Supporting Information

Modularly Assembled Upconversion Nanoparticles for Orthogonally Controlled Cell Imaging and Drug Delivery

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Materials

Cyclohexane, 1-octadecene (90%), oleic acid (OA) (90 %), $YCl_3 \cdot 6H_2O$ (99.9%), $ErCl_3 \cdot 6H_2O$ (99.9%), $YbCl_3 \cdot 6H_2O$ (99.9%),$GdCl_3 \cdot 6H_2O$ (99.9%), NaOH (98+%), NH_4F (98+%), Sodium dodecyl sulfate (SDS), Cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS) (98%), (3-aminopropyl) triethoxysilane (APTES) (99%), Zinc phthalocyanine, 4-(Phenylazo)benzoyl chloride (97%), NH_4NO_3 (98+%), dimethyl sulfoxide (99%), triethylamine (99.5%) and paclitaxel (97%) were purchased from Sigma-Aldrich and used as received without further purification. The CellMask™ green plasma membrane stain and Hoechst 33342 were purchased from Thermo Fisher Scientific.

Physical Characterization

Luminescence spectra of UCNPs were recorded on a Hitachi F-500 fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) equipped with an NIR continuous wave (CW) laser with an emission at 980/808 nm (Photonitech (Asia) Pte. Ltd, Singapore). Laser power (2 W) is kept same throughout the measurement. The DLS size distribution statistics were measured using a Malvern Zetasizer Nano Series (Malvern Instruments Ltd, Worcestershire, UK). Transmission electron microscopy (TEM) images of UCNPs were recorded on a JEOL 2010F transmission electron microscope (Jeol Ltd, Tokyo, Japan) operating at an acceleration voltage of 200 kV. Luminescence decay curves were measured on our dynamic measurement platform which was based on an Acton SpectraPro 2300i spectrophotometer (Princeton Instruments). A 2 W NIR CW laser at 980 nm, pulse modulated by an SFG-2120 synthesized function generator (GW INSTEK), was employed. The scattered excitation light and the emission light profile were monitored

using a photodetector (DET10A, Thorlabs) and a photomultiplier tube (Hamamatsu R928), respectively.

Methods

Synthesis of β-phase $NaREF_4$ (RE=Y, Gd, Yb, Tm, Er) Core Nanocrystals

$NaREF_4$ nanocrystals were synthesized by using a standard solvent thermal synthesis method. Typically, a total amount of 1 mmol $RECl_3$ aqueous solution with stoichiometric ratio was added in a 100 mL flask. After water was fully evaporated, 15 mL 1-octadecene and 6 mL oleic acid were added and the mixture was heated at 156 ^{0}C for 10 mins to form RE-oleate complexes. The resulting solution was then cooled to room temperature and mixed with a methanol solution (5 mL) containing NH_4F (4 mmol) and NaOH (2.5 mmol). After that, the temperature was raised to 120 ^{0}C for 10 mins for complete methanol removal. Then the solution was degassed for 15 mins to remove residual methanol and oxygen. Subsequently, the resulting solution was raised to 300 ^{0}C for 1 hour under argon environment. The products were precipitated down with acetone, under centrifugation at 8000 rpm for 10 mins, washed with acetone, and finally dispersed in 10 mL cyclohexane for further use.

Synthesis of $NaREF_4$@$NaREF_4$ and $NaREF_4$@$NaREF_4$@$NaREF_4$ (RE=Y, Gd, Yb, Tm, Er, Nd) Core-Shell and Core-Shell-Shell Nanocrystals

Core-shell and core-shell-shell UCNPs were prepared through epitaxial growth. The as-prepared core $NaREF_4$ nanocrystals were used as seeds for inert-shell growth. In a typical process, based on the core-shell ratio, certain amounts of an aqueous solution of $RECl_3$ were added into a 100 mL flask. After the water was fully evaporated, 15 mL 1-octadecene and 6 mL oleic acid was added. The mixture was kept at 156 ^{0}C for 10 mins to form RE-oleate complexes. Upon cooling of the RE-oleate precursors to room temperature, the as-prepared core nanoparticles dispersed in 10 mL cyclohexane were

added, and the resulting mixture was then heated at 120 ^{0}C for 20 mins to evaporate the cyclohexane. Subsequently, the solution was cooled to room temperature, followed by the addition of a methanol solution containing NH_4F (0.8 M) and NaOH (0.5 M), the overall amount of methanol is based on the $RECl_3$ precursor added, 5 mL methanol solution is required for every 1 mmol $RECl_3$ precursor added. The resulting mixture was vigorously stirred and then heated at 120 ^{0}C for 10 mins. After that, the reaction was degassed for 10 mins to evaporate the residual methanol and oxygen in the solution. Finally, the temperature was raised to 300 ^{0}C and kept under argon atmosphere for 1.5 hours. The resultant nanoparticles were precipitated down after the addition of acetone under 8000 rpm centrifugation for 10 mins, washed with acetone, dispersed in 10 mL cyclohexane for further usage.

Synthesis of UCNPs-C

Here we have used a microemulsion based method to make two types of UCNPs to assemble into a UCNPs cluster (UCNPs-C) and the procedure is as follows. The oil phase (cyclohexane, 1 mL) containing individual, well-dispersed oleic acid (OA)-capped UCNPs (total concentration is 5 mg/mL) was mixed together, then the mixed UCNPs solution was added into 10 mL of the aqueous phase containing surfactants (sodium dodecyl sulfate (SDS)). A stable O/W emulsion system was obtained under vigorous stirring and sonication, by which the well-dispersed UCNPs (one UNCPS or mixed UCNPs) were confined in the emulsion (cyclohexane) droplets (oil phase) that were stabilized by the surfactants present in the aqueous phase. Subsequently, the low-boiling cyclohexane was evaporated from the emulsion droplets by heating the solution at 70 °C for 4 hours. During the removal of cyclohexane, the droplets shrink and the two types of UCNPs within the emulsion droplets (cyclohexane) get concentrated and

pack closely with each other through hydrophobic Van der Waals interactions of OA on the surface of UCNPs, thereby assembling to form UCNPs clusters.

Synthesis of Mesoporous Silica-Coated UCNPs-C

10 μL APTES was added to 10 mL UCNPs-C solution (the concentration of UCNPs is 0.5 mg/mL), and the solution was stirred for 30 mins to let the UCNPs-C adsorb positively-charged APTES. After that, 10 mL water, containing 35 mg CTAB and 2 mL ethanol were added to the above solution. After stirring for 30 mins, 300 μL 33% ammonium hydroxide solution was added into the mixture. Then 75 μL TEOS was added dropwise into the above solution and kept string for 24 hours. After washing twice with acetone and methanol mixture (volume ratio of 1:1), the UCNPs-C with silica coating dispersed in 10 mL ethanol and mixed with 10 mL ethanol containing 300 mg NH_4NO_3. The solution was refluxed at 60 °C for 2 hours to remove the CTAB to get mesoporous silica coating. The obtained samples were then washed by acetone and ethanol mixture twice and dispersed in 10 mL ethanol for further use.

Azobenzene Modification of OP-UCNPs-C

The OP-UCNPs-C were modified with N-(3-triethoxysilyl)propyl-4-phenylazobenzamide (azobenzene) to obtain OP-UCNPs-C@azo. First, the azobenzene solution was prepared by adding 3-Aminopropyltriethoxysilane (1.22 g, 5.5 mmol) and triethylamine (0.556 g, 5.5 mmol) into 50 mL the ethanol solution of 4-phenylazobenzoyl chloride (1.22 g, 5 mmol). The solution was stirred for 12 hours under argon atmosphere at room temperature and followed by purification via filtration. The resulting azobenzene was further concentrated to a solid form using a rotary evaporator. The as-prepared azobenzene (0.08 g) was dispersed in ethanol and added drop-wise to the ethanol solution containing 10 mg OP-UCNPs-C. The suspension was stirred for 1 hour at 80 °C, and the OP-UCNPs-C@azo were obtained by centrifugation

at 12000 rpm for 20 mins and resuspended in water for subsequent studies. The modification was characterized by UV-vis spectrophotometry.

Paclitaxel Loading into OP-UCNPs-C@azo

For the loading of paclitaxel, 0.2 mg of paclitaxel was dispersed in 1 mL solution of mesoporous silica-coated OP-UCNPs-C@azo in DMSO. The solution was kept for shaking at room temperature for 8 hours followed by collection of OP-UCNPs-C@azo-P (OP-UCNPs-C@azo loaded with paclitaxel) via centrifugation at 12,000 rpm for 20 mins. To confirm loading amount, the supernatant of OP-UCNPs-C@azo-P solution was collected before and after the addition of paclitaxel and analysed by UV absorption spectra.

Cell and Spheroid Culture

HeLa cells were procured from American Type Culture Collection (ATCC) and grown in DMEM culture medium, supplemented with 10% FBS (Life Technologies), 100 units/mL of penicillin, and 100 μg/mL of streptomycin.

3D tumour spheroids were prepared by seeding cells in an ultra-low attachment 96-well microplates (Sigma) and growing them in DMEM culture media (Life Technologies), supplemented with 10% FBS (Life Technologies), 100 units/mL of penicillin, and 100 μg/mL of streptomycin. The 2D cells and 3D spheroids were maintained at 37 °C in a humidified, 5% (v/v) CO_2 atmosphere.

Cell Viability Assay

Cell viability assay was investigated for different concentrations of OP-UCNPs-C@azo-P as well as for different excitation wavelengths (phototoxicity). HeLa cells were seeded in 96-well plates for 2D culture and in ultra-low attachment 96-well plates for 3D spheres. Both the plates were maintained at 37 °C in the CO_2 incubator. After incubation of 24 hours (for 2D culture) and 72 hours (for 3D spheres) of incubations,

the plates were either treated with OP-UCNPs-C@azo-P at different concentrations (0-1 mg/mL) or subjected to 980 and 808 nm NIR irradiations for timings relevant to the study. After 24 hours of incubation, the cell viability was evaluated using MTS assay (for 2D culture) and ATPlite assay (for 3D culture), as per the manufacture's protocol.

Cellular Uptake of OP-UCNPs-C@azo-P

HeLa cells were incubated with 0.5 mg/mL of OP-UCNPs-C@azo-P and kept at 37 °C in humidfied CO_2 incubator for different time points. At the end of each incubation time, the cells were washed thrice to get rid of the excess OP-UCNPs-C@azo- P in the media and on the cells surfaces. The washed cells were trypsinized by 0.05% trypsin in 1x PBS and collected for measuring the luminescence emission of OP-UCNPs-C@azo-P under 980 nm excitation using NIR spectrophotometer. To explore the mechanism of OP-UCNPs-C@azo-P uptake, inhibitors-based blocking of the uptake pathways was carried out. HeLa cells were incubated with OP-UCNPs-C@azo-P and different inhibitors such as chlorpromazine, cytochalasin B, nystatin and filipin to block endocytosis, macropinocytosis and lipid raft-mediated uptake. After incubation, the cells were processed as mentioned before to record the luminescence emission.

The uptake was also investigated visually by counterstaining the OP-UCNPs-C@azo-P-treated cells and 3D spheroids with CellMask™ green plasma membrane stain and Hoechst 33342. The excess OP-UCNPs-C@azo-P after treatment was removed by washing cells and spheroids with 1x PBS, followed by the addition of fresh culture media. To this, 1x HBSS solution of CellMask™ green plasma membrane stain (for cell membrane) was added and incubated in the CO_2 incubator. After 5 mins, Hoechst 33342 (for cell nucleus) was added at a final concentration of 1.0 μM and the cells and spheroids were incubated for another 5 mins. After incubation, the cells and spheroids

were washed thrice with 1x PBS to get rid of excess stains and resuspended in culture medium prior to imaging.

Programmed Activation

OP-UCNPs-C@azo-P were subjected to different durations of 808 and 980 nm irradiations.

Simultaneous and Subsequent Activation

For simultaneous (non-orthogonal) activation, Control-UCNPs-C@azo-P that were only activatable by 980 nm excitation were synthesized. The subsequent (orthogonal) activation was achieved using the OP-UCNPs-C@azo-P excitable by both 980 and 808 nm radiations.

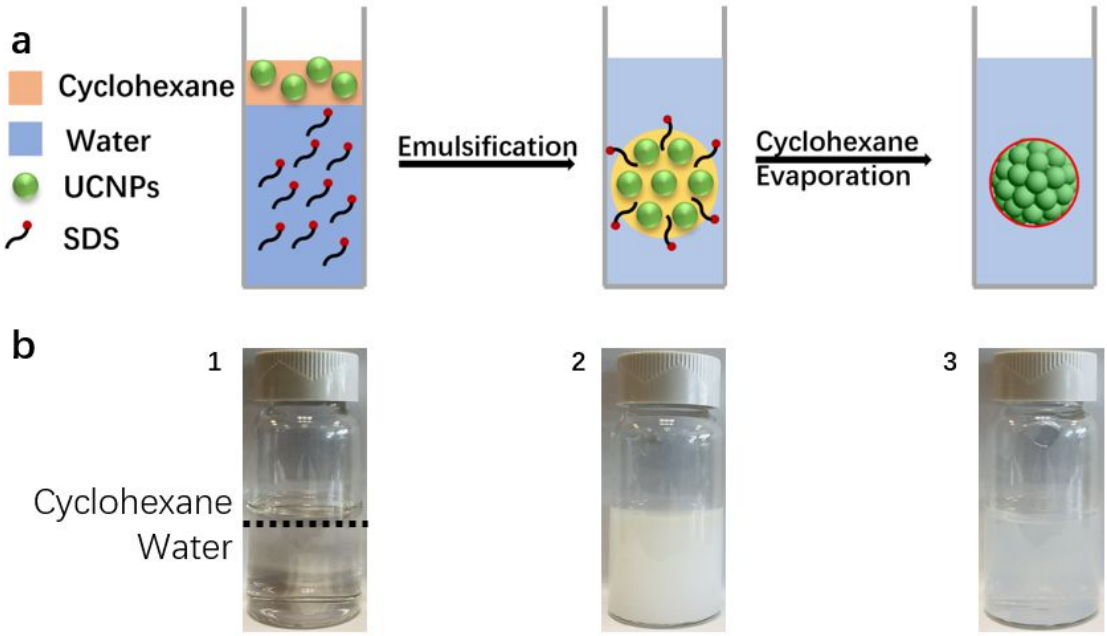

Figure S1. Emulsion-based self-assembly of UCNPs-C. **a,** A scheme showing the self-assembly of UCNPs into UCNPs-C structures via an emulsion-based process. **b,** Photos of UCNPs-C fabrication process: 1) 1 mL cyclohexane suspension of UCNPs (5 mg/mL) mixed with 10 mL water containing 7 mg SDS, forming immiscible two layers when put together. 2) After vortexing and sonication, turbid microemulsion solution was obtained. 3) UCNPs-C solution was obtained after cyclohexane evaporated form water.

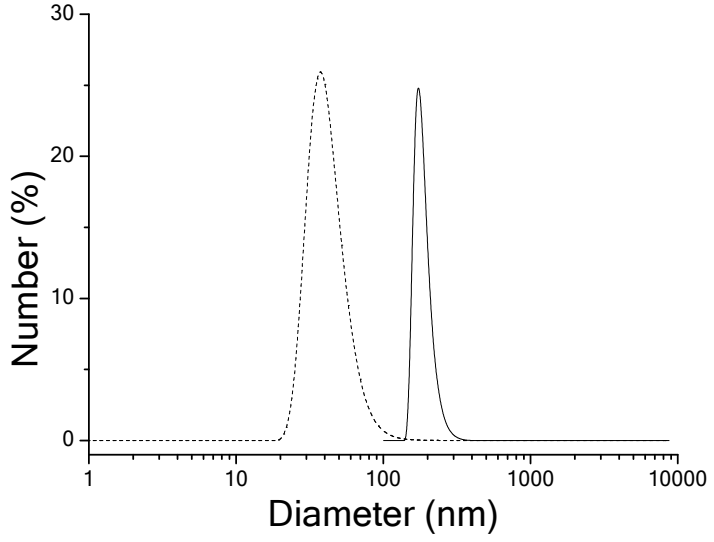

Figure S2. DLS characterization of UCNP-Er-M cyclohexane suspension (dash line) and corresponding UCNPs-C water suspensions (solid line).

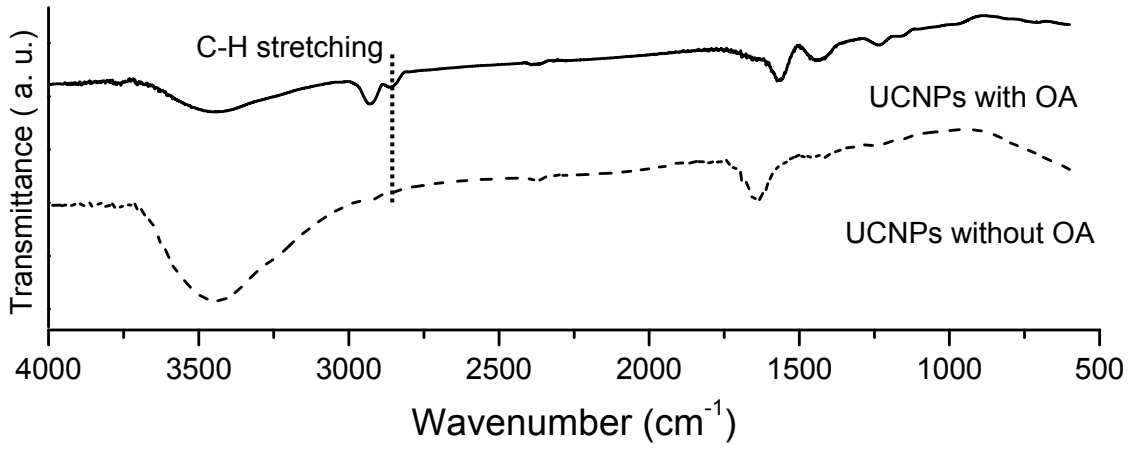

Figure S3. FTIR spectra of bare UCNP-Er-M (OA removed, dash line) and OA capped UCNP-Er-M (solid line). C-H stretching peak of OA disappeared after washing.

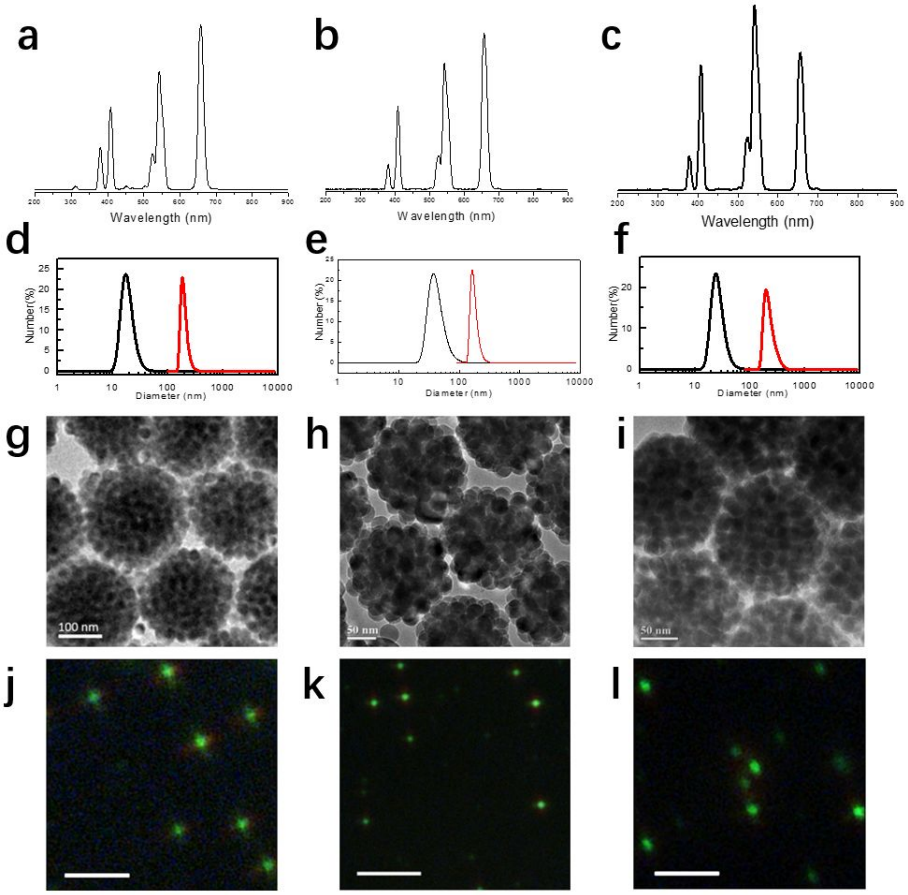

Figure S4. a-c, UCL spectrum under 980 nm laser excitation of UCNP-Er-S, M and L, respectively. **d-f,** DLS characterization results of UCNP-Er-S, M and L (dash line) and their corresponding UCNPs-C water suspension (solid line) respectively. TEM images (**g-i**) and luminescence microscopy (**j-l**) of UCNPs-C prepared from UCNP-Er-S, M and L, respectively. The scale bar in j-l is 2 μm.

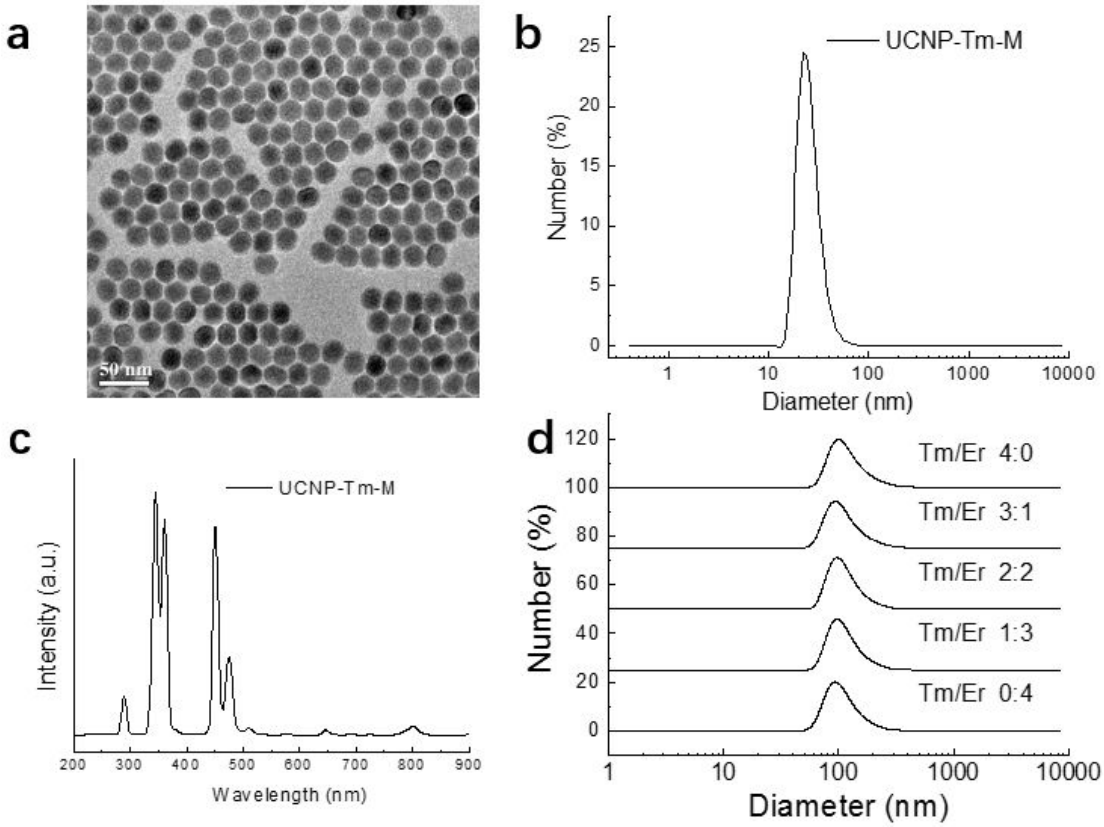

Figure S5. a-c, TEM image, DLS measurement and UCL spectrum under 980 nm laser excitation of UCNP-Tm-M used for color encoding; **d,** DLS characerization of UCNPs-C prepared with different UCNP-Tm-M to UCNP-Er-M ratios for color encoding.

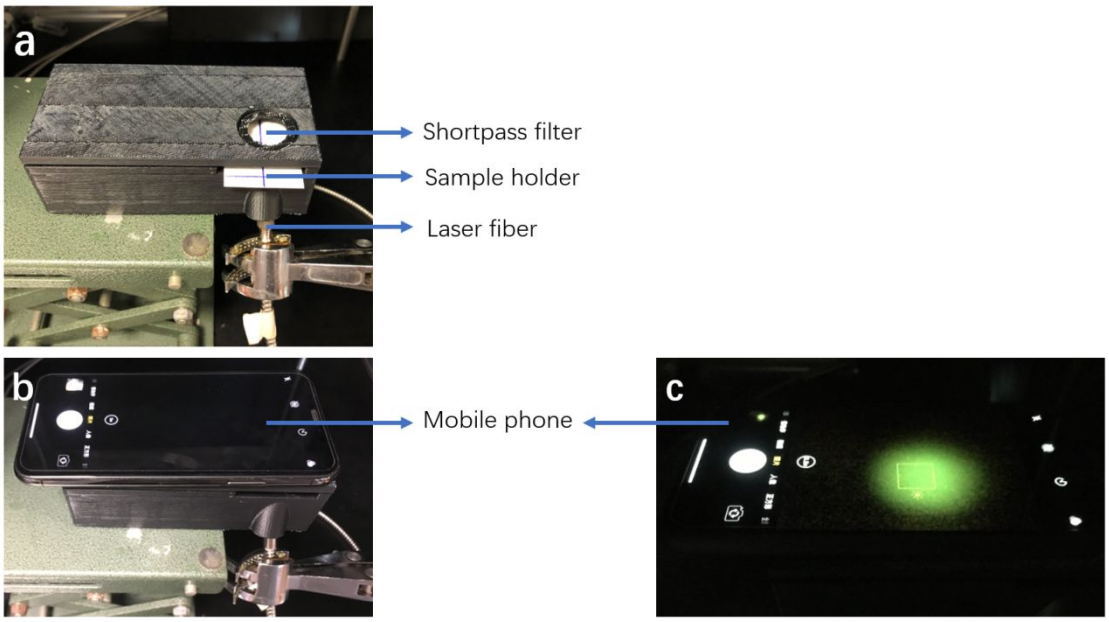

Figure S6. Details of mobile phone photography device: **a-b,** Device setup, **c,** Picture shows the device working condition.

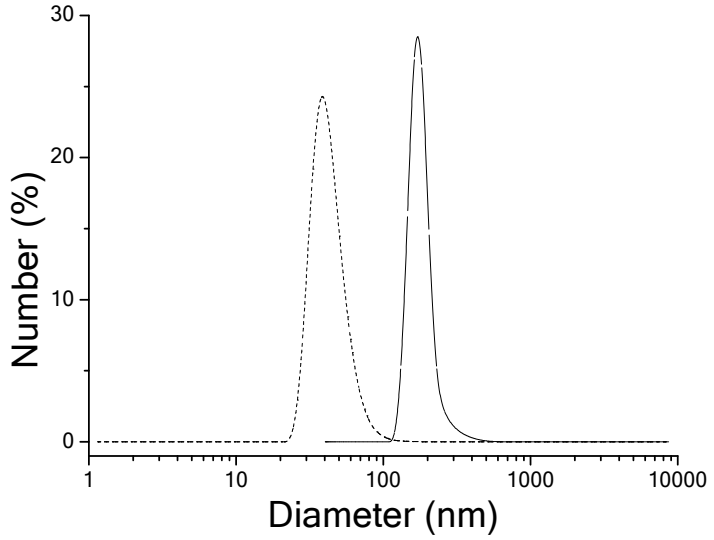

Figure S7. DLS characterization of mixed UCNPs A and B with weight ratio 1:1 cyclohexane suspension (dash line) and corresponding UCNPs-C water suspensions (solid line).

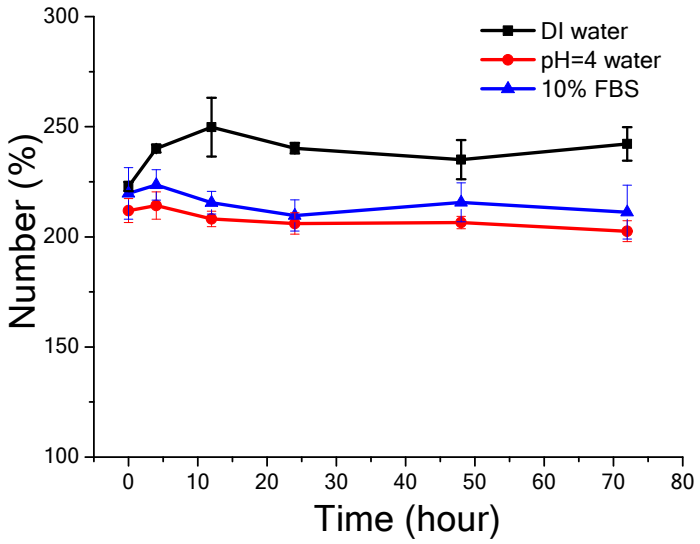

Figure S8. DLS data showing stability of OP-UCNPs-C@silica in DI water, pH=4 water solution and 10% FBS solution conducted over 72 hours.

Table S1 Abbreviations, structural and compositional details of UCNPs used in study

Name	Core	Shell	Shell	Core:Shell Ratio
UCNP-Er-S	$NaGdF_4$:20%Yb, 2%Er	$NaGdF_4$	--	1:1
UCNP-Er-M	$NaYF_4$:20%Yb, 2%Er	--	--	--
UCNP-Er-L	$NaYF_4$:20%Yb, 20%Lu, 2%Er	--	--	--
UCNR	$NaYF_4$:20%Yb, 2%Er	$NaYF_4$	--	1:1
UCNP-Tm-M	$NaYF_4$:30%Yb, 0.5%Tm	--	--	--
A	$NaYF_4$:60%Yb, 20%Gd, 2%Er	$NaLuF_4$:25%Y	--	1:1
B	$NaYF_4$:30%Yb, 0.5%Tm	$NaYF_4$:10%Yb	$NaNdF_4$:10Yb	1:0.2:1

Table S2 Variations in zeta potential after different surface modifications

	OP-UCNPs-C	OP-UCNPs-C@mSiO_2	After azobenzene conjugation
Averaged Zeta Potential(mV)	-60	38	49

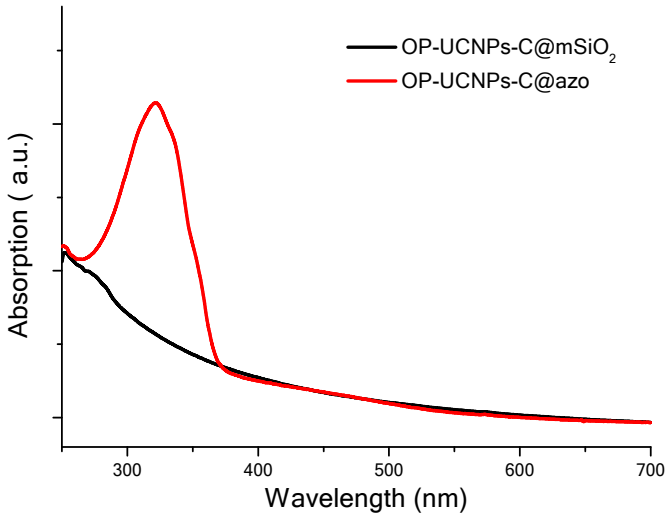

Figure S9. UV-Vis absorbance spectrophotometry of OP-UCNPs-C@mSiO$_2$ and OP-UCNPs-C@azo.

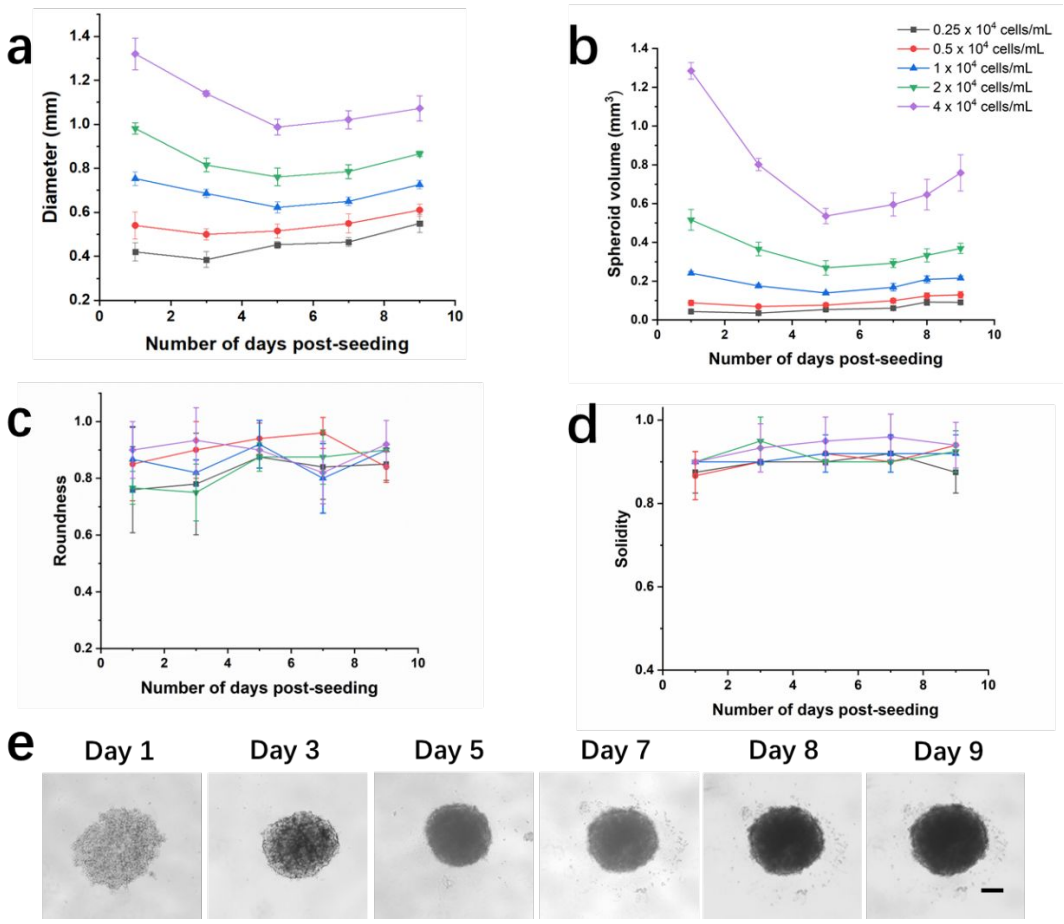

Figure S10. HeLa 3D tumor spheroids. Plots showing the spheroid (**a**) diameter, (**b**) volume, (**c**) roundness, and (**d**) solidity over the period of 9 days for different concentrations. **e,** Representative bright field images of HeLa 3D tumor spheroids from day 1 to day 9 for cell seeding density of 1 x 10^4 cells/mL (4x magnification, scale bar = 200 μm).