Nanoparticles

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Luminescent Gold Nanoparticles with Efficient Renal Clearance**

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Developing functional nanomaterials with efficient renal clearance is of fundamental importance to their in vivo biomedical applications.[1] Ideal nanomaterial-based contrast agents should be effectively cleared out of the body, have little accumulation in organs, and show minimum interference with other diagnostic tests.[1c,e,2] While significant progress has been made toward the creation of fluorescent quantum dots with efficient renal clearance,[2] in vivo applications of noble metal nanoparticles (NPs), another promising nanomedicine in biomedical imaging, drug delivery, as well as antibacterial and photothermal therapy,[3] are still severely hampered by their slow renal clearance and high, nonspecific accumulation in the organs of the reticuloendothelial system (RES; e.g. liver, spleen) after systematic administration.[4] Although NPs with hydrodynamic diameters (HDs) smaller than 10 nm are generally considered to be stealthy to the RES organs, they are still often found in the liver.[2a] For example, nearly 50% of 1.4 nm gold NPs (AuNPs) were found in the liver and only about 9% of them can be excreted into urine within 24 h after intravenous (IV) injection.[4b] Therefore, the development of metal NPs with efficient renal clearance and a fundamental understanding of the key factors in renal clearance are highly desirable.

Herein, we report renal clearance of approximately 2 nm glutathione-coated luminescent gold NPs (GS-AuNPs). We found that only (3.7 ± 1.9)% of the particles were accumulated in the liver and more than 50% of the particles were found in urine within 24 h after IV injection, which is comparable to the quantum dots (QDs) with the best renal clearance efficiency.[2b] By comparing with similarly sized AuNPs coated with cysteine, a ligand that can significantly enhance renal clearance of quantum dots in vivo,[2b] we found that glutathione has advantages over cysteine in enhancing the stability of AuNPs under physiological conditions. Real-time accumulation of luminescent GS-AuNPs in the bladder was further visualized by X-ray computed tomography (CT). The differences in quantum size confinements between metal NPs and QDs means that luminescent AuNPs are often smaller than quantum dots.[5] Consequently, coated with glutathione, luminescent AuNPs might find applications in in vivo biomedical imaging with minimized nanotoxicity.

Previous studies[2b,c] on the biodistribution of QDs suggested that QDs with purely anionic or cationic charged surfaces prefer to bind to serum proteins and are often trapped in the liver, lung, and spleen. However, small 5.5 nm QDs coated with cysteine, a ziwitterionic ligand, can be cleared effectively out of the body.[2a] To test whether cysteine could also be used to enhance renal clearance of very small AuNPs, we created (3.5 ± 0.9) nm cysteine-coated AuNPs (Figure S1a in the Supporting Information). However, these NPs were not stable and formed (220 ± 60) nm aggregates rapidly in phosphate-buffered saline (PBS) before in vivo administration (see Figures S1b and S1c in the Supporting Information), which is consistent with previous reports.[6] Citrate is another general ligand used in synthesizing AuNPs. However, approximately 3 nm AuNPs coated with citrate also form (130 ± 40) nm aggregates in PBS (Figure S2 in the Supporting Information). By using cysteine-coated AuNPs as a model we found that only (0.1 ± 0.03)% of the NPs were able to excreted in the urine and more than 50% of the particles were accumulated in the liver and spleen within 24 h after IV injection (Figure S1d in the Supporting Information). These studies suggest that neither cysteine nor citrate is suitable to minimize nonspecific accumulations of AuNPs in the liver.

Given that many small natural peptides such as glutathione (a tripeptide) have low affinities to cellular proteins[7] and are present in abundance in the cytoplasm, these natural small peptides could potentially serve as capping agents to render metal NPs with the desired stealthiness to the RES organs. Instead of using conventional nonluminescent AuNPs, we mainly investigated biodistribution and renal clearance of approximately 2 nm luminescent AuNPs coated by glutathione. The luminescence of these coated AuNPs not only offers a unique way to evaluate the biological stability and renal clearance kinetics of the AuNPs, but also could be potentially used for in vivo biomedical imaging once the luminescence is shifted to the near-IR range.

The detailed synthesis and characterization of GS-AuNPs have been reported before.[8] Briefly, a fresh 25 mM aqueous solution of reduced glutathione was added into a 25 mM aqueous solution of $HAuCl_4$ at a molar ratio of 1:1. Glutathione molecules treated with gold ions to form Au(I)-GS polymers, which dissociated after a few days into approximately 2 nm AuNPs with mixed valence states (see

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Figure S3 in the Supporting Information). By using gel electrophoresis we confirmed that the GS-AuNPs and the luminescence were co-localized (see Figure S4 in the Supporting Information) and the bright luminescence indeed originates from these approximately 2 nm nanoparticles.

While these GS-AuNPs can be readily dispersed in PBS without forming any aggregates (Figure 1 a), a determinant of biodistribution and renal clearance of NPs is their HDs in

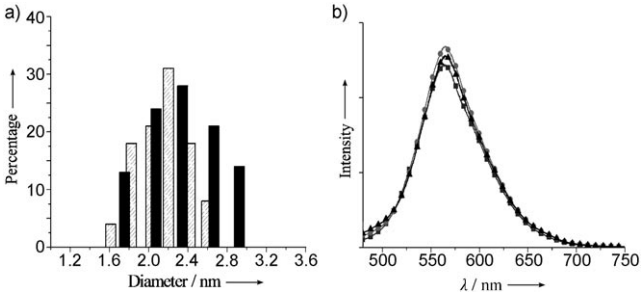

Figure 1. In vitro stability of GS-AuNPs after incubation with fetal bovine serum (FBS). a) Hydroxydynamic radius distribution of GS-AuNPs by number in PBS with (black) and without (hatched) incubation with FBS, and b) luminescence spectra of GS-AuNPs incubated with FBS (37 °C, 5 % CO_2) after 5 min (square), 24 h (circle), and 48 h (triangle): 94 % of the luminescence was preserved.

serum, which are dependent on the interactions between surface ligands of the particles and serum proteins.[2b,c,9] Purely negatively or positively charged surface ligands often have very high affinity to serum proteins, which results in a significant increase in the HDs of NPs and subsequently nonspecific accumulations.[2b] Therefore, ideal surface ligands of NPs should be inert to serum proteins. By using dynamic light scattering (DLS) we compared the HDs of GS-AuNPs in PBS with or without incubation for 48 hours with fetal bovine serum (FBS) at 37 °C. As shown in Figure 1 a, the very small changes in the HDs before and after incubation with FBS indicated that the GS-AuNPs undergo few interactions with serum proteins (see the Supporting Information for the detailed method). In contrast, with the same method, a significant increase in the HDs of approximately 3.5 nm cysteine-coated AuNPs was observed after incubation with FBS (see Figure S5 in the Supporting Information).

Although enzymes often digest small peptides in the blood,[10] glutathione shows an unusual degree of resistance to serum enzyme digestion. Figure 1 b shows that incubating the GS-AuNPs in FBS at 37 °C for 48 h had little effect on both the luminescence spectra and quantum yields (3.5 ± 0.2 %), which indicates that these GS-AuNPs are resistant to enzymatic digestion. Since the pH value of urine could be as low as pH 4.5,[11] we also investigated the chemical and luminescence stability of the particles at pH 4.5. As shown in Figure S6 in the Supporting Information, more than 80 % of the luminescence was retained in PBS without a changing in the spectral line shape when the pH value was decreased from 7.4 to 4.5. Even in FBS at pH 4.5 and 37 °C, more than 75 % of the luminescence of the GS-AuNPs was preserved. These studies further suggested that glutathione is a ligand that not only prevents adsorption of serum proteins but also protects luminescent AuNPs from degradation under biologically relevant environment.

To investigate the in vivo distribution and clearance profile of GS-AuNPs we injected 100 μL of a solution of GS-AuNPs in PBS (9 mg mL^{-1}) into three balb/c mice through the tail vein. In contrast to the 1.4 nm AuNPs coated with bis(*p*-sulfonatophenyl)phenylphosphine, which was hardly excreted in the urine (only 9 % of the particles were found within urine in 24 h after IV injection),[4b,12] the luminescent AuNPs were observed in the urine 2 h post-injection (p.i.; see Figure 2 a). While the urine has an

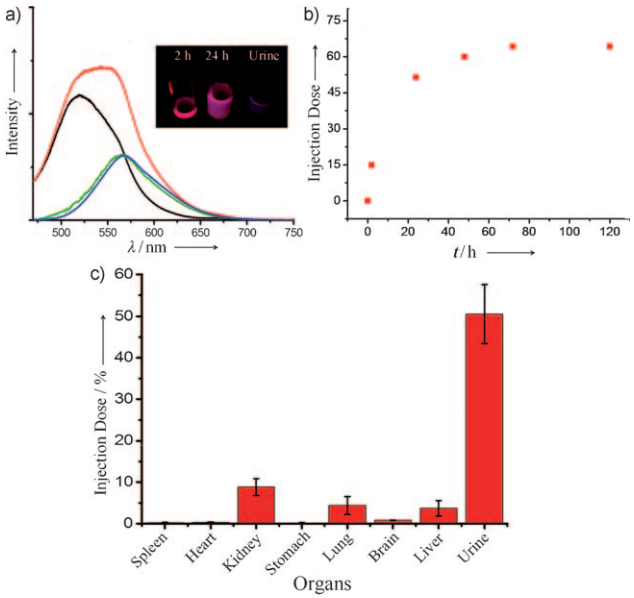

Figure 2. Renal clearance and biodistribution studies of GS-AuNPs. a) Luminescence spectra of urine (black), GS-AuNPs (blue), the urine collected 2 h post-injection (p.i.) (red), and the spectrum (green) after subtracting the urine background (excitation at 420 nm). Inset: Luminescence images of GS-AuNPs in the urine at 2 and 24 h p.i. and control urine under excitation with ultraviolet (UV) light with a 630/75 bandpass filter. b) Gold concentrations in the urine at 2, 6, 24, 48, 72, and 120 h p.i. measured by inductively coupled plasma mass spectrometry (ICP-MS). c) Biodistribution of GS-AuNPs in mice ($n = 3$) 24 h after intravenous injection. The percentage of the injected dose was calculated based on the gold concentration measured by ICP-MS.

autofluorescence background with a maximum intensity around 510 nm, the luminescence of the GS-AuNPs was still clearly observed. By subtracting the background of the urine, we were able to obtain a luminescence spectrum of the GS-AuNPs after circulating in the body; this spectrum is almost identical to that obtained in PBS (Figure 2 a). These results further indicated that GS-AuNPs and their optical properties were highly stable in vivo. By using inductively coupled plasma mass spectrometry (ICP-MS) we also studied the renal clearance kinetics of the particles by measuring the gold concentration in the urine at different p.i. time points. We found that more than 50 % of the GS-AuNPs were excreted out of the body within 24 h p.i. and up to 65 % after 72 h p.i. (see Figure 2 b).

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The biodistribution of these luminescent AuNPs in vital organs was also characterized at 24 h p.i. In sharp contrast to previously reported biodistributions of 1.4, 5, and 18 nm AuNPs, which showed 50 to 94% of the NPs present in the liver,[4b,12] only (3.7 ± 1.9)% of the GS-AuNPs were accumulated in the liver, and (8.8 ± 2.0)%, (4.4 ± 2.1)%, and (0.3 ± 0.1)% of the particles were found in the kidney, lung, and spleen, respectively (Figure 2c and Table S1 in the Supporting Information).

Since liver excretion is a general route for the clearance of most nanometer-sized objects that are not biodegradable,[2b] a significantly low accumulation of GS-AuNPs in the liver and spleen suggests that glutathione can prevent the first-pass extraction from the RES.[2b] Glomerular filtration in the kidney, which generally require that the HDs of the particles be smaller than 10 nm, becomes a major route for the clearance of these luminescent NPs, which implies that these luminescent AuNPs did not bind to large proteins or form large aggregates during blood circulation.

To further confirm that the 2 nm GS-AuNPs were cleared through kidney filtration and renal excretion we took advantage of the large X-ray absorption cross-section of the gold atom, which is nearly 2.7 times larger than iodine-based contrast agents[13] and used CT to non-invasively monitor the dynamic accumulation of AuNPs in the bladder after IV injection. Before we introduced the AuNPs through intravenous injection for CT imaging, we measured the X-ray absorption of the GS-AuNPs at different concentrations. As shown in Figure S7 in the Supporting Information, a linear relationship ($R^2 = 0.996$) between the gold concentration of the GS-AuNPs and the CT signal intensity was observed. At a concentration of 9 mg mL^{-1}, the CT intensity of GS-AuNPs was 845 HU, which is about 4 times higher than the normal tissue background.

While only bones and some food minerals in the stomach were observed before injection because of their high-density characteristics (Figure 3a), the accumulation of GS-AuNPs in the bladder became apparent by an increase in the CT intensity at 30 min p.i., (Figure 3b), which is consistent with the observation of the AuNPs in urine (Figure 2). This result further indicates that these tiny AuNPs can be cleared out by filtration from the blood through the kidney to the bladder.

While glutathione is a promising ligand for minimizing the adsorption of serum proteins, lowering nonspecific accumulation, and improving renal clearance efficiency, the origin of this efficient renal clearance might not be solely attributed to glutathione. To understand how the particle size influences the renal clearance of GS-AuNPs we synthesized nonluminescent GS-AuNPs (NGS-AuNPs) with HDs of about 6 and 13 nm (Figures 4a and 4b). While these NPs are fairly stable in PBS (insets of Figures 4a and 4b), biodistribution studies (see Table S1 in the Supporting Information) show that (4.0 ± 0.6)% and (27.1 ± 2.3)% of the 6 nm AuNPs were found in the urine and the liver while (0.5 ± 0.1)% and (40.5 ± 6.2)% of the 13 nm AuNPs were observed in the urine and the liver, respectively (Figure 4c and Table S1 in the Supporting Information) within 24 h after IV injection. Renal clearance of 6 nm GS-AuNPs is more than two to three orders better than 5 nm gold NPs coated with different polyethylene glycol (PEG) ligands (1.3×10^{-2}% to 3.8×10^{-3}% of particles in urine).[12] By using the ratio between the particle percentage in the urine and that in the liver to reflect the renal clearance efficiency we found that the clearance efficiency of AuNPs with the same glutathione coating decreases exponentially with the increase in the particle size (Figure 4d), which is consistent with previous reports on the effect of the HDs of QDs on renal clearance.[2b] To explore the origin of the decrease in renal clearance with the increase of the HD in the GS-AuNPs we further studied the stabilities of 6 and 13 nm of GS-AuNPs in FBS (Figures S8a and S8b in the Supporting Information). The surface plasmons of the NPs in PBS are red-shifted about 17 nm on addition of FBS, thus indicating the aggregation of the NPs induced by a serum protein. The red-shifts in the plasmons are consistent with the observed (31 ± 15) nm and (47 ± 19) nm aggregates from solutions of 6 and 13 nm NP in PBS after addition of FBS, respectively (Figures S8c and S8d in the Supporting Information). These results suggest that the physiological stability of the NPs decreased as the size increased in the presence of serum proteins. While glutathione has a very low affinity to serum proteins, the significant differences in the physiological stability and renal clearance between the 2 and 6 or 13 nm GS-AuNPs imply that binding between glutathione and serum proteins is strongly dependent on the particle size: glutathione on a 2 nm particle might behave more similar to free glutathione molecules during interactions with proteins while glutathione on the large particles exhibits different interactions with serum proteins. These results suggest that both the ligand and the particle size play central roles in renal

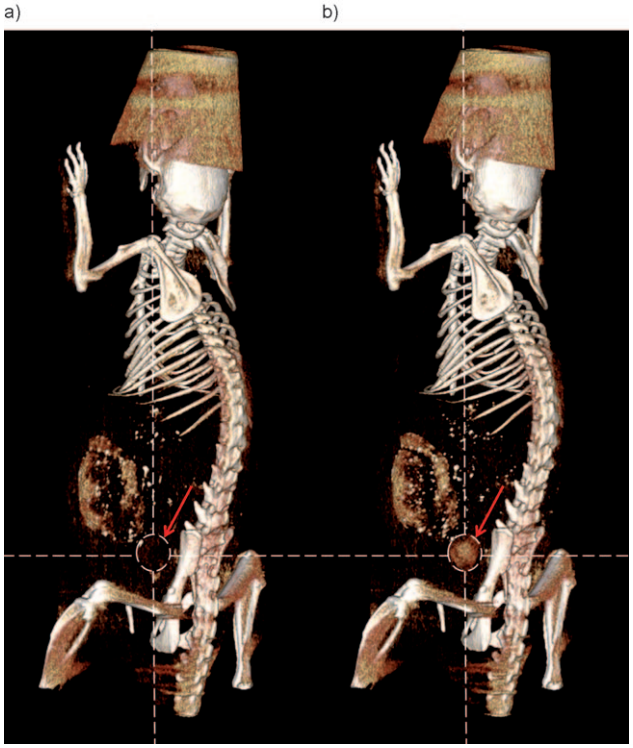

Figure 3. X-ray computed tomography (CT) images of a live mouse a) before and b) 30 min after IV injection of GS-AuNPs.

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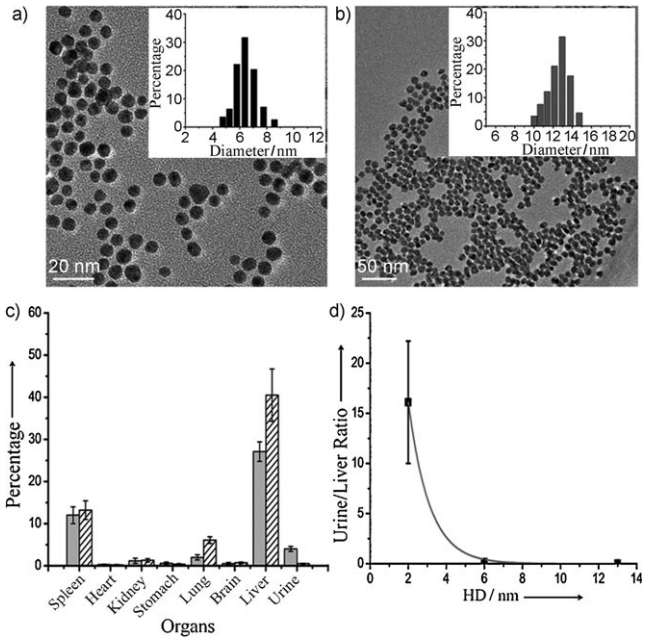

Figure 4. Characterization of the size and biodistribution of 6 and 12 nm nonluminescent GS-AuNPs (NGS-AuNPs). a) Transmission electron microscope image of 6 nm (scale bar: 20 nm) and b) 12 nm (scale bar: 50 nm) NGS-AuNPs in aqueous solution. Insets: The HDs of as-synthesized NPs in PBS are (6.3 ± 0.5) nm and (13.0 ± 0.8) nm. c) Biodistribution of 6 (gray) and 12 nm (hatched) NGS-AuNPs in different organs in mice ($n = 3$) within 24 h after IV injection. The percentage of the injected dose was calculated based on the gold concentration measured by ICP-MS. d) The relationship and exponential fitting ($R^2 = 0.995$) between the HD of different GS-AuNPs and the accumulated urine/liver ratio.

clearance and these two factors can be intertwined to affect nonspecific accumulations of metal NPs.

Taken together, we have found that the renal clearance of 2 nm glutathione-coated luminescent NPs was more than 10 to 100 times better than those of the similar-sized AuNPs coated with bis(*p*-sulfonatophenyl)phenylphosphine and cysteine. The efficient renal clearance of the luminescent particles results from the very small particle size and the glutathione ligand, which not only enables the majority of the luminescent AuNPs to be cleared out of the body through kidney filtration, but also stabilizes the luminescent AuNPs during blood circulation. In addition, the particle size can influence the renal clearance efficiency through changing the interactions between the ligands and serum proteins. With these new findings and rapid progress in developing near-IR luminescent metal NPs with diameters of only a few nanometers,[14] it will be highly promising to apply them for in vivo biomedical imaging.

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