Ultra-stable near-infrared Tm³⁺-doped upconversion nanoparticles for *in vivo* wide-field two-photon angiography with a low excitation intensity

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Two-photon luminescence with near-infrared (NIR) excitation of upconversion nanoparticles (NPs) is of great importance in biological imaging due to deep penetration in high-scattering tissues, low auto-luminescence and good sectioning ability. Unfortunately, common two-photon luminescence is in visible band with an extremely high excitation power density, which limits its application. Here, we synthesized NaYF₄:Yb/Tm@NaYF₄ upconversion NPs with strong two-photon NIR emission and a low excitation power density. Furthermore, NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs with high chemical stability were obtained by a modified multilayer coating method which was applied to upconversion NPs for the first time. In addition, it is shown that the as-prepared hydrophilic upconversion NPs have great biocompatibility and kept stable for 6 hours during *in vivo* whole-body imaging. The vessels with two-photon luminescence were clear even under an excitation power density as low as 25 mW/cm². Vivid visualizations of capillaries and vessels in a mouse brain were also obtained with low background and high contrast. Because of cheaper instruments and safer power density, the NIR two-photon luminescence of NaYF₄:Yb/Tm@NaYF₄ upconversion NPs could promote wider application of two-photon technology. The modified multilayer coating method could be widely used for upconversion NPs to increase the stable time of the *in vivo* circulation. Our work possesses a great potential for deep imaging and imaging-guided treatment in the future.

Keywords: Ultra-stable upconversion nanoparticles; two-photon luminescence; *in vivo* brain angiography; low excitation power density.

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1. Introduction

Recently, nanoparticle-assisted optical bio-imaging has attracted enormous attention in the field of biological photonics.^{1–3} The most notable nanoparticles (NPs) are those with NIR absorption or emission due to the outstanding tissue penetration abilities, the minimal photon-damage and the lower auto-luminescence of near-infrared (NIR) light compared with visible light.^{4–6} Over the last two decades, NPs with large two-photon absorption in the NIR region have been successfully developed, and two-photon luminescence has become an effective strategy to utilize the NIR excitation.^{7–9} Due to the excellent NIR excitation and special nonlinear optical properties, two-photon luminescence is naturally endowed with several advantages, for example, deeper penetration in high-scattering tissues, lower auto-luminescence, higher spatial resolution and better sectioning ability.^{10,11} Until now, the most common two-photon probes are dye-doped NPs.¹² Although dye-doped two-photon NPs are endowed with high biocompatibility, their limits are also obvious. In the process of two-photon absorption, the intermediate excited state of the dye is a ‘virtual’ state with a very short lifetime. Dye-doped two-photon NPs require a very high excitation photon flux in a very short period of time, which only the femtosecond laser can provide. High prices and the maintenance complexity of the femtosecond laser system limits the widespread use of two-photon luminescence. As the high excitation power density requires a highly focused excitation beam, the excitation mechanism of the dye limits its imaging model to the spot scanning imaging.¹³ Besides, two-photon luminescence of dye-doped NPs is mostly located in the visible range. Therefore, upconversion NPs with two-photon NIR emission would be a better choice.

In the case of upconversion NPs, the intermediate excited state of Re^{3+} is a real state with a long lifetime, which only requires very moderate excitation conditions. In this case, a cheap and portable continuous wave diode laser can offer enough photons to trigger the two-photon luminescence.^{13,14} The much lower power density of the excitation beam would further reduce photo damage and auto-luminescence in tissues. Furthermore, the imaging method could be broadened to the non-scanning

wide-field imaging system. The system is much simpler and the acquisition time is shorter. Apart from this, the two-photon NIR emission of Tm^{3+} lies within the so-called biological window ‘700–900 nm’, which results in less scattering and deeper tissue penetration.¹⁵ Therefore, the unique nonlinear optical properties with NIR excitation and NIR emission make Tm^{3+} -doped upconversion NPs ideal probes for bio-imaging. Unfortunately, current *in vivo* imaging work based on upconversion NPs were mostly imaging for liver, which could not fully demonstrate the penetration and sectioning ability of upconversion NPs.^{9,15} These imaging work were limited by the traditional coating method of upconversion NPs. In these works, poly acrylic acid (PAA) or polyethylene glycol (PEG) was chosen as the only coating polymer. The poor chemical stability could be speculated from sharp fading of vessels in several angiography work and fast aggregation in the liver in most imaging work.^{16,17} Indeed, the chemical stability of upconversion NPs in aqueous solutions with various pH is very essential, since (1) so far there is no report on the pH stability of polymer-coated upconversion NPs; (2) the pH values of biological reactions vary in a large range. Furthermore, a high chemical stability is helpful to the long *in vivo* circulation time.

In this study, $\text{NaYF}_4:\text{Yb}/\text{Tm}@\text{NaYF}_4$ upconversion NPs with strong two-photon NIR luminescence were synthesized and further functionalized via a modified multilayer coating method.¹⁸ The method has been used in coating gold nanorods and quantum dots before and stable NPs were obtained.^{19,20} Here, we applied a modified method to coat $\text{NaYF}_4:\text{Yb}/\text{Tm}@\text{NaYF}_4$ upconversion NPs and confirmed the chemical stability of as-obtained $\text{NaYF}_4:\text{Yb}/\text{Tm}@\text{NaYF}_4@\text{SiO}_2@\text{OTMS}@\text{F127}$ NPs in aqueous solutions with various pH. The ultra-stable hydrophilic upconversion NPs were then applied in the whole-body imaging with the excitation power density as low as $25 \text{ mW}/\text{cm}^2$. Furthermore, the vessels were still clear after 6 h which demonstrated the long *in vivo* circulation time. Due to the strong NIR luminescence and high chemical stability of upconversion NPs, brain angiography was carried out. Vivid visualizations of vessels and capillaries were obtained with low background noise and high contrast.

2. Experimental Section

2.1. Materials

$\text{Y}(\text{CH}_3\text{CO}_2)_3 \cdot 6\text{H}_2\text{O}$ (99.9%), $\text{Yb}(\text{CH}_3\text{CO}_2)_3 \cdot 6\text{H}_2\text{O}$ (99.9%), $\text{Tm}(\text{CH}_3\text{CO}_2)_3 \cdot 6\text{H}_2\text{O}$ (99.9%), oleic acid (OA, 90%), 1-octadecene (ODE, 90%), NH_4F (98%), NaOH (98%), Pluronic F-127 (F127), tetraethyl orthosilicate (TEOS), IGEAL CO-520 and trimethoxy (octadecyl) silane (OTMS) were purchased from Sigma-Aldrich. Chloroform, tetrahydrofuran (THF), N-butyl alcohol, cyclohexane, ammonia, buffered saline (PBS) and ethanol were bought from Sinopharm Chemical Reagent Co. Ltd. Deionized (DI) water was used in all experiments.

2.2. Characterization

The dynamic light scattering (DLS) was carried out to obtain the average size and size distribution of the NPs at room temperature on the Zetasizer Nano ZS-90 (Malvern) at a fixed angle of 90° . The transmission electron (TEM) images of the NPs were taken with a 80 kV voltage in bright-field mode on the JEM-1200EX (JEOL, Japan). Two-photon NIR emission spectra was measured by a home-built luminescence measuring system. In the system, a focused 980 nm continuous wave (CW) laser was chosen as the excitation beam and a spectrometer (PG2000, Ideaoptics) was chosen as the luminescence collecting instrument.

2.3. Synthesis of $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4$ NPs

The upconversion NPs were synthesized by a coprecipitation method.²¹ Briefly, 0.4 mmol lanthanide triacetate composed of 69.5% $\text{Y}(\text{CH}_3\text{CO}_2)_3$, 30% $\text{Yb}(\text{CH}_3\text{CO}_2)_3$, 0.5% $\text{Tm}(\text{CH}_3\text{CO}_2)_3$ were dissolved in 2 mL DI water and mixed with 3 mL OA, 7 mL ODE in a three-necked flask. The mixture was heated to 150°C and kept for 90 min to form a clear yellow solution. After the reaction system was cooled down to room temperature, the solution was heated to 50°C , 5 mL methanol containing 1 mmol NaOH and 1.6 mmol NH_4F was added and the system was kept at 50°C for another 30 min. Then the solution was heated to 100°C to remove the methanol and further heated to 280°C under the protection of N_2 . After 90 min, the reaction was cooled down naturally and 6 mL ethanol was added. The mixture was centrifuged at 10,000 rpm

for 10 min to collect the Nps. Finally, $\text{NaYF}_4\text{:Yb/Tm}$ NPs were washed twice with ethanol and dispersed in cyclohexane.

The inert shell of NaYF_4 was grown by the same method. $\text{NaYF}_4\text{:Yb/Tm}$ NPs in cyclohexane were added into the mixture of $\text{Y}(\text{CH}_3\text{CO}_2)_3$ and OA, and the solution was kept at 80°C to evaporate the cyclohexane before the reaction. The remaining reaction steps were the same as before. Finally, $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4$ NPs were dispersed in 4 mL cyclohexane.

2.4. Synthesis of $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs

$\text{NaYF}_4\text{:Yb/Tm@NaYF}_4$ upconversion NPs were caught in silica via a common reverse microemulsion coating method. In detail, 400 μL $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4$ NPs were added into the mixture of 1 mL IGEAL CO-520 and 8 mL cyclohexane under vigorous stirring. Then, 80 μL aqueous solution of ammonia (29.4%) and 45 μL TEOS were added into the dispersion in turn, and the reaction was kept stirring at room temperature. After 24 h, 3 mL N-butyl alcohol was added into the mixture to precipitate $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2$ NPs. The mixture was centrifuged at 13,000 rpm for 8 min. Then, NPs were washed twice with ethanol and dissolved in 5 mL ethanol for the next step. 55 μL ammonia solution (29.4%) and 500 μL OTMS in chloroform solution were added into the dispersion under vigorous stirring. After 24 h, the mixture were centrifuged at 13,000 rpm for 8 min to collect NPs and washed twice with ethanol. $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS}$ NPs were dispersed in 400 μL THF, and mixed with 30 mg F127. Then, the dispersion in THF was added into 1 mL DI water dropwise under mild stirring. The mixture was kept at room temperature for 24 h to evaporate THF contents. Finally, $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs were washed twice with DI water and dispersed in 800 μL DI water for future use.

2.5. Chemical stability of $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs

The stability of $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs in an aqueous dispersion

was characterized by comparing the luminescence intensities at certain time intervals. Incubated with serum and various aqueous solutions with pH = 1–13 at 37°C, the luminescence images of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs were taken under the same conditions after 0.5 h, 24 h and 9 days. The pH of aqueous solutions was adjusted by adding HCl or NaOH.

2.6. Toxicity study of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs

All animal experiments were conducted in accordance with the rules of the Zhejiang University Animal Study Committee for the care and use of laboratory animals in research.

Histology analysis and blood assay were carried out to evaluate the *in vivo* toxicity of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs.^{22,23} Twelve mice were divided randomly into four groups. Group A was intravenously injected with 200 μL PBS (1×) as control while Groups B, C and D were injected with 200 μL NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs (10 mg/mL in PBS 1×). Group B was sacrificed to check acute reactions after 24 h, Group C was sacrificed after seven days, while Group A and D were sacrificed to check the long-time reactions after 31 days. For the blood parameters assay, bloods were collected from the ophthalmic arteriae.²⁴ The blood routine examination, hepatic and renal function analyses were conducted respectively. For histology analysis, major organs (brain, heart, liver, spleen, lung, and kidney) were fixed in 4% paraformaldehyde and embedded in paraffin. The section slices were then stained with haematoxylin and eosin (H&E) and imaged on an optical microscope with a 10× or 40× objective lens.

2.7. Pharmacokinetic property of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs

The *ex vivo* luminescence images of major organs were taken to check the pharmacokinetic property of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs.²⁵ Three mice were intravenously injected with NPs (10 mg/mL in PBS 1×), while the control mouse was injected with PBS (1×). The experimental mice was sacrificed immediately, 3 days and

31 days post-injection and the major organ was harvested. The *ex vivo* luminescence images were then conducted on a home-made whole-body imaging system under the excitation of 980 nm laser.

2.8. In vivo whole-body imaging

An eight-week-old female nude mouse was injected with 200 μL NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs (10 mg/mL in PBS 1×) for *in vivo* whole-body imaging. The mouse was imaged on a home-made system under the side excitation of 980 nm laser beam. The luminescence signals were filtered by a 750 nm long-pass filter and a 900 nm short-pass filter. Two-photon luminescence images were vertically taken by EMCCD (iXon Ultra 888, Andor).

2.9. In vivo wide-field microscopy imaging

Before imaging, an eight-week-old female mouse underwent a skull-removal surgery and the wound was sealed with a glass flakelet. The mouse was then injected with 200 μL NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs (10 mg/mL in PBS 1×) and fixed on a mice holder after anaesthesia. The mouse was imaged on an upright optical microscope with the 980 nm laser beam focused on the mouse brain. The objective lens was a scan lens (Thorlabs, 5×, work distance = 25.1 mm) or a water-immersion objective lens (Olympus, 20×, work distance = 2.0 mm). The two-photon luminescence signals were collected with the same lens and filtered by a 750 nm long-pass filter and a 900 nm short-pass filter. Two-photon luminescence images were taken by EMCCD (iXon Ultra 888, Andor).

3. Results and Discussion

3.1. Preparation and characterization of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs

NaYF₄:Yb/Tm NPs were synthesized by a well-established co-precipitation method and the NaYF₄ shell was developed to protect NaYF₄:Tm from the surroundings and further increase the upconversion luminescence intensity. The Uv-Vis absorption

Fig. 1. The luminescent spectrum of $NaYF_4:Yb/Tm$ NPs, $NaYF_4:Yb/Tm@NaYF_4$ NPs and $NaYF_4:Yb/Tm@NaYF_4@SiO_2@OTMS@F127$ NPs under the excitation of the 980 nm laser. The left inset shows the normalized intensity of the NIR luminescence and the right inset shows the luminescent images of $NaYF_4:Yb/Tm$ NPs and $NaYF_4:Yb/Tm@NaYF_4$ NPs.

spectra of $NaYF_4:Yb/Tm@NaYF_4$ NPs in cyclohexane was measured and the absorption peak was located at 976 nm as shown in Fig. S1. So, the 980 nm laser was highly efficient to excite these NPs. As shown in Fig. S2, the simulation results illustrate that 980 nm laser beam has much better penetration and focusing capability than 450 nm (a commonly used visible laser) laser beam in biological tissues. As shown in Fig. 1, $NaYF_4:Yb/Tm@NaYF_4$ NPs in cyclohexane presented the sharp NIR emission at 800 nm and low visible emission at 650 nm. Besides, the luminescent intensity of core NPs was 27.3% while that of core-shell NPs was 100% according to luminescent spectrum (left inset) and the luminescence intensity of $NaYF_4:Yb/Tm@NaYF_4$ core-shell NPs were about 4 times higher than $NaYF_4:Yb/Tm$ core NPs according to luminescent images in the right inset. This phenomenon was coherent with the previous work.^{26,27} Theoretically, the upconversion luminescence of NPs could be enhanced with a $NaYF_4$ shell because the additional passivating shell could reduce nonradiative decay losses

(i)

Nanoparticles	Hydrodynamic Diameter	Solubility in Water
$NaYF_4:Yb/Tm$	37.9 ± 8.0 nm	×
$NaYF_4:Yb/Tm@NaYF_4$	52.8 ± 12.6 nm	×
$NaYF_4:Yb/Tm@NaYF_4@SiO_2$	76.3 ± 25.9 nm	✓
$NaYF_4:Yb/Tm@NaYF_4@SiO_2@OTMS$	80.0 ± 35.6 nm	×
$NaYF_4:Yb/Tm@NaYF_4@SiO_2@OTMS@F127$	84.8 ± 27.7 nm	✓

Fig. 2. (a)–(e) The DLS results (number-weighted) of $NaYF_4:Yb/Tm$ NPs, $NaYF_4:Yb/Tm@NaYF_4$ NPs, $NaYF_4:Yb/Tm@NaYF_4@SiO_2$ NPs, $NaYF_4:Yb/Tm@NaYF_4@SiO_2@OTMS$ NPs, and $NaYF_4:Yb/Tm@NaYF_4@SiO_2@OTMS@F127$ NPs respectively. (f)–(h) The TEM images of $NaYF_4:Yb/Tm$ NPs ($\times 1.2 \times 10^5$ magnification), $NaYF_4:Yb/Tm@NaYF_4$ NPs and $NaYF_4:Yb/Tm@NaYF_4@SiO_2@OTMS@F127$ NPs ($\times 1.5 \times 10^5$ magnification). (i) The size distribution and the solubility in water of the respective NPs.

Scheme 1. Schematic illustration of the fabrication of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs through a modified multilayer coating method and the chemical structures of TEOS, OTMS and F127.

of the surface luminescence.²⁸ Furthermore, according to the dynamic light scattering (DLS) measurements, NaYF₄:Yb/Tm NPs had an average hydrodynamic diameter (number-weighted) of 37.87 ± 8.0 nm and NaYF₄:Yb/Tm@NaYF₄ NPs were 52.8 ± 12.6 nm as shown in Figs. 2(a), 2(b) and 2(i). The TEM images of NaYF₄:Yb/Tm NPs and NaYF₄:Yb/Tm@NaYF₄ NPs were shown in Figs. 2(f) and 2(g).

To convert the hydrophobic NaYF₄:Yb/Tm@NaYF₄ NPs into hydrophilic probes with good biological compatibility, we adopted a modified multilayer coating method to form NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 upconversion NPs as illustrated in Scheme 1. The method was first proposed to coat gold NPs and further applied in quantum dots to serve high chemical stability and biosafety. Here, we introduced the excellent coating method to upconversion NPs for the first time, to increase the biological compatibility and *in vivo* stability. The hydrophobic NaYF₄:Yb/Tm@NaYF₄ upconversion NPs in cyclohexane were turned into hydrophilic with SiO₂ shell by the common reverse microemulsion method. The hydrophilic NaYF₄:Yb/Tm@NaYF₄@SiO₂ NPs were converted into hydrophobic again with the fabrication of OTMS. The hydrophobic NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS NPs were further combined with F127, an FDA-proved amphiphilic polymer, to disperse evenly in water. Here, F127 was used to replace the expensive polyethylene glycol (PEG) in the origin method as the last layer. During the process of coating, the changes of DLS results, TEM images and the solubility in water was recorded to verify the successful coating of each layer as shown in Fig. 2(i). The diameters enlarged

layer by layer which directly demonstrated the successful coating of SiO₂, OTMS and F127. Besides, the coating results could be indirectly judged from the solubility in water. The hydrophobic NaYF₄:Yb/Tm@NaYF₄ turned into hydrophilic NaYF₄:Yb/Tm@NaYF₄@SiO₂, and became hydrophobic (white aggregation once touched water) again with the layer of OTMS. The final NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 dispersed well in water which verify the successful coating. In addition, there wasn't any obvious difference in TEM images between NaYF₄:Yb/Tm@NaYF₄@SiO₂, NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS and NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 because OTMS and F127 was a kind of polymer presenting quite poor contrast compared with the metal core. Furthermore, the normalized NIR luminescence intensity of well-coated NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs was 16.9% while NaYF₄:Yb/Tm@NaYF₄ NPs was 100% as shown in Fig. 1 (left inset).

3.2. The chemical stability and photo-stability of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs

The chemical stability of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs was characterized by comparing the luminescence intensities of upconversion NPs in an aqueous dispersion with different pH at certain intervals. As shown in Fig S2, there were none obvious intensity changes with naked eyes between the images with different pH and different time. Thus, we calculated the

Fig. 3. Normalized NIR luminescent intensities of $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs in aqueous solutions with different pH, taking the value at 30 min (pH = 7) as 100% based on luminescent images in Fig. S3.

luminescence intensity of each image by subtracting that of pure water under the same conditions. As shown in Fig. 3, taking the intensity of dispersions with pH = 7 at 0.5 h as 100%, the changes with time and different pH were less than 30%. Considering the inevitable errors during the imaging and calculating process, the results indicated that $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs were endowed with high chemical stability which was significant for biological imaging, especially for applications involving long-term study.

To demonstrate the photo-stability of NPs, $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs in water were excited continuously for 3 h under the irradiation of a 980 nm laser (fixed at 500 mW). The luminescent spectra were measured at certain time intervals and the areas of the NIR emission were calculated to compare. The normalized results were presented in Fig. 4 by taking the value at 0 min as 100%. It was obvious that the luminescence of NPs were kept stable during the three hours' irradiation. The outstanding stability against photobleaching of upconversion NPs shown here was very important for the long time observation in bioimaging.

3.3. *In vivo* toxicity of $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs

To evaluate the *in vivo* toxicity of $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs, we carried out the histology analysis and blood parameters

Fig. 4. Normalized NIR luminescent intensities of $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs during the three hours' irradiation of the 980 nm laser, taking the value at 0 min as 100%.

assay.²⁹ As shown in Fig. 5, microscopic images of major organs (heart, liver, spleen, lung, kidney and brain) were taken with a 10 $\times$ and 40 $\times$ objective lens. Compared with PBS (1 $\times$), the mice injected with $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs presented none obvious inflammation or abnormality after 24 h, 7 days and 31 days. For the blood routine examination results, as shown in Fig. S4, there were no obvious signs of infection, allergic or toxic reactions found. For the hepatic function analyses, as shown in Fig. S5, there was no obvious difference in alkaline phosphatase, alanine transaminase, aspartate transaminase, and gamma-glutamyl transferase between the experimental group and the control group. For the renal function analyses, as shown in Fig. S5, the markers of creatinine, uric acid, and blood urea nitrogen were within the normal range. Thus, $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs were proved to be biocompatible. The high biocompatibility was beneficial to future imaging work.

3.4. Pharmacokinetic property of $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs

To demonstrate the pharmacokinetic property of $\text{NaYF}_4\text{:Yb/Tm@NaYF}_4\text{@SiO}_2\text{@OTMS@F127}$ NPs, we carried out the *ex vivo* macroscopy of major organs. As shown in Fig. S6(a), strong signals were

Fig. 5. Microscopic images of tissue sections from mice injected with PBS (1 $\times$) (top, control group), PBS (1 $\times$) dispersions of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs after 24 h, 7 days and 31 days.

observed in the lung immediately post injection as the NPs distributed mostly in the blood. After 3 days, as shown in Fig. S6(b), the signals decreased as a whole. As signals of the lung decreased a lot, signals of the liver and spleen relatively increased due to the metabolism. After 31 days, as shown in Fig. S6(c), the whole signals reached a quite low level, which demonstrated the good metabolism property of the NPs.

3.5. *In vivo* whole-body imaging

To verify the potential application of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs in biological imaging, a nude mouse was injected intravenously with 200 μ L NPs (10 mg/mL in PBS 1 $\times$) and imaged on our home-made system under the excitation of the 980 nm laser. As shown in Fig. 5(a), the mouse was excited evenly by the expanded 980 nm laser and the two-photon NIR luminescence signals were collected vertically by the EMCCD. Thanks to the special nonlinear optical properties of upconversion NPs, the vessels were clearly distinguishable under the power density as low as 25 mW/cm², as shown in Fig. S7(a). In addition, the *in vivo* stability was also demonstrated. Three images were taken immediately, 2 h and 6 h post-injection, respectively, under the same conditions with the power density 44 mW/cm². As shown in Figs. 5(b) and S7(b)–S7(d), vessels were very clear after 2 h and still recognizable after 6 h as signals in the liver increased. In our work, the prolonged

in vivo circulation time shows the potential for long-time bio-imaging research.

3.6. *In vivo* wide-field microscopy imaging

As NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs could be stable in vessels for hours, it was possible to apply the bright NIR NPs in brain angiography. As shown in Fig. 6(a), the imaging system was reconstructed based on a commercial upright microscopy. The excitation laser beam was introduced and focused on the brain of the mouse (100 mW/cm²), and luminescence signals were collected from above. As shown in Fig. 6(b), the visualization of the brain blood vessels was achieved

Fig. 6. (a) Schematic illustration of our home-made whole-body imaging system. (b) *In vivo* luminescence images (using false color) of nude mice immediately post injection of NaYF₄:Yb/Tm@NaYF₄@SiO₂@OTMS@F127 NPs.

Fig. 7. (a) Schematic illustration of wide-field microscopy imaging system. (b) The *in vivo* luminescence image of brain vessels with $5\times$ lens post injection of $\text{NaYF}_4:\text{Yb}/\text{Tm}@\text{NaYF}_4@\text{SiO}_2@\text{OTMS}@\text{F127}$ NPs. (c) The luminescence intensity distribution across a selected vessel.

via a $5\times$ lens and the imaging depth reached 1 mm. Due to the unique upconversion process and low scattering of NIR light, capillaries were very clear. The luminescence intensity distribution across a selected vessel was calculated and the diameter of the vessel was $16.4\ \mu\text{m}$, as shown in Fig. 6(c). In addition, the sectioning ability was also shown in Fig. S8. The vessels across the images disappeared and vessels in the corner became clear as the depth increased. In general, the well-defined vascular border in Figs. 6 and S8 was attributed to the strong two-photon luminescence and low scattering of NIR light. Thus, $\text{NaYF}_4:\text{Yb}/\text{Tm}@\text{NaYF}_4@\text{SiO}_2@\text{OTMS}@\text{F127}$ upconversion NPs were demonstrated to be an excellent probe with high contrast in deep tissue.

4. Conclusions

In summary, $\text{NaYF}_4:\text{Yb}/\text{Tm}@\text{NaYF}_4$ upconversion NPs with strong two-photon NIR emission have been synthesized and functionalized via a modified

multilayer coating method. The obtained $\text{NaYF}_4:\text{Yb}/\text{Tm}@\text{NaYF}_4@\text{SiO}_2@\text{OTMS}@\text{F127}$ NPs were quite stable in aqueous solutions with $\text{pH} = 1\text{--}13$ and the PBS solution. Due to the extraordinary chemical stability and biocompatibility, the as-prepared upconversion NPs in PBS were applied in the long-time whole-body imaging of the nude mouse. The stable *in vivo* circulation time was as long as 6 h and the excitation power density was as low as $25\ \text{mW}/\text{cm}^2$. The upconversion NPs have further been applied in brain angiography. Vivid visualizations of capillaries and big vessels have been demonstrated with low background noise and high contrast. Our work should have a great potential in deep imaging and imaging-guided treatment in the future.

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