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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, 1e, 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, antibacterial and photothermal therapy,^[3] are still severely hampered by their slow renal clearance and high nonspecific accumulations in the reticuloendothelial system (RES) organs (e.g. liver, spleen) after systematic administration.^[4] Although NPs with hydrodynamic diameter (HD) smaller than 10 nm are generally considered being 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 ~9% of them can be excreted into urine in 24 hours after intravenous (IV) injection.^[4b] Therefore, developing metal NPs with efficient renal clearance and fundamental understanding of key factors in renal clearance are highly desirable.

Herein, we report renal clearance of ~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 in 24 hours after IV injection, which is comparable to the known quantum dots (QDs) with the best renal clearance efficiency.^[2b] By comparing with similar 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

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conditions. Real-time accumulation of luminescent GS-AuNPs in the bladder was further visualized by X-ray computed tomography (CT). Due to the differences in quantum size confinements between metal NPs and QDs, 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, 2c] on biodistribution of QDs suggested that QDs with purely anionic or cationic charged surface prefer binding to serum proteins and are often trapped in the liver, lung and spleen. However, small 5.5 nm QDs coated by cysteine, a zwitterionic ligand, can be effectively cleared 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 (Fig. S1a). However, these NPs were not stable and formed 220 ± 60 nm aggregates rapidly in phosphate buffered saline (PBS) before in vivo administration (Fig. S1b&c), consistent with the previous reports.^[6] Citrate is another general ligand used in synthesizing AuNPs. However, ~ 3 nm AuNPs coated with citrate also form 130 ± 40 nm aggregates in PBS (Fig. S2). Using cysteine-coated AuNPs as a model, we found that only $0.1 \pm 0.03\%$ of the NPs were able to excreted from urine and more than 50% of the particles were accumulated in the liver and spleen in 24 hours after IV injection (Fig. S1d). 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 tri-peptide) are abundantly presented in the cytoplasm with low affinities to cellular proteins,^[7] these natural small peptides potentially can be served as capping agents to render metal NPs with desired stealthiness to the RES organs. Instead of using conventional nonluminescent AuNPs, we mainly investigated biodistribution and renal clearance of ~ 2 nm luminescent AuNPs coated by glutathione because luminescence property not only offers a unique way to evaluate the biological stability and renal clearance kinetics of the AuNPs over the conventional nonluminescent ones, but also could be potentially used for in vivo biomedical imaging once the luminescence is shifted to near IR range.

The detailed synthesis and characterization of GS-AuNPs have been reported before.^[8] Briefly, a fresh 25 mM reduced glutathione aqueous solution was added into 25 mM HAuCl₄ aqueous solution at a molar ratio of 1:1. Glutathione molecules reacted with gold ions to form Au (I)-GS polymers, which dissociated into ~ 2 nm AuNPs with mixed valence states after a few days (Fig. S3). Using gel electrophoresis, we confirmed that GS-AuNPs and the luminescence were co-localized (Fig. S4) and the bright luminescence indeed originates from these ~ 2 nm nanoparticles.

While these GS-AuNPs can be readily dispersed in PBS without forming any aggregates (Fig. 1a), a determinant of biodistribution and renal clearance of NPs is their HDs in serum, which are dependent on the interactions between surface ligands of the particles and serum proteins.^[2b, 2c, 9] Purely negatively or positively charged surface ligands often have very high affinity to serum proteins, resulting in a significant increase in HDs of NPs and subsequently serious nonspecific accumulations.^[2b] Therefore, ideal surface ligands of NPs should be inert to serum proteins. Using Dynamic light scattering (DLS), we compared HDs of GS-AuNPs in PBS with or without 48-hour incubation with fetal bovine serum (FBS) at 37 °C. As shown in Fig. 1a, very little changes in the HDs before and after incubation with FBS indicated that GS-AuNPs have little interactions with serum proteins (Please see SI for detail method). For direct comparison, with the same method, a significant increase in HDs of ~ 3.5 nm cysteine-coated AuNPs after FBS incubation was observed (Fig. S5).

Though enzymes often digest small peptides in the blood,^[10] glutathione shows an unusual degree of resistance to serum enzyme digestion. Fig. 1b shows that both luminescence spectra and quantum yield ($3.5 \pm 0.2\%$) of GS-AuNPs in FBS exhibited little difference after incubation at 37 °C for 48 hours, indicating that these GS-AuNPs are resistant to enzymatic digestion. Since the pH of urine could be as low as pH 4.5,^[11] we also investigated chemical and luminescence stability of the particles at pH 4.5. As shown in Fig. S6, more than 80% of luminescence was retained in PBS without changing of spectral line shape when pH decreased from 7.4 to 4.5. Even in the FBS at pH 4.5 and 37 °C, more than 75% luminescence of 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 μ l of GS-AuNPs PBS solution (9 mg/ml) into three balb/c mice via the tail vein. Different from 1.4 nm AuNPs coated with bis(p-sulfonatophenyl)-phenylphosphine, which was hardly excreted into urine (only 8% of the particles were found in urine in 24 hours after IV injection),^[4b, 12] luminescent AuNPs were observed in the urine after 2 hours post-injection (p.i.) (shown in Fig. 2a). While the urine has autofluorescence background with the maximum peak around 510 nm, the luminescence of GS-AuNPs was still clearly observed. By subtracting the background of the urine, we were able to obtain a luminescence spectrum of GS-AuNPs after circulating in the body, which is almost identical to the spectrum obtained in PBS (Fig. 2a). These results further indicated that GS-AuNPs and their optical properties were highly stable in vivo. Using inductively coupled plasma mass spectrometry (ICP-MS), we also studied the renal clearance kinetics of the particles by measuring gold concentration in the urine at different p.i. time points, and found that more than 50% of the GS-AuNPs were excreted out of the body within 24 hours p.i. and up to 65% after 72 hours p.i. (Fig. 2b).

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

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 RES.^[2b] Glomerular filtration in the kidney, which generally require HDs of the particles smaller than 10 nm, becomes a major route for the clearance of these luminescent NPs, implies that these luminescent AuNPs did not bind to large proteins or form large aggregates during blood circulation.

To further confirm that ~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 noninvasively 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 X-ray absorption of the GS-AuNPs at different concentrations. As shown in Fig. S7, a linear relationship ($R^2=0.996$) between gold concentration of GS-AuNPs and CT signal intensity was observed. At a concentration of 9 mg/ml, the CT intensity of GS-AuNPs was 845 HU, which is ~4 times higher than normal tissue background.

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

While glutathione is a promising ligand for minimizing 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 renal clearance of GS-AuNPs, we synthesized nonluminescent GS-AuNPs (NGS-AuNPs) with HD of ~6 and ~13 nm respectively (Fig. 4a&b). While these NPs are fairly stable in PBS (Fig. 4a&b inset), biodistribution studies (Table S1) show that $4.0 \pm 0.6\%$ and $27.1 \pm 2.3\%$ of 6 nm AuNPs were found in the urine and the liver while $0.5 \pm 0.1\%$ and $40.5 \pm 6.2\%$ of 13 nm AuNPs were observed in the urine and the liver respectively (Fig. 4c and Table S1) in 24 hours 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 PEG ligands ($1.3 \times 10^{-2}\%$ to $3.8 \times 10^{-3}\%$ of particles in urine).^[12] Using the ratio between the particle percentage in the urine and that in the liver to reflect renal clearance efficiency, we found that the clearance efficiency of AuNPs with the same glutathione coating exponentially decreases with the increase of particle size (Fig. 4d), which is consistent with previous reports on the effect of HD of QDs on renal clearance.^[2b] To explore the origin of the decrease in renal clearance with the increase of HD in GS-AuNPs, we further studied the stabilities of 6 nm and 13 nm of GS-AuNPs in FBS. Shown in Fig. S8a&b, the surface plasmons of the NPs in PBS are red shifted about 17 nm with addition of FBS, indicating the aggregation of the NPs induced by serum protein. The red shifts in plasmons are consistent with observed 31 ± 15 nm and 47 ± 19 nm aggregates from 6 nm and 13 nm NP PBS solutions after addition of FBS respectively (Fig. S8c&d). These results suggest that the physiological stability of the NPs decreased with the size increase in the presence of serum proteins. While glutathione has very low affinity to serum proteins, the significant differences in physiological stability and renal clearance between ~2 nm and 6 nm or 13 nm GS-AuNPs imply that binding between glutathione and serum proteins is strongly dependent on the particle size: glutathione on 2 nm particle might behave more similar to the free glutathione molecules during the interactions with proteins while glutathione on the large particles exhibit different interactions with serum proteins. These results suggest that both ligand and particle size play central roles in renal clearance and these two factors can be intertwined to affect nonspecific accumulations of metal NPs.

Taken together, we 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 by bis(p-sulfonatophenyl)-phenylphosphine and cysteine respectively. The efficient renal clearance of the luminescent particles results from the very small particle size and 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 the blood circulation. In addition, the particle size can influence renal clearance efficiency through changing the interactions between ligands and serum proteins. With these new findings and rapid progress of developing few-nm near IR luminescent metal NPs,^[14] it is highly promising to apply them for in vivo biomedical imaging.

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Figure 1.

In vitro stability of GS-AuNPs after incubated with fetal bovine serum (FBS). a) hydrodynamic radius distribution of GS-AuNPs by number in PBS with (dark) and without (slash) incubation with FBS, and b) Luminescence spectra of GS-AuNPs incubated with FBS (37 °C, 5% CO₂) at 5 min. (square), 24 hours (circle) and 48 hours (triangle): 94% of luminescence was preserved.

Figure 2.

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

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

Figure 4. Size characterization and biodistribution of 6 nm 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, respectively. c) Biodistribution of 6 nm (grey) and 12 nm (slash) NGS-AuNPs in different organs in mice (n=3) in 24 hours after IV injection. The percentage of 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 accumulated urine/liver ratio.