Supporting Information

Multiplexing strategy of rare-earth doped nanoparticles for NIR-II biomedical imaging

Xiao Zhang,[‡a,b] Shuqing He, [‡b] Bingbing Ding,[b] Chunrong Qu,[b] Hao Chen,[b,c] Yu Sun,[b] Ruiping Zhang,*[d] Xiaoli Lan,*[a] and Zhen Cheng*[b,c]

Abstract: Featured with simultaneous multicolor imaging for multiple targets, multiplexing strategy has been a promising application for fluorescence imaging. The visible and the first near infrared (NIR-I, 700-900 nm) fluorophores have been explored to multicolor imaging to achieve good multiplexing capacity, while largely hampered by narrow imaging bands available (400-900 nm, band width 500 nm), broad emission spectra of many fluorophores, shallow tissue penetration and scattering loss. With the attractive characteristic emission peaks in the second NIR window (NIR-II, 1,000-1,700 nm), narrow emission spectrum, and deeper tissue penetration capability, rare-earth doped nanoparticles (RENPs) have been considered by us to be outstanding candidates for multiplexing bioimaging. Herein, two RENPs, $NaYF_4$:Yb20Er2@$NaYF_4$ and $NaYF_4$:Nd5@$NaYF_4$, were prepared and modified with polyethylene glycol (PEG) to explore simultaneous imaging at the NIR-IIb (1530 nm, under 980 nm laser excitation) and the NIR-II (1060 nm, under 808 nm laser excitation) windows. The PEGylated-RENPs (RENPs@PEG) were able to simultaneously visualize the circulatory system, trace the lymphatic system, and evaluate skeletal system. Our study demonstrates that RENPs have high multiplexing capability in multifunctional biomedical applications using their NIR-II fluorescence. Importantly, the two RENPs@PEG are complementary to each other for higher temporal resolution in $NaYF_4$:Nd5@$NaYF_4$@PEG and higher spatial resolution in $NaYF_4$:Yb20Er2@$NaYF_4$@PEG, which may provide more comprehensive and accurate imaging diagnosis. In conclusion, RENPs are highly promising nanomaterials for multicolor imaging strategy in the NIR-II window.

Keywords: Multiplexing • Second near-infrared window • Fluorescent probes • Nanoparticles • Rare-earth doped nanoparticles

DOI: 10.1039/x0xx00000x

SUPPORTING INFORMATION

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Experimental Procedures

1. Synthesis of $NaYF_4$:Nd5@$NaYF_4$ and $NaYF_4$:Yb20Er2@$NaYF_4$ nanoparticles

The two rare-earth doped nanoparticles (RENPs) were successfully synthesized using the thermal decomposition method.[1] $Y(OAc)_3$ (1.9 mmol) and $Nd(OAc)_3$ (0.1 mmol) were added to a 100 mL three-neck round-bottom flask containing 1-octadecene (30 mL) and oleic acid (OA, 12 mL). The mixture was stirred magnetically and heated to 130–150°C under vacuum to form the lanthanide oleate complexes. The temperature of the solution was then lowered to 40 °C and a solution of NH_4F (8.0 mmol) and NaOH (8.0 mmol) dissolved in methanol (20 mL) was added dropwise to the reaction flask with stirring for 30 min at 40 °C. After the removal of the methanol through nitrogen flow, the reaction temperature was increased to 300 °C and stirred at this temperature for 90 min. Then the $NaYF_4$:Nd5 core were isolated via centrifugation at 5000 rpm and then dispersed in n-hexane for the subsequent shell growth procedure. Followed by the repeating procedure using $Y(OAc)_3$ (2 mmol) to coat the $NaYF_4$:Nd5 core. $NaYF_4$:Yb20Er2@$NaYF_4$ nanoparticles were synthesized using the same method. All reactions were performed under nitrogen flow protection.
The final synthesized $NaYF_4$:Nd5@$NaYF_4$ and $NaYF_4$:Yb20Er2@$NaYF_4$ NPs were characterized by transmission electron microscopy (TEM, Tecnai G^2 20 TWIN, FEI, the Netherlands) and Dynamic Light Scattering (DLS) on an analyzer (Malvern Zetasizer Nano ZS90, UK). Absorbance spectra of the samples were taken on Agilent spectrophotometer UV-Vis-NIR Cary 60 spectrometer. The NIR-II photoluminescence (PL) emission spectra were captured on a home-built spectroscopy setup by exciting nanoparticles with an 808 or 980 nm laser diode with a power output of 100 mW. The fluorescence quantum yield (QY) of $NaYF_4$:Nd5@$NaYF_4$ and $NaYF_4$:Yb20Er2@$NaYF_4$ was measured in dichloroethane using IR-26 (QY = 0.5%) as a reference.

2. Preparation of PEGylated-RENPs

The RENPs (10.0 mg) were dissolved in n-hexane (2 mL), and the suspension was mixed with an aqueous solution of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-5000] (DSPE-PEG, 5 KDa, 1.0 mg/mL) with a 1:8 volume ratio and sonicated for 2 min on an ice bath. The n-hexane was removed under N_2 flow. The final solution was filtered through a molecular weight cutoff filter (30 kDa) to remove the extra DSPE-PEG-5000. The aqueous suspension of PEGylated RENP was freeze-dried. The morphology and the diameter of PEGylated-$NaYF_4$:Nd5@$NaYF_4$ (named as Nd@PEG) and PEGylated-$NaYF_4$:Yb20Er2@$NaYF_4$ (named as Er@PEG) were identified by TEM and DLS. The photostability of Nd@PEG and Er@PEG was evaluated in phosphate-buffered saline (PBS) and fetal bovine serum (FBS) using the NIR-II spectroscopy setup.

3. Cell culture and cytotoxicity test

The NIH 3T3 fibroblasts were maintained in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% FBS. Approximately 1×10^4 3T3 fibroblast cells per well were planted into 96-well plates overnight, followed by different concentrations (5, 10, 25, 50, 100 and 250 μg/mL) of PEGylated-RENPs added to the cells and incubated for 24 h, and further treated with 1 mM methyl thiazolyltetrazolium (MTT) for an additional 4 h. The formazan crystals were dissolved in 150 μL of DMSO per well, and the absorbance of the samples was measured at 490 nm to calculate cell viability.

4. Animal models

All animal studies were approved by Stanford University's administrative panel on Laboratory Animal Care (Protocol ID: 11580) and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. BALB/c nude mice (female, n = 15, 4–6 weeks) were purchased from Charles River Laboratories (Wilmington, MA, USA) and the mice were used for the circulatory system, bone, and lymph node imaging.

5. NIR-II/NIR-IIb imaging for evaluation of the circulatory system, bone and lymph node

Nd@PEG (100 μL of a 5 mg/mL solution) and Er@PEG (100 μL of an 8 mg/mL solution) were co-injected into the mice through the tail vein. Imaging of Nd@PEG was performed using the home-built NIR-II small-animal imaging system in NIR-II window using a 1000 nm long-pass filter under 808 nm excitation. NIR-IIb imaging of Er@PEG was performed under 980 nm excitation using a 1400 nm long-pass filter. Blood pool phase images were acquired immediately post-injection, and bone images were performed at 4 h post-injection.

A total of 40 μL Nd@PEG (5 mg/mL) was injected intradermally into the right rear foot pad and 40 μL Er@PEG (8 mg/mL) into the other side at the same time (n = 3). The images of the mice were acquired 10 min, 1, 2, 4, 8, 24 and 32 h post-injection under anesthesia. The power density of 808 nm and 980 nm laser were kept below 100 mW cm^{-2} and the exposure times were 150 ms and 500 ms, respectively. Image J was used to analyze the luminescent signals.

6. The biodistribution study of Nd@PEG and Er@PEG

For biodistribution study, BALB/c nude mice were injected with Nd@PEG (50 μL, 1 mg/mL, n = 3), or Er@PEG (50 μL, 1 mg/mL, n = 3), or PBS (50 μL, n = 2, as blank controls) via tail vein and sacrificed at 24 h post-injection. The biological tissues of interest (i.e., heart, liver, spleen, lung, kidney, and bone) were removed and digested in the mixture of HNO_3, HCl, and $HClO_4$ (v/v/v = 3/1/2). And their metal ion Y^{3+} content was measured with decay inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7900).

7. Statistical analysis

The commercial software GraphPad Prism (version 5.0, San Diego, CA, USA) was applied in statistical analysis. All data are presented as the mean ± standard deviation (SD). Means were compared using Student's t-test with $P < 0.05$ being statistically significant.

Results

Size histograms of the NPs

The size histograms corresponding to the $NaYF_4$:Nd5@$NaYF_4$, $NaYF_4$:Yb20Er2@$NaYF_4$, Nd@PEG and Er@PEG were shown in Figure S1. The data obtained from the statistical analysis of the images was included in Figure 1C, 1F, 2A and 2B.

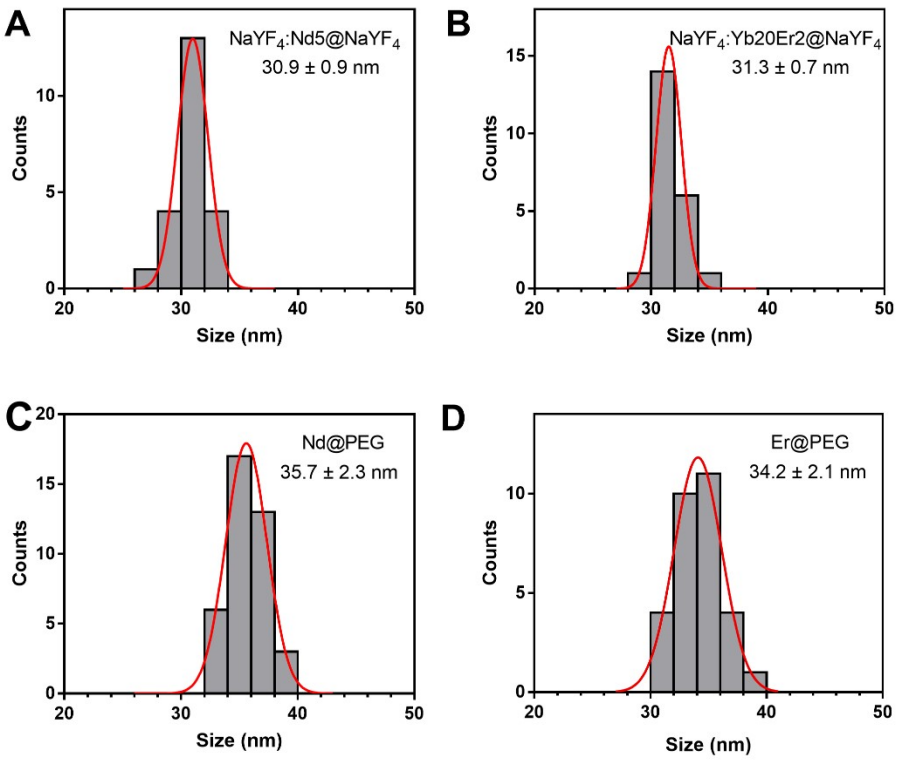

Figure S1. Size histograms corresponding to the $NaYF_4$:Nd5@$NaYF_4$ (A), $NaYF_4$:Yb20Er2@$NaYF_4$ (B), Nd@PEG (C) and Er@PEG (D).

The elemental analysis of RENPs

For the $NaYF_4$:Nd5 nanoparticles, the percentage of each element Y and Nd were measured using the Inductively Coupled Plasma Mass Spectrometry (ICP-MS). In the 200 μg/mL $NaYF_4$:Nd5 nanoparticles solution, Y amount was measured at 69.47 μg/mL, Nd

amount was measured at 5.91 μg/mL, then we can calculate that the mole ratio of Y:Nd = 19.1:1 and the percentage ratio is around 95.04:4.96. For the $NaYF_4$:Yb20Er2, we used the same ICP-MS method to measure the percentage of Y, Yb and Er. In the 200 μg/mL $NaYF_4$:Yb20Er2 nanoparticles solution, Y amount was measured at 58.63 μg/mL, Yb amount was measured at 30.20 μg/mL and Nd amount was measured at 2.89 μg/mL. then we can calculate that the mole percentage ratio of Y:Yb:Er = 77:21:2, which is close to the expected value 78:20:2.

In vivo NIR-II image-guided surgery of lymph node

A total of 40 μL Nd@PEG (5 mg/mL) was injected into the right foot pad to visualize the lymph nodes. At 10 min post-injection, a popliteal lymph node was identified under 808 nm excitation with the exposure times of 150 ms. The lymph node resection was performed under NIR-II image guidance to pattern the standard lymph node biopsy process of clinical use. In the process of excision (Video S1, Figure S2), SC was partially removed (C) and NIR-II imaging provided live feedback for the incomplete resection of the lymph node (D). The remaining lymph node tissue was subsequently removed with a size for only 1.3-mm (E and F).

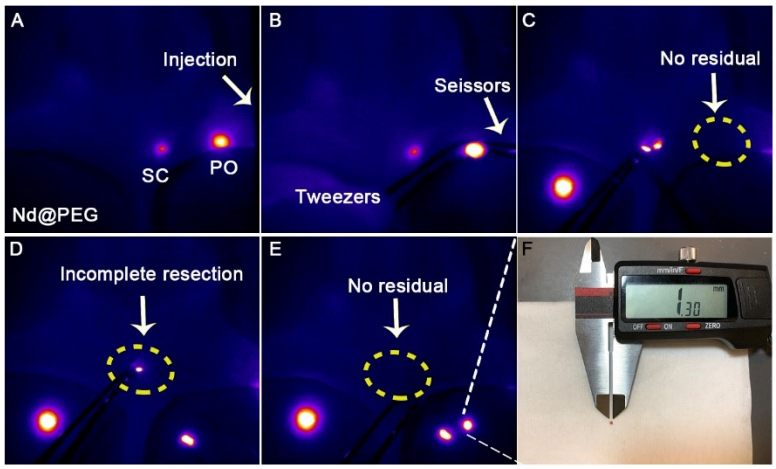

Figure S2. Lymph node resection performed under NIR-II image

The comparison of RENPs and ICG in the presence of serum proteins

Nd@PEG and Er@PEG (100 μL of 0.1 mg/mL solution) and ICG (100 μL of 0.1 mg/mL solution) was diluted into 1 mL fetal bovine serum (FBS). The fluorescence signals were measured in sequential 1000 nm long-pass filters under 808 or 980 excitation (Figure 4**#**). The brightness of the samples was recorded by the photon counts at (a) 46755, (b)18126, (c) 29653 and (d) 2327, respectively. We added detailed information and results to the supplement data.

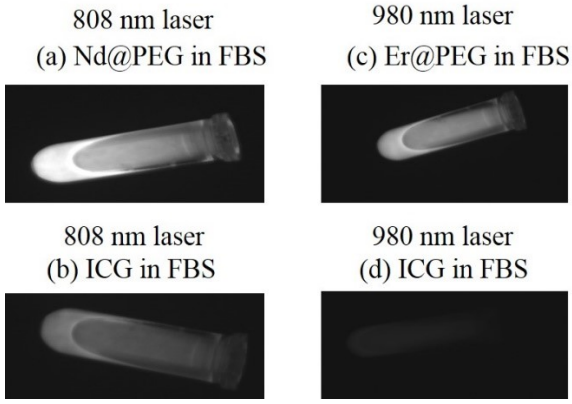

Figure S3. The comparison of the nanoparticles and ICG in the presence of serum proteins.

References

[1] R. Wang, X. Li, L. Zhou, F. Zhang, *Angew. Chem. Int. Ed.* **2014**, 53, 12086–12090.

Author Contributions

Z. C., X. L. and R. Z. conceived and devised the project. X. Z. and S. H. wrote the manuscript. B. D. and C. Q. designed the study and performed all the experiments. H. C. contributed to the modify the nanoparticles. Y. S. performed the cell and animal studies.