A simple approach for glutathione functionalized persistent luminescence nanoparticles as versatile platforms for multiple in vivo applications†

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We develop a simple method by constructing glutathione (GSH) conjugated persistent luminescence nanoparticles (PLNPs–GSH) as versatile platforms for multiple biological applications. PLNPs–GSH possess enhanced water solubility and contains a large number of active groups, which offer opportunities for further modification with different functional groups.

Persistent luminescence materials are a special kind of luminescent materials which can emit afterglow for several seconds to days after removing the external excitation light. In recent years, persistent luminescence nanoparticles (PLNPs) have attracted widespread attention in the fields of bio-imaging and therapeutics, because they can effectively avoid tissue autofluorescence interference, phototoxicity and photobleaching, which usually occur in traditional imaging agents. Despite their advantages, the applications of PLNPs in vivo are severely limited owing to their large size and difficulty in further modification. To overcome these obstacles, Yan’s group developed a three-step process to endow PLNPs with sufficient amino groups on the surface by modification with 3-aminopropyltriethoxysilane, diglycolic anhydride and polyethyleneimine successively. They also coated liposomes on the surface of PLNPs to improve their biocompatibility as luminescent trackable drug carriers for chemotherapy. Han’s group demonstrated a strategy for the direct aqueous-phase synthesis of sub-10 nm PLNPs, which can dissolve in DI water and can be modified with BSA or PEL. Moreover, many other approaches have been introduced for the preparation of water soluble and functionalized PLNPs by using polyethylene glycol (PEG), poly(lactic-co-glycolic acid) (PLGA), mesoporous silica and so on. In order to make the process more convenient and simpler, it is anticipated to construct PLNPs with excellent water solubility and active groups using a more accessible procedure, which is beneficial for multiple bio-applications of PLNPs.

GSH, an important antioxidant produced by cells, plays key roles in antioxidant defense, metabolism, cell signaling, and proliferation. Therefore, by conjugating GSH on the surface, the nanoparticles will possess excellent water solubility and biocompatibility. Besides, GSH contains one thiol group, one primary amino group and two carboxyl groups, which is superior as a bridging ligand to construct nanoparticles for further multiple modifications. What’s more, due to its easy synthesis and modification, GSH has attracted increased attention to construct multifunctional structures as a linker in the fields of biotechnology, catalysis, electrochemistry, and so on.

To fabricate water soluble nanoscale PLNPs with an active surface, herein, we developed an exceptionally simple method to construct GSH conjugated PLNPs for multiple biological applications. Under one-step of wet grinding in a solution of GSH, GSH modified PLNPs (PLNPs–GSH) can be obtained. GSH conjugation can make PLNPs possess good water solubility and biocompatibility. Meanwhile, after the reaction of thiol groups in GSH and PLNPs, the remaining carboxyl groups and amino groups endow PLNPs with sufficient active groups for further functionalization. What’s more, this physical grinding method avoided the complexity of chemical modifications. The synthesis procedure of PLNPs–GSH is depicted in Scheme 1.

Sr2MgSi2O7:Eu,Dy PLNPs were firstly synthesized using a sol–gel method according to our previous report. After one-step treatment of wet grinding in the solution of GSH, well-dispersed and water soluble PLNPs–GSH were obtained. From the transmission electron microscopy (TEM) images in Fig. 1A and B, the size of the PLNPs was found to be rather large with a diameter of about 500 nm. After being treated with GSH, the morphology of PLNPs–GSH changed obviously with an average size of about 50 nm. The size distribution result obtained from dynamic light scattering (DLS) showed that the hydrodynamic diameter was around 70 nm, which further confirmed the GSH conjugation (Fig. S1, ESI†). And the GSH content was determined by TGA, which was calculated to be 0.75 mmol mg⁻¹.
PLNPs–GSH (Fig. S2, ESI†). As shown in the X-ray diffraction (XRD) spectrum, the pattern of PLNPs–GSH was in accordance with that of the standard sample (Fig. S3, ESI†).

The coordination between GSH and PLNPs was firstly investigated. As can be seen from the Fourier transform infrared spectra in Fig. 1C, GSH displayed obvious characteristic absorption peaks at 2520 cm\(^{-1}\), 1713 cm\(^{-1}\) and 3000–3500 cm\(^{-1}\), which are assigned to the vibration absorptions of \(\nu\)(SH), \(\nu\)(C=O), \(\nu\)(OH) and \(\nu\)(NH), respectively. However, after coordinating with PLNPs, the absorption peak at 2520 cm\(^{-1}\) disappeared, which suggested that it is through thiol groups that GSH was modified on PLNPs. Moreover, X-ray photoelectron spectroscopy (XPS) experiments were also carried out to verify the coordination reaction. As shown in Fig. 1D, there is an obvious blue shift in the characteristic peaks of Sr 3d and the binding energy of Sr changed after thiol linking. The above results confirmed that GSH was anchored on the PLNPs via an Sr–S bond.

Then, the optical properties of PLNPs–GSH were evaluated. From the fluorescence spectra in Fig. 2, we can see that PLNPs–GSH has a unique fluorescence emission at 465 nm under 405 nm excitation and its persistent luminescence can last for 30 min after the removal of excitation light. And the photographs of PLNPs and PLNPs–GSH exhibit bright blue afterglow when the excitation light was removed (Fig. S4, ESI†). The rechargeability of PLNPs–GSH was verified in PBS and DMEM buffer solution. As observed from the fluorescence kinetic curve, the PL intensity was almost the same after 10 charging cycles, indicating the excellent rechargeability of PLNPs–GSH in vitro and in vivo (Fig. S5, ESI†). Then the chemical stability and photostability of PLNPs–GSH were verified in PBS buffer. As shown in Fig. S6 (ESI†), the fluorescence intensity of PLNPs–GSH at different times (0, 3, 6, 9 and 12 h) did not show an obvious change, which indicated that the chemical stability and photostability were very good. The stability of GSH modified PLNPs was further investigated at different pHs, in different buffer solutions and at different physical temperatures. Under incubation in PBS buffer with different pHs, the PL intensity of PLNPs–GSH displayed a slight increase from 5.0 to 7.0 and remained constant from 7.0 to 9.0 (Fig. S7A, ESI†). Moreover, the PL intensity of PLNPs–GSH in different buffer solutions (MES, Tris, BBS, PBS, and Hepes) was similar to that in water and there was no obvious change in the temperature range from 20 °C to 45 °C (Fig. S7B and C, ESI†). The above results demonstrated that PLNPs–GSH possessed good stability under physiological conditions. The biocompatibility of PLNPs–GSH was also verified using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay in HepG2 cells.34 As shown in Fig. S7D (ESI†), after incubation with PLNPs–GSH with different concentrations (0.2, 0.5, 0.75, 1.0, 1.25, 1.5, and 2.0 mg mL\(^{-1}\)), HepG2 cells exhibited high viability of nearly 100%, which indicated that PLNPs–GSH had no side effects.

Then the remaining active carboxyl groups and amino groups on the surface of PLNPs–GSH were verified using 6-aminofluorescein (6-AF) and 5(6)-carboxyfluorescein (5(6)-CF) via amide reactions. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), a water-soluble carbodiimide, was employed as a carboxyl activating agent for the coupling of primary amines to yield amide bonds.35,36 As can be seen from the fluorescence spectra, the emission peaks of 6-AF and 5(6)-CF appear at around 520 nm only if EDC was added into the reaction system, indicating the successful conjugation of 6-AF and 5(6)-CF on PLNPs–GSH (PLNPs–GSH–6-AF and PLNPs–GSH–5(6)-CF) (Fig. S8, ESI†). The results demonstrate that there are active carboxyl groups and amino groups on the surface of PLNPs–GSH, which provides lots of opportunities for further modifications and can act as a versatile platform for in vivo applications. What’s more, GSH conjugation through the thiol group was confirmed by fluorescence analysis. As H\(_2\)O\(_2\) could oxidize the thiol group of GSH and prevent it from reacting with PLNPs, 6-AF could not be anchored on the PLNPs through an amido bond. From the fluorescence spectra we can see that the fluorescence intensity...
was rather low when GSH was pre-treated with H$_2$O$_2$, and H$_2$O$_2$ has no influence on the fluorescence intensity of 6-AF (Fig. S9, ESI†). The results demonstrated that GSH was modified on the PLNPs via the thiol group.

**In vitro** imaging of PLNPs–GSH in HepG2 cells was then investigated using a confocal laser scanning microscope (CLSM). As shown in Fig. S10 (ESI†), a bright red signal of the HepG2 cells was observed when they were incubated with PLNPs–GSH. After the HepG2 cells were incubated with PLNPs–GSH–6-AF and PLNPs–GSH–5(6)-CF, which were synthesized in the presence of EDC, the fluorescence intensity became much higher than that without EDC (Fig. 3). Meanwhile, the green signal of PLNPs overlays well with the red signal of the fluorophore, indicating the success of GSH coordination and abundant active groups for further modifications. Because of the spectral match, the confocal images of PLNPs–GSH–6-AF and PLNPs–GSH–5(6)-CF in the HepG2 cells can be obtained via Förster resonance energy transfer (FRET). As shown in Fig. S11 (ESI†), red FRET signals of 6-AF and 5(6)-CF appeared under excitation at 405 nm, suggesting the energy transfer from the PLNPs to the fluorophore on the surface.

The feasibility of PLNPs–GSH as a versatile platform for **in vivo** applications was also investigated. As shown in the **in vivo** images of mice with intraperitoneal injection of PLNPs–GSH, the PL signal lasted for more than 1 h and became stable at 40 min post injection (Fig. S12, ESI†). And we found that the background fluorescence signal was effectively avoided. Combined with abundant active groups on the surface, PLNPs–GSH have the ability to act as a versatile platform for bio-imaging and bio-detection after further modification with targeting groups or a special probe. As an example, an abdominal inflammation mouse model was employed. As the first response of the human immune system, inflammation is associated with many diseases such as cancer and atherosclerosis.\(^3\) Due to the high concentration of superoxide anions ($O_2^{•−}$) in inflammation tissues, we anchored a luminescent probe, dihydroethidium (HE), on the surface of PLNPs–GSH, to construct PLNPs–GSH–HE and detect $O_2^{•−}$ in inflammation tissues with no excitation via FRET.\(^18,39\) Mice were administered with an intraperitoneal injection of lipopolysaccharide (LPS) to trigger inflammation\(^40,41\) and 4 h later treated with PLNPs–GSH–HE. As shown in Fig. 4, the mice pre-treated with LPS displayed a strong PL signal with no excitation. However, in the control group, the PL signal of the mice pre-treated with PBS buffer was rather weak. These results indicated that PLNPs–GSH–HE could detect $O_2^{•−}$ with no excitation **in vivo** and PLNPs–GSH had the ability to act as a versatile platform for **in vivo** applications.

In conclusion, we developed an exceptionally simple method to construct water soluble PLNPs with a simple procedure of grinding in a solution of GSH. Remarkably, GSH as a ligand can not only decrease the size of PLNPs from micrometers to nanometers, but also make the PLNPs with good water solubility and biocompatibility. Moreover, after the reaction of thiol groups in GSH with PLNPs, the remaining carboxyl groups and amino groups endow PLNPs with sufficient active groups for further functionalization through amide reactions. Optimized experimental results showed that PLNPs–GSH had the afterglow property and remains stable under physiological conditions. The confocal images indicated that PLNPs–GSH had active carboxyl and amino groups for multiple modifications. After anchoring HE on PLNPs–GSH, we detected $O_2^{•−}$ without extra excitation **in vivo**. Similarly, we predict that PLNPs–GSH modified with targeting agents will achieve real-time and **in situ** tracing **in vivo** without extra excitation. We anticipate that this synthesis...
approach will provide more opportunities to water soluble PLNPs for in vivo imaging without extra excitation.

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Conflicts of interest
The authors declare no competing financial interest.

Notes and references