Supporting Information

Targeted labeling of early-stage tumor spheroids in chorioallantoic membrane model with upconversion nanoparticles

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MATERIALS AND METHODS

Synthesis of Carboxyl Functionalized $NaYF_4$:Yb^{3+}, Er^{3+} UCNPs. Hydrophobic $NaYF_4$:Yb^{3+}(20%),Er^{3+}(2%) upconversion nanoparticles were synthesized by a modified hyper-thermal decomposition method according to literature. [1] In a typical synthesis procedure, 236.54 mg $YCl_3 \cdot 6H_2O$ (0.78 mmol), 77.48 mg $YbCl_3 \cdot 6H_2O$ (0.2 mmol), and 7.64 mg $ErCl_3 \cdot 6H_2O$ (0.02 mmol) were dissolved in 2 mL methanol before being transferred into a 50 mL three-neck flask containing 3 mL oleic acid (OA) and 7 mL 1-octadecene (ODE). The mixture was stirred at room temperature for 30 min and then slowly heated to 110 °C for 15 min under argon atmosphere. After removing methanol and water, the solution was heated to 156 °C and maintained at this temperature for 1 h. The received lanthanide precursors were cooled down to room temperature, followed by adding 10 mL methanol solution containing 148.21 mg NH_4F (4 mmol) and 100.02 mg NaOH (2.5 mmol) into the flask and were stirred for 1 h. After the methanol was evaporated at 60 °C, the solution was heated to 300 °C for 90 min followed by cooling down to room temperature. The mixture was precipitated by the addition of 20 mL ethanol, and collected by centrifugation at 5000 r/min for 15 min. Product was re-dispersed with 5 mL hexane and precipitated by adding 15 mL ethanol, then collected by the same centrifugation. The product was washed three times, and the final product was re-dispersed in 10 mL hexane.

As the OA capped $NaYF_4$:Yb^{3+}, Er^{3+} UCNPs have poor dispersibility in aqueous phase, further surface modification was performed to improve the water-solubility and biocompatibility. In detail, a two-step ligand exchange method utilizing polyacrylic acid (PAA, -COOH groups) was used to replace the OA molecules, as shown in Scheme 1. First, 5 mL of OA capped UCNPs (50 mg) dispersed in hexane was mixed with 10 mL aqueous solution. After that, 200 μL HCl solution (1 mol/L) was added to the mixture and stirred for 2 h at room temperature. The oleate ligands were easily protonated and released from UCNPs in the presence of HCl, leaving ligand free UCNPs. [2,3] These ligand free UCNPs were then collected by centrifugation and washed with water/acetone for two times to remove the excess HCl and OA, and then

were redispersed in 5 mL water. At this stage, 50 mg PAA molecules were added into the ligand free nanoparticles and stirred overnight. Because of the high surface activity of ligand free UCNPs and the strong coordination interaction between rare earth ions and carboxyl groups, PAA molecules were bonded tightly onto UCNPs. The obtained carboxyl functioned UCNPs were washed with water for at least twice to remove the free PAA molecules.

Covalent Functionalization of UCNPs-Ab Conjugates. Monoclonal antibodies of estrogen receptor alpha (ER-α) were covalently functionalized with PAA functionalized $NaYF_4$:Yb^{3+},Er^{3+} UCNPs via an EDC cross-linking method in order to acquire targeted cancer delivering abilities. Briefly, 2 mg UCNPs were dispersed in 500 μL MES buffer, 10 mg EDC and 10 mg NHS were added into the solution and shaken continuously for 90 min at room temperature. The NHS-activated nanoparticles $NaYF_4$:Yb^{3+}, Er^{3+}-NHS were collected by centrifugation and washed twice with MES buffer, and redispersed in 500 μL MES buffer for further conjugation. Afterwards 100 μg ER-α antibodies were added into UCNPs solution and shaken gently for 1 h at room temperature. The acquired UCNPs-Ab conjugates were washed with phosphate-buffered saline (PBS) twice and redispersed in 500 μL PBS. Then 3 % BSA was added in order to block the excess bonding sites. The resulting solution was stored at 4 °C for further applications.

***In Vitro* Experiments.** Two different cell lines, human breast adenocarcinoma MCF-7 (for positive control) and fibroblast NIH 3T3 (for negative control), were cultured for evaluating the biocompatibility and specificity of UCNPs-Ab conjugates. Both were bought from the American Type Culture Collection (ATCC) and cultured according to standard methods. The MCF-7 cells were cultured in DMEM/F-12 medium. The medium was supplemented with 10 % fetal bovine serum (FBS), 1 % MEM Non-Essential Amino Acids Solution (100X), 100 unit/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen). The 3T3 cells were cultured in DMEM medium. The medium was supplemented with 10 % FBS, 100 unit/mL penicillin, and 100 μg/mL streptomycin (all from Invitrogen). Both cell lines were cultivated at 37 °C, 95 % humidity, and 5 % carbon dioxide (CO_2) atmosphere.

The cytotoxicity of UCNPs-Ab conjugates was evaluated by the mitochondria activity using standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Both MCF-7 and 3T3 cells were seeded into 96-well plates at concentration of 1×10^5/well and 5×10^4/well, respectively, and incubated at 37 °C under 5 % CO_2. After 24 h, different amount of UCNPs-Ab conjugates were added into the wells at a final concentrations of 0, 5, 10, 20, 50 and 100 μg/mL in culturing medium. The cells were further incubated for 24 h. Subsequently, 10 μL of MTT solution (5 mg/mL MTT in PBS) was added to each well and incubated for 4 h. After removing the medium, the wells were washed by PBS, and the intracellular formazan crystals were extracted into 100 μL of iso-propanol. The absorbance of cell lysate was recorded at 550 nm by a plate reader, and the cellular viability could be calculated from the average value of four parallel wells.

To study the specificity of the UCNPs-Ab conjugates for targeted imaging, *in vitro* experiments were carried out on human breast adenocarcinoma MCF-7 cells (with high expression level of ER-α). Human fibroblast cells 3T3 (expressing low levels of ER-α) were used for the control experiments. Both, MCF-7 and 3T3 cells were seeded on a coverslip at a concentration of 10^4 cells/mL and then treated with UCNPs-Ab conjugates (20 μg/mL) for 8 h at 37 °C. Prior to imaging, the coverslip was washed twice with PBS in order to remove any unbound upconversion conjugates. The cells were then fixed with 4% paraformaldehyde and mounted with 95% glycerol solution. Upconversion luminescence imaging was performed using our previous described confocal microscope system.[4]

Tumor Spheroid Bearing CAM Model. In this study, a modified shell-less CAM model was developed in order to evaluate the *in vivo* targeted labeling properties of UCNPs and UCNPs-Ab conjugates. Fertilized hen's eggs (Drost Loosdrecht BV, The Netherlands) were incubated at 39 °C and 70 % humidity. At embryonic age (EA) day 3, the egg was opened and the content carefully dropped into a dry and sterile dish. The dish was then covered with parafilm and put into an incubator set to 38 °C and 60 % humidity.

Three-dimensional multicellular tumor spheroids (MCTS) of human breast

adenocarcinoma MCF-7 cells were cultured *in vitro* for transplantation. To create MCTS, single MCF-7 cell suspensions of 0.5×10^6 cells/mL in medium were added to 1.5 % agar-coated 24 well plates (200 µl/well), and incubated at 37 °C under 5 % CO_2. After 3 days of incubation, tumor cells were aggregated with each other into a spheroid. The spheroids were then transferred into regular 24 well plates (non-agar coated) for further incubation. 200 µl of medium was added into each well every day. The tumor spheroids grew into MCTS with approximately 0.4 mm in diameter in seven days.

MCTS implantation was conducted on EA day 9 or 10. Firstly in a region away from major blood vessels, a small incision was made in the CAM, using a 30 gauge needle attached to a 1 mL syringe. Afterwards, the same region was slightly scratched with the needle's tip. Any resulting sera and blood was gently aspirated with the syringe. Thereafter, the MCTS was dropped onto the prepared region and placed on the incision side. The dish was then covered with parafilm, and returned to the incubator. Successful implantation was observed when the MCTS was engulfed into the CAM.

***In Vivo* Microscopy Imaging of MCTS.** On EA day 14, 100 µL of UCNPs or UCNPs-Ab conjugates dispersed in PBS (1 mg/mL) were systematically administered into superficial micro veins by injection under a stereomicroscope. For imaging, a homemade intravital microscope system equipped with a 100 W mercury lamp, a 500 mW 980 nm laser, 890 nm short pass filter to reject the excitation light, a digital camera (QImaging, Retiga-SRV, Canada), and an 4x objective was used. The chick embryo was placed under the objective on an electrical heating plate to keep the temperature at 37 °C. One hour and 24 hours after injection, the MCTS and the surrounding tissue were imaged with white light illumination to obtain bright field images. Thereafter, upconversion luminescence images so called dark field images were obtained using only laser light. Both images were merged to localize the upconversion origin.

Histological Examination of the MCTS and CAM. On EA day 15, after imaging, areas containing the MCTS were cut out and embedded in tissue fixation gel for

cryotom sectioning. The MCTS were cut into 10 μm sections, stained with hematoxylin-eosin (H&E) and further analyzed using a standard white light microscope. In addition, upconversion luminescence confocal imaging was carried out using an inverted Olympus IX71 microscope equipped with an 100× oil immersion objective and a 980 nm Ti:Sapphire laser, as previously described.[4]

Refrences

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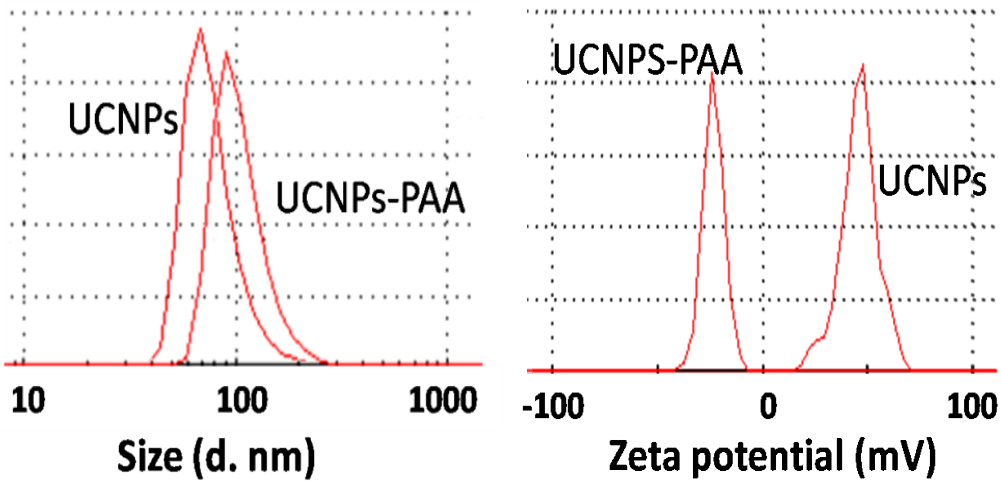

Figure S1. Hydrodynamic diameter distribution (left) and zeta potential (right) of ligand free UCNPs and PAA coated UCNPs.

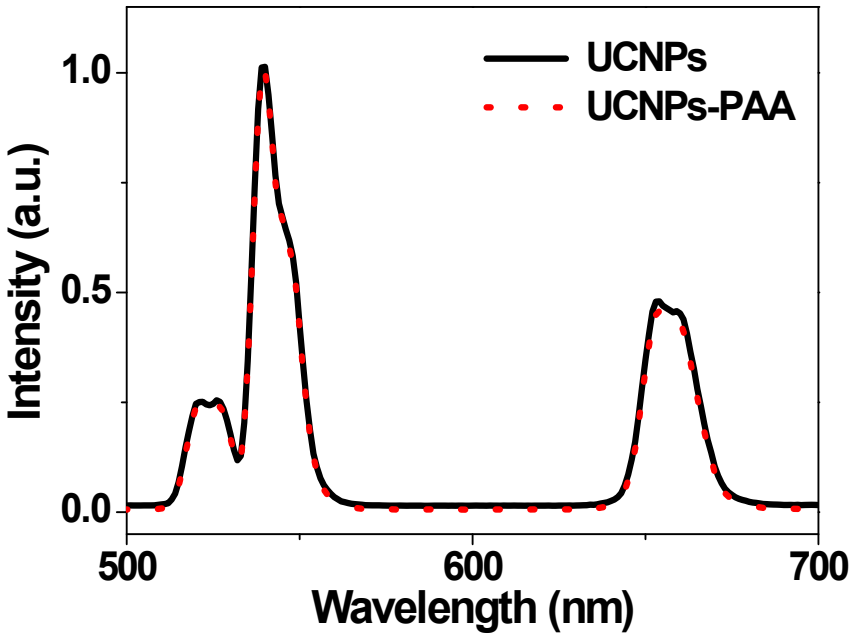

Figure S2. Upconversion luminescence spectra of ligand free and PAA coated $NaYF_4$:Yb^{3+},Er^{3+} UCNPs dispersed in water (1 mg/mL) under 980 nm excitation (400 mW).

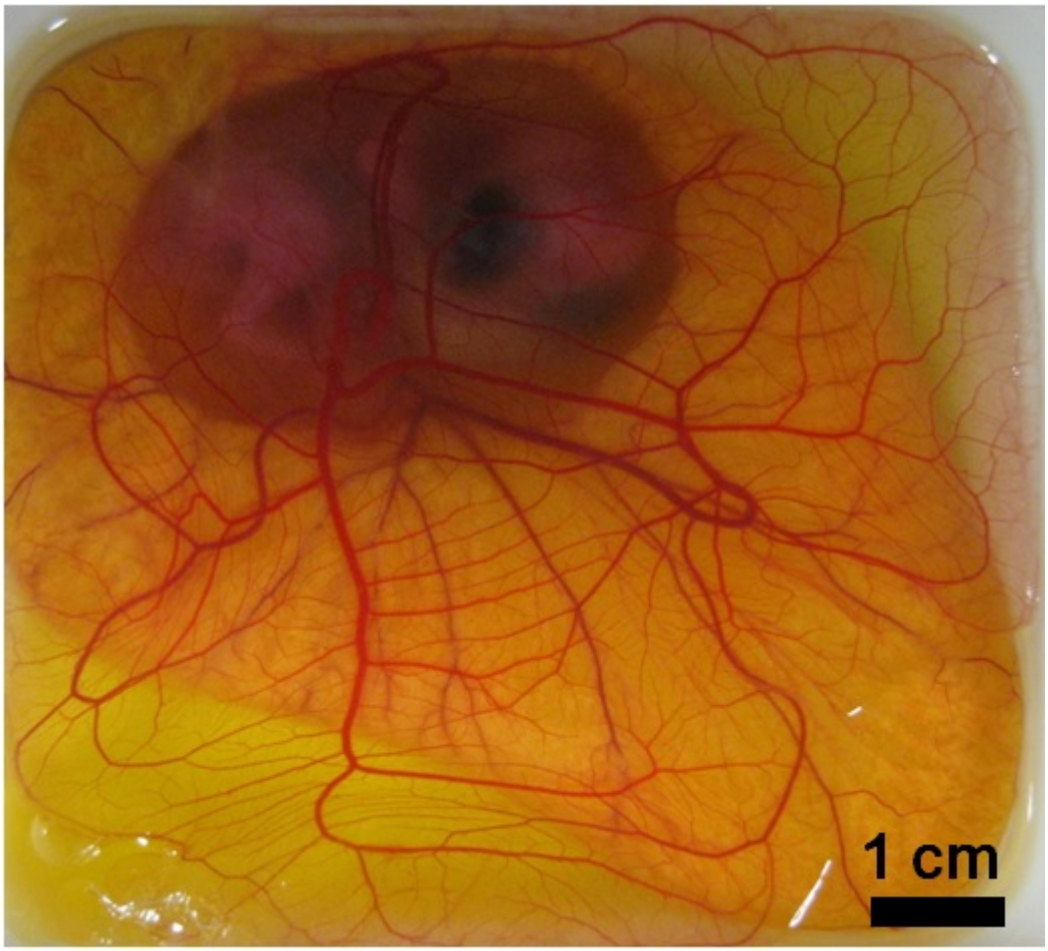

Figure S3. An entire photo of the *ex vivo* cultured chick embryo, in which the chorio allantoic membrane (CAM) is localized on the top of yolk and embryo. The CAM vein (bright red) and artery (dark red) can be easily separated from their color difference.

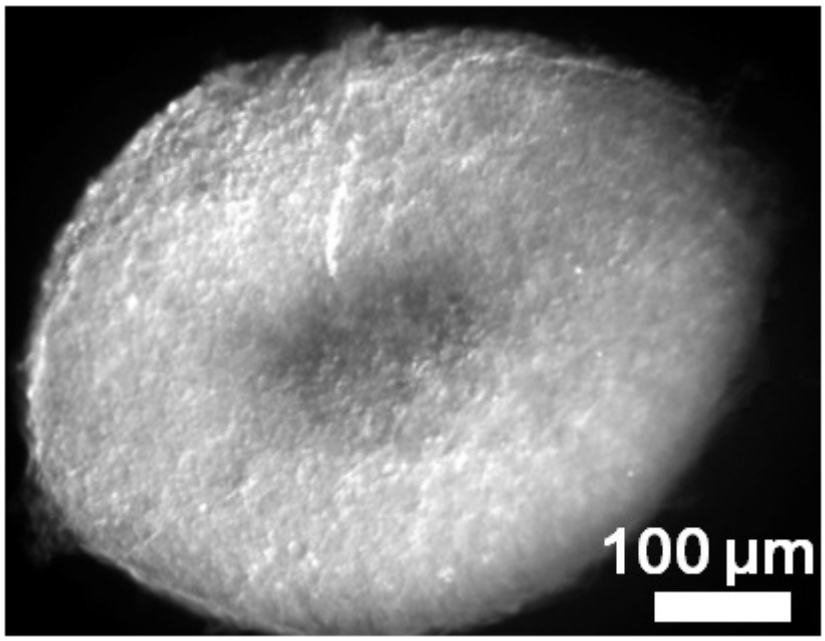

Figure S4. A typical multicellular tumor spheroids (MCTS) formed after 5 days of incubation in agarose gel coated well plates.The diameter is about 500 um.

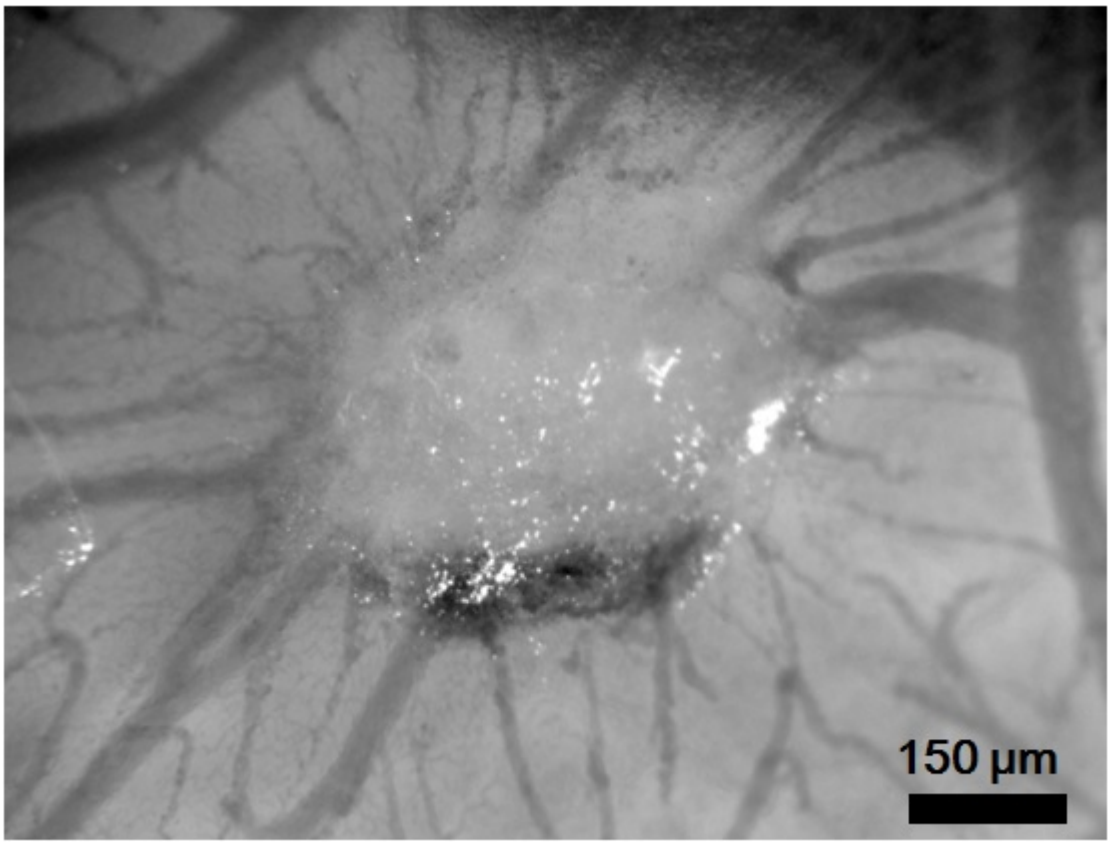

Figure S5. Multicellular tumor spheroid (MCTS) grated on the chick embryo CAM model.

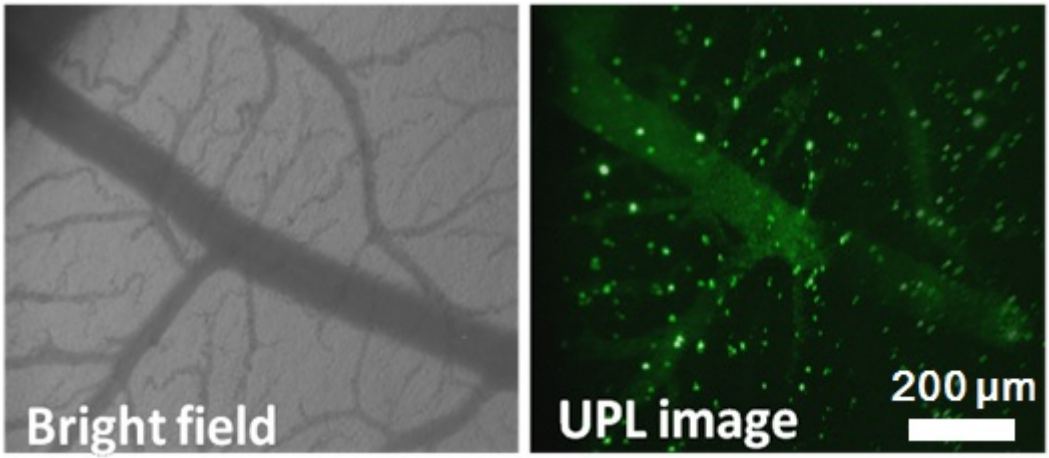

Figure S6. Microcirculating behaviors of UCNPs-Ab conjugates in chick embryo CAM. Left is the bright field microscope image, right is the upconversion luminescence (UCL) image of the same area captured after 10 min intravenous injection with 100 μg UCNPs-Ab.