Supporting Information

Facile *in Situ* Synthesis of Ultrasmall Near-Infrared-Emitting Gold Glyconanoparticles with Enhanced Cellular Uptake and Tumor Targeting

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Experimental Section

Materials and equipment

Chloroauric acid trihydrate ($HAuCl_4 \bullet 3H_2O$) and glutathione (GSH) were purchased from Sigma-Aldrich (St Louis, MO, USA). The 1-thiol-β-D-glucose (TG) was obtained from Beijing Chemsynlab Co., Ltd. Phosphate Buffered Saline (PBS) was purchased from Gibco (Thermo Fisher Scientific, Suzhou). All chemical reagents were used as received without further purification. All glassware was cleaned with fresh aqua regia before use. Ultrapure water (18.2 $M\Omega \cdot cm^{-1}$) was obtained from a Life Sciences purification system (PALL, USA). The fluorescence spectra were collected with a LS55 fluorescence spectrophotometer (PerkinElmer, US). The absorption spectra were obtained on a UV-vis Spectrophotometer UV-2600 (Shimadzu, Japan). Transmission electron microscopic (TEM) images were acquired with a transmission electron microscope (JEM2100F, JEOL, Japan) with an accelerating voltage of 200 kV. Cell viability was measured by an automatic microplate plate reader Elx800 (BioTek, USA). The fluorescence imaging experiments were performed on an inverted fluorescence microscope (Olympus IX83 with disk scanning unit IX3-DSU, Japan) with an Evolve 512 Delta EMCCD camera through Xe-lamp excitation. The Au concentrations were determined by an Inductively Coupled Plasma mass spectrometry (ICP-MS) (Agilent7700, USA). The *in-vivo* near-infrared (NIR) fluorescence imaging studies were performed on a UVP ChemStudio PLUS 815 *in-vivo* imaging system equipped with a 150 W Xe-lamp (Analytikjena, USA).

Synthesis of 1-thiol-β-D-glucose (TG) capped NIR-emitting gold nanoparticles (AuGNPs)

The NIR-emitting AuGNPs were synthesized by a thermal reduction method according to our previously reported protocol with modifications in the concentrations.[1] Typically, into a 100-mL three-necked flask were mixed with 4.8 mM TG solution and 1.2 mM $HAuCl_4$, and then heated in oil bath at 95 °C for 25 min under vigorous stirring until the

fluorescence intensity of the solution reached the maximum monitored by a fluorescence spectrometer. Once the reaction is complete, the resulting solution was centrifuged at 21000 *g* to remove the large nanoparticles before the excess of ligand were removed by ultrafiltration using a 3 kDa membrane filter (15000 *g*× 10 min × 3 cycles). After the purification of the AuGNPs solution, the concentrated AuGNPs were diluted with PBS before the tail intravenous injection.

Surface coverage of AuGNPs

The calculation process was according to our previously reported methods.[2, 3] Chemical formula of AuGNPs can be expressed as Au_xTG_y, TG = 1-Thio-β-D-glucose sodium salt containing a deprotonated thiol group ($C_6H_{11}NaO_5S$, 218.2 g/mol), x = number of Au atom, y = number of TG. The surface coverage of TG on AuGNPs could be calculated with y/x.

The Calculation of x: For a spherical metal nanoparticle, the number of metal atom in this particle (N) can calculated with following equation:

$R = r_s \bullet N^{1/3}$, R = particle radius, r_s = Wigner-Seitz radius

For the AuGNPs with average diameter = 2.4 nm (r_s = 0.145 nm for Au), $x = N$ =566.

The Calculation of y:

y could be calculated from the results of element analyses according to the following equation:

$[12 \times y \times 6] / [x \times 197 + y \times 218.2]$ = C% (for carbon content) = 8.54%

y = 178

Surface coverage (%) = 31.4%

Synthesis of the TG capped large plasmonic gold nanoparticles (p-AuGNPs, 13 nm)

The p-AuGNPs were synthesized using a two-step method. Firstly, the citrate coated AuNPs (13 nm) were prepared with a previously reported method.[4] into a 100-mL three-

necked flask was boiled with 50 mL $HAuCl_4$ (1.0 mM) for 30 min at 120 ^oC oil bath, and then added 5 mL sodium citrate solution (38 mM) under vigorous stirring. After another 20 min of boiling, the mixture solution was allowed to cool to the room temperature until the color of solution changed to wine red. Secondly, 10 mg of bis(p-sulfonatophenyl)phenylphosphine dihydrate dipotassium salt were added into 50 mL of the above AuNPs solution, followed by gently stirring at room temperature overnight. Then 1 mL TG solution (0.1 M) was introduced to the above solution and reacted for another 6 h. During the reaction, sodium solution (0.1 M) was added in batches to improve the efficiency of TG modification.

Cell culture

The human breast cancer cell line MDA-MB-231 was cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/ streptomycin at 37 ^oC in a humidified atmosphere containing 5% CO_2.

Cell imaging and cellular uptake of AuGNPs

For the cell imaging, MDA-MB-231 cells were seeded on 35-mm cell culture dish, and incubated in low glucose DMEM ($C_{glucose}$ = 1.0 mg/mL) overnight. In order to investigate whether exogenous glucose effect the cellular uptake, we then prepared a series of cell medium containing exogenous glucose for the cell culture. Into a 15-mL tapered plastic centrifuge tube was added exogenous glucose (C: 0, 1.5, 3.0, 4.5, and 6.0 mg/mL) in the low glucose DMEM, followed by replacing the original medium with the above cell medium added with different amount of glucose for 30 min incubation, respectively. The AuGNPs (20.0 μM) were then added and incubated for 6 h. Subsequently, the medium were removed, and the DPBS were added before the cell imaging. Each group was imaged at least three times to ensure data reliability.

For the cellular uptake, MDA-MB-231 cells were seeded at 10^5 cells/well in a 12-well plate and incubated overnight. Then the cells was performed with the above treated cell medium containing exogenous glucose (Concentration: 0, 1.5, 3.0, 4.5, and 6.0 mg/mL) for 30 min. The AuGNPs (20.0 μM) were then added into each well and incubated for 6 h. The medium was removed, and the cells were washed with DPBS for three times. The trypsin (0.25%, 200 μL) were added in each well before the addition of 1 mL DPBS. Finally, the collected cells were lysed in glass bottle by using fresh aqua regia, and boiled at 120 ^{o}C to evaporate the aqua regia. Once the complete evaporation was finished, the HCl (0.05 M, 2 mL) was added to dilute the samples before the measurement of Au content by ICP-MS.

For the cellular uptake inhibition studies of AuGNPs, the cytochalasin B (*C*: 0, 5 and 10 μM) was pre-added and incubated in the low glucose DMEM (exogenous glucose: 3.0 mg/mL) for 30 min as the experimental group before the addition of AuGNPs (20 μM). After 6 h incubation of AuGNPs, the cells were washed for three times with PBS and harvested in trypsin. Then cell samples were collected and lysed before the measurements of the Au content using ICP-MS.

Construction of tumor-bearing mice model

Female nude mice (5-7 week old) were purchased from Guangdong medical laboratory animal center. The mice were housed in ventilated cages with number of 3~4 each cage under standard environmental conditions (23 ± 1 ^{o}C, 50 ± 5% humidity and a 12/12 h light/dark cycle) with free access to water and standard laboratory food.

In order to investigate the *in-vivo* tumor-targeting of the AuGNPs, we constructed the MDA-MB-231 tumor-bearing mice model. The typical construction method were described as follows: MDA-MB-231 cells (~2×10^6 cells for each mouse) were suspended in 100 μL DMEM, then the matrigel with the volume ratio of 1/1 (*v*/*v*) was mixed with DMEM. Subsequently, the resulting dense suspension of 200 μL was injected into

mammary fat pad area through subcutaneous inoculation. The tumor was allowed to grow for about two weeks later with observable size of 6~8 mm in diameter.

In-vivo and *ex-vivo* tumor imaging

The nude mice for the *in-vivo* tumor imaging studies were anesthetized ~5 min with ~2% isofluorane in oxygen flowing at 0.8 L/min, and then pre-imaged before the intravenous (iv) injection. The parameters used in the imaging were described as follows: Excitation filter: 450 nm short; Emission filter: 800 nm longer; Exposure time: 45 s. The imaging parameters were kept all the same at each time point used both for the *in-vivo* and *ex-vivo* imaging. The Au concentration of the AuGNPs for the tail intravenous injection was determined to be ~27 mg/mL by ICP-MS method. The MDA-MB-231 tumor-bearing nude mouse was imaged at different time points of post injection (p.i.) for 72 h. For the *ex-vivo* imaging, the mice were dissected at 1, 24, 48 and 72 h p.i., respectively, and then the major organs as well as tumor were collected, followed by imaging with the *in-vivo* imaging system immediately.

Pharmacokinetics Studies

The blood samples of the mouse iv injected with 250 μL AuGNPs (C_{Au} = 27 mg/mL) were collected from the eye socket at 2, 5, 10, 30 min, 1, 3, 5, 8, 12, 24, 48 and 72 h p.i.. The pretreatments of blood samples were described as follows: The net weights of blood samples were obtained before lysing in a glass bottle by using the 2 mL fresh aqua regia for 5 min. The lysed blood samples were heated at 120 °C in oil bath until the aqua regia was completely evaporated. Then HCl (8.0 mL, 0.05 M) was added into the glass bottle for redissolution of the residue of each blood sample after the evaporation, followed by sonicating for 30 min. Finally, the Au concentrations of the resulting samples were measured by ICP-MS.

Biodistribution studies

To further assess the biodistributions of AuGNPs, the MB-MDA-231 tumor-bearing mice (n = 3, each group) iv injected with AuGNPs were sacrificed at 0.5, 1, 12, 24, 48 and 72 h p.i.. Then the collected organs and tumors were cut into pieces and weighted before the pretreatments. The pretreatment method was similar as described in the part of "Pharmacokinetics studies". The Au concentrations of resulting samples were determined using ICP-MS.

Purification of hepatocytes

The mice (n = 5, each group) iv injected with AuGNPs, GS-AuNPs or p-AuGNPs were anesthetized at 6 h p.i.. A heat lamp was used to maintain the mice body temperature at 37 ^{o}C during the liver perfusion. After exposing the peritoneal cavity and thoracic wall, gastrointestinal organs were softly moved to the side in order to locate the inferior vena cava. The suprahepatic inferior vena cava was clamped with hemostatic forceps to keep localized perfusion. Then the subhepatic inferior vena cava was inserted with scalp vein set attached with 50-mL syringe, the liver was first perfused with DPBS buffer. Once the perfusion started, the portal vein was rapidly cut to make DPBS flow through the liver freely. Until the blood from liver was no longer visible, the liver was excised before the gallbladder was removed. Then the whole liver in DMEM was torn apart for release of the cells from Glisson's capsule. The saturated DMEM was passed through a cell strainer (40 μm) to filter the liver remnants. Whole blood red cell lysing reagent was also added into the cellular liquid and incubated for 5 min to further lyse the red cell. Then the cellular liquid was centrifuged at 100 *g* for 3 min at 4 ^{o}C. The precipitate was resuspended with buffer solution and centrifuged again under the same condition. Thus the purified hepatocytes were counted by cytometry method and collected to determine the Au amount in hepatocytes using ICP-MS.

Supplementary Data

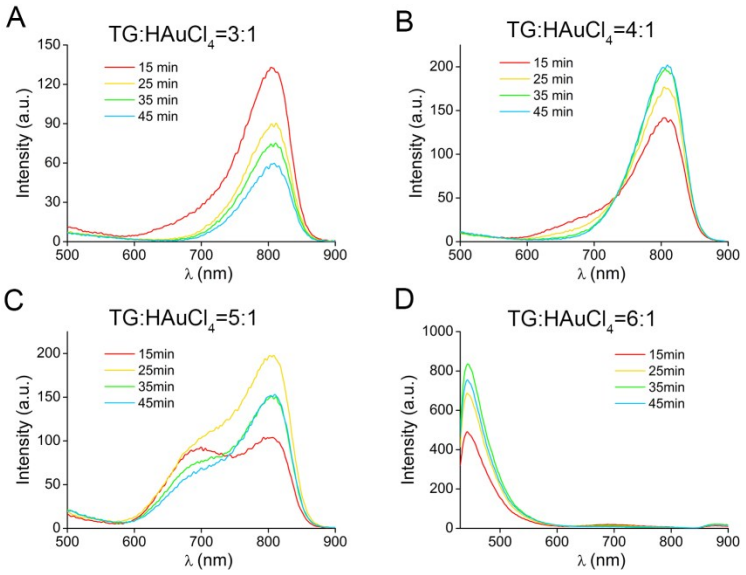

Fig. S1 The luminescence spectra of the AuGNPs synthesized at different mole ratios of TG to $HAuCl_4$. A) 3:1, B) 4:1, C) 5:1 and D) 6:1.

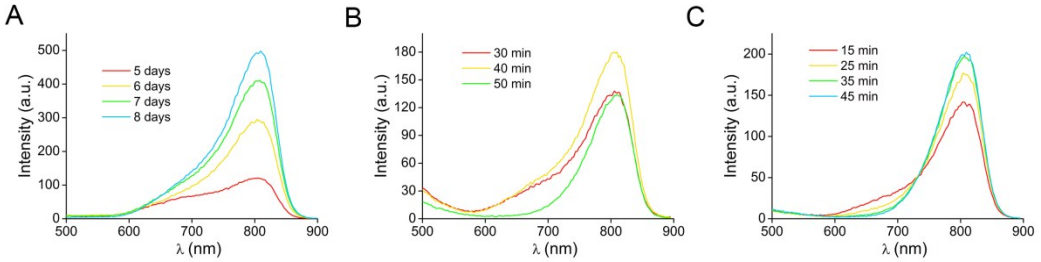

Fig. S2 The luminescence spectra of the AuGNPs synthesized at different temperatures. A) 25 ℃, B) 60 ℃ and C) 95 ℃.

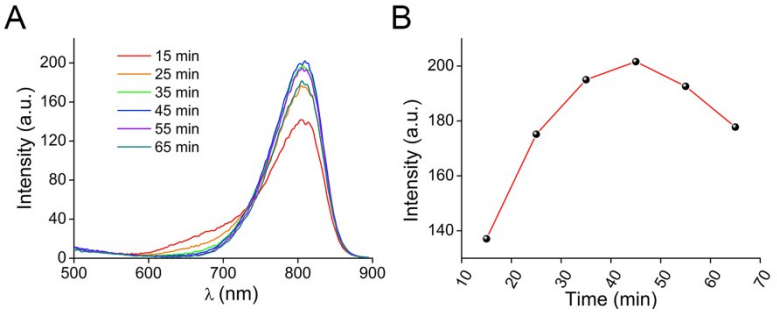

Fig. S3 A) The luminescence spectra of the AuGNPs synthesized with different reaction time. B) Effect of reaction time on the luminescence intensities of AuGNPs at 810 nm.

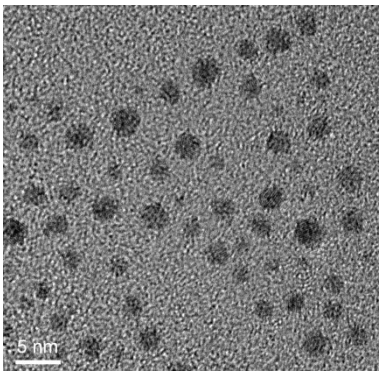

Fig. S4 A typical TEM image of the 810 nm-emitting AuGNPs.

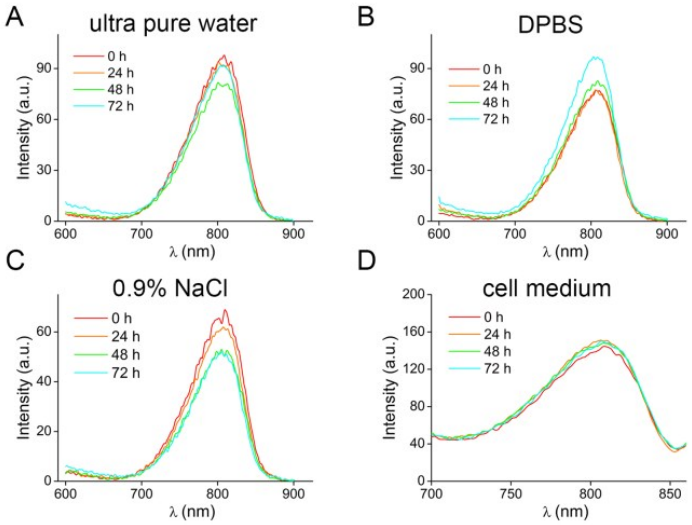

Fig. S5 Fluorescence stability test of AuGNPs at 37 °C over 72 h incubation in different mediums.

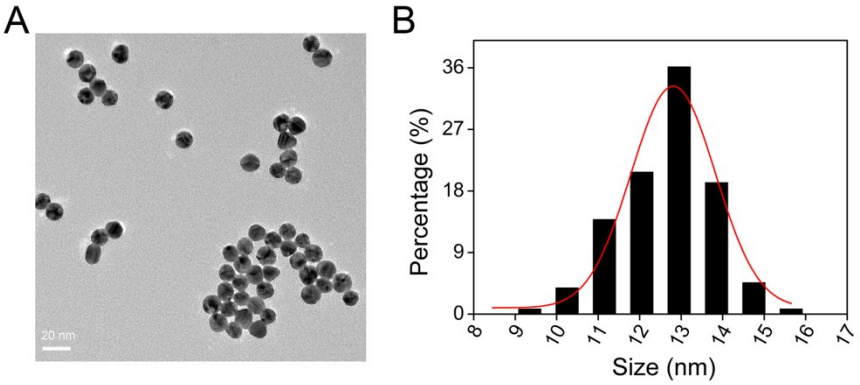

Fig. S6 A typical TEM image (A) and core-size distribution (B) of the p-AuGNPs. The average size of the synthesized p-AuGNPs was calculated to be 13.0 ± 0.9 nm.

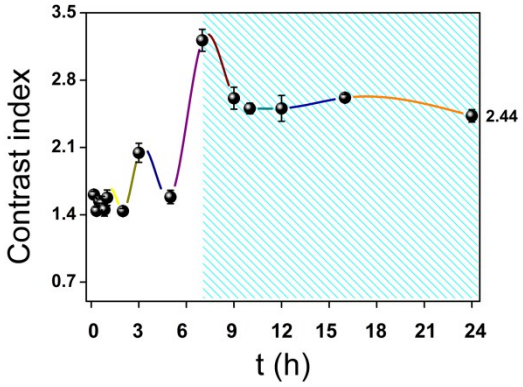

Fig. S7 The contrast index (CI) values of the tumor area injected with AuGNPs.

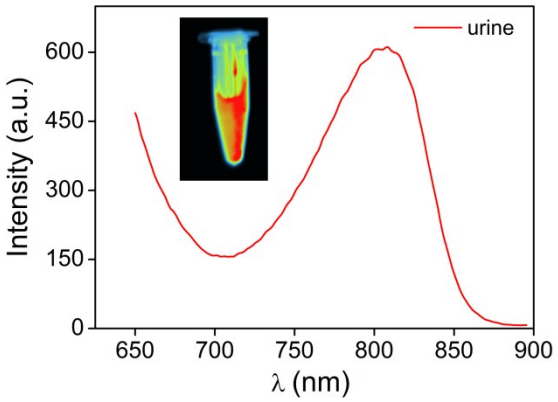

Fig. S8 The emission spectra of the urine collected from the mice iv injected with 810 nm-emitting AuGNPs after 12 h p.i.. Inset showed fluorescence imaging of the urine. The renal clearance of AuGNPs was also confirmed from fluorescence signal of the urine collected at 12 h p.i. with strong NIR emission with the same wavelength as the AuGNPs, indicating that the AuGNPs were also highly stable during the body circulation.

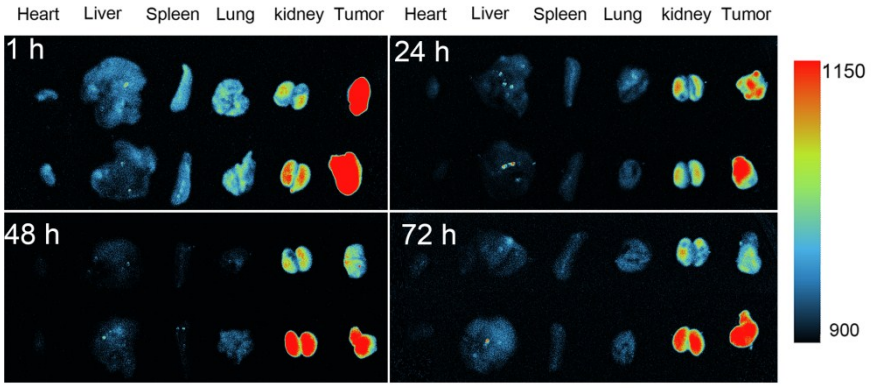

Fig. S9 The additional two groups of the *Ex-vivo* fluorescence imaging of main organs (heart, liver, spleen, lung, kidney and tumor) from the MDA-MB-231 tumor-bearing mice iv injected with 810 nm-emitting AuGNPs at 1, 24, 48 and 72 h p.i., respectively.

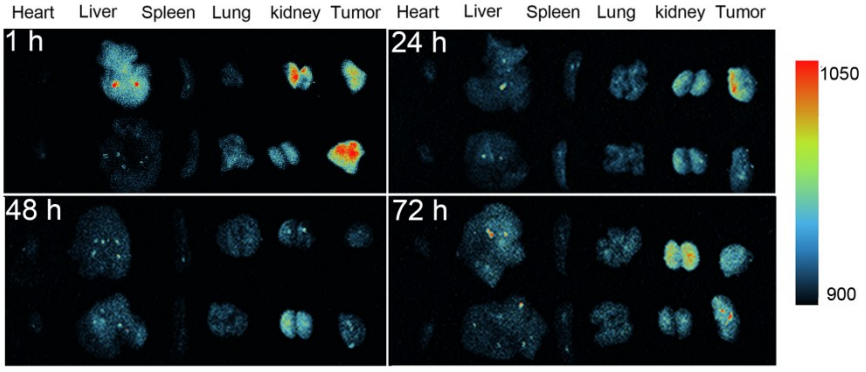

Fig. S10 Ex-vivo fluorescence imaging of main organs (heart, liver, spleen, lung, kidney and tumor) from the MDA-MB-231 tumor-bearing mice iv injected with 810 nm-emitting GS-AuNPs at 1, 24, 48 and 72 h p.i., respectively.

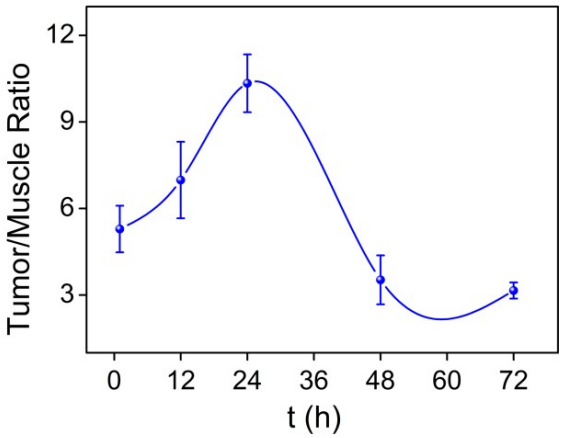

Fig. S11 The time-dependent tumor/blood ratios from the mice iv injected with AuGNPs.

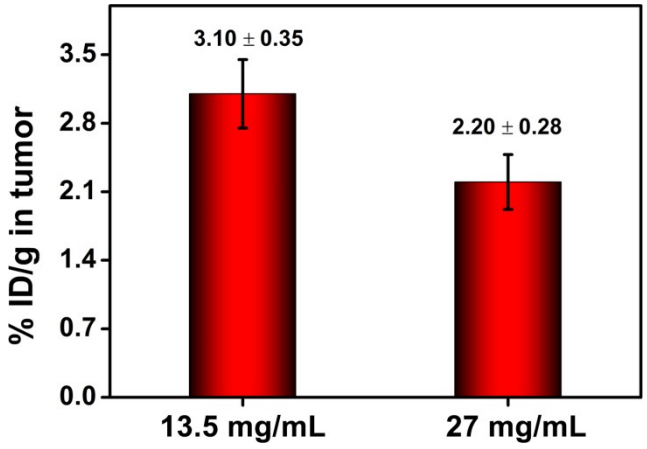

Fig. S12 The tumor targeting efficiencies (at 72 h p.i.) after iv injection of 250 μL AuGNPs with the gold atom concentration of 13.5 and 27 mg/mL, respectively.

Table S1 Comparison of biodistribution results of various nonrenal-clearable NPs.

No.	Nanoparticles	Size	Time p.i.	Liver
1.	PEG-TA-AuNPs[5]	20 nm	48 h	30.31% ID/g
2.	UCNPs[6]	18.5 nm	24 h	45% ID/g
3.	Au_{55}[7]	1.4 nm	24 h	47.5% ID/g
4.	MSNs[8]	20 nm	24 h	46% ID/g
5.	AuNPs[9]	10 nm	24 h	46.3% ID/g

Table S2 Summary of the results of biodistribution in the unit of ID%/g after iv injections of AuGNPs at different p.i. time points, respectively (n = 3 for each group).

Organ	0.5 h	1 h	12 h	24 h	48 h	72 h
Heart	7.17±2.88	5.64±0.91	1.60±0.17	1.46±0.20	1.12±0.06	1.09±0.24
Liver	10.65 ± 0.29	11.50 ± 3.69	3.93 ± 0.62	6.19 ± 0.97	14.02 ± 2.60	12.45 ± 0.79
Spleen	9.83 ± 1.04	10.75 ± 1.19	31.27 ± 5.50	17.54 ± 4.29	13.38 ± 1.59	10.10 ± 1.91
Lung	6.10 ± 0.62	9.08 ± 1.69	29.60 ± 4.59	13.67 ± 2.24	1.74 ± 0.50	1.52 ± 0.35
Kidney	10.57 ± 0.68	7.26 ± 1.06	8.44 ± 1.12	11.95 ± 2.20	11.82 ± 2.50	12.66 ± 1.54
Tumor	4.21 ± 1.56	2.74 ± 0.03	3.42 ± 0.29	3.79 ± 0.26	2.54 ± 0.40	2.20 ± 0.28
Skin	2.81 ± 0.40	1.42 ± 0.10	1.59 ± 0.17	1.79 ± 0.56	3.11 ± 0.79	2.74 ± 1.07
Muscle	1.12 ± 0.05	0.53 ± 0.08	0.55 ± 0.14	0.37 ± 0.04	1.14 ± 0.60	0.70 ± 0.05
Brain	0.31 ± 0.13	0.25 ± 0.05	0.08 ± 0.01	0.05 ± 0.002	0.04 ± 0.003	0.04 ± 0.001
Stomach	3.93 ± 0.83	3.92 ± 1.48	3.86 ± 1.23	3.97 ± 1.92	2.86 ± 0.66	1.72 ± 0.21
Intestine	6.70 ± 1.75	4.36 ± 0.94	2.95 ± 0.76	2.78 ± 0.10	2.13 ± 1.73	1.87 ±0.10
Blood	7.17 ± 2.56	6.95 ± 3.54	1.74 ± 0.48	0.79 ± 0.06	0.59 ± 0.14	1.04 ±0.51

Table S3 Summary of the results of biodistribution in the unit of ID% after iv injections of AuGNPs at different p.i. time points, respectively (n = 3 for each group).

Organ	0.5 h	1 h	12 h	24 h	48 h	72 h
Heart	0.70±0.31	0.49±0.08	0.12±0.03	0.12±0.02	0.12±0.008	0.12±0.02
Liver	9.96±1.31	11.56±3.68	3.60±0.44	6.33±1.20	11.69±1.97	12.94±0.72
Spleen	0.89±0.13	1.544±0.14	2.26±0.73	2.41±0.65	0.90±0.11	0.92±0.13
Lung	0.71±0.12	1.14±0.17	3.01±1.21	1.57±0.11	0.23±0.07	0.19±0.07
Kidney	2.53±0.05	1.92±0.25	1.96±0.27	3.47±1.12	2.68±0.54	2.94±0.52
Tumor	0.46±0.05	1.65±0.415	1.01±0.33	2.16±0.52	1.07±0.84	1.04±0.44
Skin	0.53±0.17	0.20±0.09	0.16±0.01	0.17±0.08	0.21±0.14	0.20±0.07
Muscle	0.16±0.06	0.05±0.02	0.08±0.01	0.06±0.02	0.07±0.03	0.037±0.004
Brain	0.11±0.05	0.074±0.01	0.02±0.004	0.018±0.001	0.01±0.002	0.01±0.0008
Stomach	0.42±0.12	0.40±0.14	0.37±0.09	0.37±0.09	0.14±0.04	0.13±0.02
Intestine	1.96±0.48	2.65±1.06	1.69±0.53	1.40±0.56	0.77±0.59	1.01±0.05
Blood	1.43±0.66	2.00±0.52	0.25±0.16	0.35±0.12	0.13±0.03	0.07±0.006

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