Passive Tumor Targeting of Renal Clearable Luminescent Gold Nanoparticles: Long Tumor Retention and Fast Normal Tissue Clearance

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Experimental Section

Materials and equipment

The IRdye 800CW used for this study was purchased from Li-COR Biosciences (U.S.). Hydrogen Tetrachloroaurate used for the synthesis of the NIR emitting gold nanoparticles (GS-AuNPs) was obtained from Fisher Scientific (U.S.). All the other chemicals were obtained from Sigma-Aldrich and used as received unless specified. The luminescence spectra were collected by a PTI QuantaMaster^{TM} 30 Fluorescence Spectrophotometer (Birmingham, NJ). Absorption spectra were collected using a Varian 50 Bio UV-Vis spectrophotometer. Hydrodynamic diameter (HD) of the nanoparticle (NP) in aqueous solution was analyzed by a Brookhaven 90Plus Dynamic Light Scattering Particle Size Analyzer (DLS). Transmission electron microscopy (TEM) images were obtained with a 200 kV Jeol 2100 transmission electron microscope. The elemental analysis for the carbon, hydrogen, and nitrogen of GS-AuNPs were performed using GLI procedure ME-12 method by Galbraith Laboratories, Inc. The in vivo near-infrared fluorescence imaging was performed using a Carestream Molecular imaging system In-Vivo FX PRO (U.S.).

Animals and tumor implantation

The animal studies were performed according to the guidelines of the University of Texas System Institutional Animal Care and Use Committee. The female nude mice (AthymicNCr-nu/nu, strain code: 01B74) of 6-8 weeks old, weighing 20 ~ 25 g, were obtained from the National Cancer Institute (NCI) Frederick National Laboratory. The animals were housed in ventilated cages under standard environmental conditions (23 ± 1 ^{o}C, 50 ± 5 % humidity and a 12/12 hr light/dark cycle) with free access to water and standard laboratory food.

The human breast cancer cell line MCF-7 was cultured in Minimum Essential Medium (MEM) with 10 % (v/v) fetal bovine serum (FBS) and 1 % (v/v) penicillin-streptomycin at 37 ^{o}C in humidified atmosphere containing 5 % CO_2. The cell suspension (in MEM with 10 % (v/v) FBS) was then mixed 3:1 (v/v) with matrigel and injected subcutaneously upper near the mammary fat pad (MFP) area of the mouse with 100 μL of dense suspension (containing about 1×10^6 cells) for each mouse. The tumor was

allowed to grow about two weeks to reach a palpable size (~ 6 - 8 mm) for the imaging and biodistribution studies.

Synthesis and characterization of the NIR Emitting GS-AuNPs

The NIR emitting GS-AuNPs were synthesized according to literatures and the purification method was modified.[1] Briefly, 150 μL of 1 M $HAuCl_4$ solution was added to 50 mL of 2.4 mM glutathione solution under vigorous stirring. The mixture was then heated at 90 ^oC oil bath for 35 min. The resulting solution was cooled to room temperature and centrifuged at 21, 000 g to remove the large aggregates after the reaction. The supernatant was further purified by adjusting the pH of the solution to 3 ~ 4, and adding a small amount of ethanol into the aqueous solution (2:1, $V_{H2O}/V_{ethanol}$), followed by centrifuging the solution at 4000 g for 5 min to discard the supernatant. The precipitates were then suspended in 300 μL PBS buffer, and then purified again with NAP-5 column in PBS buffer before the animal study. The FT-IR spectra of the purified GS-AuNPs showed that the peak at 2522 cm^{-1} of glutathione which corresponds to S-H stretching vibration mode disappeared in the GS-AuNPs, indicating that the free glutathione was removed during the purification process (Figure S1). Elemental analysis for the dried GS-AuNP: C, 12.10 ± 0.12 %; H, 1.77 ± 0.047 %; 3.79 ± 0.065 %. According to the elemental analysis and the core size of GS-AuNP (Figure S2C), the molecular weight of GS-AuNP was calculated to be ~ 160,000. The extinction coefficient (ε, λ = 400 nm) was estimated to be 3.5×10^6 $L \cdot mol^{-1} \cdot cm^{-1}$. The luminescence quantum yield (QY) of GS-AuNPs excited at 400 nm was measured to be ~ 0.5 % using $[Ru(bpy)_3]Cl_2$ (QY = 2.8 %) as a reference.[2]

In Vivo and Ex Vivo Tumor Imaging

The nude mice used for the in vivo tumor imaging studies were anesthetized using 1.5 - 3 % isofluorane in oxygen flowing at 0.8 L/min about 5 min prior to the intravenous (IV) injection. Each mouse was pre-imaged before the IV injection. The parameters used in the imaging for the GS-AuNP and IRdye 800CW were described as follows: GS-AuNP (Excitation: 470/10 nm; Emission: 830/20 nm; Exposure time: 30 s) and IRdye 800CW (Excitation: 710/10 nm; Emission: 790/20 nm; Exposure time: 5 s). The NIR emitting GS-AuNP for the injection was prepared in the PBS buffer with the gold atom concentration of 20 mg/mL as determined by ICP-MS method. The IRdye 800CW with a concentration of 10 μM was dissolved in PBS, and filtered through a 0.02 μm filter before IV administration. After IV injection of 200 μL GS-AuNP (20 mg/mL) (or IRdye 800 CW, 10 μM), the MCF-7 tumor-bearing nude mouse was imaged at different p.i. time points (as described in the corresponding figures) in 24 hr (Figures 2 and S5). The imaging parameters were kept all the same at each time point. For the ex vivo imaging, the mice were then dissected and collected the major organs and tumors after 1 and 12 hr p.i.

of the NIR emitting GS-AuNPs, and IRdye 800 CW, respectively, followed by imaging with the in vivo imaging system immediately (Figures 2B and 2C).

To evaluate the accumulation and retention kinetics of the particle and dye in tumors and normal tissues, the contrast index (CI) values were calculated as described in the literature.[3] The CI was measured according to the formula CI = (fluorescence intensity of tumor area – autofluorescence)/(fluorescence intensity of normal contralateral region – autofluorescence). The pixels of the tumor area were corresponding to the tumor location identified from the white light image. The fluorescence of normal contralateral region was calculated by the mean intensity value of 3 selected areas with the same pixels of the tumor area on the contralateral region of the mouse. The autofluorescence from the corresponding region measured before the IV injection of GS-AuNP or IRdye 800CW. In order to further quantitatively describe the retention kinetics of the GS-AuNP and IRdye 800CW in tumor and normal tissue, we defined (fluorescence intensity of tumor area – fluorescence intensity of normal contralateral region) as the tumor fluorescence intensity, and (fluorescence intensity of normal contralateral region – autofluorescence) as the normal tissue background intensity. The fluorescence intensity described in the kinetics study was normalized by setting the maximum fluorescence intensity of the time point to be 1.

Pharmacokinetics Studies

Nude mice IV injected with 200 μL GS-AuNP (7 mg/mL) (or IRdye 800CW, 10 mM) were blood sampled from the retro-orbital sinus at 2, 5, 10, 30 min, 1, 3, 5, 8, 12, and 24 hr p.i.. The gold atom concentrations in the blood samples injected with GS-AuNP were measured using ICP-MS method described as follows: The blood samples were weighted, and completely lysed in 2 mL freshly made aqua regia in screw capped glass bottles (10 mL) separately for 24 hr. The samples were then heated at 115 ^{o}C in an oil bath until the complete evaporation of the aqua regia. The residue for each sample was then re-dissolved in 10 mL 0.05 M HCl, followed by sonicating 10 - 30 min. The resulting samples were then further diluted with 0.05 M HCl and analyzed by a Perkin Elmer-SCIEX ELAN 6100 DRC Mass Spectrometry. As for the IRdye 800CW, 10 μL of each fresh blood sample was dissolved in 50 μL EDTA (1.5 mg/mL, 0.9 % NaCl solution). The NIR fluorescence intensity of each blood sample was assayed on an in vivo imaging system. Blank blood samples without the IRdye 800CW were measured to determine the blood auto-fluorescence level, which were subtracted from the fluorescence intensities of the IV injected samples. The concentration of the IRdye 800CW in each blood sample was evaluated based on a standard curve constructed on the fluorescence intensity of the known IRdye 800CW concentrations added to the 10 μL blank blood supplemented with 50 μL EDTA (1.5 mg/mL, 0.9 % NaCl solution) (Figure 3D).

Synthesis and characterization of the 30 nm bovine serum albumin (BSA) coated AuNPs (BSA-AuNPs)

The BSA-AuNPs with a core size diameter of ~ 30 nm was synthesized as follows: First, the "naked" AuNPs was synthesized according to our previous report.[4] Briefly, into a 1.5 mL centrifuge tube containing 500 μL H_2O were added 1 mM $NaBH_4$. The solution became reddish after the addition of 0.25 mg/mL KAuCl4, indicating the formation of the plasmonic AuNPs. Secondly, the synthesized "naked" AuNPs solutions were then centrifuged at 6500 g, and the pellets were then redispersed by 2.5 mg/mL BSA solution, followed by an overnight incubation at room temperature. Finally, the BSA-AuNPs were purified by centrifuging the solution at 6500 g, and the precipitate was washed several times with PBS solution. The BSA-AuNPs solutions were then centrifuged again at 3000 g to remove the large AuNPs before the animal study. The gold atom concentration of the BSA-AuNPs was measured to be ~ 1 mg/mL by ICP-MS method. The size distributions of BSA-AuNPs were characterized by TEM and DLS, respectively (Figure S10).

Biodistribution studies

To assess tissue distributions of the GS-AuNP, IRdye 800CW and BSA-AuNP, the MCF-7 tumor-bearing mice were sacrificed at 1 and 12 hr (n = 3 each group) after IV injection of GS-AuNP, IRdye 800CW or BSA-AuNP. The collected organs and tumors were weighted and the concentrations of gold (% injection dose/g, %ID/g) were determined using the ICP-MS method as described in the "Pharmacokinetics studies" part (Figures 4A and 4C). As for the IRdye 800CW, dissected organs were subjected to NIR imaging by the in vivo system.[5] In order to minimize errors resulted from the penetration depths of the light, the organs such as tumors, livers and kidneys were cut into small pieces before the imaging, and each small piece of organ was weighted after the imaging. A standard curve was constructed based on the fluorescence emission intensity imaged as the same conditions as the organs with the dye added to a PBS solution at the concentrations of 0%, 0.1%, 0.25%, 0.5%, and 1%ID. The dye concentration in organs (% ID/g) was calculated based on the established standard curve (Figures S9 and 4B).

Supplementary Data

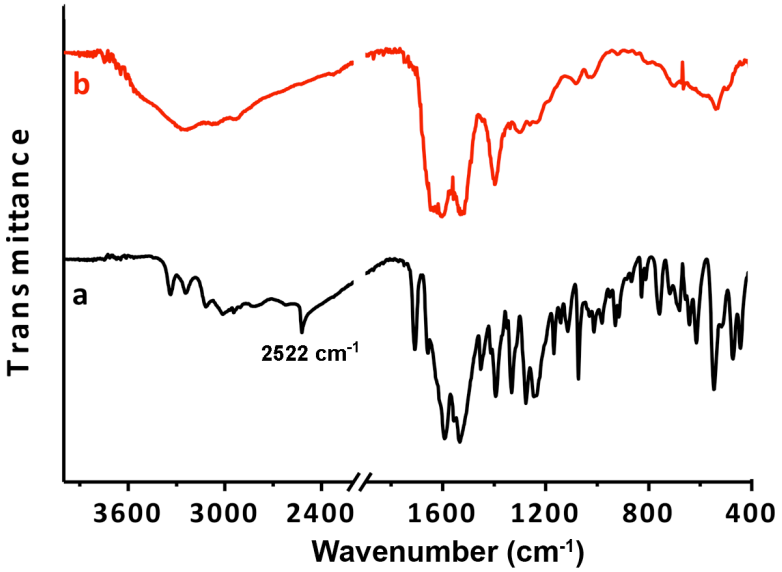

Figure S1. FT-IR spectra of glutathione (a) and purified GS-AuNPs (b). The peak at 2522 cm^{-1} of glutathione corresponding to S-H stretching vibration disappeared in the GS-AuNPs due to the formation of Au-S bond on the nanoparticle surface.

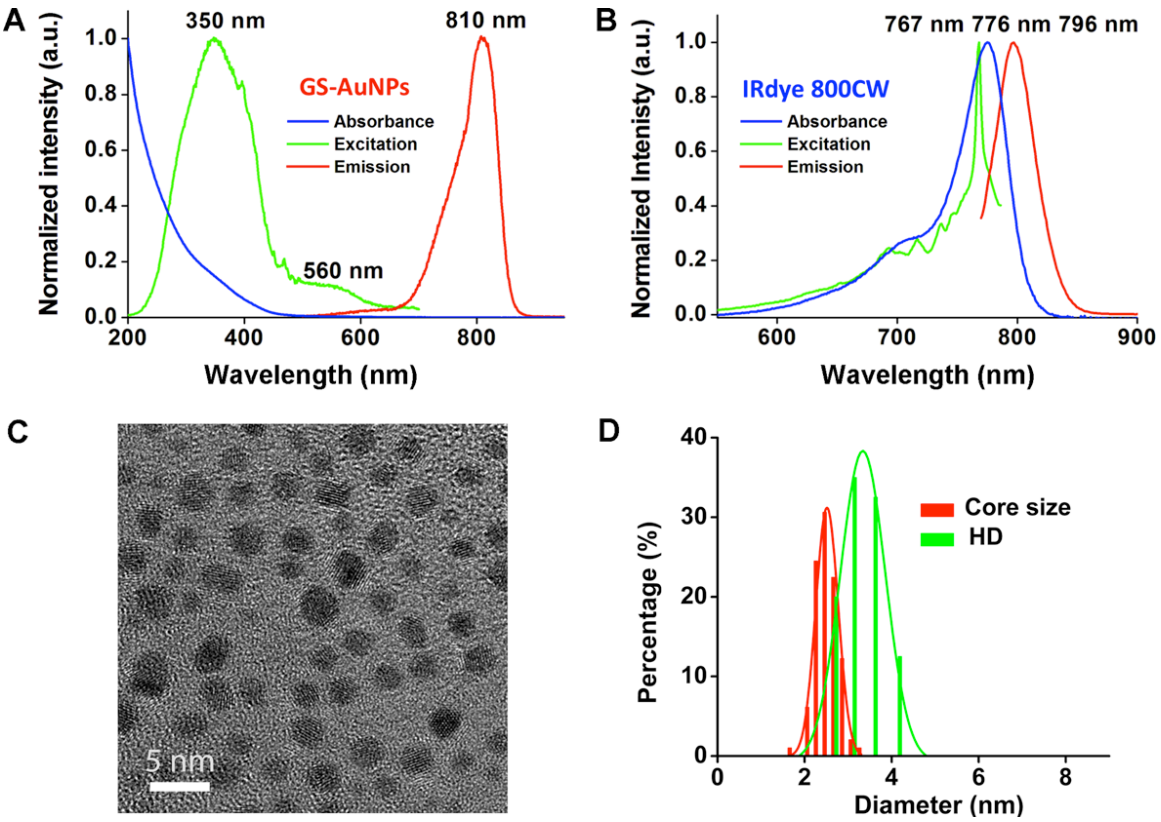

Figure S2. Characterization of the GS-AuNP and IRdye 800 CW. A) The absorption and fluorescence spectra of GS-AuNPs in PBS solution with maximum emission at 810 nm, and excitation peaks at 350, and 560 nm, respectively. B) The optical spectra of IRdye 800CW in PBS solution with maximum emission and excitation at 796 and 767 nm, respectively. C) Typical TEM image of the synthesized GS-AuNP showing that the GS-AuNPs were monodispersed. D) Histogram of the size distribution of the GS-AuNP showing that a core size of 2.5 ± 0.3 nm calculated from the TEM images, and a hydrodynamic diameter (HD) of 3.3 ± 0.4 nm in PBS solution measured by dynamic light scattering (DLS).

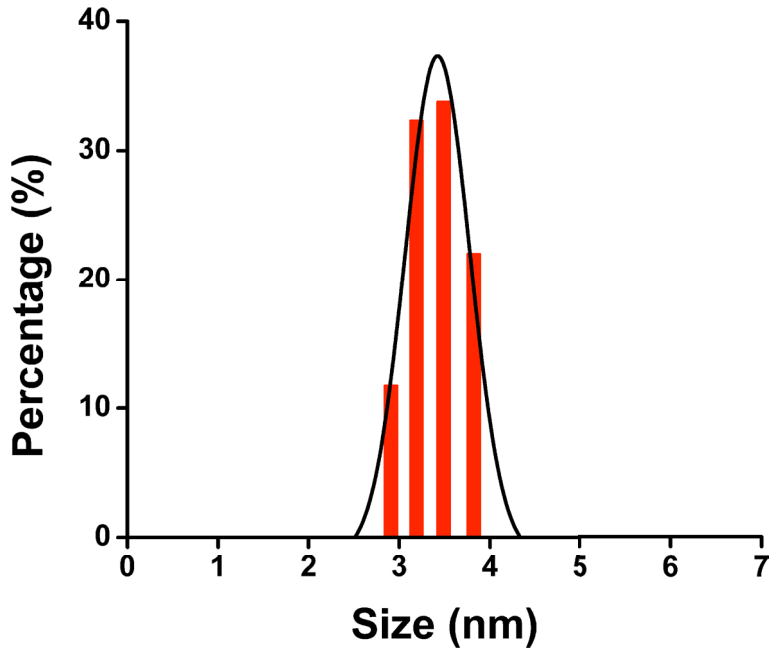

Figure S3. Characterization of the size distribution of GS-AuNP after the incubation at 37 ^{o}C in PBS solution supplemented with 10 % (v/v) fetal bovine serum (FBS) for 30 min by DLS. The results showed that HD of GS-AuNP (3.4 ± 0.3 nm) after the FBS incubation was very close to the HD of GS-AuNP (3.3 ± 0.4 nm) before the FBS incubation, indicating that GS-AuNP was resistant to the serum protein adsorption, which was responsible for the renal clearance. The HD of GS-AuNP was measured after the remove of the unbounded FBS with sephadex LH-20 and NAP-5 columns, respectively.

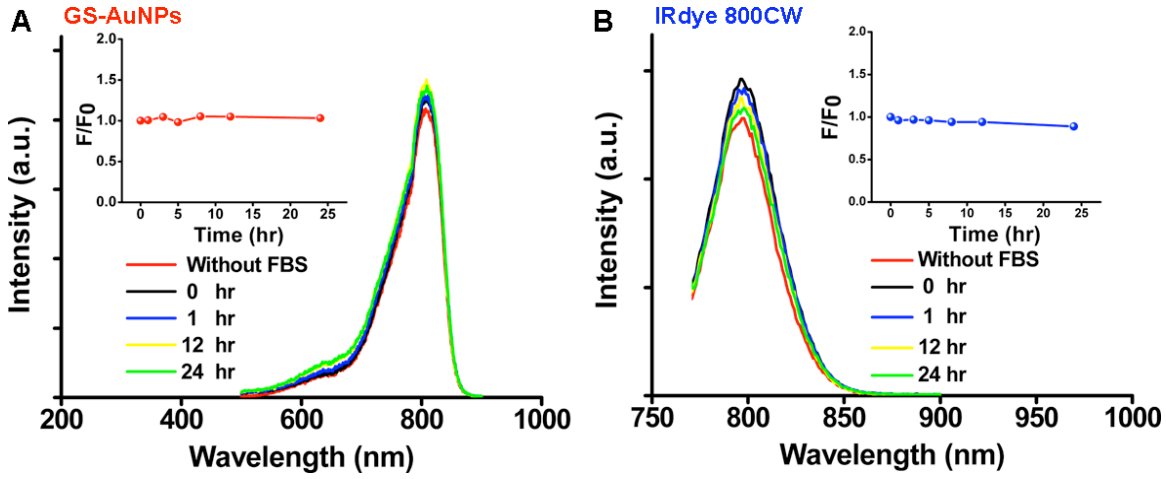

Figure S4. Stability studies of GS-AuNP and IRdye 800CW over 24 hr incubation at 37 ^{o}C in PBS solution supplemented with 10 % (v/v) FBS. The fluorescence spectra of the GS-AuNPs (A), and IRdye 800 CW (B) after incubation with 10 % (v/v) FBS at 0, 1, 12 and 24 hr. Inset showing the time-dependence of the ratio between the fluorescence intensity (F) at different incubation time and the fluorescence intensity of GS-AuNPs (A) or IRdye 800 CW (B) right after incubated with FBS (F_0). Interestingly, fluorescence intensities of both probes in PBS containing 10 % (v/v) FBS were slightly increased by 5 % compared to those in PBS solution without FBS, indicating that no aggregation-induced self-quenching was observed. After 24 hr incubation with 10 % (v/v) FBS at 37 ^{o}C, little change was observed in fluorescence intensity of GS-AuNPs and only 10 % decrease in fluorescence was detected from IRdye 800CW, implying that both probes exhibit high photophysical stability in physiological environment.

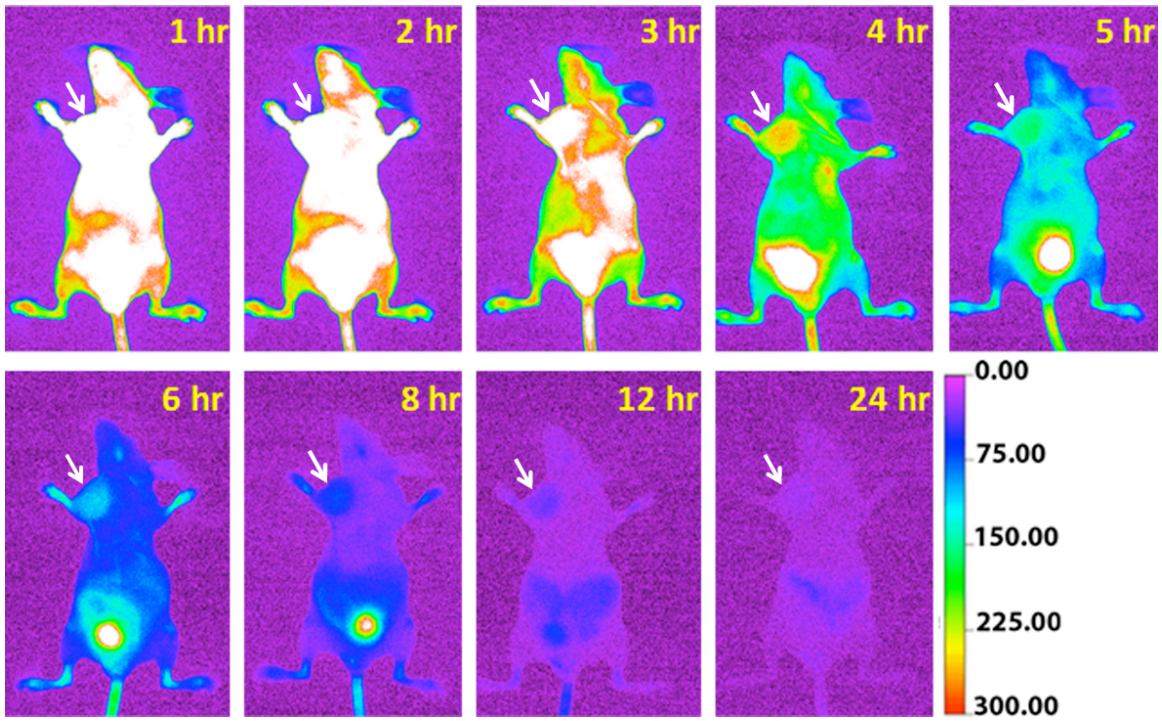

Figure S5. In vivo NIR fluorescence images of a nude mouse injected with IRdye 800CW taken at different p.i. time points showing the tumor targeting of the IRdye 800CW. The tumor arears were indicated with arrows.

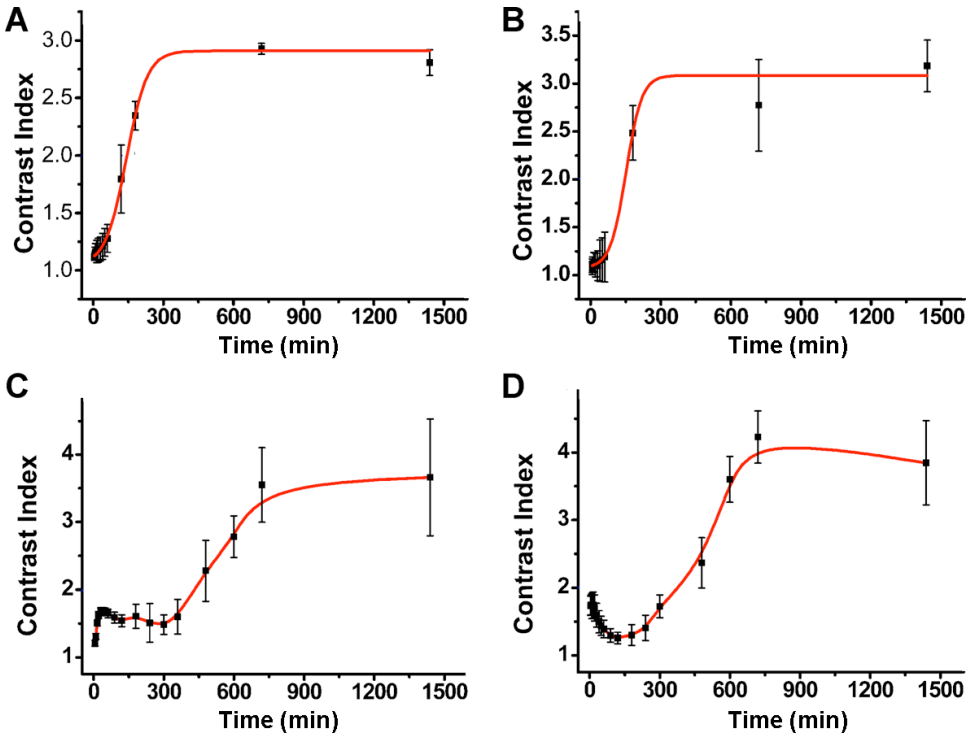

Figure S6. Additional time dependence contrast indexes of GS-AuNP (A, B) and IRdye 800CW (C, D) obtained from the second and third mouse (The data obtained from the first mouse were shown in Figure 3A), respectively.

Table S1. Summary of the results of tumor contrast index after the injections of GS-AuNP and IRdye 800CW, respectively (n = 3 for each probe).

Administered content	Group	Contrast Index = 2 .5	
		t (hr)	Average Value
GS-AuNP	1	3.0	**3.1 ± 0.2**
	2	3.3	
	3	3.0	
IRdye 800CW	1	8.0	**8.2 ± 0.6**
	2	8.9	
	3	7.8	

Note: In the reference 12b, it was reported that the positive (active targeting)-to-negative (passive targeting) tumor ratio was 2.8 and the tumor-to-background ratio was 5.0 for the renal clearable cysteine coated QDs. Therefore, the CI value of passive tumor targeting of QDs was calculated to be CI = 5.0 / 2.8 = 1.8.

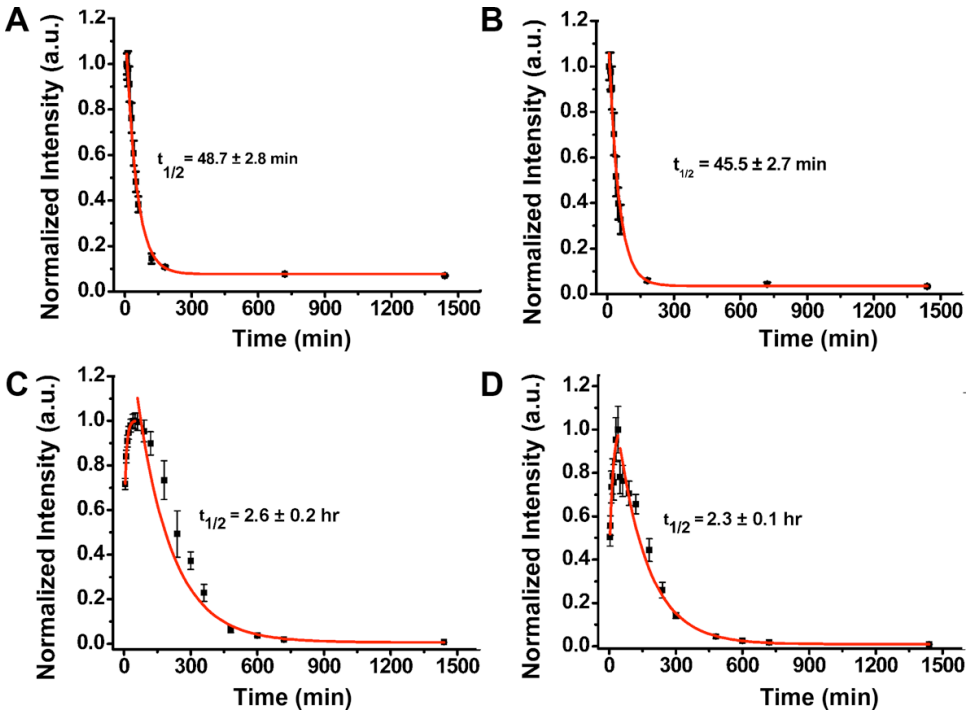

Figure S7. Additional retention kinetics of GS-AuNP (A, B), and IRdye 800CW (C, D) in normal tissues obtained from the second and third mouse (The data obtained from the first mouse were shown in Figure 3B), respectively. All the normal tissue background decay was fitted by a monoexponential decay equation and the R-square values were listed in Table S2.

Table 2. Summary of the normal tissue background decay kinetics results from the mice IV injected with GS-AuNP and IRdye 800CW in 24 hr (n = 3 for each probe), respectively.

Administered content	Group	Time of background increase to maximum		Half-life of background decay		
		t (min)	Average Value	$t_{1/2}$	R-Square	Average Value
GS-AuNP	1	10	**8.7 ± 1.2**	36.0 ± 2.5 min	0.9877	**43.4 ± 6.6 min**
	2	8		48.7 ± 2.8 min	0.9871	
	3	8		45.5 ± 2.7 min	0.9917	
IRdye 800CW	1	50	**46.7 ± 5.8**	2.1 ± 0.1 hr	0.9567	**2.3 ± 0.3 hr**
	2	50		2.6 ± 0.2 hr	0.9671	
	3	40		2.3 ± 0.1 hr	0.9754	

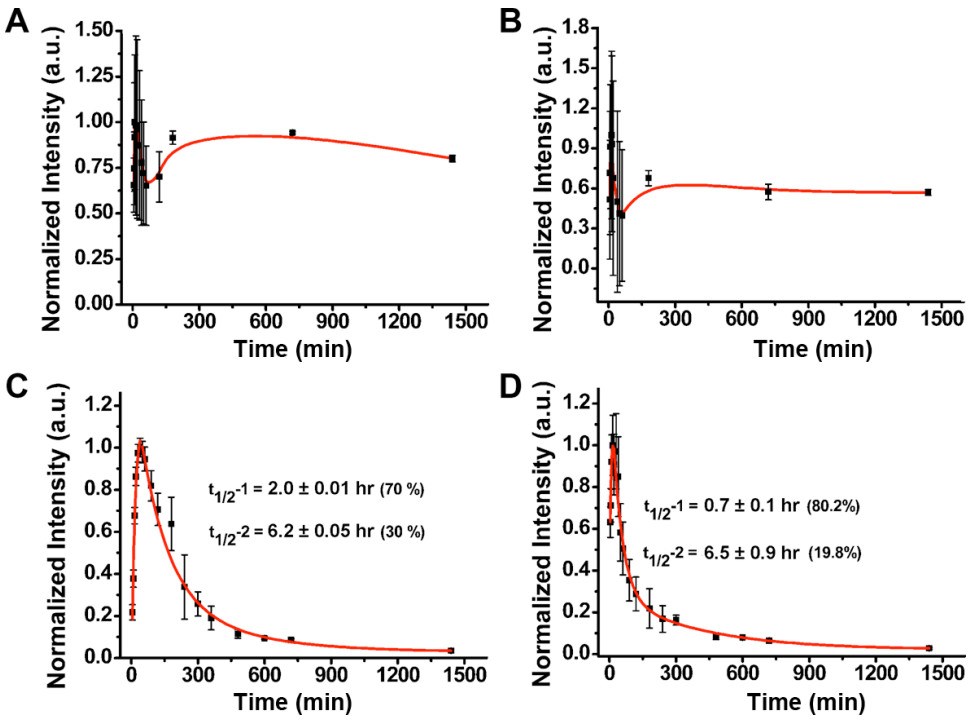

Figure S8. Additional tumor targeting kinetics of GS-AuNP (A, B), and IRdye 800CW (C, D) obtained from the second and third mouse (The data obtained from the first mouse were shown in Figure 3C), respectively. The retention kinetics of the IRdye 800CW in the tumor followed biexponential decay, and the first mouse (Figure 3C) with half-lives of 1.4 ± 0.7 hr (61.9%, $t_{1/2}$-1) and 5.9 ± 1.5 hr (38.1%, $t_{1/2}$-2). The average tumor retention half-lives of IRdye 800CW calculated from the three mice were 1.4 ± 0.6 hr (70.7 ± 9.2 %) and 6.2 ± 0.3 hr (29.3 ± 9.2%), respectively. The tumor targeting kinetics of IRdye 800CW were fitted by a biexponential decay equation, and the R-Square values for the three mice were as follows: Mouse 1 (0.9803); mouse 2 (0.9875) and Mouse2 (0.9858).

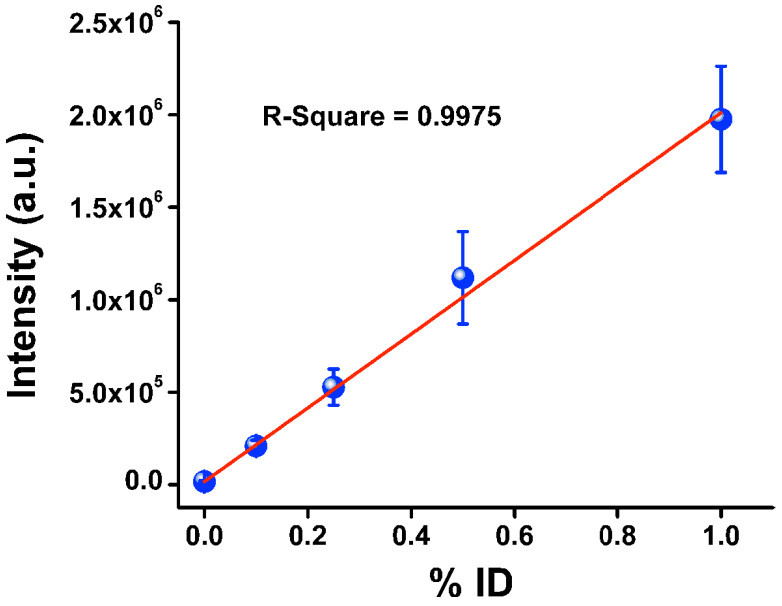

Figure S9. A standard curve for calibrating the dye concentration. The fluorescence intensity of the dye is linearly dependent on its concentration of 0 %, 0.1 %, 0.25 %, 0.5 %, and 1 %ID in PBS solution. The correlation coefficient between the fluorescence intensity and concentration of IRdye 800CW was estimated to be R-Square = 0.9975. The fluorescence intensities of the organs and tumor in each mouse were counted and the biodistribution of dye (% ID/g) in organs and tumor was calculated based on the standard curve established above (n=3).

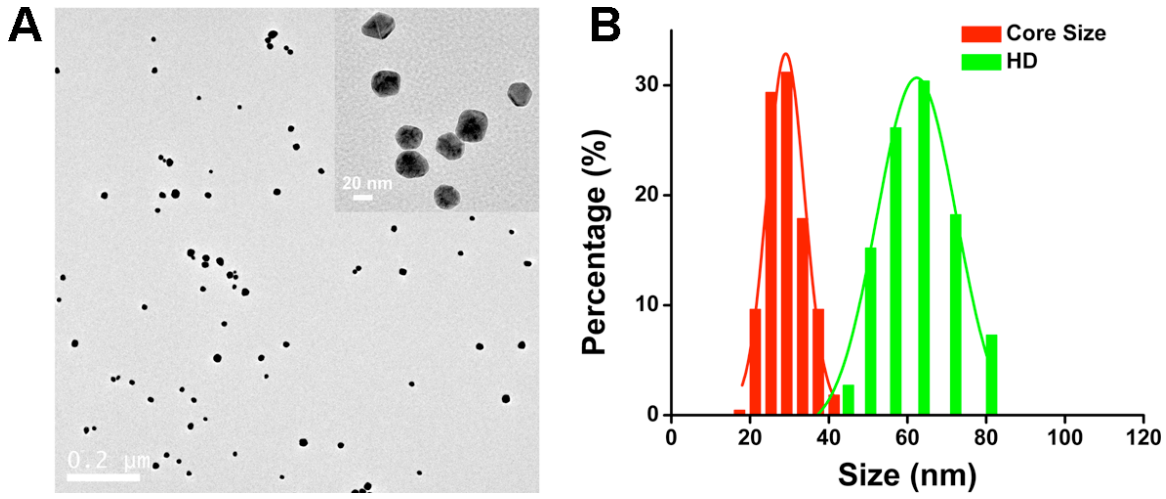

Figure S10. Characterization of the BSA coated AuNPs (BSA-AuNPs). A) Typical TEM images of the BSA-AuNPs. Inset: a high magnification TEM images of the BSA-AuNPs. B) Size distribution results showed that the core size of BSA-AuNP was 29.0 ± 4.1 nm calculated from the TEM images and the HD was 62.0 ± 7.9 nm in PBS solution measured by DLS.

Reference:

(1) (a) Tu, X.; Chen, W.; Guo, X. *Nanotechnology* **2011**, *22*, 095701; (b) Zhou, C.; Hao, G. Y.; Thomas, P.; Liu, J. B.; Yu, M. X.; Sun, S. S.; Oz, O. K.; Sun, X. K.; Zheng, J. *Angew. Chem. Int. Edit.* **2012**, *51*, 10118.
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(4) Liu, J. B.; Zhou, C.; Yu, M. X.; Zheng, J. *Sci. Adv. Mater.* **2012**, *4*, 813.
(5) (a) Gao, J.; Chen, K.; Xie, R.; Xie, J.; Lee, S.; Cheng, Z.; Peng, X.; Chen, X. *Small* **2010**, *6*, 256; (b) Gao, J.; Chen, K.; Luong, R.; Bouley, D. M.; Mao, H.; Qiao, T.; Gambhir, S. S.; Cheng, Z. *Nano Lett.* **2012**, *12*, 281.