Supporting Information

Dual Activity of Rose Bengal Functionalized to Albumin-Coated Lanthanide-Doped Upconverting Nanoparticles: Targeting and Photodynamic Therapy

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Experimental Section

1- Synthesis of $NaGdF_4$: Yb^{3+}/Er^{3+} (Ln-UCNPs)

Monodisperse upconverting $NaGdF_4$:Yb^{3+}/Er^{3+} nanoparticles were synthesised by thermal decomposition technique established by our group [1]. Erbium oxide (Er_2O_3, 99.99%), Gadolinium oxide (Gd_2O_3), Ytterbium oxide (Yb_2O_3), trifluoroacetic acid (CF_3COOH, 99%), Sodium trifluoroacetate ($NaCF_3COONa$, 98%), oleic acid (technical grade,90 %), and 1-octadecene (technical grade, 90%) were all purchased from Sigma-Aldrich and were used without any further purification. First, lanthanides precursors are prepared by dissolving Gd_2O_3 (0.534 g, $9.75x10^{-4}$ mole, 78 mol %), Er_2O_3 (0.0096 g, $2.5x10^{-5}$ mole, 2 mol %), and Yb_2O_3 (0.0985 g, $2.5x10^{-4}$ mole, 20 mol %) in a 10 ml mixture of distilled water and trifluoroacetic acid (1:1) at 80 $^{\circ}$C for 12 hours under reflux. The solvent was evaporated slowly at 60 $^{\circ}$C for 16 hours to produce a white powder. Sodium trifluoroacetate (0.34 g, 2.5 $x10^{-3}$ mole) was added over the dried precursor and the solution is gradually heated up to 125 $^{\circ}$C under vacuum in the presence of 7.5 mL oleic acid and 7.5 mL 1-octadecene. Meanwhile, a receiving flask containing 12.5 mL oleic acid and 12.5 mL 1-octadecene heated up to 150 $^{\circ}$C under vacuum for 30 minutes, followed by a direct temperature increase up to 310 $^{\circ}$C under inert atmosphere using gentle flow of argon gas. Heated precursors were injected to the receiving flask at rate of 1.5 mL/min using syringe and pump system and left stirring for an additional hour. The solution was cooled down to room temperature, and $NaGdF_4$: Yb^{3+}/Er^{3+} nanoparticles were precipitated with ethanol. Nanoparticles were isolated by centrifugation 3400 rpm for 15 minutes. For additional purification, collected pellet were washed twice with hexanes/ ethanol (1:4).

2- Transmission electron microscopy and negative staining

To determine the particle size and morphology, high resolution transmission electron microscopy using a Philips CM200 operated at 200kV equipped with a charge coupled device (CCD) camera (Gatan) was used. A solution of 0.1 wt % Ln-UCNPs was prepared in toluene and 15 μL of the solution was evaporated on a formavar/carbon film supported on a 200 mesh copper grid**.** Samples for the negative stain were prepared by adding 5 μL of BSA coated Ln-UCNPs in water (0.5 mg/mL) to copper grid, and sample left to air dry. Later, 5 uL of 1.5% uranyl formate solution in 5 M NaOH solution was added, incubated for 1 min and allowed to dry.

3- X-Ray powder diffraction

X-ray powder diffraction (XRPD) measurements was performed using Scintag XDS-2000 diffractometer equipped with Si(Li) solid state Peltier detector, a Cu Kα source using a generator power of 40mA and 45 kV, receiving beam slits (0.2-0.5 mm), and beam divergent (2 mm and 4 mm). The range of the scan was set between 20-80^{o} 2θ with a 0.02^{o} step size and a count time of 1 s.

4- Bovine serum albumin (BSA) coated $NaGdF_4$: Yb^{3+}/Er^{3+}

A modified version of BSA nanoparticle synthesis protocol [2], was used to produce water-monodisperse BSA coated $NaGdF_4$: Yb^{3+}/Er^{3+} (BSA-Ln-UCNPs). To prepare the coated nanoparticles, 2 mg of Ln-UCNPs were dispersed in 7 mL ethanol, sonicated for 10 minutes and injected using a syringe and pump system to a BSA (Bovine Serum Fraction V, Bioshop) solution 1mg/mL at a rate of 1 mL/min. Once the injection was terminated, different volumes (4, 15, 20, 30 μL) of (8%) glutaraldehyde solution was added, and the reaction stirred for an additional 4

hours. BSA-Ln-UCNPs were centrifuged (12470 g, for 5 minutes) and the white pellet washed 3 times with ultrapure water and stored in water at -20 °C.

5- Thermogravimetric Assay (TGA)

TGA was used to estimate the actual mass of BSA on the surface on of the Ln-UCNPs. The mass of oleate was quantified by measuring the mass loss of $NaGdF_4$: Yb^{3+}/Er^{3+} between 250-600 °C. Initial mass of the Ln-UCNPs used was 9.845 mg and showed a total weight loss of 2.65%. This represents $5.63x10^{16}$ molecules of oleate per 1 mg Ln-UCNPs, which corresponds to a surface coverage of approximately 15 % , based on the surface area of 0.4 nm^2 for the oleate molecule [3], and 15290 nm^2 area of single Ln-UCNPs. The area of the Ln-UCNPs is calculated by measuring the average length of the hexagon prism shown by TEM. Similarly, TGA measurement was carried out for BSA-Ln-UCNPs and percent mass loss was recorded between 200 °C and 800 °C, showing a 15.24 % mass loss which represents 120.64 µg for 1 mg Ln-UCNPs. BSA has a molecular weight of 66 463 kDa, this will imply to 112 BSA molecules per single Ln-UCNP.

6- Primary amine quantification

Trinitrobenzenesulfonic acid (TNBS) a well-established method for primary amines quantification, and protein detection, was used to quantify free amine on BSA-Ln-UCNPs [4]. Briefly, 0.25 mL of TNBS solution in 0.01% $NaHCO_3$ was mixed with lysine standard solutions between 1-10 µg, for a total volume of 2 mL per sample to generate a standard curve. Mixtures were incubated for 2 hours at 37 °C, followed by addition of 0.25 mL 10% SDS, and 0.125 mL of 1M HCl generating an orange color that was quantified by measuring the absorbance at 346 nm. BSA-Ln-UCNPs quantification was obtained using the lysine standards. Based on calibration curve we estimated that 2128 primary amine sites per single BSA-Ln-UCNPs.

7- Luminescent intensity $NaGdF_4$: Yb^{3+}/Er^{3+}

To study the influence of water on the luminescence of the Er^{3+} ions of the BSA conjugated nanoparticles the emission spectra were recorded using ultra pure water and D_2O as the solvent. A 980 nm diode laser (F6 series fiber coupled, 0.5 W power) was used to excite the sample solution (1 wt %). The emission spectra were collected using a double monochromator (Jarrell-Ash Czerny Turner), photomultiplier tube (R943-02, Hamamatsu), SR440 (Standard research amplifier), SR400 (Gated photon counter), and SR465 (computer data acquisition software).

8- Rose Bengal linker addition, and surface conjugation

RB (Rose Bengal) was modified by adding a hexanoic acid linker to facilitate coupling with the primary amines of BSA that is coated on the nanoparticles surface [5]. In brief 50 mM RB and 125 mM 6-bromohexaonic acid were mixed in 70% acetone solution (20 mL) and refluxed at 60 $^{\circ}$C for 18 hours. The reaction mixture was acidified with H_2SO_4 and extracted three times using dichloromethane (30 mL). The sample was dried under vacuum for 24 hours. Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) was used to confirm the modification to RB. In order to couple modified RB to BSA-Ln-UCNPs, 20 mM modified RB was mixed with 50 mM EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, Sigma-Aldrich) and 50 mM NHS (N-Hydroxysuccinimide, Sigma-Aldrich) in DMSO and stirred for three hours at room temperature. Different concentration of nanoparticles were added over the mixture and stirred overnight (in dark). Dialysis was performed using ultrapure water for 4 hours, and 16 hours using 12-14 kD membrane (Spectrum labs). RB-BSA-Ln-UNCPs are collected via centrifugation (14000 rpm), and washed three times using ultra pure water and the sample was stored at -20 $^{\circ}$C.

9-Energy transfer measurements

The concentration of modified RB coupled to BSA-Ln-UCNPs was determined using RB calibration curve. Three different concentrations (30, 20, and 3 μM) were conjugated to BSA-Ln-UCNPs. For Luminescent intensity measurements, same concentration of Ln-UCNPs were optimized to 1 mg/ ml in water using ICP-MS (inductively coupled plasma mass spectrometry, Agilent 7500ce). BSA-Ln-UCNPs were used as standard emission under 980 nm excitation. Energy transfer is attributed to the decrease in the intensity of $^4S_{3/2}$-$^4I_{15/2}$ transition at 540 nm for RB-BSA-Ln-UCNPs emission compared to BSA-Ln-UCNPs.

10-Singlet oxygen measurement

DPBF (1,3 dipenylisobenzofuran, Sigma-Aldrich) probe was used to detect singlet oxygen production [6]. RB-BSA-Ln-UCNPs (1mg/ml) are dispersed in 1:1 ratio in deuterated solvents of Dimethyl sulfoxide-d_6 (Sigma Aldrich), and D_2O (Sigma Aldrich). Absorbance of DPBF (35 μM) at 415 nm was measured prior to 980 nm laser irradiation. 980 nm excitation of RB-BSA-Ln-UCNPs was performed at 15 minute time intervals for a total time of 90 minutes. Assuming 1:1 ratio of singlet oxygen and DPBF, approximately 28 % of DPBF was consumed which was used to calculate the production of molecules of singlet oxygen ($5.9x10^{21}$).

11- In vitro localization assay

Cellular localization of the nanoconstruct was tested using A549 lung cancer cell lines. 35 mm cell culture dish cells are seeded with a density of $2.5x10^3$ cells. After 24 hours, different concentration of RB-BSA-Ln-UCNPs were mixed with 2 mL cell culture medium that contained F-12k (Wisent Bioproducts) with 10% FBS (Thermofisher Scientific), and 1% Penicillin/ streptomycin

(Thermofisher Scientific) (10, 000 U)/mL. RB-BSA-Ln-UCNPs were incubated for 4 hours, followed by isotonic saline washing using PBS (Thermofisher Scietific). Furthermore, cells are fixed with 10 % formalin (Sigma Aldrich) for 15 minutes, followed by washing with PBS twice. Fixed cells are stored at 4 $^{\circ}$C, and imaged using a Zeiss LSM780 confocal microscope.

12- Cell viability assay

A549 lung cancer cells were seeded on 96-well plate with a density of 5000 cells/ well. The cells allowed to grow for 18 hours, followed by PBS wash and the addition of 100 µL cell culture medium (F-12k medium with 10% FBS and 1% Penicillin/streptomycin (10, 000 U)/mL). Different concentrations of RB-BSA-Ln-UCNPs were incubated with A549 cells for 4 hours at 37 $^{\circ}$C. The cells are washed with PBS and incubated for additional hour at 37 $^{\circ}$C in cell culture medium. After this, cells are irradiated with 980 nm for a period of 10 minutes. A549 cells are allowed to grow for additional 18 hours. Later MTT ((3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyltetrazoliumbromide, Thermo-Fisher Scientific) reagent (0.2 mg/mL in cell culture medium) was added to cells and incubated for 4 hours at 37 $^{\circ}$C. Formazan crystals formed are dissolved by the addition of 150 µL of DMSO, and MTT absorbance at 590 nm measured using 96-well plate (BioTeK). Same procedure as above was done for RB-BSA-Ln-UCNPs but without irradiation to estimate the toxicity of nanoconstruct by itself for A549 cells.

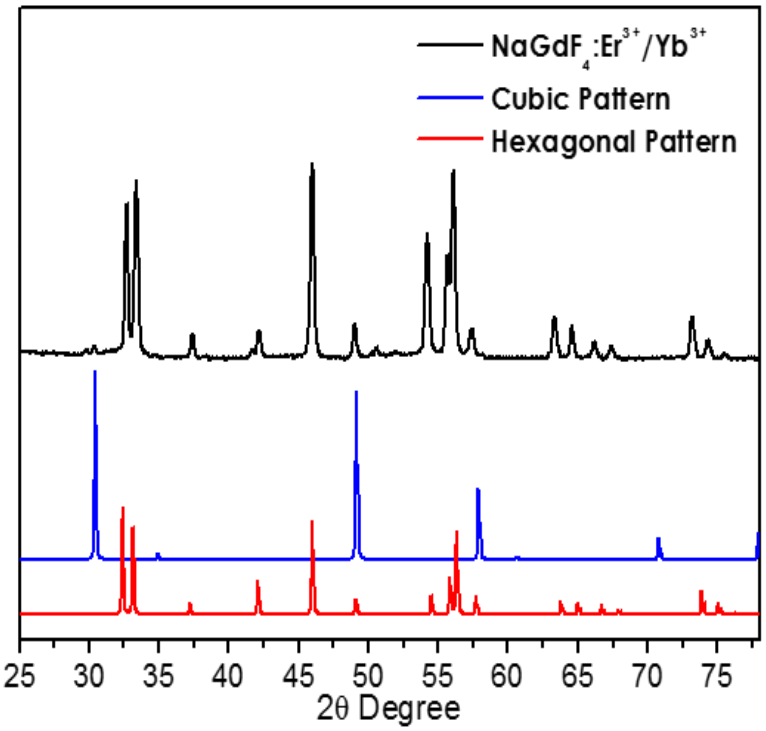

Figure S1. X-ray powder diffraction pattern of $NaGdF_4$: Yb^{3+}/Er^{3+} (Black). Spectrum comparisons are drawn to reference hexagonal and cubic phases shown in red and blue respectively.

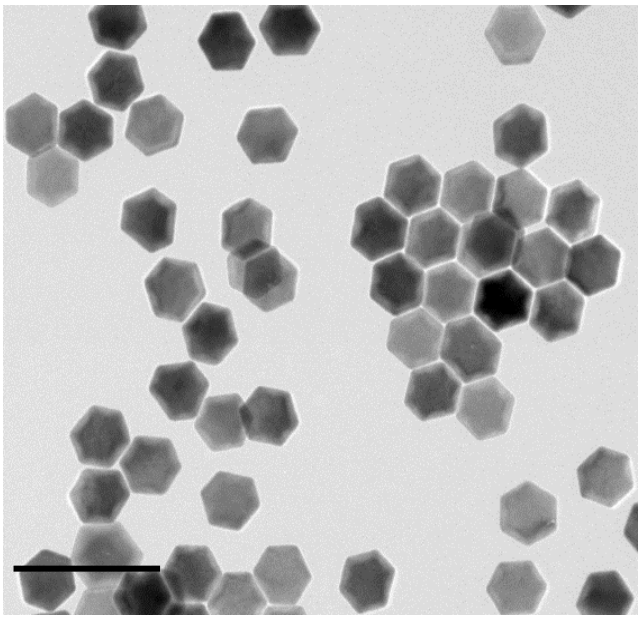

Figure S2. TEM image of $NaGdF_4$: Yb^{3+}/Er^{3+} ,1% solution in Toluene. Average size of the particle is 78 nm +/-2.9 nm (scale 500 nm)

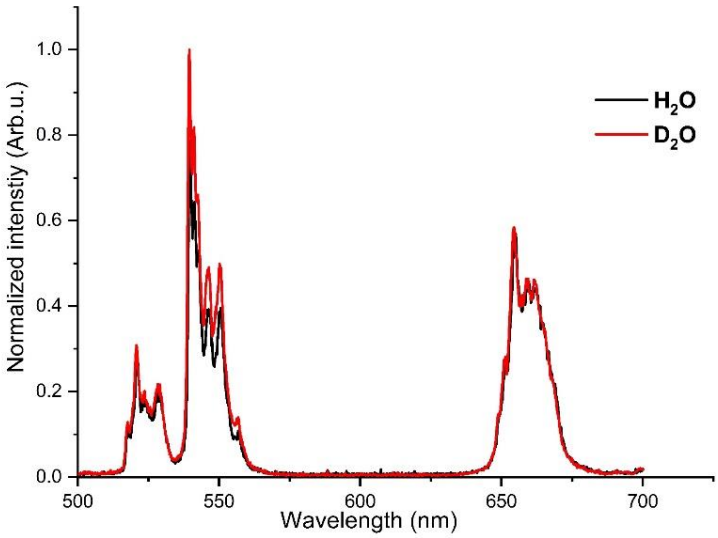

Figure S3. Luminescent intensity measurements using 980 nm excitation. Same concentration of BSA coated $NaGdF_4$: Yb^{3+}/Er^{3+}used for both measurements (1mg/ml). In red sample in D_2O, and black in H_2O.

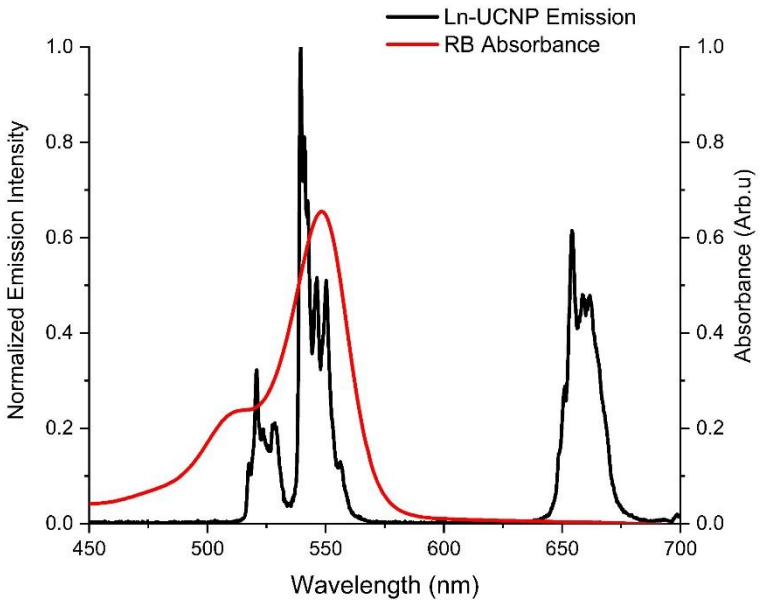

Figure S4. Overlap spectrum of $NaGdF_4$: Yb^{3+}/Er^{3+} and RB. Luminescence emission using 980 nm excitation shown in black, and RB absorption shown in red.

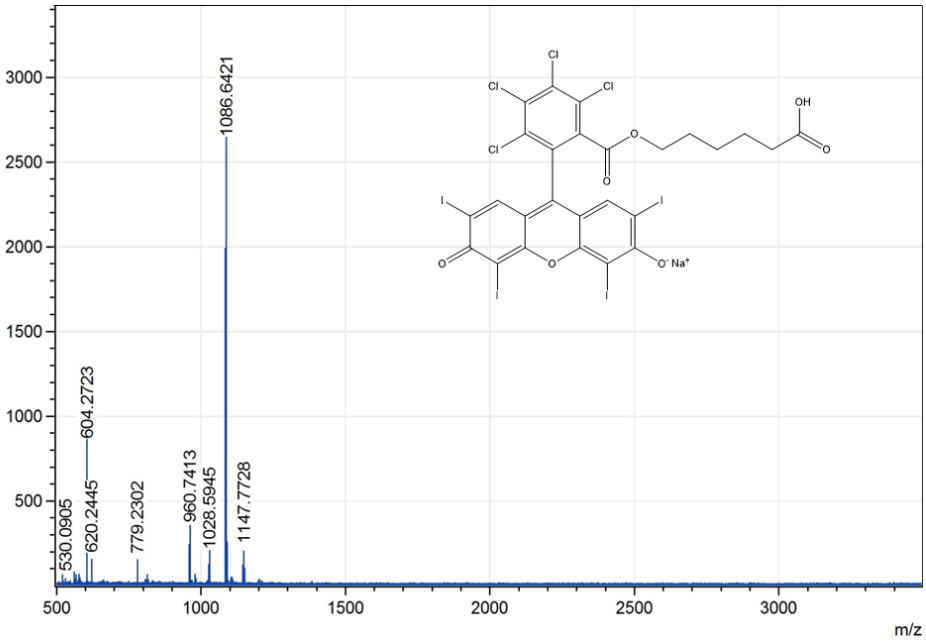

Figure S5 : MALDI-MS for hexanoic acid modified RB. (M-H) ionization peak at 1086.6421 represents the expected molecular weight for the modified RB.

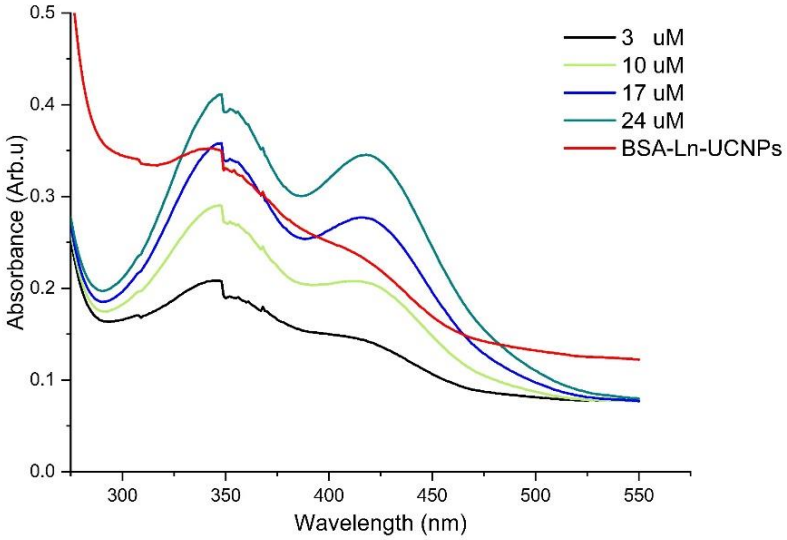

Figure S6. TNBS assay for primary amine quantification. Lysine used as standard with different concentrations. BSA-Ln-UCNPs (1 mg/ml, H_2O) unknown primary amine concertation shown in red.

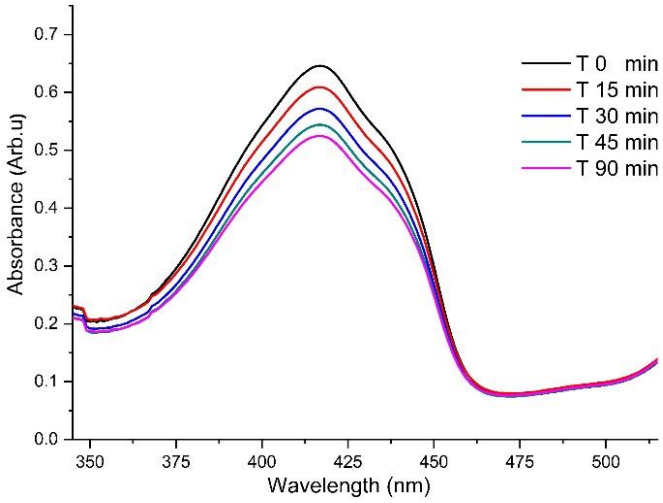

Figure S7. DPBF absorption decrease as a function of irradiation time using 980 nm. 1mg/ml nanoparticles in deuterated DMSO and D_2O (1:1) with 35 µM DPBF.

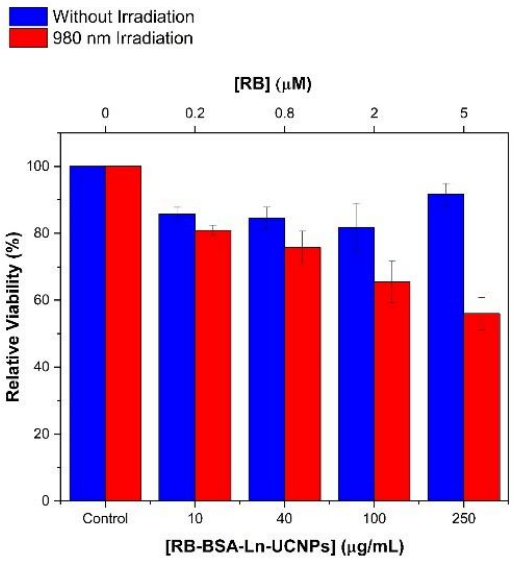

Figure S8. Viability assay of A549 cell using different concentrations of RB-BSA-Ln-UCNPs. Blue columns without irradiation, and red columns under 980 nm excitation for 10 minutes period, using A549 cells. Four different concentrations 10, 40, 100, and 250 μg of nanoconstruct used.

References

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