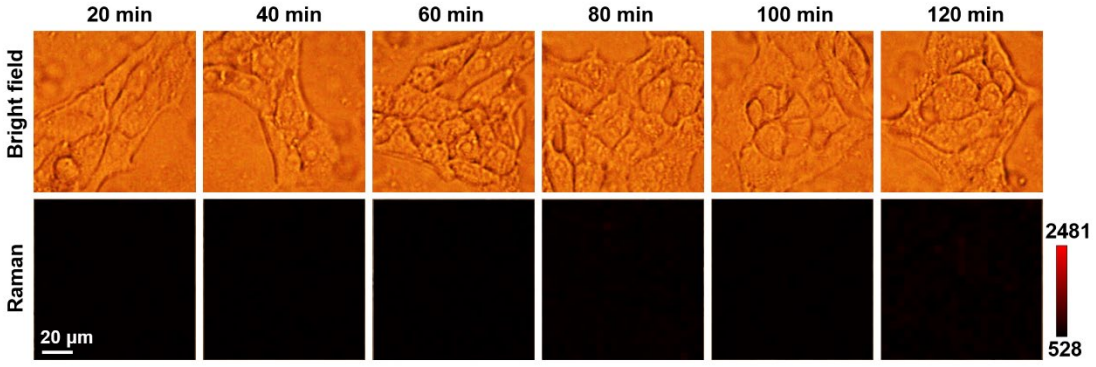

Fig. S6 Raman imaging of 4T1 cells incubated with 10 nM Au10-DTTC/PEG-PBA for 20 - 120 min without washing. All images have same scale.

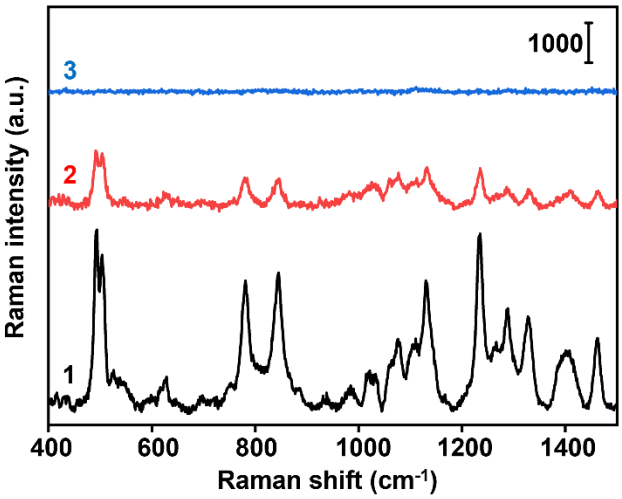

Fig. S7 Raman spectra of (1) the incubating solution of 4T1 cells with 10 nM Au10-DTTC/PEG-PBA for 1 h, (2) the first PBS washing solution, and (3) the second PBS washing solution, which were all mixed with 5 nM Au40-PEG-SA repectively before Raman detection.

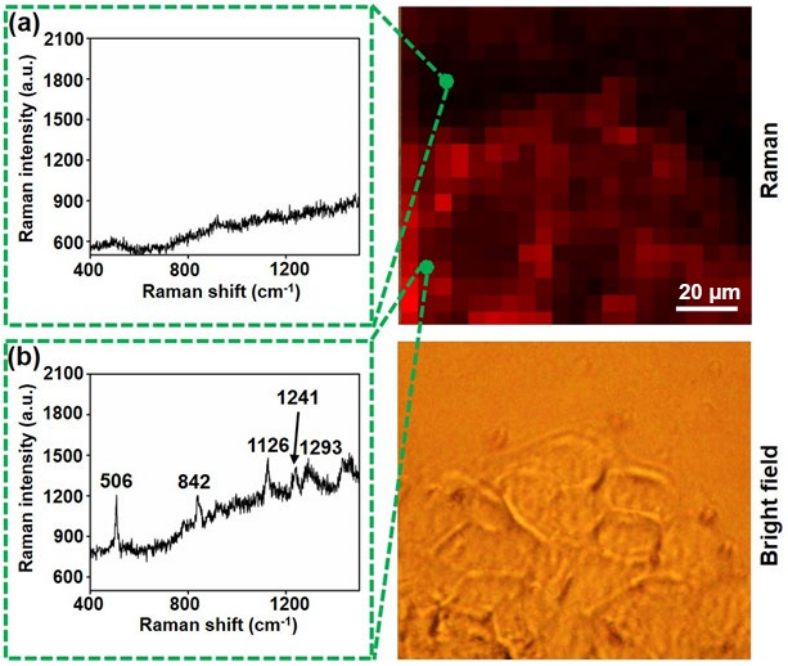

Fig. S8 Representative Raman spectra at different locations on Raman images of 4T1 cells.

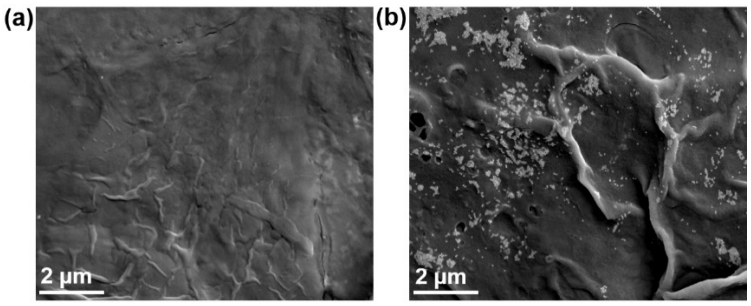

Fig. S9 SEM images of 4T1 cell incubated with (a) Au10-DTTC/PEG-PBA, and (b) Au10-DTTC/PEG-PBA and then Au40-PEG-SA.

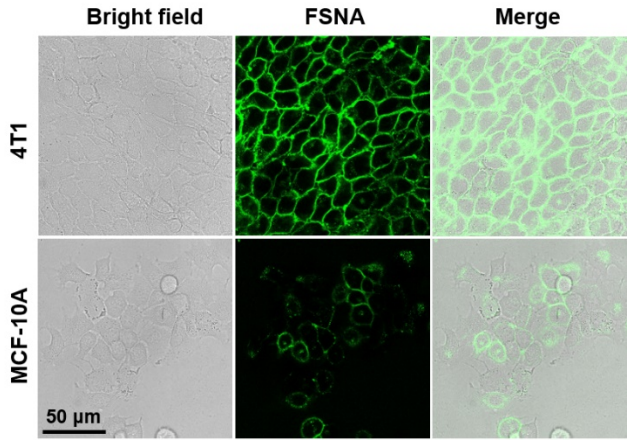

Fig. S10 Confocal fluorescence images of 4T1 and MCF-10A cells incubated with 5 μg/ml FSNA solution. All images have same scale.

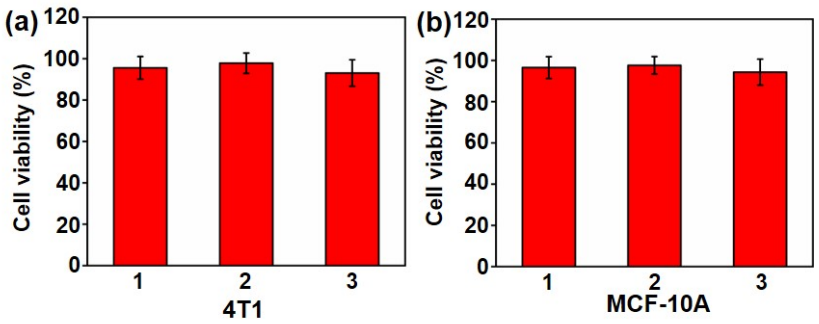

Fig. S11 CCK8 assay of (a) 4T1 and (b) MCF-10A cell viability after incubation with 10 nM Au10-DTTC/PEG-APBA for 6 h (1), 1 nM Au40-PEG-SA for 6 h (2), and 10 nM Au10-DTTC/PEG-APBA for 3 h and then 1 nM Au40-PEG-SA for 3 h (3), respectively. Error bars represent ± S.D. (n=3).

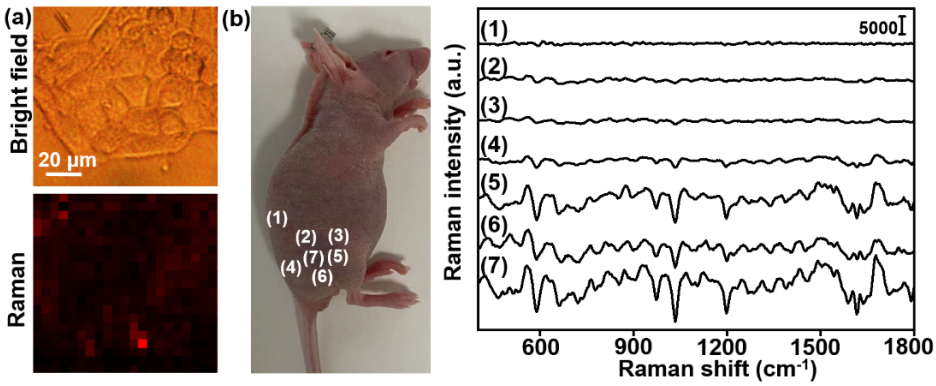

Fig. S12 (a) Bright field and Raman imaging of 4T1 cells incubated with 1 nM Au40-DTTC/PEG-PBA for 1 h. (b) Raman spectra obtained from different locations around the injection position of normal mice after injections of Au40-DTTC/PEG-PBA for 4 h.

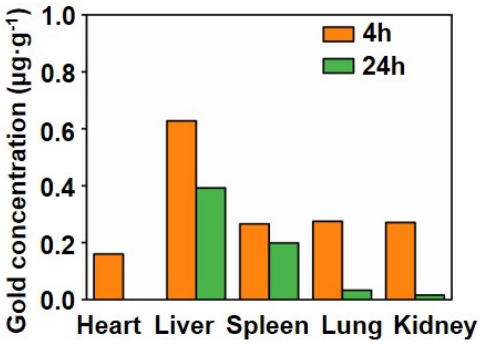

Fig. S13 Gold concentrations remained in different tissues from 4T1 tumor xenograft mice after injected with 100 μL Au10-DTTC/PEG-PBA (10 nM) and Au40-PEG-SA (1 nM) (with an interval time of 2 h) for 4 and 24 h.

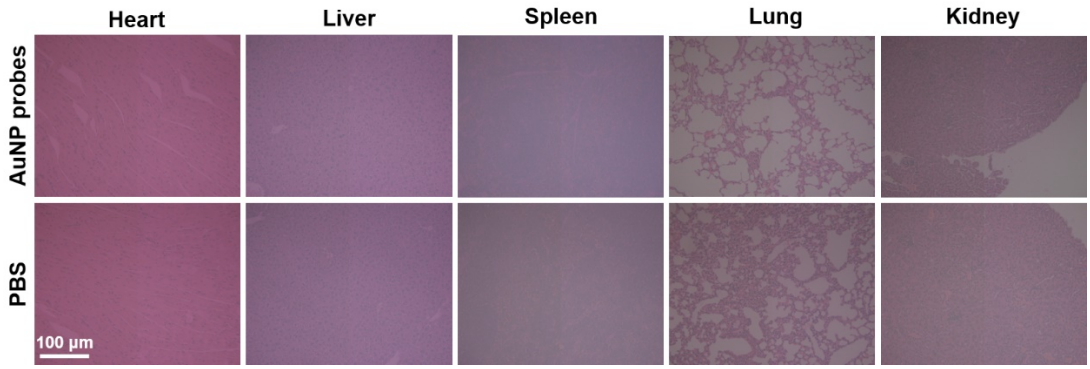

Fig. S14 Histological observations of the normal tissues from the 4T1 tumor xenograft mice after injection of two AuNP probes for 7 days. All images have same scale.

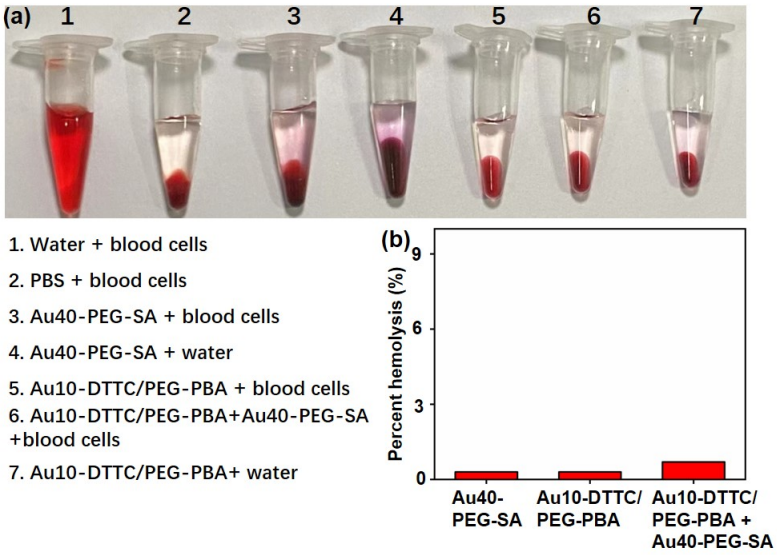

Fig. S15 (a) Photographs of the 4T1 tumor xenograft mice blood incubated with water, PBS, and different AuNP probes. Samples 3 and 4 were centrifuged at 3500 rpm for 15 min to precipitate blood cells and Au40 probes. Samples 5, 6 and 7 were firstly centrifuged at 3500 rpm for 15 min to precipitate blood cells. The corresponding supernatant were collected and centrifuged at 11000 rpm for another 15 min to precipitate Au10 probes. (b) Histograms of the percent hemolysis. Sample 1 and 2 in (a) were used as the positive and negative controls, respectively. The hemoiysis rate was determined by (Abs_{test} - $Abs_{negative}$ / $Abs_{positive}$ - $Abs_{negative}$) × 100% using the absorbances (Abs) values of each sample at 570 nm.

Supplementary References

S1 G. Frens, *Nat. Phys. Sci.,* 1973, **241**, 20–22.

S2 W. Haiss, N. T. K. Thanh, J. Aveyard and D. G. Fernig, *Anal. Chem.,* 2007, **79**, 4215–4221.