Supporting Information for

Rare earth doped fluoride nanoparticles with engineered long luminescence lifetime for time-gated in vivo optical imaging in the second biological window

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1. Experimental Procedures

Materials: All the materials were used as received without further purification. Rare earth oxides (Y_2O_3 , Yb_2O_3 , Nd_2O_3), sodium trifluoroacetate (NaTFA), poly(acrylic acid) (PAA, MW=18000), and trifluoroacetic acid (TFA) were purchased from Aladdin Chemistry Co. Ltd. Calcium Oxide (CaO), hexane and ethanol were purchased from Xilong Scientific Co. Ltd. Oleic acid (OA, >90%), oleylamine (OM, >70%), and 1- octadecence (ODE, >90%), were purchased from Sigma-Aldrich.

Synthesis of the NaGdF₄:Yb³⁺,Nd³⁺,Tm³⁺ NPs: The NaGdF₄:Nd³⁺(3 mol%), Yb³⁺(2mol%), Tm³⁺(0.2 mol%) nanoparticles were synthesized following a thermal decomposition procedure previously described.^[S1] In a first step gadolinium and neodymium trifluoroacetates were prepared by the reaction of appropriate amounts of Nd₂O₃, Gd₂O₃, with a mixture of distilled H₂O (5 mL) and trifluoroacetic acid (5 mL). The cloudy solution was refluxed at 80 °C until it was clear, and then dried at 60 °C. Afterwards 2.50 mmol of sodium trifluoroacetate (0.340 g) was added to the dried precursor, together with 20 mL of oleic acid and 20 mL of 1-octadecene. The solution was degassed at 120°C under vacuum for 30 min. Subsequently the temperature was rapidly increased to 320 °C under Ar flow; the solution was left to react for 2 hours and then left to cool down. The nanoparticles were precipitated using absolute ethanol, isolated via centrifugation and washed twice with a 1:6 hexane/ethanol mixture.

Synthesis of the NaDyF₄:Yb³⁺,Nd³⁺,Tm³⁺ NPs: The synthesis of NaDyF₄: nanoparticles was performed by thermal decomposition of oleate precursors following a procedure previously reported.^[S2] For the preparation of 1 mmol of sample, adequate amounts of DyCl₃·6H₂O NdCl₃·6H₂O, YbCl₃·6H₂O and TmCl₃·6H₂O were added in a triple-neck round bottom flask, together with 3.5 mL oleic acid and 15 mL 1-octadecene. The mixture was

stirred and heated to 120° C for 3h under vacuum in order to prepare the rare earth-oleate precursors. After the solution cooled down to room temperature, a 10mL of methanol solution containing 2 mmol (0.07999g) NaOH and 2.5 mmol (0.09259g) NH₄F was added. The mixture was kept under agitation at room temperature for 1 h, and then slowly heated to 70 °C in order to remove the methanol. After this, the mixture was heated to 295 °C at 15 °C/min and kept at this temperature for 2 h under argon atmosphere. The obtained NPs were then collected by centrifugation at 6000 rpm for 15 min. The precipitate was washed with ethanol and hexane and centrifuged at 6000 rpm (~15 min) for several times.

Synthesis of α -NaYF₄: 10% Yb³⁺, x% Nd³⁺ (x=10, 20, 30, 50, 80) core nanoparticles: The α -NaYF₄: Yb³⁺, Nd³⁺ core nanoparticles (NPs) were prepared via decomposition of the metal trifluoroacetate at high temperature. In a typical procedure, 0.05 mmol Yb₂O₃, x mmol Nd₂O₃ (x=0.05, 0.1, 0.15, 0.4) and (0.45-x) mmol Y₂O₃ were loaded into a 250 ml flask containing 5 ml deionized water and 5 ml TFA, and heated to 90 °C for 1 hour to yield a clear solution. Then, the resulting clear solution was evaporated at this temperature under argon purge to get muddy powdered RE(TFA)₃. Subsequently, 8 ml OA, 8 ml OM, 12 ml ODE and 2 mmol NaTFA were added into the flask. The solution was heated to 120 °C and kept at that temperature for 30 min, followed by heating up to 300 °C for 30 min before naturally cooling down room temperature. An argon environment was as applied during the whole synthesis process. The resulting nanoparticles were precipitated by adding 20 mL ethanol to the cooled reaction flask. After centrifugal washing with ethanol for three times, the collected white powder was finally dispersed in 10 ml hexane for further uses.

Synthesis of α -NaYF₄: 10% Yb³⁺, x% Nd³⁺ (x=10, 20, 30, 50, 80)@CaF₂ core-shell nanoparticles: The core-shell nanoparticles were prepared via a seed-mediated epitaxial

growth process, involving the use of α -NaYF₄: 10% Yb³⁺, x% Nd³⁺ core as the seed and the corresponding growth in the shell precursor solution. To prepare the shell precursor, firstly, 2 mmol CaO with 5 ml deionized water and 5 ml TFA were added to a 250 ml flask and heated at 90 °C for 1 hour to produce a clear solution. This solution was then evaporated at this temperature to yield the shell precursor of calcium trifluoacetate (Ca(TFA)₂). Next, 0.5 mmol NaYF₄: 10% Yb³⁺, x% Nd³⁺ (x=10, 20, 30, 50, 80) core nanoparticles, 7 ml OA, and 7 ml ODE were all added to the flask. The solution was then heated to 120 °C for 30 min, followed by heating up to 300 °C for 60 min before naturally cooling down. The whole process was carried out under an argon environment. The resulting core-shell nanoparticles were precipitated by adding 20 mL ethanol to the cooled reaction flask. After centrifugal washing with ethanol for three times, the collected core-shell NPs was finally dispersed in 10 ml hexane for further uses.

Synthesis of α -NaYF₄: x% Yb³⁺, 30% Nd³⁺ (x=10, 20, 30, 40, 60) core nanoparticles: The synthesis procedure of α -NaYF₄: x% Yb³⁺, 30% Nd³⁺ (x=10, 20, 30, 40, 60) NPs is identical to that used for α -NaYF₄:10% Yb³⁺, x% Nd³⁺ NPs, except for the amount of rare-earth oxide.

Synthesis of α -NaYF₄: x% Yb³⁺, 30% Nd³⁺ (x=10, 20, 30, 40, 60)@CaF₂ core-shell nanoparticles: The synthesis procedure of α -NaYF₄:x% Yb³⁺, 30%Nd³⁺ (x=10, 20, 30, 40, 60) @CaF₂ core-shell NPs is identical to that used for the growth of α -NaYF₄: 10% Yb³⁺, x% Nd³⁺@CaF₂ core-shell NPs. The only difference is that the α -NaYF₄: 10% Yb³⁺, x% Nd³⁺ core NPs is now replaced by the α -NaYF₄:x% Yb³⁺, 30%Nd³⁺ (x=10, 20, 30, 40, 60) core NPs.

Synthesis of aqueous NPs@PAA: In the first step, 5 ml NPs dispersed in hexane were mixed with a 5 ml N,N-Dimethylformamide (DMF) solution of nitrosonium tetrafluoroborate (NOBF₄, 0.01 M) at room temperature. The mixture was then gently shaken until the

observation of NPs' precipitation. Subsequently, toluene and hexane (1:1, volume) were added into the solution, which was centrifuged at 10000 rpm for 10 minutes. The precipitate was collected and dispersed in 5 ml DMF for the next step. In the second step, 250 mg poly(acrylic acid) (PAA) (MW=18, 000) was added into the 5 ml DMF solution of NOBF₄-treated NPs, which was then heated to 80 °C and kept at this temperature for 30 min under vigorous stirring. After that, the NPs were precipitated by adding acetone, washed with ethanol, and finally dispersed in purified water to yield a stable dispersion.

Surface modification of the NaDyF₄:Yb³⁺,Nd³⁺,Tm³⁺ and NaGdF₄:Yb³⁺,Nd³⁺,Tm³⁺ NPs for water dispersibility: After the synthesis the nanoparticles were covered with folic acid functionalized PEG following the procedure: firstly, transfer the NPs from the hexane to the chloroform, then a chloroform solution of DSPE-mPEG-2000 phospholipid was mixed with the NPs at a 1:4 (NPs:DSPE-mPEG-2000) weight ratio in a 1 dram vial and was left overnight to evaporate chloroform. Finally, centrifugal washing with ethanol for several times, dispersed in purified water to yield a stable dispersion.

Cytotoxicity Assay: Cell viability was measured by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5diphenyltetrazolium bromide (MTT) assay. HeLa cells were incubated in a 96-well flatbottomed microplate with 100 μ L growth medium at 37 °C and 5% CO₂ for 24 h. Then, the culture medium was changed and the cells were washed with PBS and fresh culture medium. Subsequently, cells were incubated with NaYF₄:10% Yb³⁺, 30%Nd³⁺@CaF₂ @PAA at different concentrations of 0, 31.25, 62.5, 125, 250, 500 and 1000 μ g/ml for 24 h. Finally, the number of the viable cells was calculated by measuring the activity of NADH-dependent cellular oxidoreductase in cells. Characterization: The as-prepared NPs were applied dropwise to the surface of a copper grid for transmission electron microscope (TEM, Tecnai G2 Spirit Twin 12) to measure their size and morphology. Powder X-ray diffraction (XRD) measurement was performed on a Rigaku D/max- γ B diffractometer at the scanning rate of 1 ° min⁻¹ in the 2 θ range of 10 – 80 °. The photoluminescence spectra were collected by a lens-coupled monochromator (Zolix Instruments Co. Ltd, Beijing, China), under laser excitation with an 800 nm continuous-wave laser diode (Hi-Tech Optoelectronics Co. Ltd, Beijing, China), capable of generating an output power of up to 5 W. The absorption spectra were acquired with a Perklin-Elmer lambda 950 spectrophotometer. Fourier transform infrared (FTIR) spectra were measured with a Perkin-Elmer 580B infrared spectrophotometer. The fluorescence lifetime of the infrared emission at 978 nm from the core-shell NPs (aqueous dispersion) was measured by a Fluorolog-3 spectrofluorometer (Horiba) under pulsed LED excitation (Spectro LED 355 nm, Horiba), and then detected by a TE-cooled near infrared photomultiplier tube (NIR PMT, Hamamatsu H10330-75). The absolute quantum yield of core/shell NPs was evaluated using an integrating sphere equipped in UV-Visible-NIR spectrofluorometer (Eidingburg, FLS 1000).

Fluorescence time-gated imaging: For imaging experiments, the sample (cuvette or mouse) was placed in an optical setup specially designed for fluorescence imaging in the infrared. It consists of a fiber coupled 808 nm laser diode (LIMO) acting as an excitation source. This laser can provide up to a total power of 10 W in an area of 10 cm², therefore leading to a maximum power density of 1 W/cm². The NIR emission of the sample was then imaged by an InGaAs camera (Xenics Xeva 17) with a spectral sensitivity from 900 to 1700 nm. Any scattered laser radiation was blocked using an 850 nm long pass filter. For the time-gated experiments, the laser diode (LIMO) was operated in pulsed mode (generating 1 ms long pulse with a period of 40 ms). The trigger output of the laser was used to act as a trigger for

the camera acquisition. An electronic system based on two RC phases and inverter logic gates was employed to generate a delay time (adjustable between 1 and 100 μ s) before the start of the acquisition of the images. The details of this electronic system have been reported in detail in our previous work (Advanced Materials 28 (46), 10188-10193, 2016).

In vivo experiments: The in vivo experiments carried out in this work were approved by the Ethics Committee from Universidad Autónoma of Madrid (CEIT) in the frame of the project MAT2010-21270-C04-01 supported by the Spanish Ministerio de Economía y Competitividad. The in vivo fluorescence images were obtained in two C57BL/6 mice anesthetized by isoflurane inhalation (4-5% induction, 2% maintenance). For the subcutaneous injection 100 μ L of an aqueous dispersion (13.3 mg/mL) of NaYF₄: 10% Yb³⁺, 30%Nd³⁺@CaF₂ NPs was used.

2. Supporting Figures



Figure S1. Energy transfer processes in NaGdF₄:Yb³⁺,Nd³⁺,Tm³⁺ responsible for the enhancement of the lifetime and the emission intensity of Yb.



Figure S2. XRD patterns of NaYF₄: 10% Yb³⁺, 30%Nd³⁺ core and NaYF₄: 10% Yb³⁺, 30%Nd³⁺@CaF₂ core/shell NPs. The standard patterns of α -NaYF₄ (JCPDS No. 77-2043) and CaF₂ (JCPDS No. 77-2096) are included for reference.



Figure S3. Energy dispersive spectrum (EDS) of NaYF₄: 10% Yb³⁺, 30%Nd³⁺ @CaF₂ core/shell NPs. The presence of Na, Y, F, Yb, Nd, Ca and F are confirmed, implying the successful synthesis of the designated core/shell NPs.



Figure S4. Absorption (solid black) and excitation (dashed blue) spectra of NaYF₄:10%Yb³⁺, 30% Nd³⁺@CaF₂ NPs dispersed in hexane. Absorption peaks from Nd³⁺ and Yb³⁺ are indicated with red circles and green squares, respectively. The excitation spectrum was acquired by monitoring the emission at 980 nm.



Figure S5. TEM images of the as-prepared NaYF₄: 10%Yb³⁺, x% Nd³⁺@CaF₂ core/shell NPs, with (a) x=10, (b) x=20, (c) x= 50, and (d) x=80 (scale bar, 100 nm). All the synthesized core/shell NPs have almost identical shape and size, indicating that the dopant concentration of Nd³⁺ has negligible effects on the resulting particle morphology.



Figure S6. a. Emission spectra of NaYF₄:10%Yb³⁺, x% Nd³⁺@CaF₂ (x=10, 20, 30, 50, 80) under 800 nm laser excitation; b. Integrated emission intensity in the spectral range of 950-1050 nm as a function of Nd³⁺ doping concentration.



Figure S7. TEM images of our as-prepared NaYF₄: x% Yb³⁺, 30% Nd³⁺@CaF₂ core/shell NPs, with (a) x=20, (b) x=30, (c) x=40), (d) x=60. Scale bars in all TEM images are 100 nm. The change of in Yb³⁺ doping concentration does not affect the shape and size of the resulting particles.



Figure S8. a. Emission spectra of NaYF₄:x% Yb³⁺, 30%Nd³⁺@CaF₂ (x=10, 20, 30, 40, 60) NPs under 800 nm laser excitation; **b.** Integrated emission intensity as a function of Yb³⁺ doping concentration.



Figure S9. Emission spectra of NaYF₄:10%Yb³⁺, 30%Nd³⁺@CaF₂ and NaYF₄:10%Yb³⁺@ CaF₂:30% Nd³⁺ core/shell NPs (dispersed in hexane) under 800 nm laser excitation. Separation of Nd³⁺ and Yb³⁺ ions in a core/shell structure prevents an efficient energy transfer from Nd³⁺ to Yb³⁺ ions, thus leading NaYF₄:10%Yb³⁺, 30%Nd³⁺@CaF₂ to emit a much lower infrared emission at 1000 nm (from Yb³⁺ ions) than the NaYF₄:10%Yb³⁺, 30%Nd³⁺@CaF₂ core/shell NPs.



Figure S10. FTIR spectra of hexane-dispersed NPs (black line) and water-dispersed PAAcoated NPs (red line). The bands at 1450 and 1560 cm⁻¹ arise from the asymmetric and symmetric vibration modes of the -COOH group, respectively, indicating the adsorption of oleate on the surface of the as-synthesized NPs through a bidentate bond. The intensities of these two bands become much weaker after performing the replacement of the original ligand of oleate with PAA. The weak stretching mode of the -C=O group at 1724 cm⁻¹ is significantly after PAA coating, suggesting an substantial increase in the number of -COOH group. Moreover, the shoulder at 2927 cm⁻¹, associated with the asymmetrical stretching mode of -CH₃ group, became weaker after PAA coating, showing that the number of the -CH₃ groups is reduced on the NPs surface. The broad band (3100-3600 cm⁻¹) peaking at 3454 cm⁻¹ arises from the O-H stretching vibration of -COOH group, which becomes much stronger after the PAA coating. Since the PAA ligands have numerous COOH groups, their increase indicates the successful replacement of the original oleate ligand by PAA.



Figure S11. Dynamic light scattering (DLS) spectrum of NaYF₄:Yb³⁺10%, Nd³⁺30%@CaF₂@PAA NPs in water. Their hydrodynamic diameter of PAA-coated NPs was determined to be about 37 nm, small enough for biomedical applications.



Figure S12. Emission spectra of NaYF₄:10% Yb³⁺,30% Nd³⁺ @CaF₂ NPs dispersed in hexane and the corresponding PAA-coated core/shell NPs dispersed in water under 800 nm laser excitation. The molar concentration of both samples was set to be equal to 0.1 mmol/mL.



Figure S13. The viability of Hela cells incubated with different concentration of PAA-coated core/shell NPs for 24 h. The viability was virtually unaffected even at the highest NP concentration (1000 μ g/mL), indicating the low toxicity of our PAA-coated core/shell NPs at the cellular level.



Figure S14. Emission spectrum of an aqueous dispersion of Ag_2S NPs as obtained under 800 nm excitation.



Figure S15. a. Optical image of our NaYF₄:10%Yb 30% Nd@CaF₂ and commercially available Ag₂S NPs at a concentration of 10 mg·mL⁻¹; **d.** NIR fluorescence images of NaYF₄:10%Yb 30% Nd@CaF₂ and Ag₂S NPs without any delay time; **e.** NIR images of these





Figure S16. NIR fluorescence images of the stomach and the intestine of a mouse after oral administration of NaGdF₄:Yb³⁺,Nd³⁺,Tm³⁺ NPs without any delay time and with a delay time of 1 μ s.

References

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