# Electronic Supplementary information

## Mitochondriotropic Lanthanide Nanorods: Implications for Multimodal Imaging

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### Synthesis of the nanorods.

In a typical procedure for the synthesis of lanthanide-doped $NaYF_4$ nanorods, a solution of NaOH (0.5 g) in deionized water (2.0 ml) of was mixed with 6 ml of ethanol and 6 ml of oleic acid under stirring. To the resulting mixture were selectively added 2 ml of $LnNO_3$ (0.2 M, Ln = Y, Yb and Gd) and 1.5 ml of $NH_4F$ (2 M). Then the solution was transferred into a 30 ml of Teflon-lined autoclave and heated at 200 $^oC$ for 3 h. The obtained nanorods were collected by centrifugation, washed with ethanol several times, and finally re-dispersed in cyclohexane.

### Surface Modification of the nanorods.

In a 100 mL flask, 100 mg of β-$NaYF_4$:Yb,Gd,Eu UCNRs was dispersed in 100 mL of ethanol via ultrasonic suspension. Thereafter, 10.0 mL of ammonia solution and 30 mL of water were added into the flask under vigorous stirring at 50°C. To this reaction mixture, a solution of 100μL of tetraethyl orthosilicate in 30 mL of isopropanol was slowly added over a period of 1 h. The resultant mixture was stirred for another 5 h. The solution containing 0.5 mL of (3-aminopropyl)triethoxy silane and 30 mL isopropanol was then added slowly into the subsequent reaction mixture. After 1h, the resultant precipitates were collected by

centrifugation and washed with ethanol several times and dried at 60°C overnight to get amino-modified β-$NaYF_4$: Yb,Gd,Eu UCNRs.

The free acid group of triphenyl phosphine derivative was conjugated to the amino group of the β-$NaYF_4$: Yb, Gd,Eu UCNRs using standard carbodiimide chemistry. To activate the free carboxyl groups of TPP derivative,1.0 g triphenyl phosphine derivative (having free COOH group) was mixed with 100 mg (0.85 mmol) of EDC and 50 mg (0.25 mmol) of sulfo-NHS. After stirring for 30 min, $NH_2$-terminated $NaYF_4$:Yb,Gd,Eu UCNRs were added to this reaction mixture and the stirring was continued under ambient conditions for another 10 hour. The resultant reaction mass was then centrifuged at 10000 rpm for 30 min (KUBOTA) to eliminate the residuals of EDC and sulfo-NHS. The supernatant was removed and the pellet was redispersed in demineralized water and dialyzed (MWCO: 10000 g·mol−1) in order to remove residues of non-reacted triphenyl phosphine derivative.

**Powder X-ray diffraction (XRD)**

Powder X-ray diffraction patterns were collected in the range of 5°–90° by using 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 nanorods were measured at 25°C by DLS (Brookhaven instruments Zetapals).

**Fourier transform infrared spectroscopy (FT-IR)**

The FT-IR spectroscopic data were obtained by using a PerkinElmer GX spectrophotometer. The spectra were recorded in the range 400–4000 $cm^{-1}$ in KB rpellate.

**Transmission electron microscopy (TEM)**

TEM images were recorded using a JEOL JEM 2100 microscope operated at 200 kV.

The morphology of the nanorods were collected by putting a dilute solution of the sample on TEM grid (lacey carbon formvar coated Cu grids (300 mesh)) by using transmission electron microscopy. No additional staining was used to perform the experiments.

**Up conversion fluorescence measurement**

The Up conversion fluorescence spectra of dried TTP conjugated β-$NaYF_4$:Yb,Gd,Eu UCNRs were measured by LS-55 fluorescence spectrophotometer (Make: PerkinElmer Co., USA). The maximum power of the laser was 120 mW (980 nm).

**Cell Culture Experiments**

The 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 around 70% confluency was achieved and the cells were washed with DMEM culture medium. Cells were then washed thrice with culture medium. Afterward the cells were washed again with phosphate buffer saline (4 times). After carrying out the live cell uptake of the UCNRs of diverse concentrations (50 ug to 100 ug) for 6 hours, the cells were washed with DMEM media, fixed with 4% PFA for 15 minutes and then washed with PBS buffet for two times and then the coverslips were mounted using mounting medium (Vectashield h-1000). The coverslips were sealed using nail varnish before imaging.

**Cytotoxicity of UCNRs by MTT assay**

The cytotoxicity of UCNRs on human RAW cells was determined by conventional MTT assay. The cells were grown at a density of $10^5$ /well in 96-well plates. When the cells grew around 70%, they were rinsed with phosphate-buffered saline (PBS), and placed into serum-free medium for 12 hours prior to treatments with UCNRs. After 12 hours incubation, the cells were treated with UCNRs at the concentration of 20, 40, 60, 80 and 100 µg/ml. After 24 h, the medium was removed, and 100 µL of fresh medium was added along with 10 µL of MTT (5 mg/mL in PBS) and incubated for another 4 h at 37 °C. Thereafter, MTT solution was slowly removed, and the purple crystals were solubilized into 2.0 mL of DMSO. The absorbance was measured at test wavelength of 550 nm in Elisa Plate Reader. The experiment was done in triplicate.

**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 UCNRs were performed for diverse concentrations from 20 μg to 200 μg. The cell uptake was carried out over 6 hours. They were excited at 488 nm and emission was collected at 600 to 650 nm. 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 UCNRs were carried out using the Zeiss LSM 510 Meta confocal microscope. The UCNRs were excited at 980 nm and emission collected between 600 to 650 nm. Two photon images (Single colour) were acquired to confirm the uptake of UCNRs (20 μg, 40 μg/mL, 100 μg/mL) by RAW cells. The data processing was again carried out using FIJI and LSM software.

**Colocalization Experiments (Multi colour imaging)**

Colocalization experiments were performed using Lyso Tracker Deep Green or Mito Tracker Deep Green in the respective experiments. For these colocalization experiments, RAW cells were incubated with UCNRs (50 μg/mL) for 12 hours. Thereafter, cells were incubated with LTDG or MTDG (750 nM) for 60 minutes. Subsequently, the cells were washed with DMEM culture media (three times) and PBS (two to three times), fixed with 4% PFA and mounted. The UCNRs were excited at 488 nm and the emission was collected in the FITC Channel (600 to 650 nm) and the Lyso or Mito Tracker Green was excited at 488 nm and emission was collected in the Alexa Fluor 550 Channel.

**SIM Microscopy - Single colour, Colocalization and Dual colour experiments**

Structured illumination microscopy (SIM) was 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 UCNRs were excited at 488 nm and the emission was collected at FITC Channel (600 to 650 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).

**MRI Experiments**

All *in vitro* and *in vivo* MRI studies were conducted using a Bruker 9.4 T/30-cm bore small animal MRI system. The procedures for animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, as adopted by the National Institutes of Health, and approved by IACUC, UNC. For relaxometry studies, a quadrature transceiver volume coil with 35 mm inner diameter was used as the imaging transceiver. The samples to be tested were prepared in syringe phantoms at 7 different $Gd^{3+}$ concentrations: 0 (saline), 2.655, 5.31, 7.965, 10.62, 13.275 and 26.55 mM. The amount of $Gd^{3+}$ concentration present per unit volume of the nanoparticle suspension was determined by inductively coupled plasma mass spectrometry (ICP-MS). A custom home-made sample holder was used to secure the phantoms in the volume coil. We used a standard spin-echo sequence with various repetition time (TR) and echo time (TE) featuring an acquisition matrix of 256*256, field of view (FOV): 40 mm2, section thickness: 1 mm, 1 average, variable TRs: 5000, 3000, 1500, 800, 400 and 200 ms and variable TEs: 11, 33, 55, 77 and 99 ms.

*In vitro* relaxometric analyses were performed using a custom-written MATLAB (Math-Works, Natick, MA) script. Signal intensity corresponding to each Fe concentration at different TR and TE was extracted by placing identical size of region of interests (ROIs) on each phantom image. Relaxation curves for $T_1$ calculation was obtained by plotting signal intensities (SI) against variable TRs corresponding to the shortest TE. The $T_1$ recovery was plotted using a one phase exponential association fitting algorithm: SI = a*(1-exp (-b*TR)) + c where a, b, c denotes the fitting parameters. Similarly, the $T_2$ values were obtained by plotting signal intensities (SI) against variable TEs corresponding to the longest TR. The $T_2$ decay was plotted using a one phase exponential decay algorithm: S1 = a*exp(-b*TE). The relaxivity values ($R_1$= d(1/$T_1$)/d [Gd] and $R_2$ = Delta $R_2$*/d [Gd]) were determined by linear fitting to the relaxation rates (1/$T_1$ and 1/$T_2$) vs concentration [Gd].

High resolution microangiography was performed in a C57B6 wild-type mouse anesthetized with isoflurane. The animal was sent inside the magnet affixed on the commercial mouse holder. A 72 mm quad-transmit only volume coil and a quad-receive only mouse brain coil was used for imaging. To determine if UCNRs can serve as an effective blood pool agent for high resolution micro-MRA, 3D FLASH images were acquired before and after injection of the contrast agent with the following parameters: acquisition matrix size: 128×128×36; FOV: 25.6 mm× 25.6 mm× 7.2 mm; TR = 50 ms; TE = 3.067 ms; spatial resolution: 200μm3. Three

repetition scans were corrected for spatial drift and averaged to improve SNR. The final image was processed and displayed using Amira (FE1, V5.3.3 or Avizo software, TGS, San Diego, CA, USA) The post-contrast image underwent Euclidean affine registration to the pre-contrast image using the multi-planar viewer of Amira. The brain and prominent surface vessels were masked in the segmentation editor of Amira and arithmetically isolated. UCNRs-enhanced three-dimensional cerebroangiography was visualized using the volume rendering tools in Amira by overlapping the pre-contrast (in white) and post-contrast (in orange) images.

## Powder XRD

The phase and equivalent lattice parameter determination were performed by using X-ray powder diffraction analysis (Philips X'pert MPD System) with CuKα1 radiation, 1.5406 Å, operating at 40 kV and 30 mA, in the 2θ range 10° to 90° with a 0.01° step size in the reflection scanning mode. Here, the recorded XRD patterns (Fig. S1) indicate that ($Yb^{3+}$ /$Eu^{3+}$ /$Gd^{3+}$)-doped $NaYF_4$ nanorods crystallized in hexagonal β-phase (space group: P63/m). [ICDD (2016) card No. 00–16–0334].

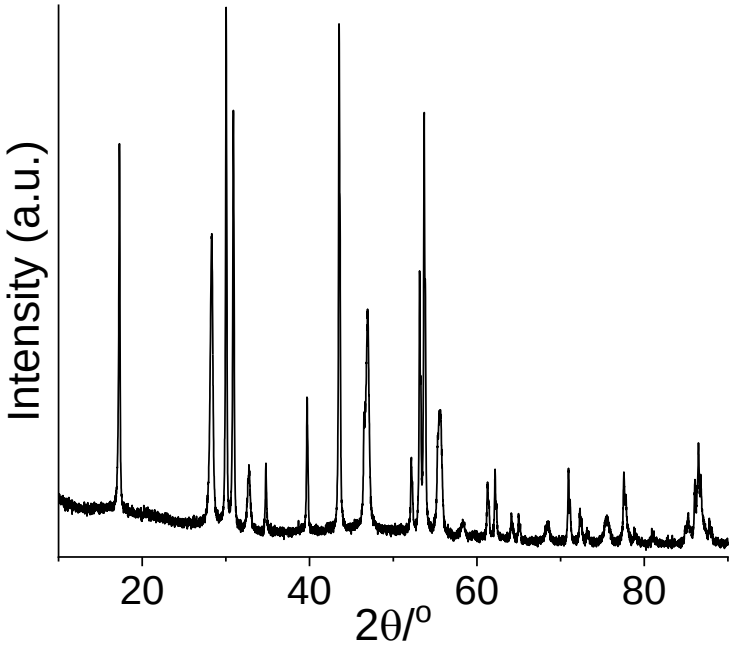


**Fig. S1** Powder XRD patterns of as-prepared β-$NaYF_4$:Yb,Gd,Eu UCNRs. The overall peak profile demonstrates an excellent crystallinity of the UCNRs.

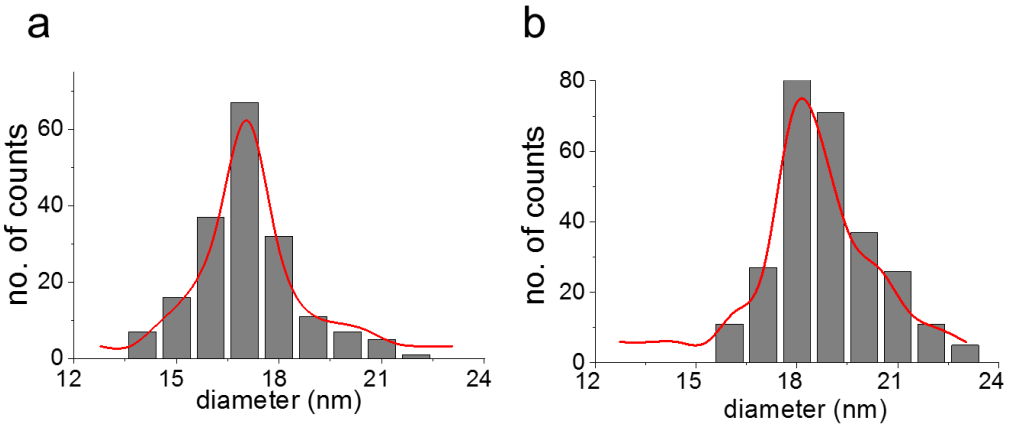


**Fig. S2** The size distribution of the UCNRs obtained from TEM images for β-$NaYF_4$:Yb,Gd,Eu UCNRs (a) and TTP- conjugated β-$NaYF_4$:Yb,Gd,Eu UCNRs (b). Image J software was employed for quantitative analysis. Data were obtained by measuring more than 300 UCNRs for both the case.

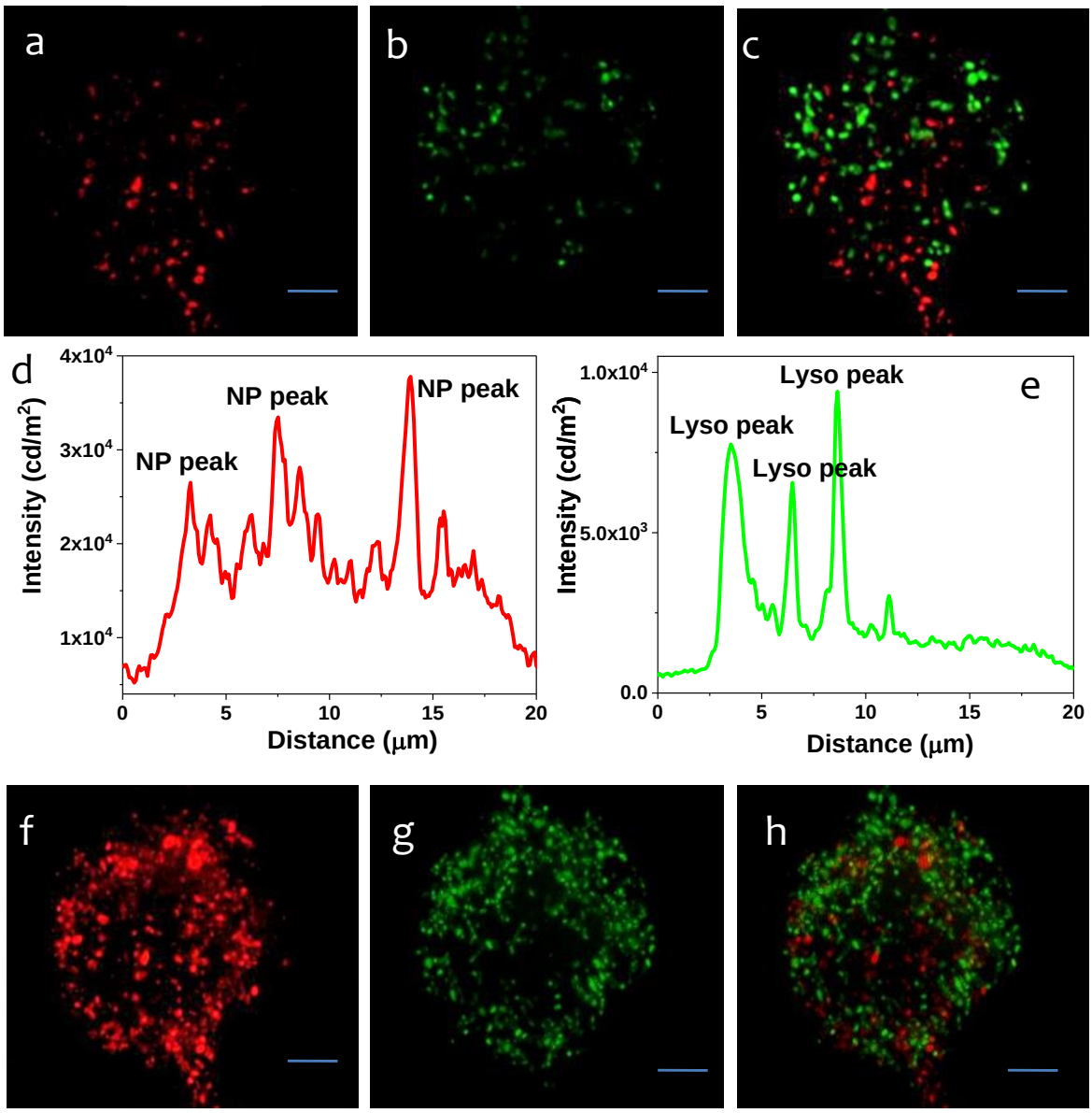


**Fig. S3** Colocalization experiments (SIM Microscopy) of intracellular localization of UCNRs using lysotraker probes: Wide field microscopy images of in cellulo-emission of UCNRs (panel a) with intensity along traced line shown underneath. Emission from lysotraker green (panel b) and intensity along the same line shown below. The overlap of the intensity is shown in panel c. panel c shows there is no overlap of the green and red fluorescence, indicating the UC-NRs are not localised over lysosome. Panel f, g and h are 3d view.

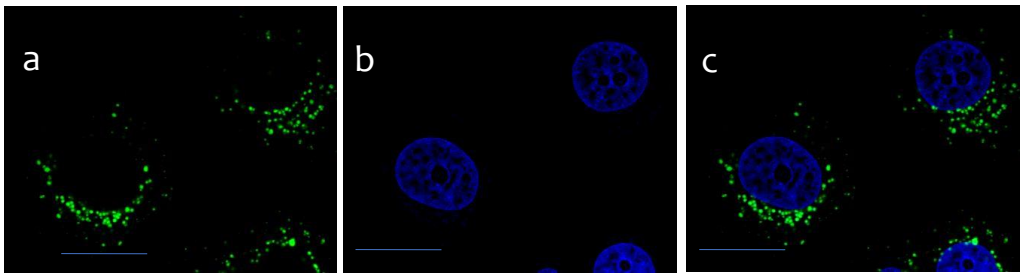


**Fig. S4** Colocalization experiments of intracellular localization of UCNRs using DAPI: Confocal Laser Scanning Microscopy images of cellular emission of UCNRs (panel a). Emission from DAPI (panel b). Panel c shows the overlap of the green and blue fluorescence, indicating no localization of UCNRs at mitochondria. Scale bar 10 µm.

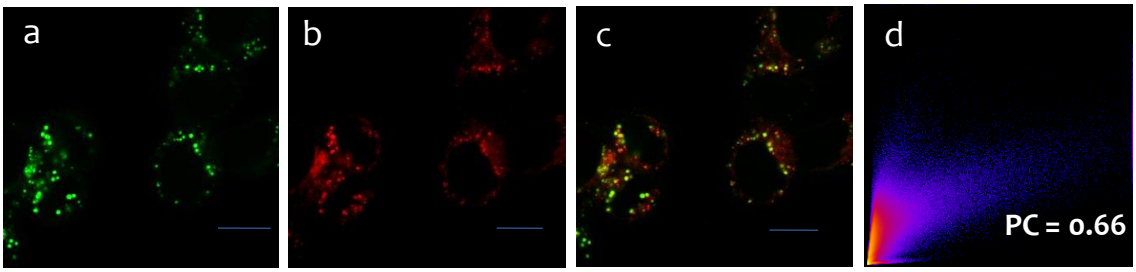


**Fig. S5** Colocalization experiments of intracellular localization of bare UCNRs (without triphenyl phosphine functionalized) using Lyso Tracker probes: Confocal Laser Scanning Microscopy images of cellular emission of bare UCNRs (panel a) and emission from Lyso Tracker Green (Panel b). The overlap of the of green and red fluorescence is shown in Panel c. Panel c shows significant overlap of the green and red fluorescence indicates that the bare UCNRs are localized over lysosomes. Panel d shows the Pearson coefficient = 0.66, also supports that. Scale bar 10 µm.

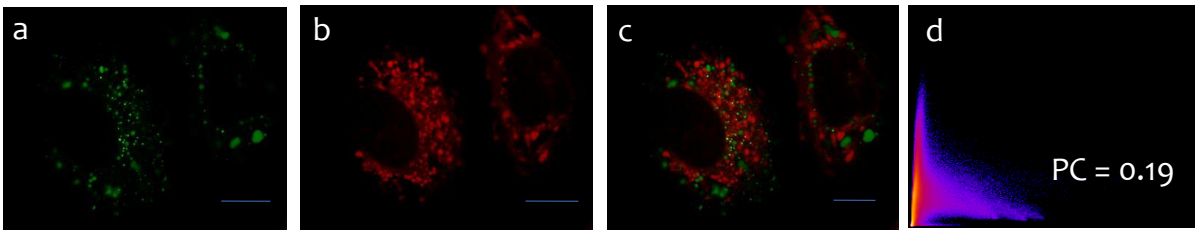


**Fig. S6** Colocalization experiments of intracellular localization of bare UCNRs (without triphenyl phosphine functionalized) using MitoTracker probe: Confocal Laser Scanning Microscopy images of cellular emission of bare UCNRs (panel a) and emission from MitoTracker Green (Panel b). The overlap of the of green and red fluorescence is shown in Panel c. Panel c shows no overlap of the green and red fluorescence indicates that the bare UCNRs are not localized over mitochondria. Panel d shows the Pearson coefficient = 0.19, also supports that. Scale bar 10 µm.

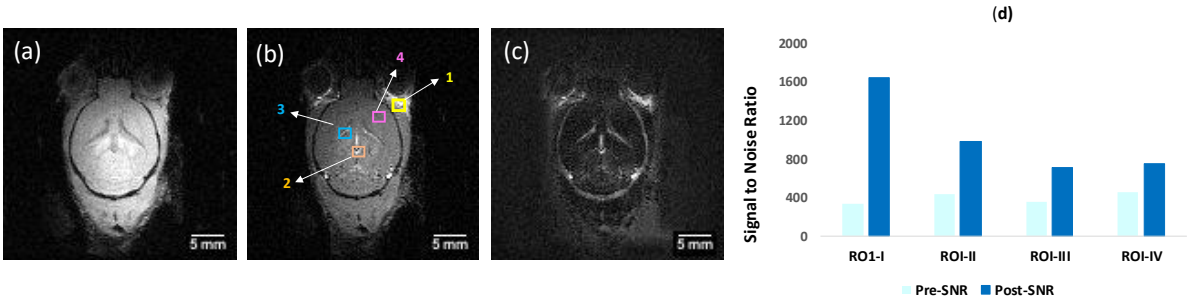


**Fig. S7** FLASH-3D MR images of a representative mouse brain slice (axial orientation) captured at 200 $\mu m^3$ spatial resolution a) before and b) after injection of UCNRs. c) The pre-contrast image was subtracted from the post-contrast image to determine the contrast enhancement. d) A few representative ROIs were picked up as shown in b) and the SNR was determined. Our results showed that UCNRs prepared in the study offers robust $T_1$ contrast enhancement in the ROIs analysed.

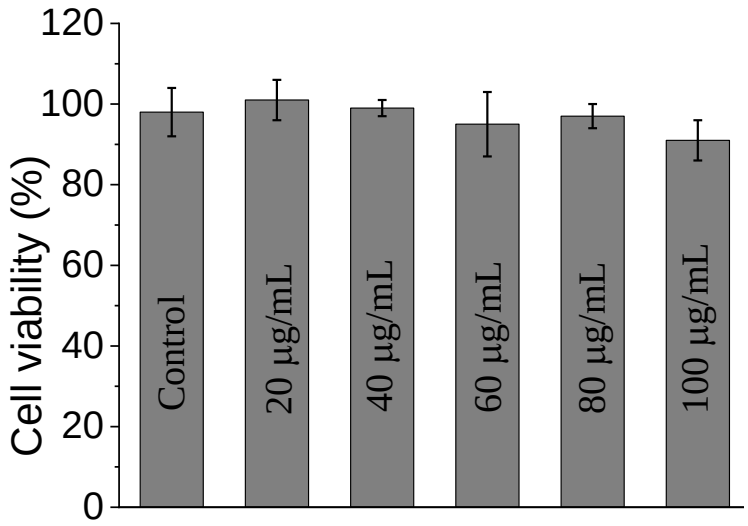


**Fig. S8** Cell viability experiment on RAW cells: Cells after 24 h exposure to a concentration range of TTP- conjugated β-$NaYF_4$:Yb,Gd,Eu UCNRs, determined using the MTT assay. Data represent mean ± standard deviation of three replicates. The cell viability levels remained stable as compared to a control group, no decrease below 97 % was observed after exposure (24 h) to different concentrations of TTP- conjugated β-$NaYF_4$:Yb,Gd,Eu UCNRs.

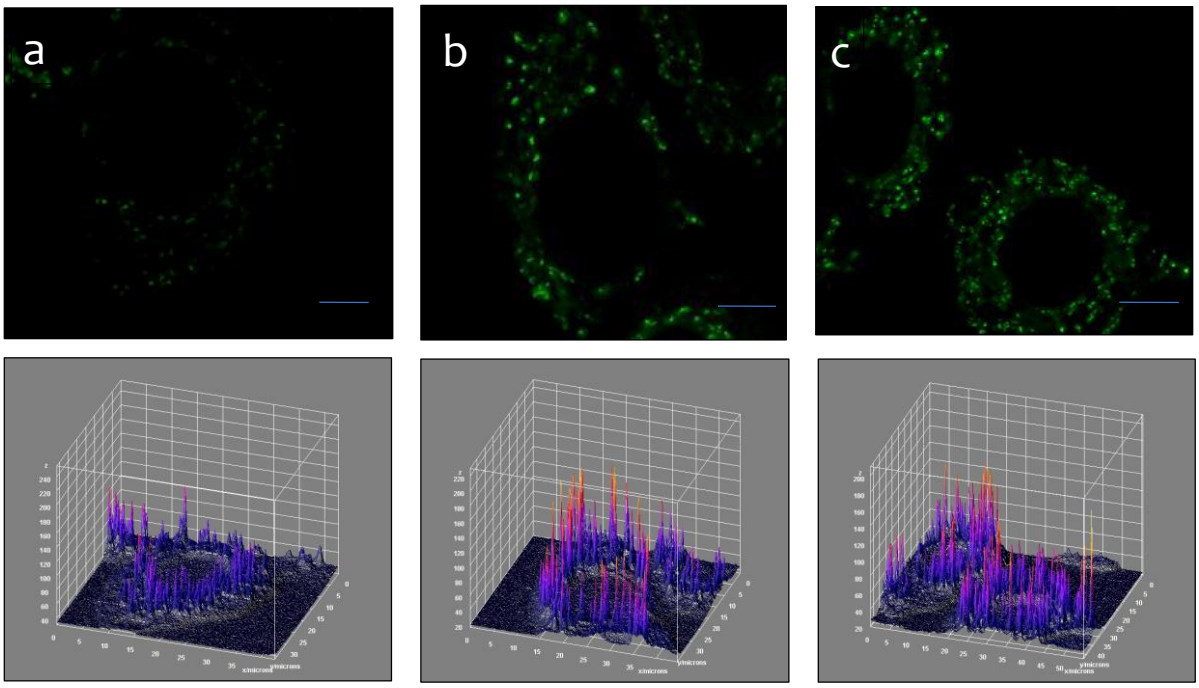


**Fig. S9** Time dependent uptake of UCNRs in RAW cells (a) after 2 hours, (b) 4 hours and (c) 6 hours. Intensity maps are shown underneath. Scale bar 10 µM.

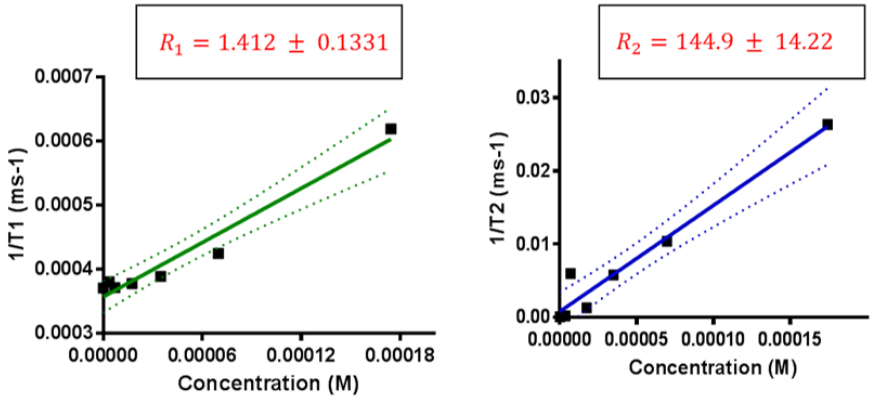


**Fig. S10** Relaxometric properties of Feraheme at 9.4 T.