Multiplexed Near-Infrared *In Vivo* Imaging Complementarily Using Quantum Dots and Upconverting $NaYF_4:Yb^{3+},Tm^{3+}$ nanoparticles

Sanghwa Jeong, Nayoun Won, Jinsik Lee, Jiwon Bang, Jeongsoo Yoo, Sang Geol Kim, Jeong Ah Chang, Joonghyun Kim, and Sungjee Kim*

Materials

yttrium oxide (Aldrich, 99.99%), ytterbium (III) oxide (Aldrich, 99.99%), thulium (III) oxide (Aldrich, 99.99%), trifluoroacetic acid (Aldrich, 98%), sodium triofluoroacetate (Aldrich, 98%), cetyltetraammonium bromide (CTAB) (Acros, 99+%), chloroform (Samchun, 99.5%), 1-octadecene, technical grade (Aldrich, 90%), oleic acid, technical grade (Aldrich, 90%).

Synthesis of α-NaYF: Yb^{3+},Tm^{3+} nanoparticles (NaYF NP)

This synthesis is based on the processes reported by Nyk *et al.*[s1] A solid mixture of 0.025 mmol thulium(III) oxide, 0.25 mmol ytterbium(III) oxide, and 1.

95 mmol yttrium oxide in 1:1 (w/w) trifluoroacetic acid aqueous solution was prepared with stirring (Y:Yb:Tm=78:20:2). The mixture was heated at 80 °C for 3 h in a 4-neck round bottom flask (r.b.f.), then dried in rotary evaporator at 60 °C for 20 min. Purple solid trifluoroacetate salt remained. The 4.5 mmol sodium trifluoroacetate was added to the dried trifluoroacetate salt, and the mixture was dissolved in 30 mL 1-octadecene and 30 mL oleic acid in the 4-neck r.b.f. The mixture was degassed with stirring vigorously at 110 °C for 1 h or more in vacuum. After water and other gases had been removed thoroughly, the solution was heated at 300 °C under nitrogen environment and vigorous stirring for 1 h. The solution was cooled to room temperature with stirring in air. To precipitate the obtained NPs, 20 mL acetone was added to the 30 mL product

solution in a 50 mL conical centrifuge tube, and the solutions were centrifuged at 5000 rcf for 15 min. The product was washed three times with 4 mL ethanol, then dissolved in 5 mL chloroform.

Encapsulation of α-NaYF: Yb^{3+},Tm^{3+} by using CTAB

A clear 10 mL 0.1 M CTAB aqueous solution was prepared and 0.5 mL of a chloroform solution of α-NaYF NPs was dropped into the aqueous solution at 0.5 mL/h with vigorous stirring. The resulting white opaque solution was sonicated for 30 min, then degassed in vacumn in room temperature to remove liquid chloroform. The yellow-green clear aqueous solution remained after the degas process. Finally, the product was dialyzed to 1/1000 with 5000 rpm for 15 min to remove remaining CTAB with dialysis tube of 30k M.W.C.O. (molecular weight cut off).

Synthesis and surface modification of CdTeSe QDs

CdTeSe alloyed QDs were synthesized under cadmium-rich condition by pyrolysis procedures reported previously.[s2] Tellurium and selenium precursors were prepared by dissolving 179 mg of tellurium shot in 2.8 ml TOP and 268 mg of selenium pellet in 3.4 ml TOP, respectively, in glove box. The selenium precursor was mixed with tellurium precursor and additional 2 ml of TOP was added. 163 mg of cadmium acetate dihydrate, 5 ml of oleylamine and 20 g of TOPO were mixed in a 3-neck flask, and heated to 300 ℃ under nitrogen gas flow. Upon reaching the temperature, the mixture of selenium and tellurium precursors was quickly injected into the reaction flask and was kept stirred until alloyed CdTeSe QDs of desired size were obtained. The size of colloidal quantum dot was controlled by the reaction time. After the synthesis of CdTeSe QDs by this pyrolysis method, the surfaces of QDs are surrounded by hydrophobic ligands like trioctyl phosphine oxide and stearic acid. For the biological application, this hydrophobic surface

of QDs was modified by dihydrolipoic acid (DHLA) which has a thiol group as the moiety to bind the QD surface and a carboxylic acid group as a charged moiety to make QDs soluble in water.[s3] Excess amount (typically more than million times the number of QDs) of the precursor of DHLA is dissolved in mixed solvent of $CHCl_3$ and MeOH (1:2, vol/vol, 3 mL). Two equimolar amounts of sodium borohydride is added to the solution and vigorously stirred for 20 min under N_2 gas flow at room temperature. Chloroform QD solution (1 mL) is added to the solution and further stirred for 20 h at room temperature. QDs are transferred to pH 3.7 acetate buffer aqueous layer by extraction. To remove excess free DHLAs, the QD solution is dialyzed twice using Amicon 50 kDa MW cutoff centrifugal filter. For the efficient delivery of QDs into cells, cationic lipids, Lipofectamine 2000 (Invitrogen), were used as delivering agents. Before treatment to cells, 100 pmol of QDs are mixed with 2 μL of Lipofectamine 2000 in a culture medium.

Characterization of α-$NaYF_4$: Yb^{3+},Tm^{3+} nanoparticles and CdTeSe QDs

TEM images were recorded using a JEOL JEM-1011 microscope. XRD patterns of the products were obtained using a Rigaku Max-2500V X-ray diffractometer equipped with graphite monochromatized CuKα radiation (λ = 1.54056 Å). Photoluminescence spectra were taken using HORIBA Jobin Yvon Symphony CCD detectors with HORIBA Jobin Yvon TRIAX 322 monochromator. Absorption spectrums were recorded using an Agilent 8453 UV-visible spectrophotometer to detect visible light and a SHIMADZU UV-vis-NIR spectrophotometer to detect NIR. Absorption spectrum of NaYF NP aqueous solution could not be obtained due to the strong and inevitable absorbance of water around 1000 nm. *In vitro* cellular images were taken by using a ZEISS Axioplan 2 imaging microscope equipped with a Hamamatsu ORCA ER digital camera. Shanhai Dream Lasers SDL-980-LM-2000T diode laser was used as an excitation

source for 980 nm light. The crystalline property of α-NaYF NP was determined using powder XRD. (Fig. S1) The NaYF NPs have an average size of 18 nm, and indeterminate morphology. (Fig. S2)

Cell culture and in vitro cellular experiment

HeLa cells (human cervical cancer cells) were cultured in Dulbecco's Modified Eagle Medium (DMEM, HyClone) supplemented with 10% (v/v) fetal bovine serum (HyClone) and 1% (w/v) penicillin/streptomycin. Cells were incubated in a humidified incubator at 37 °C with 5 % CO_2. Cells were grown in 24-well plates (Corning) at a density of 5 × 10^4 cells/well for 1 day.

Mixture of 10 nM CdTeSe QDs and 100 nM α-$NaYF_4$: Yb^{3+},Tm^{3+} NPs were diluted with DMEM with 1% (w/v) penicillin/streptomycin. The cells were treated with the QDs, α-$NaYF_4$: Yb^{3+}, Tm^{3+} , and the mixture of QDs and α-$NaYF_4$: Yb^{3+},Tm^{3+} NPs and incubated at 37 °C. After 1 h of incubation, cells were washed with phosphate buffered saline (PBS) and fixed with 4% formaldehyde/PBS for 10 min at room temperature. Images of cells were acquired using the ZEISS Axioplan 2 imaging microscope equipped with Hamamatsu ORCA ER digital camera. The excitation sources were a mercury lamp with an 650 nm short pass filter (SPF) for QDs and a Shanhai Dream Lasers SDL-980-LM-2000T diode laser for α-$NaYF_4$: Yb^{3+},Tm^{3+}.

Cell viability test

To determine the cytotoxicity of NaYF NP and CdTeSe QD, HeLa cells were treated with NaYF NP, CTAB, and CdTeSe QD. After 1 h, 6 h, and 24 h treatment, Cell Counting Kit-8 (CCK-8, Dojindo molecular technologies, Inc.) assay were performed.

HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (vol/vol) fetal bovine serum and 1 % (wt/vol) penicillin/streptomycin. 100 μL cell suspension (5000 cells/well) was dispensed in a 96-well plate and incubated for 24 hours in a humidified atmosphere of 5 % (vol/vol) CO_2 and 95 % (vol/vol) air at 37 °C. CdTeSe QD (emission center at 800 nm) aqueous solution with lipofectamine 2000 and NaYF NP aqueous solution were prepared in sterile condition. To compare the cytotoxicity of NaYF NP core and the surfactant of CTAB, the CTAB aqueous solution with the equivalent concentration in the dialyzed NaYF NP aqueous solution was also prepared. 10 μL of probe solutions with various concentration were added into the culture media in the plate. 3, 10, 30, 100 nM CdTeSe QD and NaYF NP solution, and 0.3, 1, 3, 10 μM (x100 eq. of each NaYF NP concentration) CTAB were used. The plate was incubated in the incubator for 1 h, 6h, 24 h. After appropriate length of time, 10 μL of CCK-8 solution was added to each well of the plate. After further incubation for 1 hour in the incubator, the absorbance of each plate at 540 nm was measured using a microplate reader (MULTISCAN EX, Thermo electron corporation). Results were expressed as a percentage in comparison to the absorbance of non-treated cells. (Fig. S3) To eliminate the effect of nanoparticles including absorption and dye reduction, absorption of control samples without cells were subtracted.

In vivo mouse multiplexing image experiment

HeLa cells were grown in culture dishes (Corning) at a density of 5×10^6 cells/dish for 1 day. 10 nM CdTeSe QDs and 100 nM α-$NaYF_4$: Yb^{3+},Tm^{3+} were diluted with DMEM with 1% (w/v) penicillin/streptomycin. The cells were treated with the mixture of QDs-lipofectamine 2000 , and the α-$NaYF_4$: Yb^{3+},Tm^{3+}-CTAB PBS aqueos solution then incubated at 37°C. After 1 h of

incubation, cells were washed with PBS and detached from the culture dish using cell scrapers. Two female nude mice (BALB-c-nu/nu, 8 week old, ~ 25 g, Orient Co. Ltd, Korea) were used in accordance with our institution's guidelines on animal care and use. The mice were anesthetized with 0.015 mL/g of intraperitoneal Avertin. After anesthesia, one mouse was subcutaneously injected with 20 μL of QD800-incubated HeLa cell PBS solution, α-$NaYF_4$: Yb^{3+},Tm^{3+}-incubated HeLa cell PBS solution, QD800+α-$NaYF_4$: Yb^{3+},Tm^{3+}-incubated HeLa cell solution, and non-labelled HeLa cell PBS solution into the back of mouse. The other mouse was injected in the similar way with 30 μL of QD750-incubated HeLa cell PBS solution, QD900-incubated HeLa cell PBS solution, and non-labelled HeLa cell PBS solution.

Thermogravimetric analysis (TGA) of lanthanide nanoparticles

TGA analysis was performed to determine the concentration of α-$NaYF_4$:20%Yb^{3+}, 2%Tm^{3+} NPs in chloroform solution. Before TGA, the solution was dried in vacuum oven at 70 °C for 6 h to evaporate chloroform. The range of analysis temperature was between 25 °C and 500 °C and the speed of temperature increase was 10 °C/min. (Fig. S4) Almost all organic molecules that are expected to exist in NaYF NP solution have boiling temperature < 400 °C. The concentration of NaYF NP was calculated assuming that remaining substances in chamber at 500 °C consist of pure NaYF NP. Calculation of concentration requires the density and volume of NaYF NP. The volume of NPs was calculated by using the average particle size obtained from TEM images, and density was calculated from the reference data of sodium yttrium triofluoride. To acquire the density of NPs, we assumed that the ratio of yttrium to ytterbium to thulium in NPs is the same as that of precursor, and that the lattice constant of NPs is the same as that of sodium yttrium trifluoride.

TGA results were verified using simple calculation using the doping condition and the absorption property of free Yb ions. Simple summation of Yb absorptions in our NaYF NP results in 14,600 M^{-1} cm^{-1}. The extinction coefficient of free Yb^{3+} ion is 3 M^{-1} cm^{-1}[s4], and our NaYF NPs include on average 4900 Yb. The ratio between La elements was assumed to be unchanged from the precursors in reaction to the actual NP composition; this assumption is known to hold quite well.[s5] Interactions of La elements within NPs such as self-quenching were neglected.

Specification of multiplexed NIR imaging setup

Our multiplexed NIR imaging setup (Fig. S5) follows a previously-reported setup.[s6] A halogen lamp (Light Bank LS-F150HS) was used to excite CdTeSe QDs and a 980 nm diode laser (SDL-980-LM-2000T) was used to excite NaYF NPs. Exchanging the excitation sources was performed manually with delay time of several seconds. A 650 nm short pass filter (SPF) was placed in front of halogen lamp to cut off background signals. The narrow beam size of the 980 nm diode laser was expanded using a plano-concave lens (Edmund Optics, NT45-030). A Hitachi model HV-D37 3CCD camera was used to record visible color-video images, and a Hamamatsu model ORCA-AG camera was used to obtain NIR fluorescence images. Fluorescence from the sample was split to the cameras using a 700 nm dichroic mirror (Chroma Technology Corp.) (Fig. S5). A motorized filter wheel was placed in front of NIR camera for multispectral imaging. A 750/40-nm band pass filter (BPF) and a 830-nm long pass filter (LPF) were used to obtain fluorescence images of QD750 and QD900, respectively. A 800/40-nm BPF was also used to obtain fluorescence images of QD800 and NaYF NP. The monochromatic signals of NIR camera were mapped to visible color images using control software written in LabVIEW 8.5 (National Instruments).

Figures

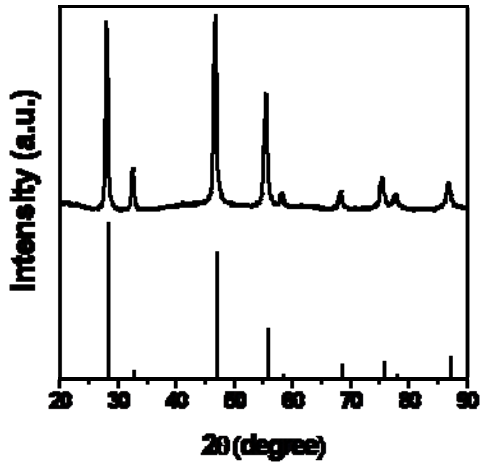

Fig. S1 (top) powder XRD spectrum of synthesized colloidal $NaYF_4:Yb^{3+},Tm^{3+}$ NP. (bottom) reference powder XRD spectrum of α-$NaYF_4$ face-centered cubic crystal (JCPDS no. 77-2042).

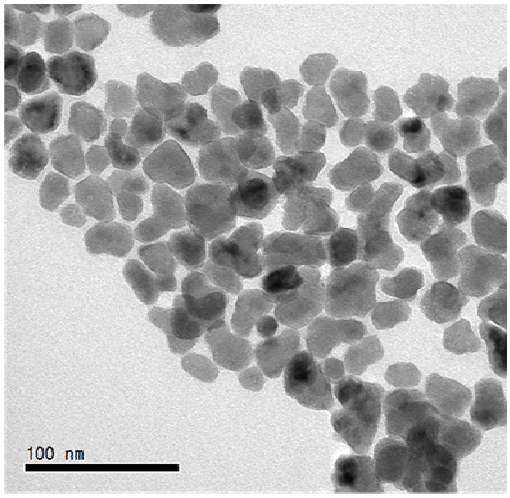

Fig. S2 TEM image of colloidal $NaYF_4:Yb^{3+},Tm^{3+}$ NPs.

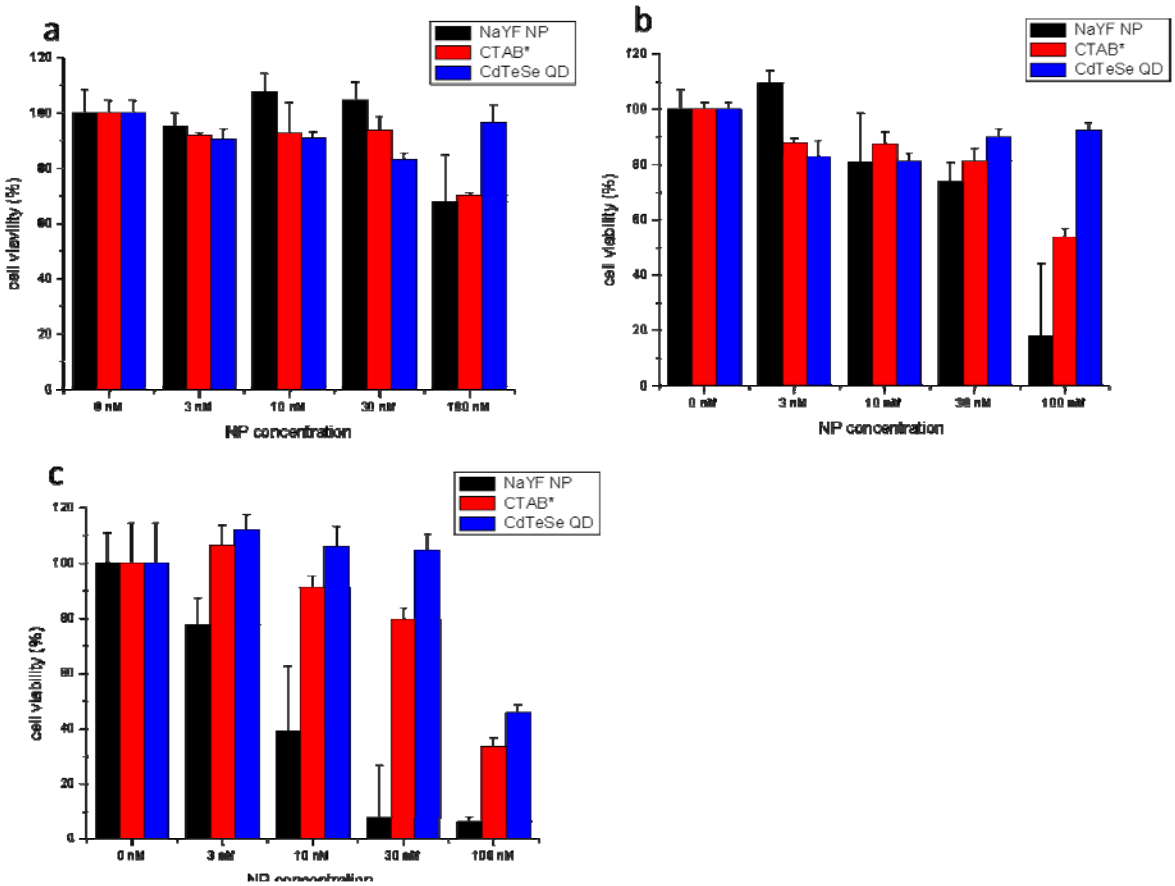

Fig. S3 Cell viability test for NaYF NP, CdTeSe QD, and CTAB in HeLa cell using CCK-8. 0, 3, 10, 30, 100 nM NaYF NP-CTAB aqueous solution, CdTeSe QD-lipofectamine aqueous solution, and 0.3, 1, 3, 10 μM of CTAB aqueous solution were investigated for the three incubation times of 1 h (a), 6 h (b), and 24 h (c). Each data was obtained for three equivalent plate for the average and standard deviation.

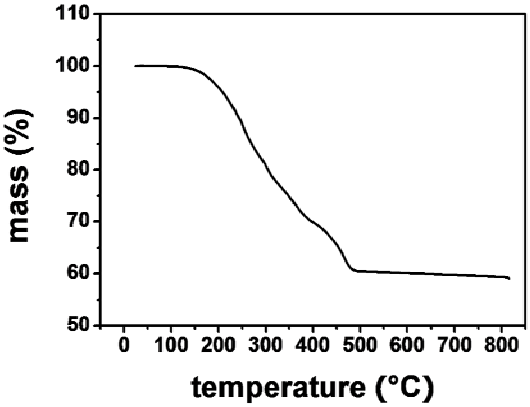

Fig. S4 TGA diagram of dried $NaYF_4$:20%Yb^{3+}, 2%Tm^{3+} nanoparticles.

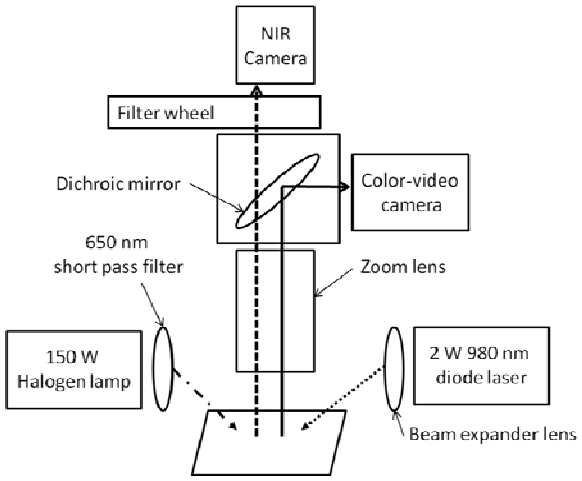

Fig. S5 Outline of real-time multiplexed NIR imaging setup.

Reference

[S1] M. Nyk, R. Kumar, T. Y. Ohulchanskyy, E. J. Bergey and P. N. Prasad, *Nano Lett.* **2008,** *8*, 3834-3838.
[S2] T. Pons, N. Lequeux, B. Mahler, S. Sasnouski, A. Fragola and B. Dubertret, *Chem. Mater.*

2009, *21*, 1418-1424.
[S3] S. H. Bhang, N. Won, T.-J. Lee, H. Jin, J. Nam, J. Park, H. Chung, H.-S. Park, Y.-E. Sung, S. K. Hahn, B.-S. Kim and S. Kim, *ACS Nano*, **2009**, *3*, 1389-1398.
[S4] Y. Cheng, X. Yang, Z. Xin, S. Cheng, D. Cao, X. Xu and J. Xu, *Laser Physics* **2009,** *19*, 2168-2173.
[S5] G.-S. Yi and G.-M. Chow, *Chem. Mater.* **2007,** *19*, 341-343.
[S6] A. M. De Grand and J. V. Frangioni, *Technol. Cancer Res. Treat.* **2003,** *2*, 553-562.