# **Supporting Information**

Separately Doping Upconversion-C<sub>60</sub> Nanoplatform for NIR

Imaging-Guided Photodynamic Therapy of Cancer Cells

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Note added after first publication: This Supplementary Information file replaces that originally published on 4th March 2013, in which the data in Fig. S9 were incorrect. Although this did not affect the conclusions of the original article, the authors have now corrected the time resolved data. The correct data are now presented as Fig. S9 in this updated ESI file.

# **Part 1: Experiment section**

**Reagents.** YCl<sub>3</sub>•xH<sub>2</sub>O (99.9%), YbCl<sub>3</sub>•xH<sub>2</sub>O (99.9%), ErCl<sub>3</sub>•xH<sub>2</sub>O (99.9%), TmCl<sub>3</sub>•xH<sub>2</sub>O (99.9%), NaOH (98%), NH<sub>4</sub>F (98%), 1-octadecene (90%), Oleylamine (OM), Poly(allylamine) (PAAm) were purchased from Sigma-Aldrich. All chemicals were used as received without further purification.

Characterization. The structure and morphology of the nanoparticles were characterized by using a Brucker D8-advance X-ray diffractometer (XRD) with Cu Ka radiation ( $\lambda$ = 1.5418 Å). The transmission electron microscopy (TEM) was performed on a Tecnai G2 F20 S-TWIN D573 electron microscope operated at 300 kV TEM. Ultraviolet-visible (UV-VIS) absorption was measured at room temperature by a UV-3101 spectrophotometer. The fluorescent emission spectra were measured at room temperature by a Hitachi F-4500 fluorescence spectrofluorimeter. The luminescence kinetics was recorded with a 500 MHz Tektronix digital oscilloscope and the excitation was realized by a nanosecond pulse train at 980 nm from an optical parametric oscillator. Cellular imaging was done using a Motic AE31 microscope equipped with the Andor GNIR CCD camera (iXon3 888-BV), which is capable of imaging in the range of 500-850 nm. A fiber coupled laser diode (*n*light, NL-PPS50) emitting at 980 nm was used as the light source, and the fiber was introduced through the entrance port of the microscope. The emitted light was passed through a 900nm short-wave pass and 740 nm long-wave pass filters and recorded by CCD camera.

#### (1) General procedure for the synthesis of core nanoparticles.

In a typical procedure to the synthesis of NaYF<sub>4</sub>: Yb/Er nanoparticles, LnCl<sub>3</sub> (0.2 M, Ln = Y, Yb, and Er) was added to a 50-mL flask containing 3 mL of oleylamine and 7mL 1-octadecene . The mixture was heated at 150 °C for 30 min to remove the water content from the LnCl<sub>3</sub>•xH<sub>2</sub>O. After cooling down to 50 °C, 5 mL of methanol solution containing NH<sub>4</sub>F (1.36 mmol) and NaOH (1 mmol) was added and the resultant solution was stirred for 30 min. After the methanol was evaporated, the solution was heated to 305 °C under argon for 1.5 h and then cooled down to room temperature. The resulting nanoparticles with a yield of 65 mg were precipitated by addition of ethanol, collected by centrifugation at 6000 rpm for 5 min, washed with ethanol several times, and re-dispersed in 6 mL of cyclohexane.

## (2) General procedure for the synthesis of core-shell nanoparticles.

The NaYF<sub>4</sub>:Yb/Tm shell precursor was first prepared by mixing LnCl<sub>3</sub> (0.2 M, Ln = Y, Yb and Tm), 3 mL of oleylamine and 7mL 1-octadecene in a 50-mL flask followed by heating at 150 °C for 30 min. After cooling down to 50 °C, NaYF<sub>4</sub>: Yb/Er core nanoparticles (20 mg) dispersed in 2 mL of cyclohexane were added along with a 5-mL methanol solution of NH<sub>4</sub>F (1.36 mmol) and NaOH (1 mmol). The resulting mixture was stirred at 80 °C for 30 min to remove the methanol and cyclohexane. Then the solution was heated to 305 °C under argon for 1 h and then cooled down to room temperature. The resulting nanoparticles were precipitated by addition of ethanol, collected by centrifugation at 6000 rpm for 5 min, washed with ethanol several times, and re-dispersed in 6 mL of cyclohexane.

#### (3) Phase transfer

The ligand exchange process was carried out to transfer hydrophobic upconversion nanoparticles into hydrophilic ones using Poly(allylamine) as ligand. 0.1mL of Poly(allylamine) 20% solution in water was dispersed in 10 mL ethanol, The hydrophobic UCNPs solution (~5mg, purified and dispersed in 2mL of cyclohexane) were mixed with the Poly(allylamine) solution and stirred vigorously over 24 h at 30 °C. After centrifugation, the obtained nanoparticles were redispersed in water. After phase transfer, the Poly(allylamine) terminated UCNPs give amino group at the end which can be used for covalently coupling with carboxyl ended molecules.

### (4) Synthesis of Monomalonic Fullerene (C<sub>60</sub>MA)

Monomalonic derivative of fullerene ( $C_{60}MA$ ) was prepared by Bingel cyclopropanation reaction. To start, 0.5 g of  $C_{60}$  was dissolved in 300 mL toluene, in which 230 µL 1,8-diazabicyclo[5,4,0]undec-7-ene and 139 µL diethyl bromomalonate were then added dropwise. The reaction proceeded for 4 h at room temperature under argon. The resulting blackish residue was dried on Na<sub>2</sub>SO<sub>4</sub> and chromatographed on silica gel (200-400 mesh) using in order hexane, hexane-toluene (4:1, 2:1, and 1:1, v/v) and toluene. The dark brown band containing  $C_{61}(CO_2Et)_2$  was collected in toluene and. To this solution, 74 mg of NaH was added and the resulting mixture was refluxed at 60 °C for 1 h under argon. The heating bath was removed afterwards and 10 mL MeOH was immediately added. The resulting brown amorphous precipitate was collected by centrifugation and further washed by MeOH, 4M HCl, and water, twice

for each solvent. The final product, monomalonic fullerene ( $C_{60}MA$ ), was dried first by rotary evaporation and then at 120 °C overnight. The formation of this product was confirmed by ESIMS and the yield is ~24%.

# (5) Covalent Conjugation of UCNPs with C<sub>60</sub>MA

To covalently conjugate C<sub>60</sub>MA to UCNPs, 5mL of dimethyl formamide (Sigma Aldrich) solution containing 0.5 mg of  $C_{60}MA$ , 1 mg 1-ethylof 3-(3-dimethylaminopropyl) carbodiimide (Sigma Aldrich), and 1 mg of N-hydroxysulfosuccinimide sodium salt (Sigma Aldrich) was incubated at room temperature for 2 h, and then 0.5 mg of amino-functionalized UCNPs was added into the solution and stirred vigorously for 24 h. UCNPs-C<sub>60</sub>MA conjugates were then centrifuged and washed with water to remove any unreacted C<sub>60</sub>MA. The amount of  $C_{60}MA$  attached to UCNPs was calculated from the  $C_{60}MA$  absorption spectrum.

### (6) Surface functionalized with PEG-SC

To surface coated UCNPs-C<sub>60</sub>MA with PEG-succinimidyl carbonate (PEG-SC) molecules, 4mL of ethanol solution containing 1 mg PEG-SC and 0.5 mg UCNPs-C<sub>60</sub>MA nanocomposites was stirred vigorously for 10 h. UCNPs- C<sub>60</sub>MA conjugates were then centrifuged and washed with water to remove any unreacted PEG-SC.

#### (7) Singlet Oxygen Measurements

In a typical FCLA experiment, 20  $\mu$ L of a FCLA/ethanol solution (10 mmol/L) was added to 2 mL of a UCNPs- C<sub>60</sub>MA solution and transferred into a 10 mm cuvette. The solution was kept in the dark and irradiated with a 980 nm laser for 18 min, and the emission intensity of FCLA at 542 nm was recorded every 2 min. For the control experiments, FCLA emission was also recorded for comparison at the same conditions in the absence of UCNPs-  $C_{60}MA$  or at 980 nm irradiation.

# (8) Target Cancer Cell Imaging

In order to increase the specificity, folic acid was covalently linked with the UCNPs in a similar way to  $C_{60}MA$  cross-linking. The FA was then mixed with 0.5mg of UCNPs-C<sub>60</sub>MA and stirred for 24 h in the dark. The resulting nanoconjugates were collected by centrifugation, washed with water three times, redispersed in 5 mL of phosphate buffer, and stored in the dark at 4  $\,^{\circ}$ C for further application. Hela cells that overexpress folate receptors (positive control) and A549 cells that have a low expression of folate receptors (negative control) were purchased from School of Basic Medical Sciences, Jilin University. Hela and A549 cells were cultured in folate-free RPMI-1640 medium. All the mediums were supplemented with 20% fetal bovine serum, 100 unit/mL penicillin, and 100 µg/mL streptomycin. Cells were cultivated in medium at 37  $\,^{\circ}$ C in a humidified 95% air and 5% carbon dioxide (CO<sub>2</sub>) atmosphere. For confocal imaging, both Hela cells and A549 cells were seeded on a petri dish at a concentration of 104 cells/mL and then treated with UCNPs-C<sub>60</sub>MA/FA nanoconjugates (100 µg/mL, 50µl) for 12 h at 37 °C. Prior to imaging, the petri dish was washed thrice with phosphate-buffered saline (PBS) in order to remove any unbound upconversion nanoconjugates. To further study the specificity of the nanoconjugates, another negative control experiment was also carried out with Hela cells by supplementing 100 times more folic acid (100 mg/L) in the culture medium to

saturate the folate receptors on the cell membrane before incubating with UCNPs- $C_{60}MA$  /FA nanoconjugates.

# (9) Photodynamic therapy

To carry out photodynamic therapy of cancer cells, Hela cells were collected through centrifugation and diluted to a density of 100000 cells/mL in the complete 1640 culture medium and then seeded onto 96-well plates (100 µL per well). After 24 h culturing, 100µl UCNPs-C<sub>60</sub>MA /FA nanoconjugates were added to the culture medium at different concentrations from 100 to 800 µg/mL, with four parallel wells for each concentration (100, 200, 300, 400, 500, 800 µg/mL). Before being exposed to NIR irradiation, the cells were allowed to incubate for another 24 h at 37 °C and then washed thrice with PBS. A power adjustable 980 nm fiber laser with maximal output power of 30 W (n-LIGHT Corporation) was collimated and employed as area light source to irradiate the 96-well plate. After 10 min exposure of 980 nm light at 1.37W/cm<sup>2</sup>, the cells were allowed to incubate for an additional 48 h. Cell viability was measured according to the standard MTT (Sigma Aldrich) assay method. Typically, 10 µL of MTT solution (5 mg/mL MTT in PBS, pH 7.4) was added to each well and incubated for 4 h at 37 °C. After removing the medium, the wells were washed by PBS, and then the intracellular formazan crystals were extracted into 100 µL of DMSO. The absorbance of cell lysate was recorded at 550 nm by a plate reader, and the cell viability could be calculated from the average value of five parallel wells. The choice of exposure power density of 1.37W/cm2 was based on the following consideration. In clinical applications and most PDT experiments the light dose is in the range of 1-1000 J/cm<sup>2</sup>, and the typical power density is below 1 W/cm<sup>2</sup>. But this value is obtained in the Stokes case, i.e., direct excitation of photosensitizers, whereas in our case it is an anti-Stokes scheme, i.e., indirect excitation (NIR light converted into UV/VIS, and the latter is then used to excite the photosensitizers) where the efficiency is less than the direct excitation and the required excitation power density should be higher. However higher excitation power density may lead to thermal decline of the cells. In the current case the highest possible excitation power density was determined to be about 2 W/cm<sup>2[1]</sup>. Therefore 1.37 W/cm<sup>2</sup> was used in our study.

# Part 2: Supplementary figures



**Figure S1**. Experimental powder X-ray diffraction (XRD) pattern for the core/shell NaYF<sub>4</sub>:  $Yb^{3+}$ ,  $Er^{3+}$ / NaYF<sub>4</sub>:  $Yb^{3+}$ ,  $Tm^{3+}$  UCNPs and the calculated line pattern for the hexagonal NaYF<sub>4</sub> phase.



**Figure S2**. TEM images of core NaYF<sub>4</sub>: Yb, Er (a) and core-shell NaYF<sub>4</sub>: Yb, Er/ NaYF<sub>4</sub>: Yb, Tm UCNPs (b).

The NaYF<sub>4</sub>: Yb, Er nanoparticles had a mean particle diameter of 30 nm (Figure S2a). Core/shell NaYF<sub>4</sub>: Yb, Er/ NaYF<sub>4</sub>: Yb, Tm UCNPs increased the size to 45 nm , corresponding to about 7.5 nm shell thickness (Figure S2b).



**Figure S3**. HRTEM image of OM-UCNP, shows distinct lattice fringes with interplanar spacing of 0.31 nm ascribed to the (110) plane of hexagonal NaYF<sub>4</sub>.



**Figure S4.** UCL spectra of colloidal nanocrystals with (1) separated model NaYF<sub>4</sub>:Yb<sup>3+</sup>(20%),  $Er^{3+}(2\%)$  / NaYF<sub>4</sub>:Yb<sup>3+</sup>(20%),  $Tm^{3+}(0.3\%)$  and (2) homogeneous model NaYF<sub>4</sub>:Yb<sup>3+</sup>(20%),  $Er^{3+}(2\%)$ ,  $Tm^{3+}(0.3\%)$  dispersed in cyclohexane (1mg/mL) under 15Wcm<sup>-2</sup> continuous excitation at 980 nm (keep the crystal size the same).

The  $Er^{3+}$  and  $Tm^{3+}$  separated doping core/shell structure was employed instead of homogeneous doping to achieve the strong multicolor. Because for  $Er^{3+}$  and  $Tm^{3+}$ homogeneously codoped nanocrystals, the fluorescence from  $Tm^{3+}$  is quenched by  $Er^{3+}$ , probably as a result of the preferential energy transfer from Yb<sup>3+</sup> to  $Er^{3+}$ . As such, only very weak  $Tm^{3+}$  fluorescence can be observed. While in the separated model, the  $Er^{3+}$  and  $Tm^{3+}$  fluorescence can both be strongly obtained. Furthermore, we preferred to dope  $Tm^{3+}$  ions in the shell region to shorten the energy transfer distance, owing to the strong absorption of  $C_{60}MA$  in the violet-blue wave band.



**Figure S5.** (a) Fluorescent emission spectra of fluorescamine upon irradiation at 390nm. (b) UCL spectra of UCNPs before and after phase transfer.

The existence of amino group on the surface of nanoparticles was proved by fluorometric method using non-fluorescent fluorescamine reagent for rapid amino assay. The reaction of primary amines with fluorescamine can result in fluorophore products, and the excess fluorescamine can be hydrolyzed into non-fluorescent products very fast. By measuring the emission band centered at 470nm ( $\lambda_{ex} = 390$  nm), the presence of amino guroup can be validated. Figure S5a illustrates the emission spectrum of phase transferred nanoparticles excited by 390nm, a strong peak centered at 470 was clearly observed, confirming the existence of amino groups.



**Figure S6**. FTIR absorption spectra of the UCNPs- $C_{60}MA$  nanoconjugate (balck) and free  $C_{60}MA$  (blue).

For a free  $C_{60}MA$ , the absorption peak at 1620 cm<sup>-1</sup> is associated with the conjugate C = C stretching vibration from the fullerene framework. The C = O stretching vibration mode of the carboxyl group is located at 1717cm<sup>-1</sup>. After conjugating with UCNPs, the peak at 1717cm<sup>-1</sup> was disappeared and two new peaks appeared at 1648 and 1556 cm<sup>-1</sup>, corresponding to the C = O stretching vibration and N-H bending vibration modes of secondary amide, respectively. The absorption peak at 2939 cm<sup>-1</sup> is associated with the = CH stretching vibration from the PAAm.



Figure S7. Photos of the UCNPs- $C_{60}$ MA nanoconjugate dissolved in water (a) and free  $C_{60}$ MA dissolved in THF (b) before and after centrifugation (10,000rpm for 10min).



Figure S8. The absorption intensities of UCNPs- $C_{60}MA$  nanoconjugates incubated with  $C_{60}MA$  of different concentration. The inset respectively shows UV-VIS absorbance spectra of UCNPs- $C_{60}MA$  nanoconjugates loading with  $C_{60}MA$  at different added values.

UCNPs at 0.5 mg/mL were mixed with various concentrations of  $C_{60}MA$ . After removal of free  $C_{60}MA$  by washing, UV-VIS spectra of UCNP- $C_{60}MA$  complexes were recorded.



**Figure S9.** Luminescence decay curves of upconversion emissions monitored (a) at 450 nm, (b) at 470nm, (c) at 540 nm, (d) at 650nm (e) at 800nm with (in black), and without (in red)  $C_{60}MA$ .

The energy transfer process was also confirmed by the temporal behavior of upconversion luminescence of both UPNPs (red) and UCNPs-C<sub>60</sub>MA conjugates (black) recorded at 450, 475, 540, 650 nm and 800nm, respectively. In all cases, the decay parts could be well fitted with single-exponential function. The instrument response function was measured to be 2 $\mu$ s. In the presence of C<sub>60</sub>MA, the average decay time decreases from 672 $\mu$ s to 371 $\mu$ s for 450nm, from 705 $\mu$ s to 487 $\mu$ s for

475nm, from 320 $\mu$ s to 155 $\mu$ s for 540nm and for 650nm, dropping down from 429 $\mu$ s to 293 $\mu$ s. While no obvious change of the average decay time at 800 nm was observed (from 968 $\mu$ s to 930 $\mu$ s) owing to the little absorption of C<sub>60</sub>MA in the near-infrared region, which validating the rationality of normalization of the steady-state UCL spectra by the intensity at 800nm. These efficient multiplexed FRET from UCNPs to C<sub>60</sub>MA ensures the high <sup>1</sup>O<sub>2</sub> generation.



**Figure S10**. The spectra of FCLA luminescence intensity without UCNPs- $C_{60}MA$  just under the illumination of 980 nm (a), and the spectra of FCLA luminescence intensity with UCNPs- $C_{60}MA$  without 980 nm illumination (b), showing negligible change over time.



**Figure S11**. FCLA assay of  ${}^{1}O_{2}$  generation by NaYF<sub>4</sub>:Yb<sup>3+</sup>,Er<sup>3+</sup> / NaYF<sub>4</sub>:Yb<sup>3+</sup>,Tm<sup>3+</sup> (denote as NCUPs (Yb, Er, Tm))-C<sub>60</sub>MA (a), NaYF<sub>4</sub>:Yb<sup>3+</sup>,Tm<sup>3+</sup> (denote as NCUPs (Yb, Tm))-C<sub>60</sub>MA (b), NaYF<sub>4</sub>:Yb<sup>3+</sup>,Er<sup>3+</sup> (denote as NCUPs (Yb, Er))-C<sub>60</sub>MA (c) and UCNPs (Yb, Er, Tm)-RB(d).

The size of UCNPs employed in these four different models keep the same. The covalent way of RB bonding to UCNPs was following the reported protocol by our group<sup>[2]</sup>. The amount of RB saturated at 7.2% (w/w) at RB concentrations above 200 $\mu$ l (Figure S12), which is lower than that of C<sub>60</sub>MA.



**Figure S12**. The absorption intensities of UCNPs (Yb, Er, Tm) -RB nanoconjugates incubated with RB of different concentration. The inset respectively shows UV-VIS absorbance spectra of UCNPs (Yb, Er, Tm) -RB nanoconjugates loading with RB at different added values.



**Figure S13**. Cell viability of Hela cells treated with the other three energy transfer models, (i) UCNPs (Yb, Er, Tm)-rose bengal (red), (ii) UCNPs (Yb, Er)- $C_{60}MA$  (blue), (iii) UCNPs (Yb, Tm)- $C_{60}MA$  (pink) and UCNPs (Yb, Er, Tm)-  $C_{60}MA$  (green).



**Figure S14**. Viability of Hela cells treated with different power densities of 980nm irradiation in the absence of UCNPs.

Reference:

[1] Niemz, M. H. Laser-Tissue Interactions: Fundamentals and Applications, 3rd ed.; Springer: Heidelberg, 2007; pp 45.

[2] Liu, K.; Liu, X. M.; Zeng, Q. H.; Zhang, Y. L.; Tu, L. P.; Liu, T.; Kong, X. G.; Lambrechts, S. A. G.; Aalders, M. C. G. Zhang, H. *ACS nano*, 2012, 6, 4054.