

Supporting information for

Bio-functionalization of Ligand-free Upconverting Lanthanide doped Nanoparticles for Bio-imaging and Cell Targeting

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Experimental procedures

All chemicals used in the synthesis of the nanoparticles were purchased from Sigma-Aldrich and used as received. Millipore water was used in the preparation of all aqueous solutions used in the characterization of the samples.

Synthesis of Oleate-Capped-Ln³⁺-UCNPs.

Oleate-capped NaGdF₄:Er³⁺ 2%, Yb³⁺ 20% nanoparticles (oleate-capped- Ln³⁺-UCNPs) were synthesized *via* the thermal decomposition procedure^{1, 2}. In the first step, the precursors (Solution A) were prepared by mixing 0.975 mmol Gd₂O₃ (99.99 %), 0.25 mmol Yb₂O₃ (99.99 %), and 0.025 mmol Er₂O₃ (99.99 %) with 5 mL trifluoroacetic acid (99 %) and 5 mL of distilled water in a 100 mL three-neck round-bottom flask. The solution was stirred and refluxed at 80 °C for 12 h or until a clear solution was obtained and the temperature was lowered to 60 °C to slowly evaporate excess trifluoroacetic acid and water.

In the second step, 2.5 mmol sodium trifluoroacetate CF₃COONa (98 %) was added to the dried lanthanide trifluoroacetate precursors and mixed with 7.5 mL each of oleic acid and 1-octadecene (Solution A). In a separate three-neck round bottom flask 12.5 mL each of the coordinating ligand oleic acid (90 %) and the non-coordinating solvent 1-octadecene (90 %) were added (Solution B). Both solutions were placed under vacuum at a temperature of 150 °C, degassed to remove residual water and oxygen with stirring for 30 minutes. Solution B was heated under argon flow at a rate of approximately 8 °C/min, to 310 °C. Solution A was added to Solution B using a mechanical pump system at a rate of 1.5 mL/min (Harvard Apparatus Econoflow). The solution was maintained at 310 °C and stirred vigorously for 2 h to form the oleate-capped NaGdF₄:Er³⁺ 2%, Yb³⁺ 20% nanoparticles.

After 2 h, the mixture was allowed to cool to room temperature, and the oleate-capped-Ln³⁺-UCNPs were precipitated by the addition of hexane/ethanol (1:4 v/v) and isolated via centrifugation at 3000 rpm for 15 minutes. The resulting pellet was then washed once with ethanol and further purified by dispersing in a minimum amount of hexane and precipitated with excess ethanol. The resulting pellet was subsequently washed with acetone and isolated *via* centrifugation. The resulting oleate-capped-Ln³⁺-UCNPs were dried.

Synthesis of Ligand-Free Ln³⁺-UCNPs

Ligand-free Ln³⁺-UCNPs were synthesized using a procedure previously reported³. Oleate-capped Ln³⁺-UCNPs (100 mg) were dispersed in a 10 mL aqueous solution. The reaction was performed with stirring for 2 h while maintaining the pH at 4 by adding a solution of HCl 0.1 M. During this reaction the carboxylate groups of the oleate ligand were protonated (to yield oleic acid). After the reaction was completed the aqueous solution was mixed with diethyl ether to remove the oleic acid by extraction with diethyl ether three times and the combined ether layers were re-extracted with water. In addition, the water layers were combined and re-extracted with diethyl ether. The ligand-free Ln³⁺-UCNPs in the water fraction were recuperated by centrifugation after precipitation with acetone. The product was redispersed in acetone and the particles were recuperated by centrifugation. Finally the particles were dispersed in water.

Synthesis of Heparin-capped Ln³⁺-UCNPs

50 mg of heparin were added to a water solution of ligand-free Ln³⁺-UCNPs (10 mL, 1 mg/mL in water) and after sonication for 2 min the mixture was stirred at room temperature for 24 h. The suspension was then transferred into a centrifuge tube and centrifuged at 3000 rpm for 15 min. Following, the removal of the supernatant, the particles were re-suspended in 10 mL water and 30 mL acetone and sonicated for 60 s. The mixture was then centrifuged and the particles were recovered. The particles were dried under vacuum.

Synthesis of Basic Fibroblast Growth Factor (bFGF)-heparin-coated Ln³⁺-UCNPs

A 10 mg solution of heparin-coated Ln³⁺-UCNPs was mixed with a 5 mL solution of bFGF (0.5 mg/mL) in phosphate buffer (PBS pH 7.4) and stirred for 6 h. The resulting solution was centrifuged and the nanoparticles were re-dispersed in 20 mL PBS. The nanoparticles were dried and stored at 4°C.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of the heparin and heparin-coated Ln³⁺-UCNPs were measured on a Nicolet FTIR spectrometer using the KBr method. The spectra in transmission mode were recorded in range 4000-500 cm⁻¹.

High-Resolution Transmission Electron Microscopy (HRTEM)

HRTEM was performed on a JEOL JEM-2011 microscope operating at 200 kV. The TEM of heparin-coated Ln^{3+} -UCNPs sample was prepared by dropping sample solutions (1 mg/mL in water) onto a 300-mesh carbon coated copper grid (3 mm in diameter) followed by the evaporation of the solvent.

Zeta potential

The surface charge of the heparin and the oleate free, heparin-coated and bFGF-heparin functionalized Ln^{3+} -UCNPs (1 mg/mL) samples in PBS solution (pH 7.4) were determined using a Zeta Plus zeta potential analyzer (Brookhaven Instruments Corporation).

Dynamic Light Scattering

The hydrodynamic radius of the free, heparin-coated and bFGF-heparin functionalized Ln^{3+} -UCNPs solutions (1 mg/mL) was measured using dynamic light scattering (DLS, Malvern Zetasizer 173°). All tubes and pipettes were treated with a flow of nitrogen gas before contact with the nanoparticle suspension. Approximately 200 μL of suspension was added to a disposable plastic cuvette used for DLS measurements. The refractive index of the dispersant (water) was set at 1.33 while the material refractive index was set at 1.59. Analysis was carried out at 25 °C using a 60 second duration per measurement. The hydrodynamic diameter obtained was 40 nm for the oleate free Ln^{3+} -UCNPs, 49 nm for the heparin-coated Ln^{3+} -UCNPs and 55 nm for the bFGF-heparin functionalized UCNPs. These results indicate that the nanoparticles do not show any significant aggregation.

Assay of Heparin Immobilized on the Surface of the Ln^{3+} -UCNPs using Nile Blue

To determine the presence of heparin immobilized on the surface of the nanoparticles, absorption spectroscopy was used to determine the changes in the absorbance intensity of the peaks at 635 nm and 538 nm. The peak at 635 nm is characteristic of Nile blue whereas the peak at 538 nm is characteristic of Nile blue molecules which are forming aggregates⁴. From a solution of 1 μM Nile blue a standard solution of 0.01 nM was prepared following the procedure of serial dilution. 1 ml of standard solution was mixed with 2 mL water and was vortex vigorously for 30 seconds. The

solution was transferred into a quartz cuvette (1 cm path length) and the absorption spectrum of Nile blue in absence of heparin was recorded using a Cary spectrophotometer (Varian 100 Bio). Water was used as the blank.

From a solution of 5.6 μM heparin five standard solutions of heparin ranging in concentration from 1.8 nM to 1.1 μM were prepared following the procedure of serial dilution. 1 ml of different standard solutions of heparin (1.8 nM to 5.6 μM) was added to each vial (six vials) plus 1 mL Nile Blue (0.01 nM) and 1 mL water and mixed vigorously for 30 seconds. To show the presence of heparin on the Ln^{3+} -UCNPs, 2 ml of a solution of 1 mg/mL heparin-coated Ln^{3+} -UCNPs solution was added to 1.0 ml of Nile blue 0.01 nM solution and mixed vigorously for 30 seconds. The solution was transferred into a quartz cuvette (1 cm path length) and the absorption spectrum was recorded using a Cary spectrophotometer. Water was used as the blank.

Assay of bFGF on the Surface of Heparin- Ln^{3+} -UCNPs.

A visual protein detection assay using Coomassie brilliant blue dye which forms a complex with the protein bFGF was performed. This assay is based on the observation that the color of the solution of Coomassie Brilliant Blue changes from pale blue to intense blue when the dye binds to the protein. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, producing a visible color change. For this assay, 10 mg of Coomassie brilliant blue was dissolved in 5 mL of methanol. The solution was added to 10 mL of 85% H_3PO_4 and diluted to 100 mL with water. The final concentration of the dye stock was 0.01 % (w/v). From this solution 0.2 mL were transferred into two vials. In the first vial corresponding to reagent blank, 0.2 mL of PBS was added. In the second vial 0.2 mL solution of bFGF- heparin-coated Ln^{3+} -UCNPs dispersed in PBS (1 mg/mL) was added.

A standard calibration curve was obtained using a series of known protein concentrations. Bovine serum albumin (BSA) was used as standard protein and 0.15 M NaCl was used as buffer. In Bradford assay⁵, 0.1 mL of protein standard solution in the range of 0-0.1 mg/ml or nanoparticle sample (1mg /mL) was pipette into test tubes containing 2 mL of dye stock. The volume in the test tube was adjusted to 5 mL with 0.15 M NaCl buffer and the solutions were mixed by vortexing and incubate for 5 min at room temperature. The UV-VIS spectrum was recorded after 5 min in 3 mL quartz cuvette against a reagent blank prepared followed the same procedure as described above

except that the protein/sample solution was replaced with 0.15 M NaCl solution. From a standard curve, absorbance measured at 590 nm plotted against the protein standard concentration, the amount of bFGF at the surface of nanoparticles was estimate.

Upconversion Luminescence Spectroscopy

Upconverted visible luminescence from the $\text{NaGdF}_4\text{:Er}^{3+}$, Yb^{3+} nanoparticles before and after modification with heparin were obtained upon excitation with 980 nm wavelength laser beam from a Coherent 6-pin fiber-coupled F6 series 980 nm laser diode (maximum power of 600 mW at 1000 mA), coupled to a 100 μm (core) fiber. For the spectroscopic studies, the samples (1 mg/mL) were dispersed in water and placed in 1 cm path length quartz cuvettes (Hellma QS). The upconversion visible emissions were collected at $\pi/2$ with respect to the incident beam and then dispersed by a 1 m Jarrell-Ash Czerny-Turner double monochromator with an optical resolution of ~ 0.25 nm. A thermoelectrically cooled Hamamatsu R943-02 photomultiplier tube detected the visible emissions from the sample exiting the monochromator. A preamplifier, model SR440 Standard Research Systems, processed the photomultiplier signals and a gated photon-counter model SR400 Standard Research Systems data acquisition system was used as an interface between the computer and the spectroscopic hardware. The signal was recorded under computer control using the Standard Research Systems SR465 software data acquisition/analyzer system.

Cell cultures

The HeLa human cervical cancer cell line and HaCaT human normal skin keratinocytes were stored frozen in liquid nitrogen. The cells were grown as a monolayer employing Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS) and 50 units/mL penicillin and 50 $\mu\text{g/mL}$ streptomycin (Sigma). The cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere and the medium was changed daily. For fluorescence observation, cells were plated onto coverslips placed into wells.

Multiphoton fluorescence microscopy

Cell cultures used for fluorescence imaging were incubated for a period of 3 h in the presence of heparin-coated Ln^{3+} - UCNPs or bFGF-heparin-coated Ln^{3+} - UCNPs at a concentration of $2.8 \cdot 10^{-2}$ μM in PBS.

The fluorescence imaging of the cells incubated with the different nanoparticles (give the nanoparticles) were obtained using a fast multiphoton microscope (Zeiss LSM510 microscope). The fluorescence images were obtained under the 920 nm excitation of a Mai Tai (Spectra Physics) ultrafast laser. The emitted intensity was detected in the range from 600 to 700 nm wavelength, corresponding to the red emission of Er^{3+} ions. All the images were obtained using the same microscope parameters.

Toxicity of heparin and bFGF-heparin-coated Ln^{3+} -UCNPs

Cell viability of HeLa cells exposed to the different nanoparticles (heparin-coated Ln^{3+} -UCNPs or bFGF-heparin-coated Ln^{3+} -UCNPs) was analyzed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) colorimetric assay. This method is based on the capacity possessing the dehydrogenases of living cells to reduce the salt of tretazolio MTT to a colored and insoluble compound called formazan. As first step, HeLa cells were incubated for 24 h with the different nanoparticles. After that, the cells were incubated with MTT (0, 1 mg/ml in DMEM with 10% SFB and 1% L-glutamine) during 4 hours at 37 °C.

Then the medium with the MTT was removed and crystallized formazan was suspended with 1 ml of dimethylsulphoxide (DMSO). Immediately later, we proceeded to measure the absorbance at 540 nm into a reader of plates (Espectra Fluor 4, Tecan). Cell viability was estimated as a percentage relative (100% viability) to the mean of absorption obtained from the control cells (not undergoing incubation with nanoparticles).

Figures

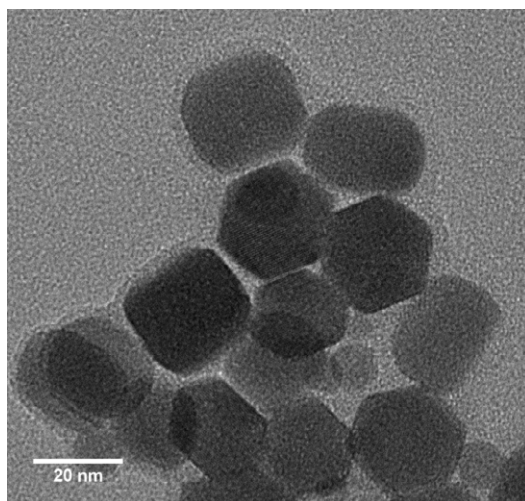


Fig. S1.- TEM image of heparin-capped UCNPs.

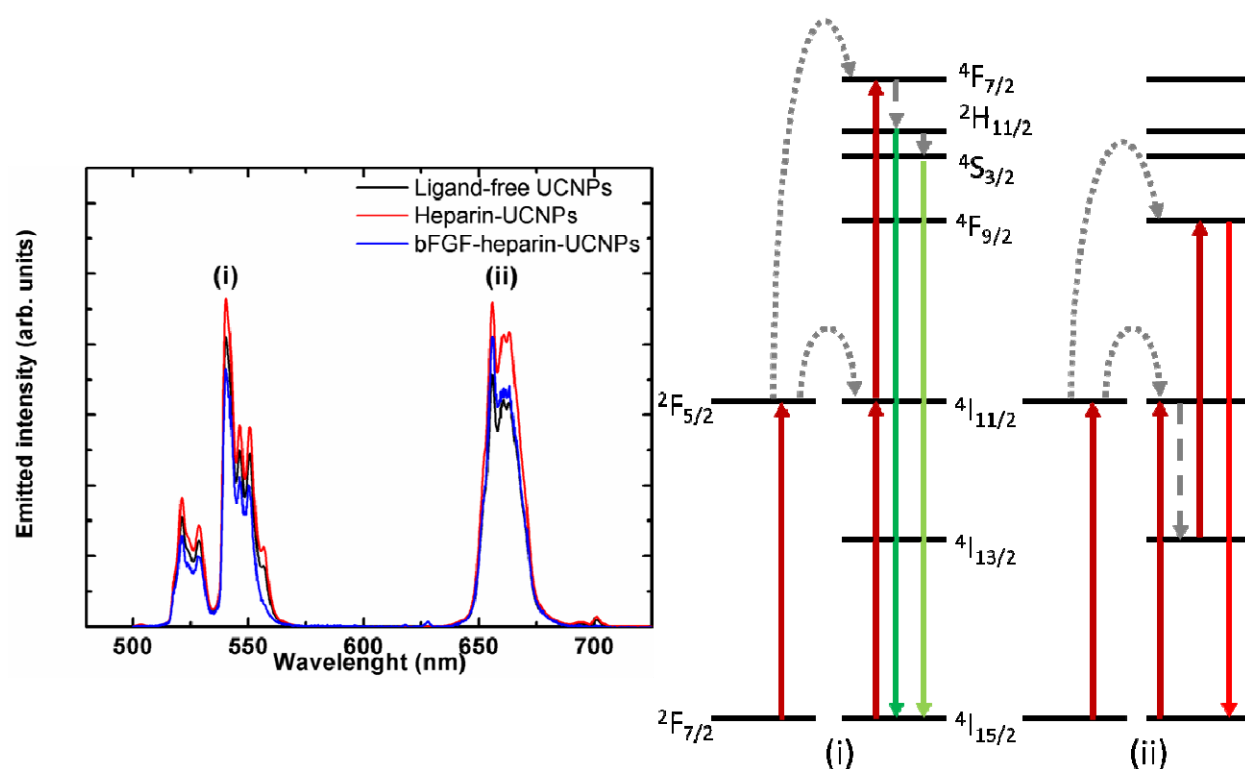


Fig. S2.- Upconversion emission spectra of lanthanide doped NaGdF₄:Er³⁺ 2%, Yb³⁺ 20% nanoparticles in aqueous solution (a) ligand-free Ln³⁺-UCNPs (b) heparin-coated Ln³⁺-UCNPs (c) bFGF-heparin functionalized Ln³⁺-UCNPs. Excitation at 980 nm wavelength.

References

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