Imaging cellular trafficking processes in real time using lysosome targeted up-conversion nanoparticles

Sumit Kumar Pramanik,[a]* Sreejesh Sreedharan,[b] Harwinder Singh,[a] Carl Smythe,[c] Jim. A. Thomas[b]* and Amitava Das[a]*

CSIR-Central Salt & Marine Chemicals Research Institute, Bhavnagar: 364002, Gujarat, India; E-Mail: sumitpramanik@csmcri.res.in (SKP); a.das@csmcri.res.in (AD)

Department of Chemistry, University of Sheffield, Sheffield, S3 7HF, UK, Email: james.thomas@sheffield.ac.uk.

Department of Biomedical Science, University of Sheffield, Sheffield, S10 2TN, UK.

Experimental Section

Reagents

Yttrium (III) nitrate hexahydrate ($Y(NO_3)_3 \cdot 6H_2O$), Ytterbium (III) nitrate pentahydrate ($Yb(NO_3)_3 \cdot 5H_2O$), gadolinium (III) nitrate hexahydrate ($Gd(NO_3)_3 \cdot 6H_2O$) of 99.8 %, DMEM culture media with L-glucose, Sodium bi carbonate, Phosphate Buffer Saline (PBS), Fetal Bovine Serum, Penicillin Streptomycin, 4% Paraformaldehyde (PFA), Vectashield h-1000 (Mounting agent), 50 mM Ammonium chloride and Lyso Tracker green, Mito Tracker Deep Red were purchased from Sigma-aldrich. Ammonium fluoride (NaF), linoleic acid, sodium hydroxide (NaOH), ammonia ($NH_3 \cdot H_2O$), nitric acid (HNO_3) were purchased from Fischer scientific (USA). 26 mm X 76 mm Microscopy glass slides and 22 mm X 22 mm (170 ± 5 µm square Cover glasses were purchased from Thor labs.

Synthesis of $NaYF_4$:Yb,Gd UC-NPs.

A mixture of 1.0 g of $Y(NO_3)_3.6H_2O$, 0.293 g of $Yb(NO_3)_3.6H_2O$, 0.030 g of $Gd(NO_3)_3.6H_2O$, 10 g of hypermer B246 (a polymeric surfactant with outstanding dispersion and emulsion stability properties) and 60 mL of ethanol were mixed in a 250 mL two-neck flask to form a transparent solution under vigorous stirring at 78 °C. After that 1.5 g of NaOH (30 mmol) dissolved in 10 mL of water was added dropwise into the flask, and the resultant mixture was refluxed for 60 min. The precipitates coming from the reaction mixture were filtered and washed three times with ethanol. The obtained solid mass was dried at 60 °C for 24 h. Subsequently, 15 mL of water, 20 mL of ethanol, and 5 mL of linoleic acid were mixed together

under stirring condition to form a homogeneous solution, to which 1 g (1 mmol) of solid precursor and 0.20 g (5 mmol) of NH_4F were added. Then the mixture was sonicated for 20 min, transferred to a 40 mL autoclave and heated at 200 °C for 24 h. After cooling the NPs were deposited at the bottom of the teflon vessel, and a mixture of dichloromethane/ethanol (1:5,v/v) was used to collect the precipitate. The NPs were purified by centrifugation, washed with ethanol three times, and dried at 60 °C for 12 h. $NaYF_4$:Yb,Gd UCNPs were thus formed.

Surface Modification of $NaYF_4$:Yb,Gd UCNPs.

In a 250 mL flask, 100 mg of $NaYF_4$:Yb,Gd UCNPs was dispersed in 100 mL of ethanol by sonication. Then at 40°C 10.0 mL of ammonia and 20 mL of water were added into the flask and stirred vigorously. After that 100µL of tetraethyl orthosilicate mixed with 30 mL of isopropanol and added dropwise into the reaction mixture over a period of 1 h, and the reaction was stirred for another 6 h. A solution containing 0.5 mL of (3-aminopropyl)triethoxysilane and 30 mL isopropanol was then added dropwise into the resultant reaction mixture. Finally after 1 h, the resultant precipitates were separated by centrifugation and washed with ethanol three times, and dried at 60 °C for 24 h to get amino-modified $NaYF_4$:Yb,Gd UCNPs.

Synthesis of LTP conjugated $NaYF_4$:Yb,Gd UC-NPs

The free acid group of LTP reacts with the amino group of the $NaYF_4$:Yb,Gd UCNPs. First 0.5 g LTPLTP (having free COOH group) was mixed with 70 mg (0.45 mmol) EDC and 20 mg (0.09 mmol) sulfo-NHS to activate the free carboxyl groups. After stirring for 30 min NH2-terminated $NaYF_4$:Yb,Gd UCNPs were added continued for stirring for another 12 hour under ambient conditions. The reaction mass was then centrifuged at 12000 rpm for 30 min (KUBOTA) to remove residuals of EDC and sulfo-NHS. The supernatant was removed and the pellet was resuspended in demineralized water and dialyzed (MWCO: 12000 g·mol−1) in order to remove residues of non-reacted COOH-terminated LTP.

Powder X-ray diffraction (XRD)

Powder X-ray diffraction patterns were collected in the range of 5°–60° with a Philips X'pert X-ray powder diffractometer using Cu Kα (λ = 1.54178 ´˚A) radiation.

Dynamic light scattering (DLS)

The average size and size distribution of the nanocarriers were measured at 20°C by DLS using a Brookhaven instruments Zetapals.

Fourier transform infrared spectroscopy (FT-IR)

The FT-IR spectroscopic measurements were carried out using a PerkinElmer GX spectrophotometer. The spectra were recorded in the range 400–4000 cm^{-1} in KBr media.

Transmission electron microscopy (TEM)

Transmission electronic microscope (TEM) images were collected using a JEOL JEM 2100 microscope operated at 200 kV. The morphology of the nanocapsules were studied by placing a dilute sample on the TEM grids (lacey carbon formvar coated Cu grids (300 mesh)) using transmission electron microscopy. No additional staining was used.

Up conversion fluorescence measurement

UC fluorescence spectra of dried LTP conjugated $NaYF_4$:Yb,Gd UC-NPs were measured by LS-55 fluorescence spectrophotometer (Make: PerkinElmer Co., USA) with an external 980 nm laser (as a light source) instead of the internal excitation source. The maximum power of the laser was 120 mW.

Tissue Culture Experiments

RAW cells were seeded on Cover slips (22 mm X 22 mm, 170 ± 5 µm square Cover glasses) placed in six well plates in DMEM culture medium containing (10% FBS and 1% Penicillin Streptomycin) for 24 hours at 37°C, 5% CO_2. After 24 hours when 70% confluency was achieved the cells were washed with DMEM culture medium. Cells were then washed thrice with culture medium. After that cells were washed again with phosphate buffer saline (2 times). After carrying out the live cell uptake of the UC-NPs of diverse concentrations (20 ug to 200 ug) for 12 hours, the cells were washed with DMEM media, then the cells were fixed with 4% PFA for 15 minutes and then washed thrice with PBS and two times and then the coverslips were mounted using mounting medium (Vectashield h-1000). The coverslips were then sealed using nail varnish before imaging.

Cytotoxicity of LTP conjugated $NaYF_4$: Yb,Gd UC-NPs by MTT assay

Cytotoxicity of LTP conjugated $NaYF_4$:Yb,Gd UC-NPs on human RAW cells was determined by conventional MTT assay. The cells were seeded at a density of 10^5/well plated in 96-well plates. Cells were typically grown to 60–70% confluence, rinsed in phosphate-buffered saline (PBS), and placed into serum-free medium overnight prior to treatments. After overnight incubation, the cells were treated with LTP conjugated $NaYF_4$:Yb,Gd UC-NPs at the concentration of 10, 20, 50, 75 and 100 µg/ml. After 24 h, the medium was removed, and 50 µL of fresh medium was added along with 10 µL of MTT (5 mg/mL in PBS) and incubation was prolonged for 4 h at 37 °C. After that MTT solution was slowly removed, and the purple crystals were solubilized in 1.5 mL of DMSO. The absorbance was measured at test wavelength of 550 nm in Elisa Plate Reader. Cell viability was quantified by MTT assay where the viable cells were determined by the reduction of the yellow MTT into purple formazan product by mitochondrial dehydrogenase present in metabolically active cells. The experiment was done in triplicate.

Characterization of UC-NPs: XRD and EDS

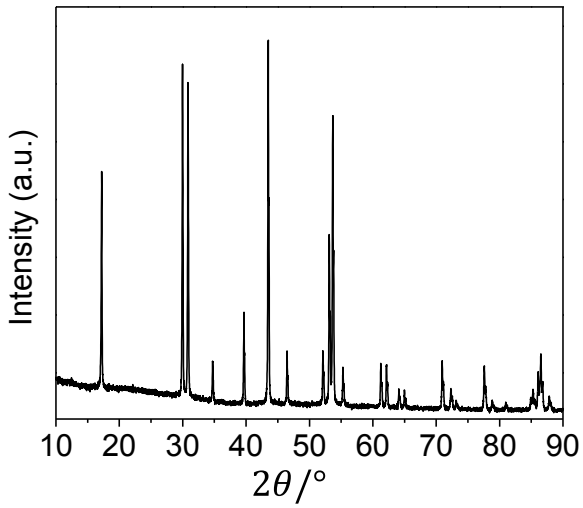

Fig. S1. Powder XRD patterns of as-prepared β-$NaYF_4$:Yb,Gd NPs. The overall peak profile demonstrates an excellent crystallinity of the UCNPs.

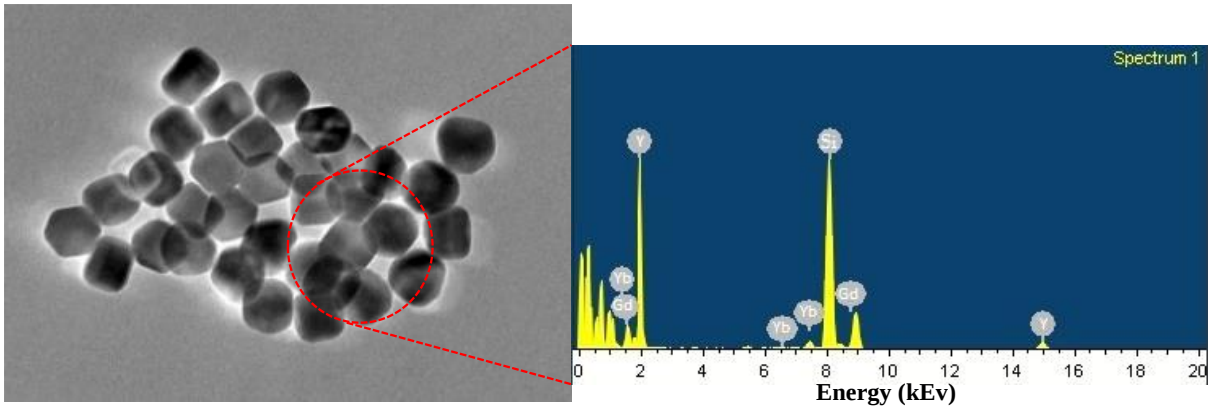

Fig. S2. Energy-dispersive X-ray analysis (EDX) patterns of β- $NaYF_4$:Yb,Gd NPs showing elemental composition.

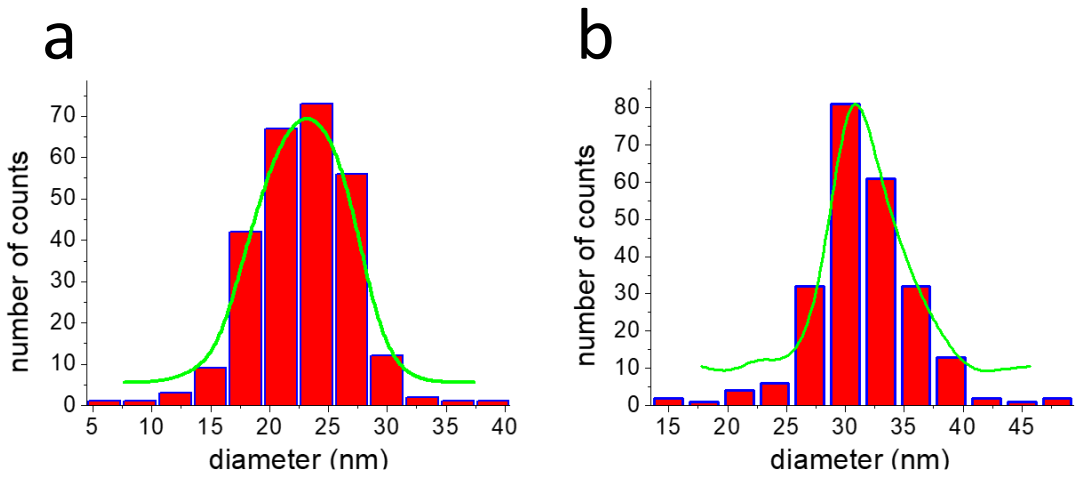

Fig. S3. The size distribution of the UC-NPs obtained from TEM images for $NaYF_4$:Yb,Gd NPs (a) and LTP- conjugated β-$NaYF_4$:Yb,Gd NPs (b). Image J software was employed for quantitative analysis. Data were obtained by measuring more than 200 UC-NPs for both the case.

MICROSCOPY EXPERIMENTS:

Deconvolution Wide field Microscopy

To remove the out of focus blur from stacks of acquired Z-Stack images, deconvolution of raw widefield images obtained using the OMX-SIM (Conventional Wide Field Microscopy mode) was carried out using the Soft Worx software.

Cellular uptake of UC-NPs (Single colour Imaging)

Single colour widefield experiments of UC-NPs were performed for diverse concentrations from 20 ug to 200 ug. The uptake was carried out over 12 hours. They were excited at 488 nm and emission was collected at 500 to 550 nm. The widefield microscopy conditions maintained were; Z stack thickness (Sections 40 to 80), section spacing (0.250 to 0.500), and thickness of the sample (8 to 11). Exposure times were between 10 to 30 and the %T was in the range of 30 to 60.

Two Photon Microscopy

Two photon microscopy experiments for UC-NPs were carried out using the Zeiss LSM 510Meta confocal microscope. Live cell uptake was carried out over 12 hours. The UC-NPs were excited at 975 nm and emission collected between 500 to 550 nm. Two photon images (Single colour) were acquired for UC-NPs (20 ug, 50 ug, 100 ug) cellular uptake on to RAW cells. The data processing was again carried out using FIJI and LSM software.

Colocalization Experiments (Multi colour imaging)

Nanoparticles (NP's) and Lyso and Mito Tracker Deep Red and Hoechst

Colocalization experiments were performed using Lyso Tracker Deep Red or Mito Tracker Deep Red in the respective experiments. For these colocalization experiments, RAW cells were incubated with UC-NPs (50 ug) for 12 hours and then the cells were incubated with LTDR or MTDR (750 nM) for 30 minutes. The cells were washed regularly three times with DMEM culture media and PBS (two to three times), fixed with 4% PFA and mounted. The UC-NPs were excited at 488 nm and the emission was collected in the FITC Channel (500 to 550 nm) and the Lyso or Mito Tracker Deep Red or Hoechst was excited at 644 nm and emission was collected in the Alexa Fluor 647 Channel (> 650 nm).

SIM Microscopy - Single colour, Colocalisation and Dual colour experiments

Structured illumination microscopy (SIM) carried out by using the Delta Vision OMX-SIM. The Z stacks acquired during the imaging were post-processed by using the reconstruction option of Soft Worx. For single colour experiments, the UC-NPs were excited at 488 nm and the emission was collected at FITC Channel (500 to 550 nm). The Structured Illumination (SI) experimental condition employed for running the SI experiment for single colour Experiments were mainly dependent on the thickness of the Z stack (Sections 80 to 100), section spacing (0.125 to 0.250), thickness of the sample (8 to 10). The dual colour experiments were performed in combination with Hoechst.

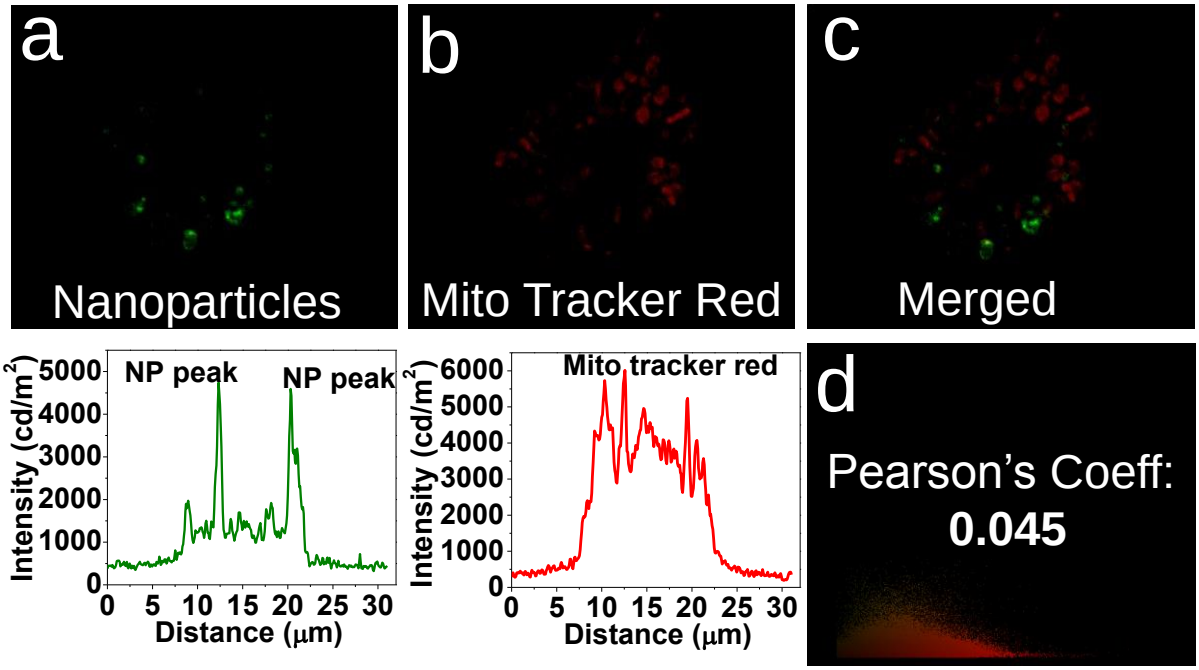

Fig. S4. Colocalization experiments of Intracellular localization of UC-NPs using Mito Traker probes: Wide field microscopy images of in cellulo emission of UC-NPs (Panel a) with intensity along traced line shown underneath. Emission from Mito Traker Deep Red (Panel b) and intensity along the same line shown below. The overlap of the intensity is shown in Panel c. Panel c shows no overlap of the green and red fluorescence, indicates that the UC-NPs are not localised over mitochondria. Panel d shows the Pearson co-efficient = 0.045, also supports that.

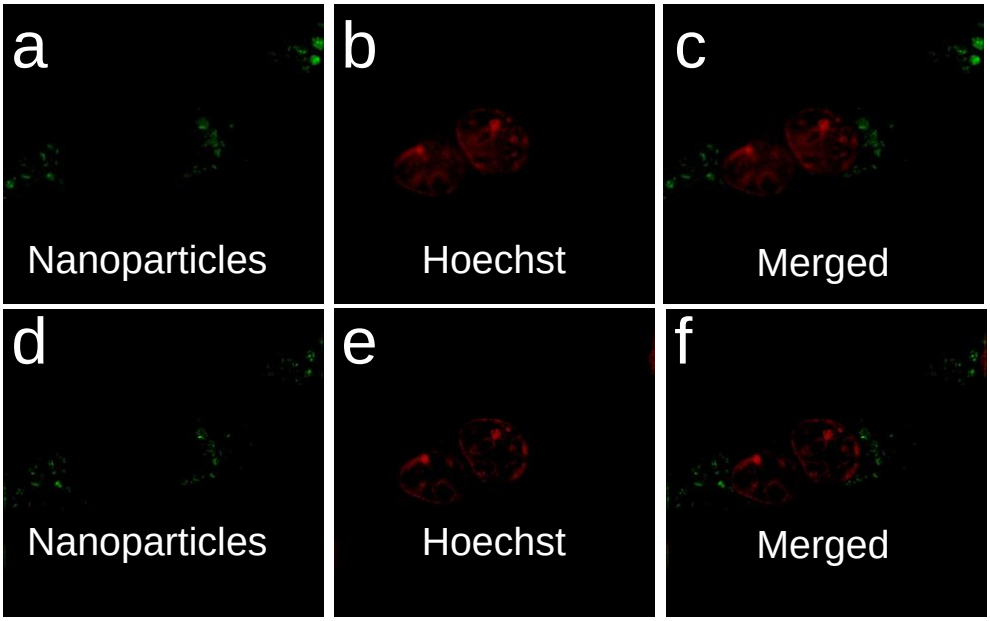

Fig. S5. Colocalization experiments of Intracellular localization of UC-NPs using Hoechst: Wide field microscopy images of in cellulo emission of UC-NPs (Panel a); nuclei tracker Hoehst (Panel b) and and merge (panel c). The panel d, e and f are similar with a, b and c respectively of Structured illumination microscopy (SIM) images.

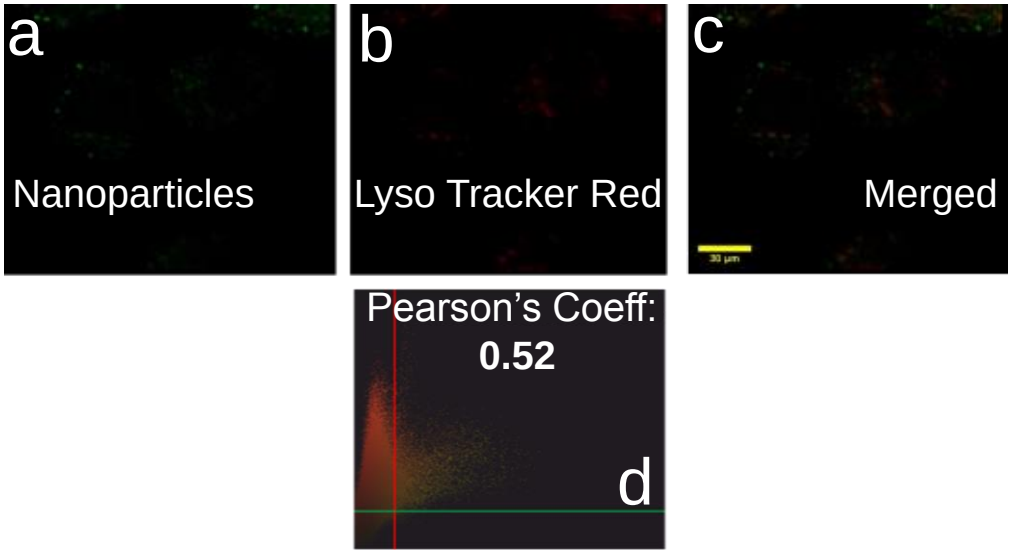

Fig. S6. Colocalization experiments of Intracellular localization of bare UC-NPs using Lyso Traker probes: Wide field microscopy images of in cellulo emission of bare UC-NPs (Panel a) with intensity along traced line shown underneath. Emission from Lyso Traker Deep Red (Panel b). The overlap of the intensity is shown in Panel c. Panel c shows not complete overlap of the green and red fluorescence, indicates that the bare UC-NPs are not localised over lysosomes. Panel d shows the Pearson co-efficient = 0.052, also supports that.

MTT ASSAY:

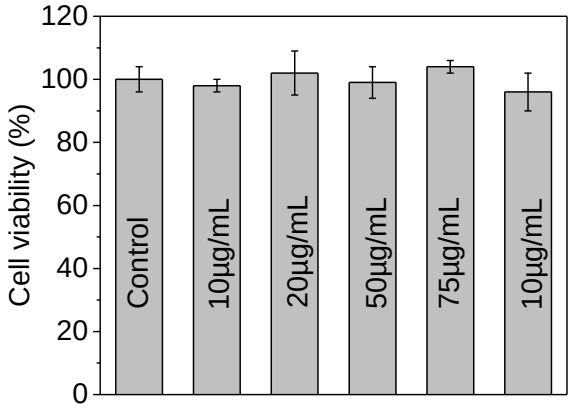

Fig. S7. Cell viability of RAW cells: Cells after 24 h exposure to a concentration range of LTP-conjugated β-$NaYF_4$:Yb,Gd NPs, determined using the MTT assay. Data represent mean ± standard deviation of three replicates. The cell viability levels remained stable as compared to a control group, no decrease below 95 % was observed after exposure (24 h) to different concentrations of LTP-conjugated β-$NaYF_4$:Yb,Gd NPs. The concentrations used are notably higher than the range used in medical applications such as drug delivery or bio imaging, encouraging that these type of nano-carrier composition are usable for biomedical applications. This confirms that the synthesized LTP- conjugated β-$NaYF_4$:Yb,Gd NPs are biocompatible.