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Dual-modality *in vivo* imaging using rare-earth nanocrystals with near-infrared to near-infrared (NIR-to-NIR) upconversion luminescence and magnetic resonance properties

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ABSTRACT

Upconversion luminescence (UCL) imaging is expected to play a significant role in future photoluminescence imaging since it shows advantages of sharp emission lines, long lifetimes, superior photostability and no blinking. To further improve penetration depth, herein, near-infrared to near-infrared (NIR-to-NIR) UCL and magnetic properties were combined into a nanoparticle, and NIR-to-NIR UCL and MRI dual-modal bioimaging *in vivo* of whole-body animal were developed. Hydrophilic and carboxylic acid-functionalized $Tm^{3+}/Er^{3+}/Yb^{3+}$ co-doped $NaGdF_4$ upconversion nanophosphors (AA-NPs) were synthesized and showed both NIR-to-visible and NIR-to-NIR luminescence under excitation of 980 nm. Collecting the signal of the upconversion emission from AA-NPs in the visible and NIR range, all UCL imaging of cells, tissues and whole-body animals with different penetration depth showed high contrast. Moreover, AA-NPs showed a high relaxivity of 5.60 s^{-1} $(mM)^{-1}$ and were successfully applied as contrast agents for magnetic resonance imaging (MRI) *in vivo*. By means of the combination of UCL imaging and MRI, the distribution of AA-NPs in living animals was studied, and the results indicated that these particles mainly accumulate in the liver and spleen without undesirable stay in the lungs. Therefore, the concept of UCL and MR dual-modality imaging *in vivo* of whole-body animals using $Tm^{3+}/Er^{3+}/Yb^{3+}$ co-doped $NaGdF_4$ with NIR-to-NIR upconversion luminescent and magnetic resonance properties can serve as a platform technology for the next-generation of probes for bioimaging *in vivo*.

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1. Introduction

Upconversion luminescence (UCL) of rare-earth nanophosphors is a process whereby continuous-wave (CW) low-energy light in the near-infrared (NIR) region (typically 980 nm) is converted to higher energy visible light through multiple photon absorption or energy transfer [1,2]. Such a unique luminescence mechanism excludes conventional fluorescent labels and endogenous fluorescent substances, which makes UCL imaging to be a unique visualizing tool without autofluorescence of biological samples [3]. Moreover, rare-earth upconversion nanophosphors (UCNPs) show some attractive chemical and optical features, such as sharp emission lines, long lifetimes, superior photostability and no blinking [3–20]. As a result, there is increased interest in bioimaging based on UCNPs as biological luminescence labels [11–15].

Recently, *in vivo* imaging has been rapidly becoming an important tool for biomedical applications because it provides a basis for studying the relationship between the properties of the probes and their *in vivo* behavior and more importantly could lead to predictive models for potential application in clinical research procedures. UCL imaging *in vivo* is expected to be the next-generation photoluminescence imaging technique. However, due to the low penetration depth of biological samples, *in vivo* imaging based on UCNPs as luminescent probes is still limited. Most of these reported UCNPs were doped with Er or Ho and showed NIR-to-visible upconversion luminescence, resulting in a limited penetration depth in bioimaging *in vivo* [21]. Compared with NIR-to-visible UCL, near-infrared to near-infrared (NIR-to-NIR) UCL shows higher penetration depth because of the "optical transmission window" of the biological tissues in the NIR range (750–1000 nm). As a result, Tm^{3+}-doped UCNPs with NIR-to-NIR UCL are expected to be used as nanoprobes for bioimaging *in vivo*. Very recently, Prasad et al. [17] and our group [22] reported Tm^{3+}/Yb^{3+} co-doped $NaYF_4$ for *in vivo* bioimaging of whole-body animal.

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To compensate for the deficiencies caused by the low penetration depth of UCL imaging, another strategy is to develop magnetic/fluorescent dual-modal imaging because magnetic resonance imaging (MRI) has the advantage of being a non-invasive technique for *in vivo* imaging and three-dimensional tomography [23,24]. Very recently, Prasad et al. [25] and Park et al. [19] reported $Gd^{3+}/Er^{3+}/Yb^{3+}$ co-doped $NaYF_4$ nanoparticles and Er^{3+}/Yb^{3+} co-doped $NaGdF_4$ nanoparticles, respectively. These two types of nanoparticles showed T_1-positive-contrast enhancement and NIR-to-visible upconversion luminescence and were considered as luminescence and MRI probes. Unfortunately, the application of these two nanoparticles in bio-imaging *in vivo* was not further investigated.

Herein, we are interested in the further development of new nanoprobes for MR and NIR-to-NIR UCL dual-modal imaging of living whole-body animals, because NIR-to-NIR UCL imaging and magnetic resonance imaging (MRI) bring out the best in each other. To improve the penetration depth of UCL bioimaging *in vivo*, we focused on NIR-to-NIR upconversion nanophosphors doped with Tm^{3+}/Yb^{3+} ions. Moreover, to meet the needs of combined application of UCL imaging with different penetration depth in cells, tissues and whole-body, both NIR-to-visible and NIR-to-NIR UCL were achieved by co-doping with Er^{3+}, Tm^{3+} and Yb^{3+}. To access the higher relaxivities for MRI, we chose $NaGdF_4$ as a host rather than $NaYF_4$ doped with Gd^{3+} to obtain high Gd^{3+} concentration on the surface of nanoparticles because Gd^{3+} ions at or near the nanoparticles' surface are the primary contributors to their relaxivities. In the present study, we present the synthesis of hydrophilic and carboxylic acid-functionalized $Tm^{3+}/Er^{3+}/Yb^{3+}$ co-doped $NaGdF_4$ upconversion nanophosphors by an environmentally friendly hydrothermal method and their characterization. Based on the large relaxivities and versatile combination of the advantages of efficient paramagnetic and upconversion luminescent properties of the obtained nanoparticles, we further explore T_1-positive MR and UCL *in vivo* imaging and the biodistribution of the nanoparticles in mice.

2. Experimental section

2.1. Materials

Rare-earth oxides Gd_2O_3 (99.999%), Yb_2O_3 (99.999%), Er_2O_3 (99.999%) and Tm_2O_3 (99.98%) were purchased from Beijing Lansu Co., China. Oleic acid (>90%) was purchased from Alfa Aesar Ltd. NaF, K_2CO_3, $KMnO_4$, $NaIO_4$, sodium oleate, ethanol, tertbutanol, cyclohexane, hydrochloric solution were purchased from Sinopharm Chemical Reagent Co., China. Rare-earth chlorides ($LnCl_3$, Ln: Gd, Yb, Er, Tm) were prepared by dissolving the corresponding metal oxide in 10% hydrochloric solution at elevated temperature and then evaporating the water completely. All other chemical reagents were of analytical grade and were used directly without further purification. Deionized water was used throughout.

2.2. Synthesis of hydrophilic azelaic acid-functionalized $Tm^{3+}/Er^{3+}/Yb^{3+}$ co-doped $NaGdF_4$ upconversion nanophosphors (AA-NPs)

2.2.1. Synthesis of hydrophobic oleic acid-capped $Tm^{3+}/Er^{3+}/Yb^{3+}$ co-doped $NaGdF_4$ upconversion nanophosphors (OA-NPs)

All of the doping ratios of Ln^{3+} in our experiments were molar. In a typical procedure, sodium oleate (20 mmol), deionized water (7.4 mL), ethanol (15 mL), and oleic acid (26 mmol) were mixed together under agitation to form a homogeneous solution. A 1 mmol (total amount) rare-earth chloride ($LnCl_3$, Ln: 78 mol% Gd, 20 mol% Yb, 1.8 mol% Er and 0.2 mol% Tm) aqueous solution was added under magnetic stirring. Subsequently, 5 mL NaF aqueous solution (1 M) was added dropwise to the above solution. The resulting mixture was agitated for 10 min, and then transferred to a 50 mL autoclave, then sealed and hydrothermally treated at 180 °C for 6 h. The system was allowed to cool to room temperature naturally, whereupon the products were deposited at the bottom of the vessel. Cyclohexane was used to dissolve and collect the products. By adding ethanol to the sample-containing cyclohexane solution, the products were re-precipitated. The precipitates were separated by centrifugation. Pure powders could be obtained by purifying the precipitates by washing with ethanol and water several times to remove oleic acid, sodium oleic and other residual compounds, and then dried in a vacuum.

2.2.2. Converting hydrophobic OA-NPs into hydrophilic AA-NPs

Hydrophilic AA-NPs were prepared according to a simple and versatile procedure [16] previously reported by our group through directly oxidizing oleic acid ligands with the Lemieux–von Rudloff reagent to convert hydrophobic OA-NPs into azelaic acid-functionalized nanophosphors. Due to the fact that the oxidation reaction can essentially be brought to completion beyond 2 h [26], herein, the reaction time for the conversion process was reduced from 48 h to 3 h. The solubility constant of gadium trifluoride (GdF_3) is as low as 2.36×10^{-19} (25 °C), implying strong binding between Gd^{3+} and F^-. The obtained AA-NPs were dissolved in $GdCl_3$ (0.02 mol/L) and stirred overnight, separated via centrifugation and washed with water several times to remove the free Gd^{3+}, and then dried in a vacuum.

2.3. Characterization

Powder X-ray diffraction (XRD) measurements were measured with a Bruker D4 X-ray diffractometer (Cu Kα radiation, $\lambda = 0.15406$ nm). Fourier transform infrared (FTIR) spectra were measured using an IRPRESTIGE-21 spectrometer (Shimadzu) from samples in KBr pellets. The size and morphologies of OA-NPs and AA-NPs were determined at 200 kV using a JEOL JEM-2010F low- to high-resolution transmission electron microscope (HRTEM). Samples were prepared by placing a drop of dilute dispersions in cyclohexane and water on the surface of a copper grid respectively. Energy-dispersive X-ray analysis (EDXA) of the samples was also performed during HRTEM measurements. Dynamic light scattering (DLS) experiments were carried out on an ALV-5000 spectrometer–goniometer equipped with an ALV/LSE-5004 light scattering electronic and multiple tau digital correlator and a JDS Uniphase He–Ne laser (632.8 nm) with an output power of 22 mW. The size distribution was measured at 25 °C with a detection angle of 90°. To ensure the accuracy of DLS measurement, great care was taken to eliminate dust from the samples. The aqueous solution of the AA-NPs was filtered through two membrane filters with 0.45 μm nominal pore size connected in series. Upconversion luminescence spectrum was measured with an Edinburgh LFS-920 fluorescence spectrometer, using an external 0–800 mW adjustable CW laser at 980 nm (Connet Fiber Optics, China), as the excitation source, instead of the Xenon source in the spectrophotometer.

2.4. Cell culture and laser scanning upconversion luminescence microscopy (LSUCLM) imaging in vitro

A human nasopharyngeal epidermal carcinoma cell line (KB cell) was provided by Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS, China). Cells were grown in RPMI 1640 (Roswell Park Memorial Institute's medium) supplemented with 10% FBS (fetal bovine serum) at 37 °C and 5% CO_2. Cells (5×10^8/L) were plated on 14 mm glass coverslips under 100% humidity and allowed to adhere for 24 h.

Subsequently, after washing with phosphate buffer solution (PBS), the cells were incubated in a serum-free medium containing 100 μg/mL hydrophilic AA-NPs at 37 °C for 1 h under 5% CO_2, and then washed with PBS sufficiently to remove excess nanoparticles. Laser scanning upconversion luminescence imaging was performed using a modified laser scanning upconversion luminescence microscope with an Olympus FV1000 scanning unit [3]. The cells were excited by a CW infrared laser operating at 980 nm (Connet Fiber Optics, China) with the focused power of ~19 mW. A 40 × oil-immersion objective lens was used and luminescence signals were detected in the wavelength region of 640–680 nm.

2.5. Cytotoxicity of AA-NPs

In vitro cytotoxicity was measured by performing methyl thiazolyl tetrazolium (MTT) assays on the KB cells. Cells were seeded into a 96-well cell culture plate at 5×10^4/well, under 100% humidity, and were cultured at 37 °C and 5% CO_2 for 24 h; different concentrations of AA-NPs (0, 62.5, 125, 250 and 500 μg/mL, diluted in RPMI 1640) were then added to the wells. The cells were subsequently incubated for 4 h or 12 h at 37 °C under 5% CO_2. Thereafter, MTT (10 μL; 5 mg/mL) was added to each well and the plate was incubated for an additional 4 h at 37 °C under 5% CO_2. After the addition of 10% sodium dodecyl sulfate (SDS, 100 μL/well), the assay plate was allowed to stand at room temperature for 12 h. The optical density OD570 value (*Abs.*) of each well, with background subtraction at 690 nm, was measured by means of a Tecan Infinite M200 monochromator-based multifunction microplate reader. The following formula was used to calculate the inhibition of cell growth: Cell viability (%) = (mean of *Abs.* value of treatment group/mean *Abs.* value of control) × 100%.

2.6. Relaxivity measurement in vitro

The T_1-weighted MR images were obtained using a 3 T Siemens Magnetom Trio running on Siemens' Syngo software version B15 (Siemens Medical Systems, Shanghai Key Lab of MR, Shanghai, China), in conjunction with an 8 array Loop coil (Siemens Medical Systems). Dilutions of AA-NPs and chelated gadolinium (Gd-DTPA, diethylenetriaminepentaacetic acid, Magnevist®) (0.25, 0.10, 0.05, 0.025 mg/mL) in deionized water were placed in a series of 1.5 mL tubes for T_1-weighted MR imaging. The following parameters were adopted: T_1-weighted sequence: saturation recover

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