

## Supporting Information

### Detection of Lymph Node Metastasis with Near-Infrared Upconversion Luminescent Nanoprobes

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#### Experimental Section

**Materials.** GdCl<sub>3</sub>·6H<sub>2</sub>O (450855), YbCl<sub>3</sub>·6H<sub>2</sub>O (337927), TmCl<sub>3</sub>·6H<sub>2</sub>O (259256), LuCl<sub>3</sub>·6H<sub>2</sub>O (542075), oleic acid (OA, 364525), 1-octadecene (ODE, 0806), ammonium fluoride (NH<sub>4</sub>F, 216011), tris(2-carboxyethyl) phosphinehydrochloride (TCEP, C4706), and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma-Aldrich. Ethanol, cyclohexane, and tetrahydrofuran (THF) of analytical grade were purchased from Sinopharm Chemical Reagent Beijing, Co., Ltd. Herceptin (anti-HER2 monoclonal antibody) was purchased from Abcam. <sup>99m</sup>Tc purchased from Shanghai GMS Pharmaceutical Co., Ltd. PEG (dp-PEG-mal, Mw: 2000) with diphosphate group at one end of the chain and a maleimide group at the other end were customized products provided by Beijing Oneder Hightech Co. Ltd.

**Characterization.** The size and morphology of the nanoparticles were observed using a FEI Tecnai G2 microscopes operating at an accelerating voltage of 120 kV. The hydrodynamic size was measured at 25°C with a Malvern Zetasizer Nano ZS90 equipped with a solid-state He-Ne laser (λ = 633 nm). The concentration of the rare-earth elements was determined by using an inductively coupled plasma atomic emission spectrometer (ICP-AES). The upconversion fluorescence spectra were recorded on an Edinburgh fluorescence spectrophotometer (FLS 980) equipped with a 980 nm laser serving as the excitation source.

**Preparation of Hydrophobic NaGdF<sub>4</sub>:Yb,Tm,Ca@NaLuF<sub>4</sub> Core@Shell Nanoparticles.** GdCl<sub>3</sub>·6H<sub>2</sub>O (0.80 mmol), YbCl<sub>3</sub>·6H<sub>2</sub>O (0.18 mmol), TmCl<sub>3</sub>·6H<sub>2</sub>O (0.02 mmol), and CaCl<sub>2</sub>·6H<sub>2</sub>O (0.02 mmol) were mixed with 14 mL OA and 16 mL ODE in a 100 mL flask. After forming a homogeneous solution under nitrogen protection by heating to 150°C, the solution was cooled down to 50°C and 10 mL of a methanol solution with NaOH (2.5 mmol) and NH<sub>4</sub>F (3.6 mmol) was added dropwise. After stirring at this temperature for 30 min, the methanol in the system was removed under vacuum at 100°C. Subsequently, the reaction mixture was heated to 300°C under atmospheric pressure for 1 h. After cooling the reaction mixture to room temperature, the

resultant nanoparticles were precipitated by ethanol, collected by centrifugation, re-dispersed in THF, and washed with ethanol for three cycles, and finally re-dispersed in THF or cyclohexane for further experiments. The deposition of NaLuF<sub>4</sub> shell followed a similar process of the preparation of NaGdF<sub>4</sub>:Yb,Tm,Ca core particles. Briefly, 0.33 mmol core particles dispersed in THF and 1 mmol LuCl<sub>3</sub>·6H<sub>2</sub>O were added to a 100 mL three-neck round-bottom flask containing 4 mL OA and 16 mL ODE. The growth of the NaLuF<sub>4</sub> shell and the following purification procedures for the core@shell particles were the same as those for the core nanoparticles.

**Ligand Exchange.** Approximately 10 mg of OA-coated NaGdF<sub>4</sub>:Yb,Tm,Ca@NaLuF<sub>4</sub> core@shell nanoparticles and 100 mg of dp-PEG-mal were dissolved in 4 mL of THF and stirring for 24 h at 40°C. Then the PEG-coated NaGdF<sub>4</sub>:Yb,Tm,Ca@NaLuF<sub>4</sub> nanoparticles were precipitated by cyclohexane and the supernatants were removed after centrifugation. After being washed with cyclohexane for three times, the final precipitate was dried under vacuum at room temperature for 4 h. The resultant particles were well dispersed in Milli-Q water, and the colloidal and optical emission stability were monitored by DLS and UCL fluorescence spectrometer, respectively.

**Cytotoxicity of Water-soluble NaGdF<sub>4</sub>:Yb,Tm,Ca@NaLuF<sub>4</sub> Core@Shell Nanoparticles.** The cytotoxicity of particles was assessed by MTT assays. SKBR3 cells were seeded into a 96-well cell culture plate by  $5 \times 10^3$  cells per well and cultured at 37 °C in an atmosphere containing 5% CO<sub>2</sub> for 24 h. Then, the culture medium was replaced by a fresh culture medium containing PEGylated NaGdF<sub>4</sub>:Yb,Tm,Ca@NaLuF<sub>4</sub> nanoparticles in a series of gradient concentrations (0, 0.1, 0.2, 0.5, 1, 2.5, 5, and 10 mmol/L, 100  $\mu$ L/well). After 24 h of further incubation, the supernatants containing NPs were removed from the wells and the cells were washed by 1×PBS for 3 times. Then, 20  $\mu$ L MTT with a concentration of 5 mg/mL was added to each well, and the plate was incubated for 4 h before the addition of 150  $\mu$ L dimethyl sulfoxide (DMSO) for dissolving the purple formazan that had been formed. Finally, the absorbance of each well was measured by using a PerkinElmer EnSpire® Multimode Plate Reader.

**Preparation of NP-mAb Probes.** 4  $\mu$ L of anti-HER2 monoclonal antibodies (mAb, 1 mg/mL in 10×PBS) were firstly subjected to mild reduction at 37°C for 30min by TCEP to convert the disulfide groups in the Fc fragments to thiol groups. The partially reduced mAb was purified by using 30 K MWCO centrifugal devices and then mixed with 1 mg of PEGylated NaGdF<sub>4</sub>:Yb,Tm,Ca@NaLuF<sub>4</sub> particles in Tris-buffered saline (TBS, pH 7.04). After reacted for 30 min, the resultant NP-mAb conjugates was transferred into 1×PBS buffer by using 30-K MWCO centrifugal devices and then stored at 4°C for further use.

**Binding Specificity of NP-mAb Probes.** Fluorescence microscopy was used for intuitively and quantitatively evaluating the targeting ability of NP-mAb conjugates to SKBR3 cells and MCF7 cells. In detail, about  $5 \times 10^4$  SKBR3 and MCF7 cells were seeded in confocal dishes for incubating overnight at 37°C under 5% CO<sub>2</sub> to allow a firm adherence, respectively. After being rinsed with PBS buffer, the cells were incubated with NP-mAb conjugates or mother nanoparticles (100  $\mu$ g/mL) at room temperature for 2 h. After that, the cells were rinsed three times with PBS buffer and then fixed with 4% paraformaldehyde. Subsequently, cells were further incubated with Hoechst 33342 (5  $\mu$ g/mL) for nuclei staining. After being rinsed three times with PBS buffer, the fluorescence micrographs were captured using a confocal microscope (Olympus FV 1200) equipped with 980 nm laser and 40 times magnification, and then the UCL signal in cells is quantitatively analyzed by Image J.

In order to further confirm the binding specificity of NP-mAb conjugates, similarly to above, SKBR3 cells and MCF7 cells were co-incubated with NP-mAb conjugates or mother nanoparticles respectively. After fixed with 4% paraformaldehyde, the cells were washed with PBS buffer for three times and then stained with Chlorophosphonazo III at room temperature for 30 min. Then the cells were washed three times with sterile water and the micrographs were captured using an ordinary optical Leica microscope (DM750).

**Establishment of Lymphatic Metastasis Model.** Lymphatic metastasis models used were established upon subcutaneous injection of  $5 \times 10^5$  SKBR3 cells into left foot pads of 4-5 weeks old specific pathogen free (SPF) grade BALB/c female mice which were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. and used under protocols approved by the Laboratory Animal Center of Soochow University. About 70% of the mice occurred lymphatic metastasis four weeks after inoculation of tumor cells.

**Upconversion Luminescence Imaging of Lymphatic Metastasis.** NP-mAb probes and mother nanoparticles were intravenously injected into mice bearing lymphatic metastasis model with a dosage of 9 mg Gd per kg body weight, respectively. *In vivo* upconversion imaging was performed on a customized upconversion imaging system (IVIS Lumina XR, Perkin Elmer) equipped with a 980 nm CW laser.

**Radiolabeling and SPECT/CT Imaging.** Radioactive Technetium-99m ( $^{99m}\text{Tc}$ ) was labeled onto NP-mAb probes and mother nanoparticles through the chelating effect of the phosphate group of PEG ligand anchoring on the particle surface. Briefly, 10  $\mu\text{L}$  of  $\text{SnCl}_2$  ( $1.0 \text{ mg mL}^{-1}$ ) solution in 0.1 M HCl was introduced into  $\text{Na}^{99m}\text{TcO}_4$  solution with a radioactivity of 1 mCi. 5 min later, NP-mAb or NPs solution ( $3 \text{ mg mL}^{-1}$ , total 180  $\mu\text{g}$  Gd) was introduced into the above mixture. After being kept under stirring at room temperature for 30 min, the resulting solution was subjected to ultrafiltration for 3 cycles with 30 kDa MWCO centrifugal filter. In this way, the radionuclide-labeled NP-mAb probes or mother nanoparticles were purified and concentrated to about 200  $\mu\text{L}$ . The final radiolabeling yield is higher than 60% for both NP-mAb probes and mother nanoparticles.

For SPECT/CT imaging, the obtained  $^{99m}\text{Tc}$ -labeled NP-mAb probes or  $^{99m}\text{Tc}$ -labeled nanoparticles were injected into nude mice *via* tail vein with a dose of 25 mCi per kg weight, corresponding to 9 mg Gd per kg body weight. And then, the SPECT-CT images were acquired at various time points on an animal SPECT/CT scanner (MILabs, the Netherlands) (scan time: 10 min/frame; FOV:  $26 \times 26 \times 70 \text{ mm}^3$ ; resolution: 0.6 mm). After 24 h imaging, the main organs were harvested and detected by a gamma counter. The acquired SPECT/CT images were reconstructed by a software package provided by MILabs and then fused with PMOD software. Quantification was performed by selecting the desired organs as volume of interest using the quantification tool of the PMOD software.

**Histological Study.** To verify the lymphatic metastasis model, lymph nodes harvested were fixed with 10% formalin soaking, and then embedded with paraffin after washing and dehydrating. Tissue sections of 7  $\mu\text{m}$  were sliced, stained with hematoxylin-eosin (HE) and mounted. Finally, the sections were imaged and analyzed by optical Leica microscope.

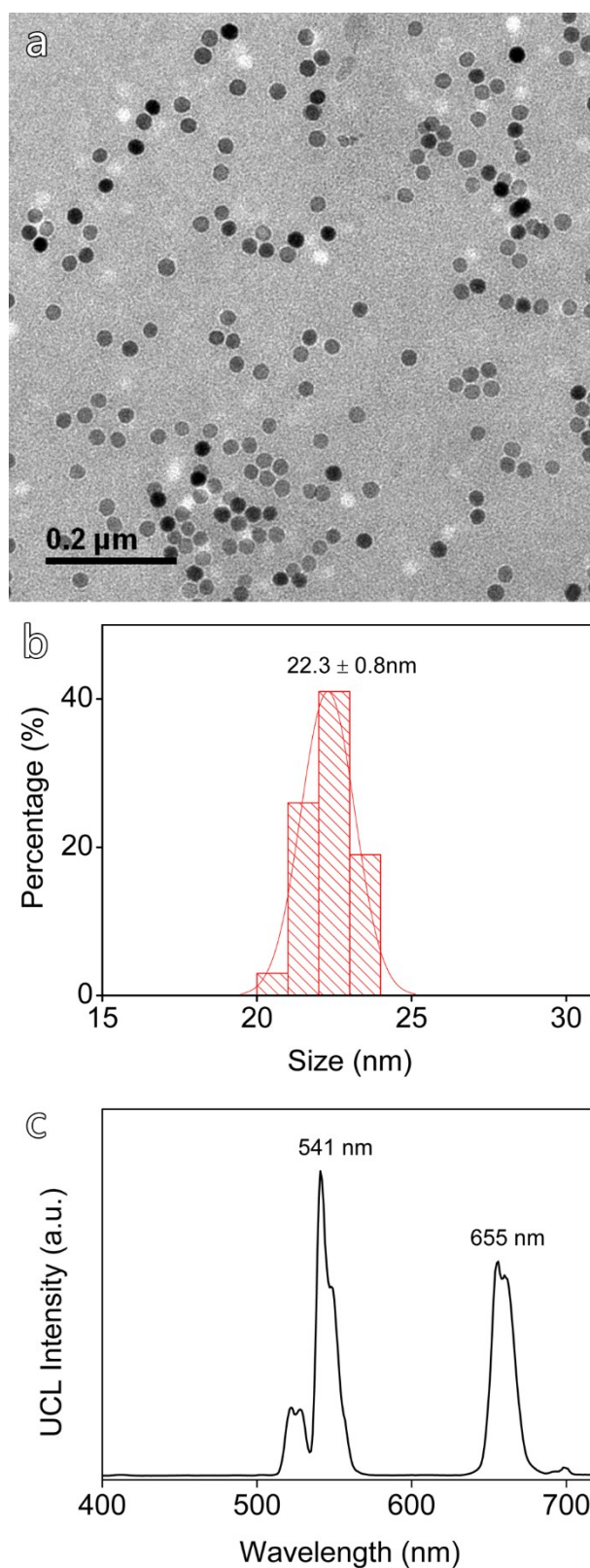


Figure S1. TEM image (a), size histograms (b), and upconversion luminescence spectrum (c) of NaGdF<sub>4</sub>:Yb, Er@ NaGdF<sub>4</sub> core@shell nanoparticles.

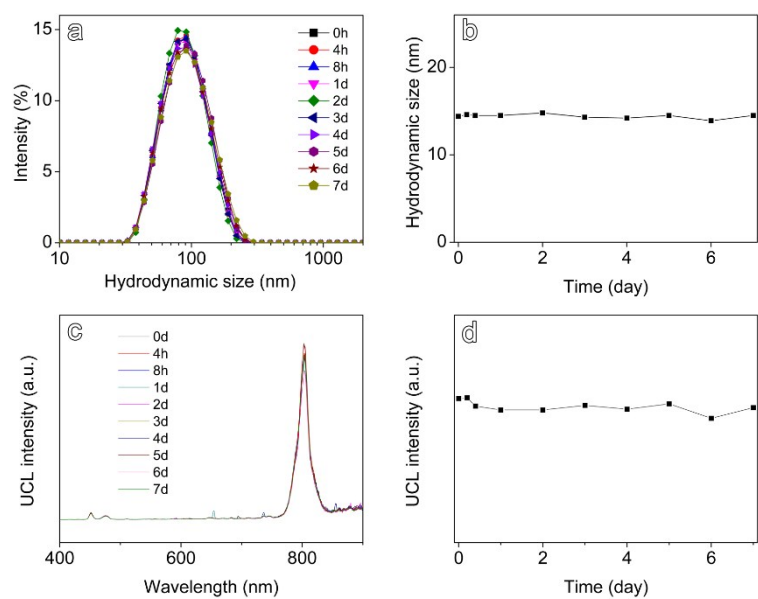


Figure S2. Hydrodynamic size distribution profiles (a) and upconversion luminescence spectra (b) of aqueous solution of NaGdF<sub>4</sub>:Yb,Tm,Ca@NaLuF<sub>4</sub> nanoparticles recorded against time for showing the temporal hydrodynamic size (c) and upconversion fluorescence emissions 804 nm (d).

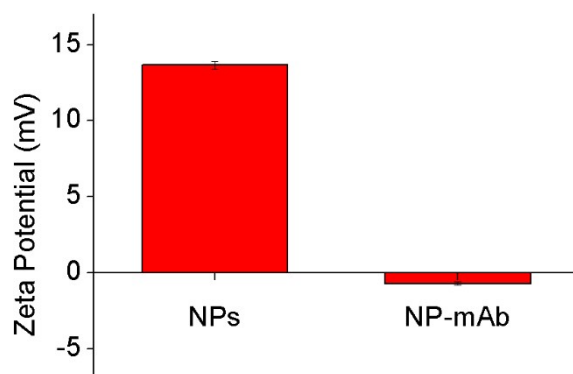


Figure S3. Zeta potential histogram of the mother particles and NP-mAb probes.

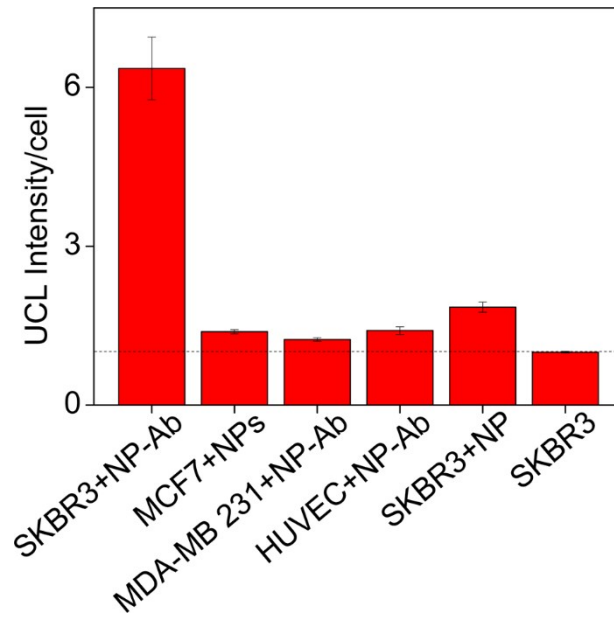


Figure S4. Quantified UCL signals of the cells shown in Figure 4.

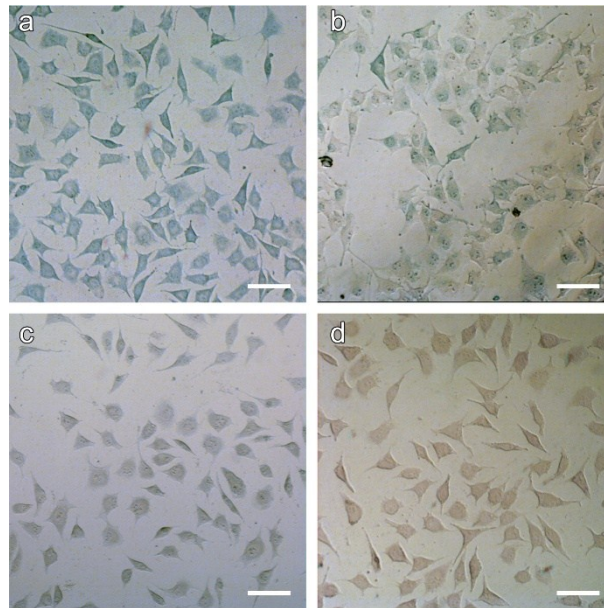


Figure S5. Microscopy images of cells stained with Chlorophosphonazo III for showing the binding affinity of NP-mAb and the mother particles to different cells: a) SKBR3 cells incubated with NP-mAb probes, b) MCF7 cells incubated with NP-mAb probes, c) SKBR3 cells incubated with the mother nanoparticles, and d) SKBR3 cells incubated with no nanoparticle or probe (the embedded scale bars correspond to 50  $\mu$ m).

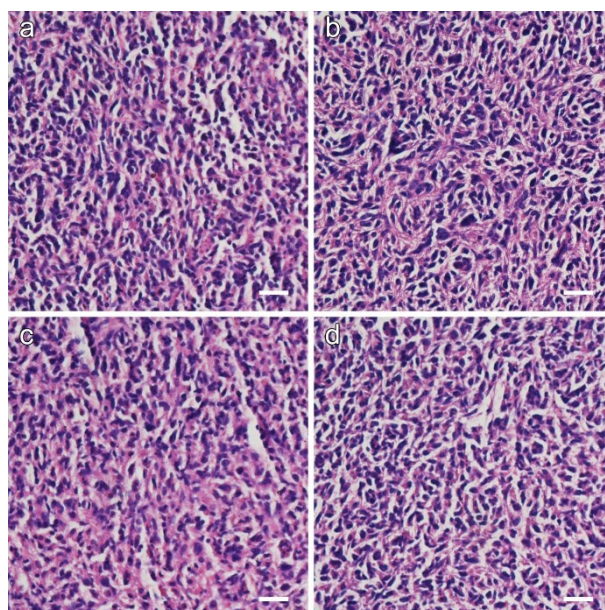


Figure S6. Pathological analysis of lymph nodes harvested from mice presented in Figure 6a and Figure 7a-b. (a) 24 h postinjection of NP-mAb nanoprobe, (b) 24 h postinjection of mother NPs, (c) 1.5 h postinjection of NP-mAb nanoprobe, and (d) 1.5 h postinjection of mother NPs (the scale bars correspond to 100  $\mu$ m).

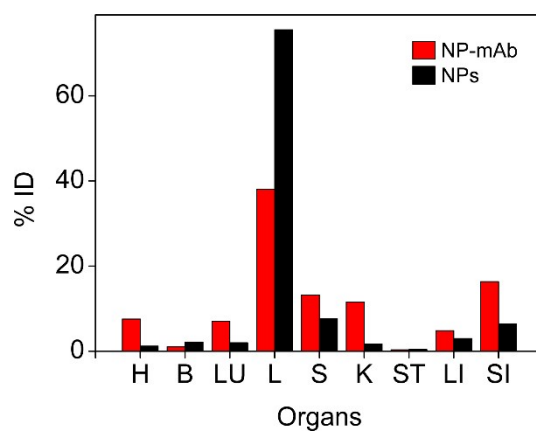


Figure S7. The biodistribution of NP-mAb nanoprobe and mother nanoparticles obtained by ICP-MS at 1.5 h post-injection. H, heart; B, brain; L, liver; S, spleen; LU, lung; K, kidney; ST, stomach; LI, large intestine; SI, small intestine.

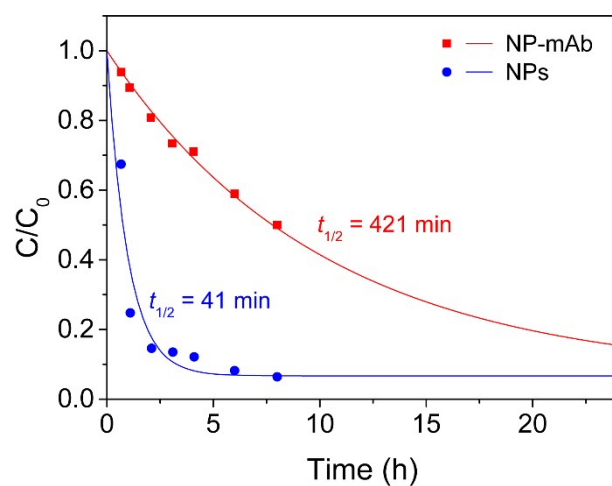


Figure S8. Blood biodistribution profiles of  $^{99m}\text{Tc}$ -labeled NP-mAb probes and mother nanoparticles fitted with one-compartment model.

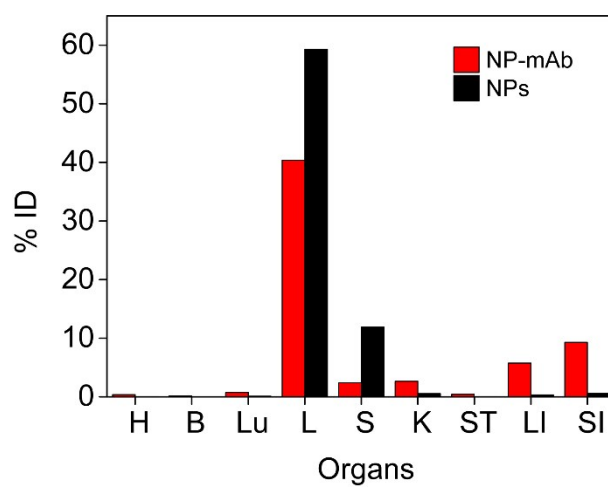


Figure S9. Biodistribution of  $^{99m}\text{Tc}$ -labeled NP-mAb nanoprobe and mother nanoparticles in main organs of mice at 24 h postinjection determined by gamma counter.