NIR-to-NIR rechargeable *in vivo* bioimaging using upconversion persistent luminescent nanoprobes

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Long-lasting persistent luminescent nanoparticles (PLNPs) with efficient near-infrared (NIR) emission are emerged as a new generation of probes for *in vivo* optical bioimaging owing to their advantages of zero-autofluorescence benefited from the self-sustained emission after excitation, deep penetration depth, and high signal-to-noise ratio. However, most of the PLNPs are charged by ultraviolet or visible light, remarkably limiting their applications for *in vivo* long-period bioimaging. Here we design a new type of 980 nm laser activated up-converted-PLNPs (UC-PLNPs) with efficient NIR emission. The NIR-emitting UC-PLNPs (ZnGaGeO: Yb/Er/Cr) were synthesized by a sol-gel method with subsequent calcination. Owing to the efficient energy-transfer between Er and Cr ions, these UC-PLNPs present long-lasting up to 15 h NIR emissions at 700 nm after the excitation of 980 nm NIR laser, both excitation and emission bands fall within biological transparent window. More importantly, the synthesized UC-PLNPs can be effectively recharged by 980 nm light to restore *in vivo* bioimaging signals. This is the first time to demonstrate the rechargeable UC-PLNPs for NIR-

to-NIR *in vivo* bioimaging. We believe that the synthesized UC-PLNPs by incorporating of UC and persistent luminescent properties into a single host may have potential applications in bioimaging area and pave the way for widely using PLNPs for *in vivo* renewable long-lasting bioimaging.

1. Introduction

Optical bioimaging has triggered great research interests and emerged as promising detection approach in biomedicine owing to its advantages of high sensitivity, and providing high resolution molecular information of bio-tissues.[1,2] Up to now, many kinds of optical nanoprobes, such as organic dyes, fluorescent protein, and quantum dots are widely developed and used in bioimaging field.[3-8] However, most of conventional optical probes need *in-situ* excitation by high energetic ultra-violet (UV) light, resulting in high autofluorescence and subsequently reducing the imaging sensitivity. Recently, lanthanide doped upconversion (UC) nanoprobes with efficient NIR-to-NIR emission have increasingly attracted attention and been considered as promising *in vivo* bioimaging agents owing to remarkably reduced autofluorescence,[9] but *in situ* light excitation is still required for *in vivo* bioimaging. Therefore, in order to achieve high sensitive, deep tissue penetration and non-autofluorescence bioimaging, it is significantly important to develop optical probe with no need of in situ excitation during bioimaging and efficient NIR emission located in biological transparent window [10] of 700-1000 nm.

Hence, NIR-PLNPs with a long-lasting and NIR emitting afterglow nature[11] are emerged as ideal alternative optical probes for *in vivo* whole body bioimaging owing to their high signal-to-noise ratio, deep tissue penetration, and no need for *in situ* excitation. Scherman and co-workers[12] demonstrated a pioneering NIR-emitting PLNPs ($Ca_{0.2}Zn_{0.9}Mg_{0.9}Si_2O_6:Eu^{2+}$, Mn^{2+}, Dy^{3+}) for *in vivo* persistent bioimaging for 1 h after ceasing excitation light. The same group further reported a new PLNPs ($CaMgSi_2O_6:Eu^{2+},Mn^{2+},Pr^{3+}$) with improved optical

characteristics.[13] Recently, ultra-long NIR afterglow emitted Cr^{3+}-doped gallate persistent phosphors with bulky size, such as $Zn_3Ga_2Ge_2O_{10}:Cr^{3+}$, were prepared by a solid-state method.[14] More recently, a new type of Cr^{3+}-doped $LiGa_5O_8$ NIR persistent phosphor was demonstrated for *in vivo* imaging for more than 4 h in mouse without an external illumination source.[15] NIR-emitting Cr^{3+}/Pr^{3+} co-doped zinc gallogermanate PLNPs with superlong afterglow was reported for *in vivo* bioimaging for more than 7 h after intravenous injection.[11] Although NIR-PLNPs present excitation-free imaging nature, subsequently eliminating background noise, this probe for bioimaging application is still restricted by its short detection time, hindering its applications for *in vivo* long-term tracking/biodistribution.[12] Moreover, most of the PLNPs were charged by UV and visible light, which can't be reactivated *in vivo*, owing to the limited tissue penetration depth of UV and visible light.[14,16,17] Recently, $ZnGa_2O_4:Cr^{3+}$ (ZGC) probes were demonstrated for *in vivo* bioimaging and these probes can be recharged by red-right for bioimaging according to the reports from Han's group and others.[18-20] In spite of such achievements in designing rechargeable NIR-emitting PLNPs for *in vivo* bioimaging, it is still a great challenge to achieve NIR-to-NIR PLNPs, namely both excitation and emission bands are located in biological transparent NIR window for *in vivo* bioimaging. Therefore, to address this problem and make NIR-PLNPs more suitable for *in vivo* tracking and bioimaging, the probes should be re-charged *in vivo* by an excitation light with high penetration depth, such as 980 nm laser. Note that some type of UC persistent luminescence with NIR emission[21] was observed in powder phosphors synthesized by traditional solid-state reaction, enable combining the unique advantages of UC and persistent luminescence processes. Apparently, the bulky size of these phosphors is not applicable to *in vivo* bioimaging.

Herein, we report that the nano-sized $Zn_3Ga_2GeO_8:Cr^{3+}$, Yb^{3+}, Er^{3+} UC-PLNPs with intense long-lasting NIR persistent luminescence. These probes can be activated under 980 nm laser before being injected into mice owing to the efficient energy transfer between Er^{3+}

and Cr^{3+}, as shown in **Scheme 1**. We have demonstrated that these UC-PLNPs could be used as a sensitive optical probe for *in vivo* bioimaging for the first time. Interestingly, the rechargeable nature of these UC-PLNPs activated by NIR light was also applied for *in vivo* bioimaging application, paving the way for designing NIR-activated NIR-emitting persistent probes in biomedicine applications.

2. Results and Discussion

2.1. Phase and Microstructure Characterization

The UC-PLNPs possess novel UC persistent luminescence as shown in **Scheme 1.** By combining UC and persistent luminescence into a single host, the persistent luminescence is activated by low photon energy excitation light of 980 nm laser, making UC-PLNPs ideal and competitive probes for rechargeable and afterglow *in vivo* bioimaging with the merits of eliminated background noise, high sensitivity and high signal to noise ratio. To achieve the goal, we have synthesized UC-PLNPs by doping rare-earth ions pair of Yb^{3+}/Er^{3+} (5 mol%/0.2 mol%) into $Zn_3Ga_2GeO_8$ 1%Cr^{3+} host with NIR persistent luminescence (hereafter referred to as ZnGaGeO: Yb/Er/Cr UC-PLNPs). The nano-sized ZnGaGeO: Yb/Er/Cr UC-PLNPs were synthesized by a sol-gel method with a subsequent calcination. The diffraction peaks of the synthesized UC-PLNPs (**Figure 1**) match the standard structure of $ZnGa_2O_4$ (JCPDS: 38-1240) and Zn_2GeO_4 (JCPDS: 25-1018) well. And no other impurity peaks were observed, indicating the formation of a pure zinc gallogermanate solid solution structure. As shown in **Figure 2**a, UC-PLNPs present particle-like shape and selected area electron diffraction (SAED) result (the inset of Figure 2a) further reveals the formation of spinel phase of the synthesized compound, according with the aforementioned X-ray diffraction data. The main elements of Zn/Ga/Ge were detected as an atomic percentage of 3/2/1 by energy dispersive X-ray spectra (**Figure S1**), further validating the formation of zinc gallogermanate solid solution structure.

2.2. NIR-activated UC persistent luminescence

In order to reveal the energy transfer between the two dopant ions of Cr^{3+} and Er^{3+}, the UC luminescent process of the synthesized UC-PLNPs was studied. As demonstrated in **Figure 2b** (red line), under the excitation of 980 nm laser, the typical UC emissions of Er^{3+} located at 525/545 and 655 nm were observed, corresponding to the electron transitions of $^2H_{11/2}/^4S_{3/2}$ -- $^4I_{15/2}$ and $^4F_{9/2}$ -- $^4I_{15/2}$ of Er^{3+}, respectively. Moreover, a weaker UC emission band centered at 700 nm (indicated by red arrow) was also found, ascribed to the energy transition of ^2E -- $^4A_2$ from Cr^{3+}. To understand the UC emission mechanism associated with Cr^{3+} doping, the excitation spectrum (green line in Figure 2b) of the synthesized UC-PLNPs was measured by detecting the typical 700 nm emission of Cr^{3+}. As demonstrated by excitation and UC spectra, an obvious spectral overlap between the excitation band of Cr^{3+} and green UC emitting band of Er^{3+} was observed, verifying the presence of energy transfer between Cr^{3+} and Er^{3+}. To further confirm the energy transfer between Cr^{3+} and Er^{3+}, lanthanide-free $Zn_3Ga_2GeO_8$ 1%Cr^{3+} sample was synthesized by the identical process and the UC spectra (**Figure S2**) of lanthanide-free and Yb/Er doped samples were measured under the excitation of 980 nm laser. The results clearly present that the lanthanide-free sample presents no any UC signal while Yb/Er doped sample has intense UC emissions of Er^{3+} and Cr^{3+}, further illustrating the occurrence of energy transfer between Cr^{3+} and Er^{3+}. Therefore, based on the above analysis, the UC emission located at 700 nm of Cr^{3+} under excitation of 980 nm laser, is mainly ascribed to the efficient energy transfer between Er^{3+} and Cr^{3+}, indicating the possibility of designing NIR-laser activated NIR-emitting UC persistent probes. Figure 2c shows the schematic diagram of 980 nm laser-activated UC persistent luminescence. The energy of $^2F_{5/2}$ level of Yb^{3+} through the absorption of 980 nm photon is transferred to the adjacent Er^{3+}, resulting in subsequently populating excitation state levels of $^2H_{11/2}/^4S_{3/2}$ and $^4F_{9/2}$ of Er^{3+} via phonon-assisted UC process. Owing to the energy transfer between Er^{3+} and

Cr^{3+}, the $^4T_2$ excited state of Cr^{3+} can be populated. As a result, the NIR UC emission of Cr^{3+} is obtained. Apart from the efficient UC emission, the absorpted energy of $^4T_2$ in Cr^{3+} ions can be also transferred and filled into the electron traps in the $Zn_3Ga_2GeO_8$ host matrix.[21] Therefore, after stopping 980 nm excitation, the UC NIR persistent luminescence of Cr^{3+} can be realized. As shown in Figure 2d, the nano-sized ZnGaGeO: Yb/Er/Cr UC-PLNPs present NIR persistent emission at 700 nm recorded immediately after irradiating the sample for 10 min by a 980 nm laser with power density of 2 W/cm^2, unambiguously verifying the 980 nm laser-activated UC persistent luminescence. To illustrate the long-lasting NIR persistent luminescence, the time-dependent persistent luminescence imagings (inset of Figure 2d) after charging the sample for 10 min with excitation source of 980 nm laser (2 W/cm^2) were studied. When the excitation is stopped, the UC persistent luminescence signals were gradually decreased with the elapsed time. Notably, a long-lasting NIR luminescence signal up to 15 h was obtained and visualized.

To assess the feasibility of rechargeable NIR persistent luminescence activated by 980 nm laser, the four repeated cycles of repeated activation and corrosponding persistent emission properties are evaluated as shown in **Figure 3**a. Moreover, rechargeable *in vitro* NIR imagings of pseudo-colors are taken by a CCD camera (Figure 3b). As demonstrated, the afterglow NIR persistent luminescence of the nano-sized ZnGaGeO: Yb/Er/Cr UC-PLNPs is renewable by NIR light after activation. The result provides an experimental evidence that these nano-sized UC-PLNPs can be readily activated by NIR laser and therefore present the long-lasting NIR UC emission, which is greatly promising for *in vivo* rechargeable bioimaging.

2.3. *In vivo* persistent bioimaging

To test the feasibility of *in vivo* bioimaging based on the UC-PLNPs, UC-PLNPs solutions with different concentrations (1 mg mL^{-1} and 0.2 mg mL^{-1}) were initially activated for 10 min by a 980 nm laser with power density of 0.5 W/cm^2 before injection. After activation, the

charged UC-PLNPs solutions (200 μL) were subcutaneously injected into a live mouse (lest sites: 1 mg mL^{-1}, right site: 0.2 mg mL^{-1}). **Figure 4** shows the *in vivo* UC persistent bioimaging detected by a modified multimodal imaging system (Bruker *In Vivo* FX Pro.). As illustrated, a strong persistent luminescence signal was observed at 5 min after injection of charged UC-PLNPs. With increasing the decay time, the signal was gradually decreased. Notably, an obvious signal can be detected even after 10 h decay from left site injection (high concentration), indicating the long-lasting bioimaging ability of these UC-PLNPs.

2.4. NIR-to-NIR rechargeable *in vivo* bioimaging

In-situ rechargeable ability is crucial for afterglow bioimaging based on persistent luminescence nanoprobes. Differing from previously reported long-lasting bioimaging results[18,20], our developed UC-PLNPs probes possess effective NIR UC persistent optical bioimaging triggered by NIR light at 980 nm, making them competitive and promising for renewable bioimaging owing to the deep tissue penetration of NIR light. Prior to *in vivo* renewable persistent bioimaging, conventional UC optical bioimaging (**Figure S3**) by detection of green light region was performed. As illustrated, these UC-PLNPs present efficient UC signal from mouse under the excitation of 980 nm laser, which is consistent with aforementioned analysis of UC spectrum. Moreover, the UC persistent signals were successfully detected after activation by 980 nm laser with different rechargeable times (**Figure 5**) and power densities (**Figure 6**). These results reveal that the synthesized UC-PLNPs can be efficiently renewed by 980 nm laser with power density of 150 mW/cm^2 for 120 s, verifying the rechargeable ability of UC-PLNPs by 980 nm laser. To shed more lights for *in-situ* rechargeable bioimaging, a mouse was subcutaneously injected with UC-PLNPs solution (1 mg mL^{-1}). The mouse was *in-situ* activated by a 980 nm laser with power density of 150 mW/cm^2 equipped on the modified multi-modal imaging system (Bruker *In Vivo* FX Pro). The bioimaging for the repeated four cycles at different decaying times was obtained. As shown in **Figure 7**, after *in situ* excitation, these UC-PNLPs present strong persistent

signal from mouse and the signal is gradually decreased with the decay time. While, after 2 h decaying, the persistent signal was dramatically reduced, implying the depletion of the trapped electrons in the host used in our nanoprobes. Through recharging the UC-PLNPs by *in situ* excitation (Figure 7), the UC-PLNPs were completely renewable and strong persistent signal was achieved after ceasing excitation. These findings reveal that our synthesized UC-PLNPs can be used as ideal optical probes for renewable bioimaging owing to their advantages of NIR UC persistent luminescence.

3. Conclusion

In summary, ZnGaGeO: Yb/Er/Cr UC-PLNPs at nanoscale were synthesized, possessing NIR UC persistent luminescence when activated by 980 nm laser. Such a feature is mainly ascribed to the efficient energy transfer between Er^{3+} and Cr^{3+} according to the UC and excitation spectra. The obtained *in vitro* bioimaging reveals that our synthesized UC-PLNPs can be readily recharged by 980 nm laser. And, the synthesized UC-PLNPs present superior properties used for afterglow *in vivo* persistent bioimaging up to 10 h. More importantly, *in vivo* rechargeable NIR persistent imaging is realized for the first time by using high-tissue penetrating light of 980 nm laser, where both excitation and emission bands fall within biological transparent window. These findings provide a new possibility for designing a new type of NIR-to-NIR imaging probes by combining the advantages of both UC and persistent luminescence processes.

4. Experimental Section

Chemicals and materials. Ga_2O_3 (99.999%) and GeO_2 (99.999%) were purchased from Sigma-Aldrich. $Zn(NO_3)_2$, $Cr(NO_3)_3$, $Yb(NO_3)_3$, $Er(NO_3)_3$, citric acid, nitric acid, and ammonium hydroxide were ordered from Sinopharm Chemical Reagent Co., China.

Synthesis of ZnGaGeO: Yb/Er/Cr UC-PLNPs: $Zn_3Ga_2GeO_8$[21] was selected as a host material for constructing upconverted persistent luminescent probes by tri-doping Yb/Er/Cr

ions. The ZnGaGeO: Yb/Er/Cr UC-PLNPs were synthesized by a sol-gel method with a subsequent calcination. In a typical synthesis[11], $Zn(NO_3)_2$, $Cr(NO_3)_3$, $Yb(NO_3)_3$, and $Er(NO_3)_3$ were dissolved in de-ionized (DI) water. Ga_2O_3 and GeO_2 were dissolved in dilute nitric acid and ammonium hydroxide solutions, respectively. These aqueous solutions with a total amount of 1 mmol metal ions were mixed together with the synthesized chemical composition of $Zn_3Ga_2GeO_8$: 1% Cr/5%Yb/0.2Er%. Then, citric acid solution (1.5 mmol) was slowly added to the above mixture. After that, ammonia hydroxide was added to adjust the pH to 5. The obtained mixture was vigorously stirred for 2 h and heated at 80 °C. The solution was gradually transformed into a sol and finally changed to a gel. Followed by the step, the formed gel was dried at 130 °C for 4 h with subsequent heating at 200 °C for 12 h to form black porous materials. Then, the black porous materials were annealed at 1000 °C for 3 h. As a comparable experiment, the lanthanide-free ZnGaGeO: 1%Cr PLNPs were synthesized by a similar method as UC-PLNPs without doping Yb^{3+} and Er^{3+} ions.

The nanosized UC-PLNPs were obtained by wet ground and centrifugation method. The annealed UC-PLNPs were wet ground by adding a small amount of ethanol for 1 h. The obtained powders were then dispersed into NaOH solution (5 mM) and vigorously stirred for 24 h. The obtained colloidal solution was then centrifuged at 5000 rpm for 30 min to remove large sized particles. The upper supernatant was centrifuged at 10000 rpm for 10 min to collect the precipitate. The finally obtained particles were dispersed in DI water and used as contrast agents for further bioimaging applications.

Characterization. The crystal phase of the as-prepared UC-PLNPs was detected by a X-ray diffractometer (XRD, Bruker D8 Discovery) at 40 kV and 40 mA with Cu-Ka radiation - = 1.54056 Å).The size and shape of the nanosized ZnGaGeO: Yb/Er/Cr UC-PLNPs were characterized by field emission transmission electron microscopy (FEI Tecnai F20) equipped with an Oxford Instrument energy dispersive X-ray spectroscopy system using an accelerating voltage of 200 kV. The UC luminescence and excitation spectra were detected by a Zolix

spectrophotometer (fluoroSENS 9000A) equipped with an external 980 nm laser as light source at room temperature. The NIR UC persistent luminescent imaging was detected by a multi-modal imaging system (Bruker *In Vivo* FX Pro) equipped with a cooled CCD camera.

***In vivo* UC persistent bioimaging.** *In vivo* UC persistent luminescence images were acquired using a multi-modal imaging system (Bruker *In Vivo* FX Pro). Aqueous solutions containing different concentrations of the nanosized UC-PLNPs (1 mg/mL and 0.2 mg/mL) were first activated for 10 min by a 980 nm laser with power density of 2 W/cm^2 before injection. The 200 μL charged UC-PLNPs solutions with concentrations of 1 mg/mL and 0.2 mg/mL were subcutaneously injected into an anesthetized Kunming mouse (intraperitoneal injection with 150 μL of 10 wt% pentobarbital sodium aqueous solution) at left and right site, respectively. *In vivo* UC persistent luminescence images were acquired after injection at different time intervals from 5 min to 15 h. The emission filter was set as 700 nm, and the exposure time was set as 120 s.

NIR-recharged *in vivo* repeating bioimaging. *In vivo* renewable bioimaging induced by 980 nm laser was also conducted on the modified multi-modal imaging system (Bruker *In Vivo* FX Pro) equipped an external 980 nm laser as pumping source. A Kunming mouse was first anesthetized via intraperitoneal injection with 150 μL of 10 wt% pentobarbital sodium aqueous solution. 200 μL UC-PLNPs solution with concentration of 1 mg/mL was then subcutaneously injected into the Kunming mouse. After in situ illumination by 980 nm laser with power density of 150 mW/cm^2, the UC persistent fluorescent signals were detected by using the same parameter as aforementioned *in vivo* bioimaging. When the persistent luminescent signals were almost completely decayed, another round in situ activation using 980 nm laser was performed. And the bioimaging was repeatedly carried out for four cycles.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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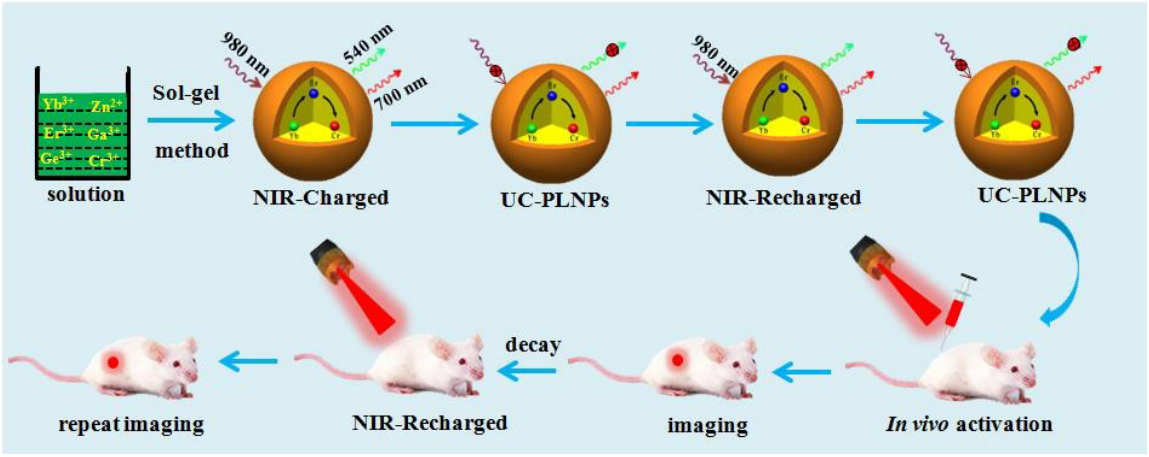

Scheme 1. Schematic illustration of the sol-gel method and NIR-to-NIR rechargeable *in vivo* bioimaging based on 980 nm laser activated UC persistent NIR-emitting UC-PLNPs.

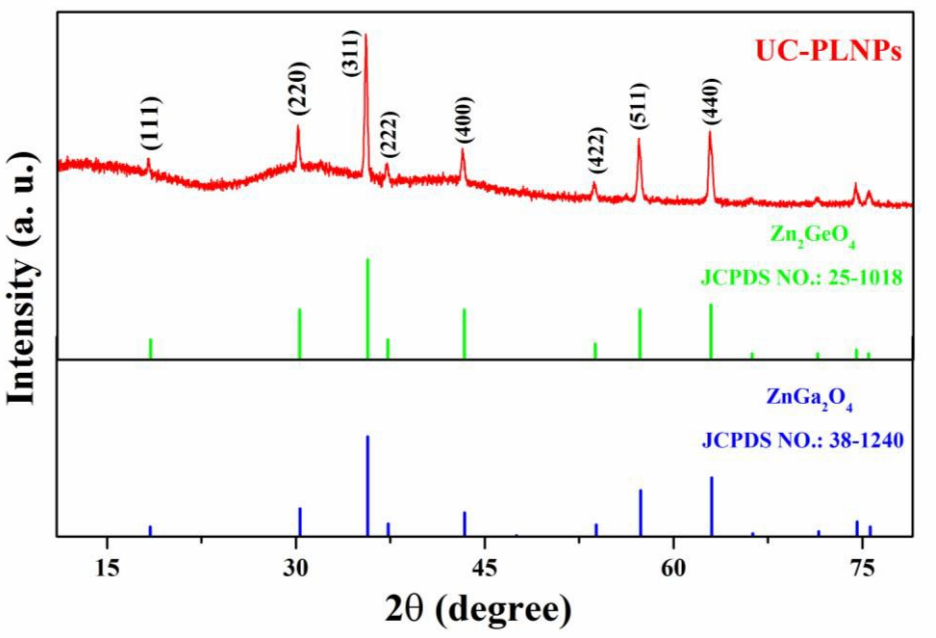

Figure 1. XRD pattern of the as-prepared ZnGaGeO: Yb/Er/Cr UC-PLNPs, the blue and green lines indicate the corresponding standard data of $ZnGa_2O_4$ (JCPDS No. 38-1240) and Zn_2GeO_4 (JCPDS No. 25-1018), respectively.

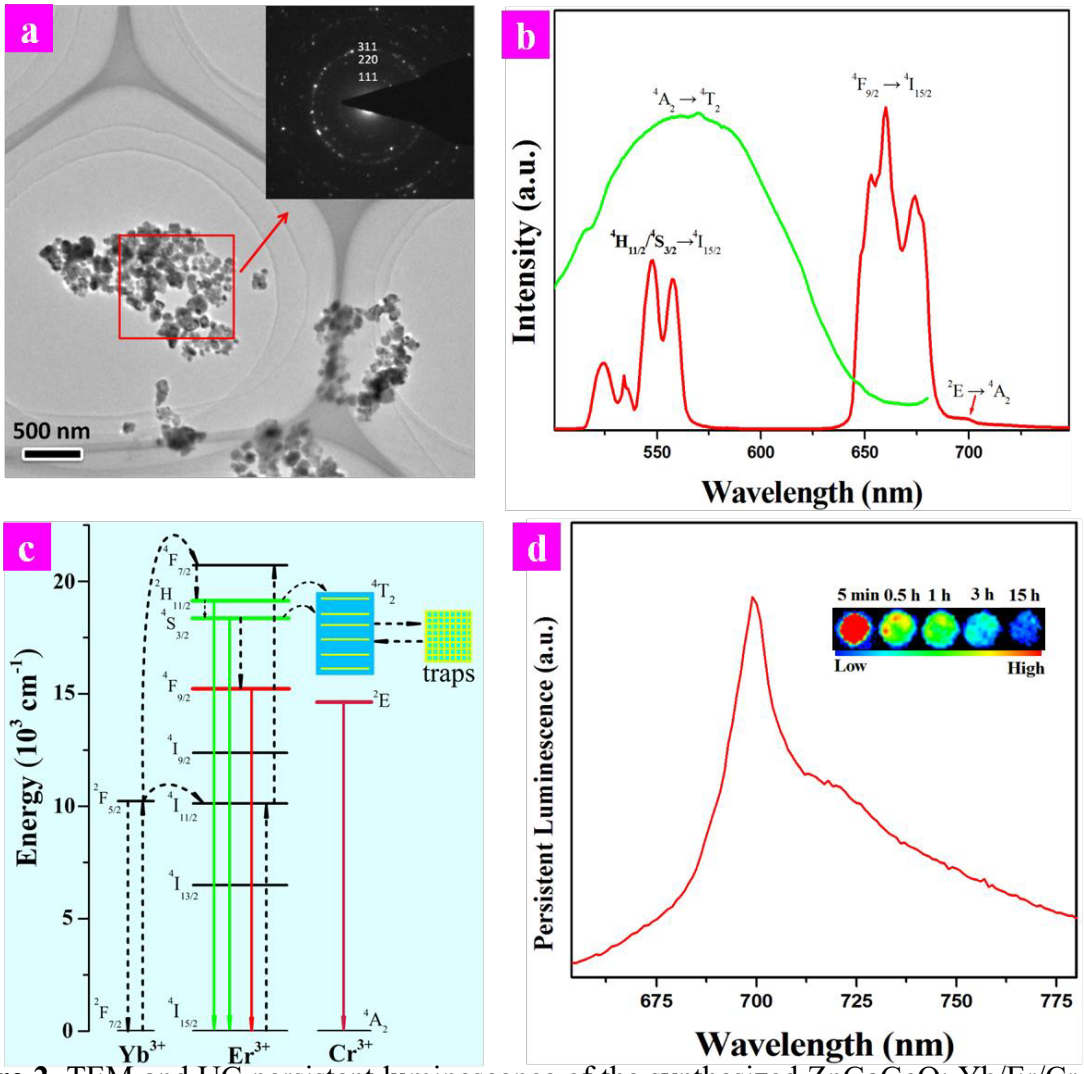

Figure 2. TEM and UC persistent luminescence of the synthesized ZnGaGeO: Yb/Er/Cr UC-PLNPs. (a) TEM image, (b) UC emission of Er^{3+} and Cr^{3+} (red line) under the excitation of 980 laser and excitation spectrum by monitoring the 700 nm emission of Cr^{3+}, (c) Schematic diagram of UC emission and NIR UC persistent luminescence of Cr^{3+} activated by 980 nm laser, (d) NIR UC persistent luminescence spectrum of Cr^{3+} detected after ceasing 980 nm laser. The inset of Figure 1a indicates the corresponding SEAD pattern. The inset of Figure 1d presents the *in vitro* phantom NIR UC persistent luminescence imaging (pseudo-color pictures) taken by a CCD camera at different decay times from 5 min to 15 h after stopping 980 nm excitation.

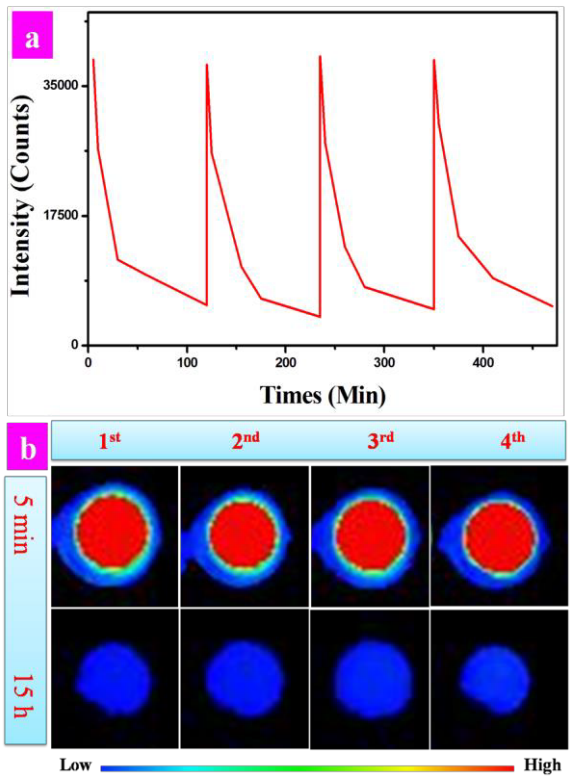

Figure 3. Rechargeable NIR UC persistent luminescence properties of the nanosized ZnGaGeO: Yb/Er/Cr UC-PLNPs. (a) The decay nature of persistent luminescent intensity after reactivation by 980 nm laser for 6 min, (b) the corresponding rechargeable *in vitro* phantom NIR imagings (pseudo-color) taken by a CCD camera at different decay times of 5 min and 15 h after the stoppage of 980 nm excitation source.

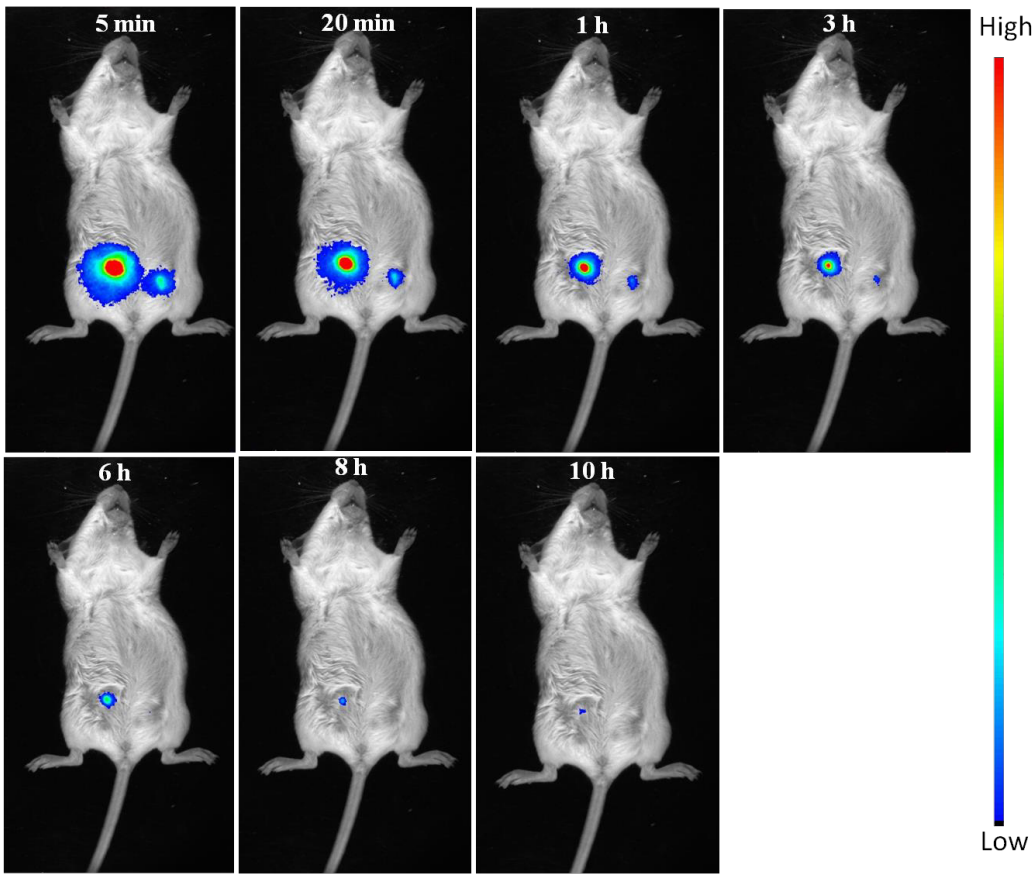

Figure 4. *In vivo* NIR UC persistent luminescence bioimaging of a mouse after subcutaneous injection of different concentrations of UC-PLNPs solution at different time intervals from 5 min to 10 h (left site: 1.0 mg mL^{-1}, right site: 0.2 mg mL^{-1}, using 980 nm laser to activate for 10 min before injection).

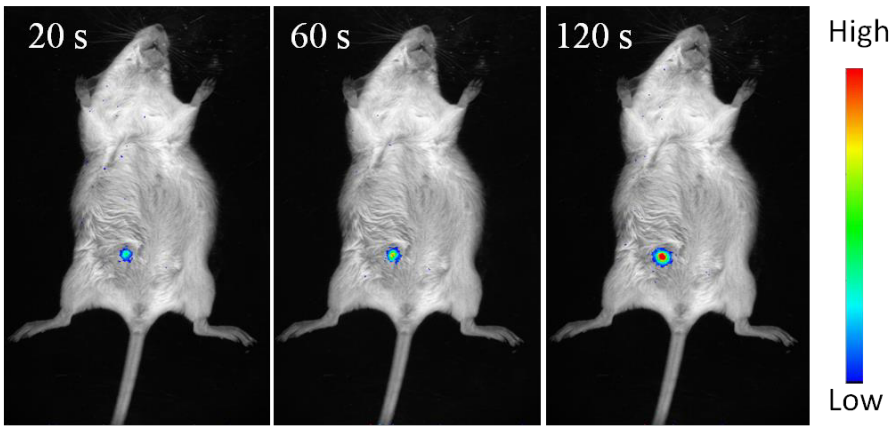

Figure 5. *In vivo* UC-persistent bioimaging with *in situ* activation of UC-PLNPs for different time (20 s, 60 s, 120 s) by a 980 nm laser with power density of 150 mW/cm^2. The results reveal that after 120 s in situ activation by 980 nm laser, these UC-PLNPs present efficient NIR persistent luminescence for bioimaging.

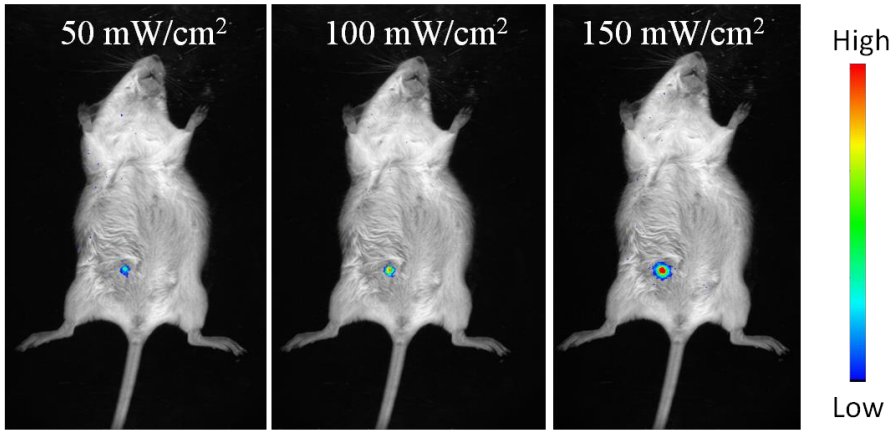

Figure 6. *In vivo* UC-persistent bioimaging with in situ activation of UC-PLNPs for 120 s by modifying the power density of a 980 nm laser from 50-150 mW/cm^2. The results reveal that the synthesized UC-PLNPs can be successfully and efficiently recharged by a whole body illumination of 980 nm laser with power density of 150 mW/cm^2.

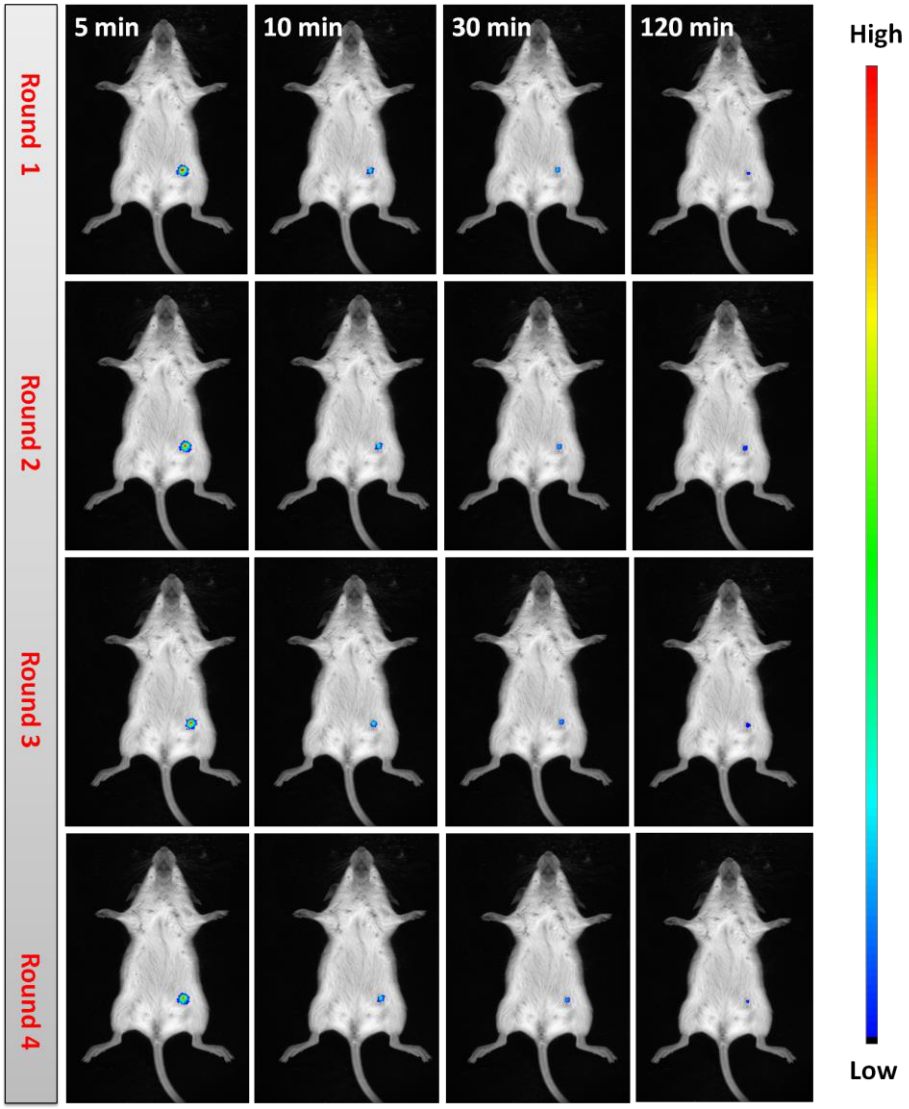

Figure 7. *In vivo r*echargeable bioimaging based on the nano-sized ZnGaGeO: Yb/Er/Cr UC-PLNPs for four cycles at different decay times (5 min, 10 min, 30 min, 120 min) after *in situ* excitation by a 980 nm laser. *In situ* excitation was performed using the 980 nm laser as light source with power density of 150 mW/cm^2 for 120 s.

A new type of upconverted NIR persistent luminescent nanoprobe is reported by incorporating upconversion and persistent luminescent properties into a single host. The synthesized nanoprobes can be in situ recharged by high-tissue penetrating light of 980 nm laser for *in vivo* bioimaging, providing a new possibility for widely using persistent luminescent nanoparticles for *in vivo* renewable bioimaging.

upconverted persistent emission, NIR emission, *in vivo* bioimaging, rechargeable persistent bioimaging

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NIR-to-NIR rechargeable *in vivo* bioimaging using upconversion persistent luminescent nanoprobes