Boosting the down-shifting luminescence of rare-earth nanocrystals for biological imaging beyond 1500 nm

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In vivo fluorescence imaging in the near-infrared region between 1500-1700 nm (NIR-IIb window) affords high spatial resolution, deep-tissue penetration, and diminished auto-fluorescence due to the suppressed scattering of long-wavelength photons and large fluorophore Stokes shifts. However, very few NIR-IIb fluorescent probes exist currently. Here, we report the synthesis of a down-conversion luminescent rare-earth nanocrystal with cerium doping (Er/Ce co-doped NaYbF4 nanocrystal core with an inert NaYF4 shell). Ce doping is found to suppress the up-conversion pathway while boosting down-conversion by ~9-fold to produce bright 1550 nm luminescence under 980 nm excitation. Optimization of the inert shell coating surrounding the core and hydrophilic surface functionalization minimize the luminescence quenching effect by water. The resulting biocompatible, bright 1550 nm emitting nanoparticles enable fast in vivo imaging of blood vasculature in the mouse brain and hindlimb in the NIR-IIb window with short exposure time of 20 ms for rare-earth based probes.

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In vivo fluorescence-based optical imaging provides high spatial and temporal resolution, giving researchers the unique ability to visualize biological processes in real-time (30 frames per second) down to the cellular level. For decades, one-photon fluorescence imaging in the visible (400–700 nm) and traditional near-infrared (NIR-I; 750–900 nm) regions of the electromagnetic spectrum have been plagued by the inability to clearly resolve deep-tissue structures and physiological dynamics. As a recent development, NIR-emissive fluorescent probes in the second near-infrared window (NIR-II, 1000–1700 nm) have afforded improved in vivo fluorescence imaging quality owing to suppressed scattering of photons and diminished autofluorescence. Several classes of fluorescent NIR-II probes have been reported including carbon nanotubes, conjugated polymers, small molecular dyes and inorganic-based nanoparticles of quantum dots and rare-earth nanocrystals. Indeed, progress have been made in NIR-II in vivo biological imaging owing to the development of various NIR-II fluorescent probes. Still, bright probes with emission in the long end of the NIR-II region remain scarce and are desired in order to further reduce scattering of emitted photons and maximize in vivo fluorescence imaging depth and clarity.

Recent progress has exemplified the enhanced resolution of vasculature structures in the mouse brain and hindlimb by detecting fluorescence emission in the NIR-IIb window (1500–1700 nm). The ~1600 nm spectral region resides in a local valley of water’s absorption spectrum where the minimal photon absorbance in-between water’s 1st and 2nd overtones enables deep-tissue optical access. In addition, a near zero-background achieved with NIR-II probes such as carbon nanotubes eliminates tissue auto-fluorescence by 808 nm excitation in the NIR-I region while detecting fluorescence emission in the >1500 nm NIR-IIb window. Since photon scattering scales as $\lambda^{-4}$ (where $\lambda$ is the wavelength and $a=0.2$–4 for different tissues), the NIR-IIb window provides the lowest photon scattering in the NIR-II region detectable with a 2D InGaAs camera, offering the most desirable imaging clarity and deep penetration using existing detectors.

Currently NIR-IIb fluorescent probes emitting in the ~1600 nm are still very limited. Previously synthesized semiconducting single-walled nanotubes (SWNTs) and CdSe@CdS core InAs quantum dots (QDs) emitting in the NIR-IIb region developed for imaging mouse brain vasculature require considerably long exposure times (200–5000 ms) due to their low quantum yield (QY) in aqueous biological environments. Other candidates such as PbS and PbSe QDs are promising but need to resolve issues of photo-instability in aqueous solutions. As an alternative, erbium doped rare-earth nanoparticles (Er-RENPs) show useful down-conversion (DC) luminescence in NIR-IIb region. Er-RENP probes, well known for their up-conversion (UC) luminescence, have garnered recent interest for biological imaging applications owing to their low toxicity, narrow band emission, and superior photo/chemical stability. Recently, the NIR-IIb DC emission (1550 nm) of Er-based RENPs (Yb/Er co-doped NaYF$_4$ nanocrystals) were employed for in vivo NIR-II imaging. A caveat was that a relatively low QY of the RENP in toluene was reported, which would be further quenched after transferring to aqueous solutions due to the strong energy-transfer rate from Er$^{3+}$ ions to the OH$^-$ groups in solution. Thus far, it has been challenging to boost the Er-RENP’s DC luminescence, requiring very long exposure times up to 1 s for in vivo imaging.

A typical Er-REN is comprised of an Er doped NaYbF$_4$ crystalline core containing Er$^{3+}$ ions and Yb$^{3+}$ ions surrounded...
by an inert shell of NaYF₄. The Yb³⁺ ions absorb ~980 nm light efficiently and can transfer their energy to excite the Er³⁺ ions to the Er⁴¹₁/₂ level. The excited Er⁴¹₁/₂ state can relax non-radiatively to the Er⁴¹₃/₂ level and then radiatively to the Er⁴¹₅/₂ level to produce the 1550 nm DC emission. However, two competing processes exist to the down-conversion luminescence. The first is the well-known UC emission process through simultaneous two-photon absorption that excites the Er⁴¹₁/₂ level to higher levels for subsequent UC emission. The second is quenching of the excited Er⁴¹₁/₂ state caused by the OH⁻ group when RENP is transferred to an aqueous solution. These competing processes pose challenges to obtaining highly bright NIR-Ib DC emission of Er-RENPs for biological imaging.

Here, we report an Er-based RENP with a 2% Er and 2% Ce doped NaYbF₄ core and NaYF₄ shell (named NaYbF₄:2%Er,2%Ce@NaYF₄) with high QY of DC emission at ~1550 nm under a 980 nm excitation for NIR-IIb imaging. The down-conversion 1550 nm luminescence of the Er-RENPs (Fig. 1d). The UC emission of the Er-RENPs at 540 and 650 nm by an inert shell of NaYF₄. The Yb³⁺ ions absorb ~980 nm light efficiently and can transfer their energy to excite the Er³⁺ ions to the Er⁴¹₁/₂ level. The excited Er⁴¹₁/₂ state can relax non-radiatively to the Er⁴¹₃/₂ level and then radiatively to the Er⁴¹₅/₂ level to produce the 1550 nm DC emission. However, two competing processes exist to the down-conversion luminescence. The first is the well-known UC emission process through simultaneous two-photon absorption that excites the Er⁴¹₁/₂ level to higher levels for subsequent UC emission. The second is quenching of the excited Er⁴¹₁/₂ state caused by the OH⁻ group when RENP is transferred to an aqueous solution. These competing processes pose challenges to obtaining highly bright NIR-Ib DC emission of Er-RENPs for biological imaging.

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Ideally the population of $^4I_{13/2}$ emitting level should be further improved by increasing the Ce$^{3+}$ concentration. However, we found that the 1550 nm luminescence ceased to increase under higher Ce$^{3+}$ doping concentrations (Fig. 1e), indicating a limit to the $^4I_{13/2}$ state desensitization by Ce$^{3+}$ ions. The resulting 2% Ce doped Er-RENPs (emission 1500–1700 nm; excitation 980 nm, 10 W cm$^{-2}$) was much brighter than the previous record$^{24}$ evident from much shorter exposure times (20 ms) for in vivo imaging. Note that we found a nonlinear relationship between the down-conversion emission and excitation power $p$, following a $p^{0.734}$ power law relationship while UC following a $p^{1.561}$ power law relationship (Supplementary Fig. 3). Such non-linearity should always be considered for rare-earth based luminescence involving multi-photons.

**Surface modification of Er-RENPs for biocompatibility.** For in vivo biological imaging, a hydrophilic surface must be imparted to the Er-RENPs for high dispersibility and stability in aqueous solutions. Various strategies have been established to convert rare-earth nanocrystals from hydrophobic to hydrophilic including ligand oxidation$^{31}$, ligand free$^{32}$, ligand exchange$^{33}$, and ligand interaction$^{34}$ methods. Here, we created a hydrophilic polymer shell on the surface of Er-RENPs by exploiting simple van-der-waals interactions between the alkyl chains of poly (maleic anhydride-alt-1-octadecene) (PMH; average molecular weight: 30,000–50,000) and the oleic acid molecules on the RENPs (Fig. 2a).

In the first step, PMH and the oleic acid coated nanoparticles were mixed and stirred in chloroform to allow insertion for alkyl chains on PMH into the oleic acid coating on Er-RENPs. After evaporating the solvent, an aqueous solution of 4-(dimethylamino)pyridine (DMAP) was added to re-disperse the nanoparticles through sonication. The DMAP served as nucleophilic catalyst for the esterification with the outward anhydride groups of PMH$^{35,36}$. Each of the anhydride rings on PMH was transformed into two carboxyl groups, thus rendering the Er-RENPs water dispersible (Fig. 2a). Dynamic light scattering (DLS) measurements showed an average hydrodynamic radius ($R_H$) of $\sim 26$ nm for the PMH capped Er-RENPs in pure water (Fig. 2b), corresponding to the 18 nm sized RENPs with oleic acid and PMH coating layers. To render the RENPs more biocompatible, we performed a further PEGylation step through conjugation of methoxy polyethylene glycol amine (mPEG-NH$_2$; average molecular weight: 5000) onto the nanoparticles. DLS measurements (Fig. 2b) showed that the average $R_H$ of the RENPs increased by 11 nm after PEGylation. The PEGylated Er-RENPs@PMH PEG showed excellent dispersibility in high-salt solutions (Fig. 2c), with no detectable aggregation even in 10x PBS buffer solution. High aqueous dispersion stability (Fig. 2d; Supplementary Fig. 5) and photostability of the PEG-RENPs in PBS and fetal bovine serum (Supplementary Fig. 6) were also confirmed. However, the luminescence intensity of Er-RENPs in aqueous solution seriously decreased compared with that in cyclohexane (Fig. 2e).

**Reducing the quenching effect to the Er-RENPs in aqueous solution.** Hydroxyl group has been shown to be a serious quencher for the DC emission of Er$^{3+}$–39. The energy spacing of $^{4}I_{13/2} \rightarrow ^{4}I_{15/2}$ transition for the 1550 nm luminescence is $\sim 6500$ cm$^{-1}$, giving way to a two-phonon quenching mechanism by the OH$^-$ groups (Fig. 3a) with fundamental stretching vibration frequencies in the range of 2700–3700 cm$^{-1}$. In fact, this drastic quenching effect is reported to be more than 20 times stronger than that of the Yb$^{3+}$$^{22}$, causing a remarkable intensity decrease of the 1550 nm emission after hydrophilic coating of Er-RENPs for aqueous solubility and compatibility (Fig. 3b)$^{40}$.

To reduce the aqueous quenching effect, the most common way is coating a NaYF$_4$ passive shell to increase the distance between lanthanide ions and surface quenchers$^{41}$. Usually an additional NaYF$_4$ layer of <3 nm thick is adequate to protect the UC luminescence from quenching effect originating from surface ligands and aqueous solvents$^{42,43}$. Indeed, when we synthesized a $\sim 3$ nm NaYF$_4$ shell on the Ce doped NaYbF$_4$:2%Er,2%Ce nanoparticles (confirmed by transmission electron microscopy (TEM), Supplementary Fig. 7a, b), we retained more than 90% of the UC intensity of the Er-RENPs after aqueous transfer (Supplementary Fig. 9). However, we found that the 1550 nm DC
emission intensity of these Er-RENPs decreased by as much as 20-fold (defined as the quenching rate) after transferring to water (Fig. 3b, c). This led us to grow thicker inert shells up to 8 nm to further isolate the Er$^{3+}$ ions in the core of the nanoparticle from water (see Supplementary Fig. 7 for TEM; see Supplementary Fig. 10 for DLS analysis). A gradual increase in the NIR-IIb emission of the Er-RENPs in water solution was observed as the shell thickness increased (Fig. 3b). When the NaYF$_4$ shell thickness was controlled at ~ 7 nm, the 1550 nm emission of intravenously injected Er-RENPs increased (Fig. 3b). This led us to grow thicker inert shells up to 8 nm to further isolate the Er$^{3+}$ ions in the core of the nanoparticle from water (see Supplementary Fig. 7 for TEM; see Supplementary Fig. 10 for DLS analysis). A gradual increase in the NIR-IIb emission of the Er-RENPs in water solution was observed as the shell thickness increased (Fig. 3b). When the NaYF$_4$ shell thickness was controlled at ~ 7 nm, the 1550 nm emission intensity of the Er-RENPs reached a maximum, which was 2.5 times brighter than the Er-RENPs with 3 nm shell. Further increase in the thickness of NaYF$_4$ shell afforded no further enhancement of the 1550 nm luminescence (Fig. 3b), likely due to reduced absorption of the excitation light by Yb$^{3+}$ in the core through a thicker shell$^{44}$. Indeed, thicker shells lowered the DC and UC luminescence alike in both organic and aqueous solution (Supplementary Fig. 9). This was the first investigation of the effects of inert shell thickness on the DC NIR-IIb luminescence of the core-shell Er-RENPs.

In terms of absolute QY, due to discrepancies in the reported QY of the IR-26 reference fluorophore ($\text{QY}_{\text{IR26}} = 0.05$–0.5%)$^{45,46}$, the absolute QYs of our Ce doped Er-RENPs in aqueous solutions were estimated to be in the range of 0.27–2.73% (Supplementary Fig. 12) under the laser excitation of 10 W cm$^{-2}$. Although this was the highest among reported down-conversion RENPs, the 1550 nm luminescence of our Er-RENPs was still seriously quenched by 8 times in the water phase relative to in cyclohexane (Fig. 3b, c), and remains a challenge to be further addressed.

**Fast in vivo cerebrovascular imaging in NIR-IIb window with RENPs.** With the bright Ce doped Er-RENPs and a 2D InGaAs camera (Princeton Instruments, detection range 800–1700 nm), we performed in vivo mouse brain vessel imaging (Fig. 4) by exciting the Er-RENPs with a 980 nm laser while detecting the 1550 nm luminescence of intravenously injected Er-RENPs@PMH-PEG solution. Imaging was done through the intact mouse scalp and skull in a non-invasive manner. Benefiting from the bright luminescence of Ce doped Er-RENPs, we were able to carry out dynamical imaging and tracking of arterial blood flow in the mouse brain in the 1500–1700 nm NIR-IIb window using a much shorter exposure time (20 ms) than previously possible (200–5000 ms) using rare-earth materials$^{25}$, carbon nanotubes$^{13}$, and QD$^{10}$ NIR-IIb probes.

Immediately after tail vein injection of 200 µl solution of Er-RENPs@PMH-PEG at a concentration of ~ 28 mg/ml, videorate imaging of the mouse brain (C57Bl/6 mouse) was performed (using a 980 nm laser excitation and luminescence detection in the 1500–1700 nm range) with each frame recorded under a 20 ms exposure time. With the excellent temporal resolution, NIR-IIb emission in the confluence of sinuses was observed within ~ 3 s post-injection (Fig. 4a, b; Supplementary Movie 1). At ~ 4 s post-injection, blood flow into the inferior cerebral veins (ICV), the superior sagittal sinus (SSS), the superficial veins (SV), and the transverse sinus (TS) began to show up (Fig. 4c). By plotting the distance traveled by the flow front as a function of time, we were able to obtain a spatially resolved blood-flow map in the brain (Fig. 4c; Supplementary Fig. 13; blood-flow velocity in a range of ~434–1471 mm s$^{-1}$). This was the first time that video-rate NIR-IIb through-skull imaging of mouse cerebral vessels is sufficiently fast to image and quantify cerebral blood-flow velocities.

Principal component analysis (PCA) of the dynamic images was performed by time-coursing 80 frames over a time course of ~3 s (Fig. 4e, f)$^{17}$. Various venous vessels (blue, Fig. 4d) including the ICVs, the SSS, the SV, and the TS were discriminated from the arterial vessels (red, Fig. 4f) of the middle cerebral artery (MCA).

Within tens of seconds post injection, the Er-RENPs@PMH-PEG fully perfused into the mouse brain vessels and clearly outlined cerebral vascular structures at a depth of >1.3 mm under the intact scalp and skull (Fig. 4d). By plotting the cross-sectional intensity profiles, we measured the vessel signal-to-background...
were then dispersed in 2 ml MES solution (10 mM; 4-Morpholinolinesulfonic acid; the pH was tuned to 6.5 with sodium hydroxide solution). 4 mg mPEG-NH₂ (5 kDa; Methoxy polyethylene glycol amine, Laysan Bio) dissolved in 2 ml MES solution (10 mM; pH = 6.5) was added into above solution and shaken for 10 min. 2 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) dissolved in 200 μl water was then added; and the solution was shaken for 3 h. 20 μl Tris-HCl solution (1 M; Thermo Fisher Scientific) was added; and the solution was shaken for another 1 h. The solution was centrifuged at 4400 rpm for 30 min; and the supernate containing RENP@PMH-PEG was washed with centrifugal filter (100 K) for 2 times to remove excess EDC and mPEG-NH₂. The afforded RENPs@PMH-PEG were ready to disperse in water, PBS solution, and PBS solution.

Mouse handling. All vertebrate animal experiments were performed under the approval of the Stanford University’s Administrative Panel on Laboratory Animal Care. C57Bl/6 male mice were obtained from Jackson Farms. Before brain and hindlimb vessel imaging, a rodent anesthesia machine with 21 min⁻¹ O₂ gas flow mixed with 2.5% isoflurane was used to anesthetize the mice. The hair over the scalp and hindlimb skin was carefully removed using Nair to avoid causing wounds to the skin. The tail vein injection of the RENPs@PMH-PEG contrast agent was carried out in dark and synchronized with the camera that started continuous image acquisition simultaneously. For brain and hindlimb imaging in the NIR-IIb window, a 1x PBS solution (200 μl) of 28 mg/ml RENPs@PMH-PEG was injected. During the dynamic imaging the mouse was kept anesthetized by a nose cone delivering 21 min⁻¹ O₂ gas mixed with 2.5% isoflurane. The sample sizes of mice were selected based on previously reported studies. No blinding was performed. Mice were randomly selected from cages for all experiments. All groups within study contained n = 5 mice.

Dynamic fluorescence imaging in the NIR-IIb window. A liquid-nitrogen-cooled, 320 x 256 pixel two-dimensional InGaAs array (Princeton Instruments) was used to carry out in vivo imaging of mouse brain and hindlimb. The excitation light was provided by a 980 nm continuous-wave laser coupled to a collimator (F240SM-A980; Thorlabs). The excitation power density at the imaging plane was 150 mW cm⁻². The emitted fluorescence was allowed to pass through a 1100 nm and a 1500 nm filter (Thorlabs) to ensure the NIR images taken in the NIR-IIb region of 1500–1700 nm. The upper bound at 1700 nm was determined by the sensitivity profile of the InGaAs detector. A lens pair consisting of two achromats (200 and 75 mm; Thorlabs) was used to focus the image onto the detector with a field of view of 25 x 20 mm. The exposure time for each image acquisition was 20 ms, while the overhead time of the camera is – 19 ms. Therefore, the frame rate we used for dynamic imaging is 1/(20 + 19 ms) = 25.6 Hz (the temporal resolution is 39 ms). To perform the PCA, early image frames immediately after injection (200 μl of RENPs@PMH-PEG at a concentration of 28 mg/ml) were loaded into an array using MATLAB software.

Data availability. The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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Discussion

Down-conversion NIR-Ib emissive rare-earth Er-based nanoparticles were engineered to enhance the 1550 nm luminescence through Ce³⁺ doping and optimization of the inert shell coating. These led to a bright Er-RENPs with a 1550 nm emission under 980 nm excitation in aqueous solution. Owing to the strong 1550 nm emission of the Er-RENPs, fast imaging of mouse cerebral-vasculatures in NIR-IIb window was achieved with a short exposure time of 20 ms per frame and high spatiotemporal resolution. Such non-invasive NIR-IIb imaging could facilitate real-time monitoring and visualization of cerebrovascular abnormalities toward the diagnosis and therapy of the cerebral diseases.

Methods

Synthesis of β-NaYF₄:Ce,Er:NaYF₄ nanoparticles. In a typical four-step synthetic procedure, 1 mmol of CeF₄·COONa and 1 mmol of REi(CF₃COO)₂ (RE: 96% Yb, 2% Ce, 2% Er) were added to a mixture of OA (10 mmol; oleic acid), OM (10 mmol; oleylamine), and ODE (20 mmol; 1-octadecene) in a two-necked flask at room temperature. The solution was pre-degassed for 30 min with vigorous magnetic stirring then heated to 120 °C under vacuum for 30 min to remove water and oxygen. The solution was then heated to 325 °C at 10 °C/min and maintained for 1 h under argon protection. After cooling to room temperature, an excess amount of ethanol was poured into the solution. The resultant nanocrystals were centrifuged at 4400 rpm for 30 min, washed with ethanol several times, and dispersed in 2 ml of cyclohexane. The second step was similar to above procedure, except that 1 mmol Ce(CF₃COO)₂, 1 mmol of Yb(CF₃COO)₃, and the prepared nanocrystals were added to a mixture of OA (20 mmol) and ODE (20 mmol) and maintained at 305 °C for 75 min and 310 °C for another 20 min under argon protection. Repeat the second step two more times; and the final resultant nanocrystals (oleic acid-capped) were dispersed in 3 ml of cyclohexane for further hydrophilic treatment.

Preparation of PMH coated rare-earth nanoparticles. The as-prepared oleic acid-capped RENPs (20 mg) were dried at 60 °C to evaporate cyclohexane and then dissolved in 1 ml chloroform. 80 mg PMH (30–50 kDa poly(maleic anhydride-alt-1-octadecene); Sigma-Aldrich) dissolved in 3 ml chloroform was then added; and the mixed solution was stirred for overnight. Chloroform was then evaporated by rotovap. 40 mg DMAP dissolved in 5 ml water was added to disperse the residue. The residue was sonicated for 30 min at room temperature to form a clear RENPs@PMH solution.

Preparation of PEGylated RENPs@PMH nanoparticles. The above RENPs@PMH solution were centrifuged (15,000 rpm, 1.5 h) and washed with water two times to remove excess PMH and DMAP. The afforded RENPs@PMH particles were engineered to enhance the 1550 nm luminescence downstream with mice injected with the RENPs, indicating the potential biocompatibility and chemically inert, though a systematic discussion was needed before any clinical application in human trials.

Data availability. The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Received: 19 March 2017 Accepted: 4 August 2017
Published online: 29 September 2017
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Acknowledgements
This study was supported by National Institutes of Health R01 HL127113-01A1, the Shenzhen Peacock Program Grant KQTD2014063016085828, the National High Technology Research and Development Program of China (No. 2015AA020408), the National Natural Science Foundation of China (No. 21503054), and the Beijing Municipal Natural Science Foundation (No. 2172056).

Author contributions
H.D. and Yeteng Z. conceived and designed the experiments. Yeteng Z., Z.M., S.Z., Jingying Y., M.Z., Jie Y., R.C., and Ying Z. performed the experiments. Yeteng Z., Z.M., S.Z., Jingying Y., A.A., Sverchkov, Y. E. & Syrtlanov, M. Mechanism and parameters of the quenching of luminescence of rare-earth ions by hydroxyl impurity groups in laser phosphate glass. Quantum Electron. 11, 1101–1103 (1981).

Additional information
Supplementary Information accompanies this paper at doi:10.1038/s41467-017-00917-6.

Competing interests: The authors declare no competing financial interests.

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