Electronic Supplementary Information

NIR laser scanning microscopy for photophysical characterization of upconversion nanoparticles and nanohybrids

Juan Ferrera-González, Laura Francés-Soriano, Nestor Estébanez, Enrique Navarro-Raga, María González-Béjar and Julia Pérez-Prieto

1 Instituto de Ciencia Molecular (ICMol), Departamento de Química Orgánica, University of Valencia, C/ Catedrático José Beltrán, 2, Paterna, Valencia 46980, Spain
2 nanoFRET.com, Laboratoire COBRA (Chimie Organique, Bioorganique, Réactivité et Analyse), Université de Rouen Normandie, CNRS, INSA, 76821 Mont-Saint-Aignan Cedex, France
3 Servicio Central de Soporte a la Investigación Experimental (SCSIE). University of Valencia, Burjassot, Valencia 46100, Spain

CONTENT

Instruments and methods ................................................................. 2
Experimental .................................................................................. 2
Estimation of lifetime window .......................................................... 3
Estimation of excitation energy .......................................................... 3
Figure S1. EDX spectra from \( UC_{Er,2}, UC_{Er,20}, UC_{Tm} \) and \( UC_{Ho} \) .......................................................... 4
Table S1. Composition of \( UC_{Er,2}, UC_{Er,20}, UC_{Tm} \) and \( UC_{Ho} \) from EDX .......................................................... 4
Figure S2. TEM images of \( UC_{Er,2}, UC_{Er,20}, UC_{Tm} \) and \( UC_{Ho} \) .......................................................... 5
Figure S3. XRD diffractogram \( UC_{Er,2}, UC_{Er,20}, UC_{Tm} \) and \( UC_{Ho} \) .......................................................... 5
Figure S4. Absorption spectra of \( UC_{Tm}@Rh101 \) and \( UC_{Tm}@Rh110 \) .......................................................... 6
Figure S5. SEM images of the \( UC_{Er,2} \) .......................................................... 6
Figure S6. NIR-LSM image of \( UC_{Er,20} \) in channel 2 .......................................................... 7
Figure S7. Fitted kinetic of \( UC_{Tm}@Rh110 \) agglomerate .......................................................... 7
Table S2. Decay lifetimes of \( UC_{Er,2}, UC_{Er,20}, UC_{Tm} \) and \( UC_{Ho} \) .......................................................... 7
Figure S8. NIR-LSM image of C1 and C2 of \( UC_{Tm}@Rh110 \) exciting at 975 nm .......................................................... 8
Table S3. Decay lifetimes obtained for bare-\( UC_{Tm} \) and \( UC_{Tm}@Rh110 \) .......................................................... 8
Figure S9. NIR-LSM images exciting at 975 nm and at 1030 nm \( UC_{Tm}@Rh101 \) sample .......................................................... 8
Figure S10. Colocalization analysis of \( UC_{Tm}@Rh101 \) .......................................................... 9
Figure S11. Colocalization analysis of \( UC_{Tm}@Rh110 \) .......................................................... 9
Figure S12. NIR-LSM Intensity profile of Tm emission vs. dye emission of \( UC_{Tm}@Rh110 \) .......................................................... 10
Estimation of spatial resolution .......................................................... 10
Figure S13. Visualization of pixel, laser spot, nanoparticles and aggregates sizes .......................................................... 10
References ..................................................................................... 10
**UV-vis spectroscopy**

The UV-visible-NIR spectra of the colloidal UCNHs were recorded in a UV/Vis/NIR spectrophotometer Lambda 1050 Perkin Elmer.

**TEM images**

TEM images were obtained using a Jeol 1010 microscope operating at 100 kV equipped with an AMT RX80 (8 Mpx) charge-coupled device (CCD) camera. For the preparation of the UCNPs samples, 10 µL of a 0.5 mg·mL⁻¹ solution of the UCNPs was left to dry under vacuum at room temperature on a formvar/carbon film supported on a 300-mesh copper grid. High-resolution transmission electron microscopy (HRTEM) images were recorded using a TECNAI G2 F20 microscope operating at 200 kV (point resolution of 0.24 nm) and equipped with a Gatan Multiscan 794 (1 Mpx) CCD camera.

**SEM images**

Scanning electron microscopy (SEM) images were obtained using Field emission microscope HITACHI S-4800, working at 20 kV. The UCNPs samples were deposited in a glass slide by spin coating as explained above.

**EDX analysis**

The energy dispersive X-ray spectroscopy (EDX) analysis was acquired using a scanning electron microscope HITACHI S-4800 equipped with XFlash 5030 Bruker detector and acquisition software QUANTAX 400. The UCNPs samples were deposited on adhesive carbon tape.

**XRD**

XRD diffractograms were registered on a Bruker D8 Advance A25 diffractometer using CuKα (λ = 1.54060 Å) radiation at a voltage of 40 kV and 30 mA, and a LynxEye detector. The powder diffraction pattern was scanned over the angular range of 2-80° (2θ) with a step size of 0.020°, at room temperature.

**Image processing and statistical analysis in colocalization**

Image processing was performed with the open source software ImageJ/FIJI. Background levels were equilibrated in the whole image by subtracting a median filtered image. Colocalization coefficients and significance tests were calculated by the ImageJ GDSC Plugin which performs the Confined Displacement Algorithm (CDA). The ROI of each image was obtained by applying the Otsu threshold and the confined compartment is the entire image (Figs. S10 and S11). Clipping signal (saturated) was discarded for the analysis. For visualization purposes the brightness of the images showed in the main subscript has been magnified.

**Experimental**

**Materials**

**Chemicals**: The chemicals used for the synthesis of the nanoparticles were: lanthanide chlorides (YCl₃·6H₂O, YbCl₃·6H₂O, ErCl₃·6H₂O, TmCl₃·6H₂O and HoCl₃·6H₂O (>99.9%, all of them)), 1-octadecene (95%), oleic acid (70%), NaOH and NH₄F (99.99%). All these chemicals were purchased from Sigma-Aldrich used as received without previous purification.

**Synthesis**

*Synthesis of oleate-capped NaYF₄:Yb³⁺(15%), Er³⁺(2%) and NaYF₄:Yb³⁺(16%), Er³⁺(18%) nanoparticles (UCₓₓ,₂, UCₓₓ,₂₀)*

NaYF₄:Yb,Er nanoparticles were synthesized by following a previously reported protocol with some modifications. In a 50 mL round-bottom flask, oleic acid (8 mL) and octadecene (15 mL) were added. Then, a solution containing YCl₃·6H₂O (0.78 mmol or 0.60 mmol), YbCl₃·6H₂O (0.20 mmol for the two syntheses) and ErCl₃·6H₂O (0.02 mmol or 0.20 mmol) dissolved in methanol (2 mL) was added to the flask and the mixture was stirred at 160 °C under N₂ until everything was dissolved. Next, the solution was cooled to 100 °C and 10 mL of a methanol solution containing NaOH (2.5 mmol) and NH₄F (4.0 mmol) were slowly added into the flask during 5 min. Then the solution was heated until 125 °C under N₂ flow and continuous stirring to remove completely methanol and water traces. Finally, the reaction was heated at 305 °C under N₂ flux for one hour. After that, the solution was cooled to room temperature and the nanoparticles were precipitated by centrifugation (9000 rpm, 15 min, 25 °C). Later on, the oleate-capped UCNPs were washed three times with (43.5:40.5:16 v/v) hexane/acetone/methanol solution and once with ethanol. As usual, the Y:Yb ratio used in the preparation (78, 20, 2 and 66, 16, 20 % respectively) is slightly different than the proportion obtained in EDS analyses of the final UCₓₓ,₂ (83, 15, 2 and 66, 16, 18 %).

*Synthesis of oleate-capped NaYF₄:Yb³⁺(17%), Tm³⁺(0.2%) nanoparticles (UCₓₓ)*

NaYF₄:Yb,Tm nanoparticles were synthesized by following a previously reported protocol with some modifications. In a 50 mL round-bottom flask, oleic acid (8 mL) and octadecene (15 mL) were added. Then, a solution containing YCl₃·6H₂O (0.80 mmol), XRD
YbCl₃·6H₂O (0.20 mmol), TmCl₃·6H₂O (0.002 mmol) dissolved in methanol (2 mL) was added to the flask and the mixture was stirred at 160 °C under N₂ until everything was dissolved. Next, the solution was cooled to 100 °C and 10 mL of a methanol solution containing NaOH (2.5 mmol) and NH₄F (4.0 mmol) were slowly added into the flask during 5 min. Then the solution was heated until 125 °C under N₂, flow and continuous stirring to remove completely methanol and water traces. Finally, the reaction was heated at 305 °C under N₂, flux for one hour. After that, the solution was cooled to room temperature and the nanoparticles were precipitated by centrifugation (9000 rpm, 15 min, 25 °C). Later on, the oleate-capped UCNPs were washed three times with (43.5:40.5:16 v/v) hexane/acetone/methanol solution and once with ethanol. As usual, the Y: Yb:Tm ratio used in the preparation (80, 20 and 0.2 % respectively) is slightly different than the proportion obtained in EDS analyses of the final UCNPs (83, 17 and <1).

Synthesis of oleate-capped NaYF₄:Yb³⁺(19%), Ho³⁺(1.0%) nanoparticles (UCNPs)

NaYF₄·Yb, Ho nanoparticles were synthesized by following the same protocol used in the synthesis of the UCNPs doped with erbium and thulium described above. In a 50 mL round-bottom flask, oleic acid (8 mL) and octadecene (15 mL) were added. Then, a solution containing YCl₃·6H₂O (0.79 mmol), YbCl₃·6H₂O (0.20 mmol), HoCl₃·6H₂O (0.10 mmol), and ethanol (2 mL) was added to the flask and the mixture was stirred at 160 °C under N₂ until everything was dissolved. Next, the solution was cooled to 100 °C and 10 mL of a methanol solution containing NaOH (2.5 mmol) and NH₄F (4.0 mmol) were slowly added into the flask during 5 min. Then the solution was heated until 125 °C under N₂ flow and continuous stirring to remove completely methanol and water traces. Finally, the reaction was heated at 305 °C under N₂ flow for one hour. After that, the solution was cooled to room temperature and the nanoparticles were precipitated by centrifugation (9000 rpm, 15 min, 25 °C). Later on, the oleate-capped UCNPs were washed three times with (43.5:40.5:16 v/v) hexane/acetone/methanol solution and once with ethanol.

Estimation of lifetime window

The minimum and maximum lifetime (τ) can be estimated with NIR-LSM by assuming a monoexponential decay by the following equations:

\[
\tau_{\text{min}} (\mu s) = \frac{d_{\text{min}} \cdot P_x}{t_w}
\]

\[
\tau_{\text{max}} (\mu s) = \frac{d_{\text{max}} \cdot P_{x\text{max}}}{t_w}
\]

Where \(d_{\text{min}}\) and \(d_{\text{max}}\) are minimum and maximum dwell times, \(P_x\) is the minimum number of points (pixels) needed to describe an exponential decay (we assumed 10), \(P_{x\text{max}}\) is the maximum number of pixels per line (4096) and \(t_w\) is the optimum temporal window (10); i.e. temporal window should not be shorter than 10 times the lifetime of the emitter.

Estimation of excitation energy

The excitation energy is expressed as the total energy density (fluence) delivered during the dwell time \(F_d\). It depends on the laser average output power, the excitation wavelength \(\lambda_{\text{exc}}\), the effective numerical aperture, the particle size \(d\), the objective numerical aperture \(NA\), the sample thickness \(t\), the objective transmission \(OT\), and the laser transmittance \(LT\). The excitation energy is calculated by:

\[
F_d = \frac{P \cdot LT \cdot OT \cdot d}{\pi \cdot 0.61 \cdot \lambda_{\text{exc}} \cdot 1.1 \cdot NA}
\]

\(P\): Laser average power at a certain excitation wavelength (W)
\(LT\): Laser transmission, software controlled (%)
\(OT\): Objective transmission, from specifications (%)
\(d\): dwell time (s/pixel)
\(\lambda_{\text{exc}}\): excitation wavelength (cm)
\(NA\): objective numerical aperture
Figure S1. EDX spectra of (A) UC_{0.2}: NaYF_{4}:Yb (20%), Er(2%); (B) UC_{0.2}: NaYF_{4}:Yb (20%), Er(20%); (C) UC_{0.2}:NaYF_{4}:Yb (20%), Tm(0.1%) and (D) UC_{0.2}: NaYF_{4}:Yb (20%), Ho(1%).

Table S1. Composition of the samples from EDX (20 Kv)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Yttrium</th>
<th>Ytterbium</th>
<th>Erbium</th>
<th>Thulium</th>
<th>Holmium</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaYF_{4}:Yb (20%), Er (2%)</td>
<td>83.3 ± 1.1</td>
<td>15.2 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NaYF_{4}:Yb(20%),Er(20%)</td>
<td>66.6 ± 2.3</td>
<td>15.7 ± 1.2</td>
<td>17.7 ±1.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NaYF_{4}:Yb(20%),Tm(0.1%)</td>
<td>82.7 ± 0.9</td>
<td>17.0 ± 0.6</td>
<td>-</td>
<td>&lt;LOD(&lt;1)</td>
<td>-</td>
</tr>
<tr>
<td>NaYF_{4}:Yb(20%),Ho(1%)</td>
<td>79.8 ± 2.1</td>
<td>19.1 ± 0.5</td>
<td>-</td>
<td>-</td>
<td>1.1 ± 0.2</td>
</tr>
</tbody>
</table>
Figure S2. TEM images of (A) UC₆r₂, (B) UC₆r₂₀, (C) UC₆m, and (D) UC₆m. Scale bar: 50 nm.

Figure S3. X-ray diffraction (XRD) diffractogram of (A) UC₆r₂, (B) UC₆m, (C) UC₆m, and (D) hexagonal NaYF₄ standard (JCPDS PDF number 16-0334).
Figure S4. Absorption spectra of the UCNH in DMF (1 mg/mL): (A) UC\textsubscript{Tm}@Rh101 and (B) UC\textsubscript{Tm}@Rh110.

Figure S5. SEM images of the UC\textsubscript{Er,2} sample displaying different agglomerates and individual UC\textsubscript{Er,2} on the glass surface. The microscope slide edges showed a higher concentration of UCNPs while intermediate areas were optimal to focus on the NIR-LSM. For comparative purposes, the pixel size (0.5 x 0.5 \(\mu\)m\(^2\)) is included in each of the NIR-LSM images.
Figure S6. (A) NIR-LSM image of UC$_{Er,20}$ in channel 2 (C2: 515-580 nm; 2 μs·pixel$^{-1}$; λ$_{exc}$ = 975 nm; F$_{d}$ = 0.2 J·cm$^{-2}$). Scale bar: 50 µm. (B) Emission spectrum of UC$_{Er,20}$ (λ$_{exc}$ = 975 nm; I = 93 W·cm$^{-2}$). (C) Kinetic profile obtained of a tail in figure A.

Figure S7. Fitted kinetic of UC$_{Er}$@Rh110 (C1: 420-500 nm, λ$_{exc}$ = 975 nm, F$_{d}$ = 18.4 J·cm$^{-2}$).

Table S2. Comparison of the acquisition conditions and decay lifetime values obtained from the different UCNPs at λ$_{exc}$ = 975 nm (F$_{d}$ = 0.2 J·cm$^{-2}$).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Emission (nm)</th>
<th>Dwell time (μs·pixel$^{-1}$)</th>
<th>Decay lifetime (μs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UC$_{Er,2}$</td>
<td>515-580</td>
<td>2</td>
<td>68.5 ± 1.1</td>
</tr>
<tr>
<td>UC$_{Er,20}$</td>
<td>515-580</td>
<td>2</td>
<td>22.5 ± 1.5</td>
</tr>
<tr>
<td>UC$_{Ho}$</td>
<td>515-580</td>
<td>2</td>
<td>220.2 ± 2.5</td>
</tr>
<tr>
<td>UC$_{Tm}$</td>
<td>420-500</td>
<td>4</td>
<td>549.4 ± 26.8</td>
</tr>
</tbody>
</table>
Figure S8. Images of the UCNH UC\textsubscript{Tm}Rh\textsubscript{110} excited at 975 nm at 8µs/pixel dwell time in (A) C1, (B) C2 and (C) the composite of both images ($F_d=20.8$ J·cm\textsuperscript{-2}).

Table S3. Decay lifetimes obtained for bare-UC\textsubscript{Tm} and UC\textsubscript{Tm}Rh\textsubscript{110} ($\lambda_{\text{exc}}=975$ nm; $F_d=18.4$ J·cm\textsuperscript{-2}).

<table>
<thead>
<tr>
<th>Detection Channel</th>
<th>bare-UC\textsubscript{Tm}</th>
<th>UC\textsubscript{Tm}Rh\textsubscript{110}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Decay (SD)</td>
<td>Decay (SD)</td>
</tr>
<tr>
<td>C1</td>
<td>403.1 (12.4)</td>
<td>379.9 (15.9)</td>
</tr>
<tr>
<td>C2</td>
<td>213.7 (10.0)</td>
<td>213.7 (10.0)</td>
</tr>
</tbody>
</table>

SD: standard deviation.

Figure S9. Brightness magnified images of the same region of UC\textsubscript{Tm}Rh\textsubscript{101} obtained under (A) 975 nm excitation and 10 µs/pixel dwell time ($F_d=21.7$ J·cm\textsuperscript{-2}), and (B) 1030 nm excitation and 10 µs/pixel dwell time ($F_d=22$ J·cm\textsuperscript{-2}). (C) Shows both images merged; scale bar 50 µm.
Figure S10. Colocalization analysis of UC\textsubscript{Tm}@Rh\textsubscript{101}. (A) Raw image obtained in the C1 while exciting at 975 nm and 100 μs/pixel dwell time ($F_d = 218.5$ J·cm$^{-2}$). (B) Raw image obtained in the C3 while exciting at 1030 nm and 12.5 μs/pixel dwell time ($F_d = 27.5$ J·cm$^{-2}$). (C) Region of confined compartment (white) used for significance testing. Saturated agglomerates were discarded (black areas). Behavior of individual coefficients PCC (D), M1 (E) and M2 (F) versus the radial displacement (translations) performed by the CDA algorithm. The probability density function of the coefficients PCC (G), M1 (H) and M2 (I) between 10 to 22 radial displacement. The 95% of confidence interval is shown between colored arrows (blue, red and green for PCC, M1 and M2, respectively) while the original value appears as a pink line. Values obtained were significant.

Figure S11. Colocalization analysis of UC\textsubscript{Tm}@Rh\textsubscript{110}. (A) Raw image obtained in the C1 while exciting at 975 nm and 100 μs/pixel dwell time ($F_d = 260.5$ J·cm$^{-2}$). (B) Raw image obtained in the C2 while exciting at 1020 nm and 2 μs/pixel dwell time ($F_d = 4.4$ J·cm$^{-2}$). (C) Region of confined compartment (white) used for significance testing. Saturated agglomerates were discarded (black areas). Behavior of individual coefficients PCC (D), M1 (E) and M2 (F) versus the radial displacement (translations) performed by the CDA algorithm. The probability density function of the coefficients PCC (G), M1 (H) and M2 (I) between 15 to 25 radial displacement. The 95% of confidence interval is shown between colored arrows (blue, red and green for PCC, M1 and M2, respectively) while the original value appears as pink line. Values obtained were significant.
Figure S12. Intensity profile of a random agglomerate of UC\textsubscript{Tm}@Rh101 in the scan direction which causes an underestimation of M1. Black profile was obtained in the detection channel 1 (C1: 420-500 nm) by exciting at 975 nm at 100 µs/pixel and red profile was obtained in the detection channel 3 (C3: 590-650 nm) by exciting at 1030 nm at 12.5 µs/pixel.

Estimation of spatial resolution

The spatial resolution has been determined according to the Rayleigh criterion of confocal microscopes and afforded a lateral resolution of 473.6 nm. We are exciting with a spot which covers 2 pixels in the x-axis (1 pixel and two half pixels, Fig. S13), therefore this will be the maximum lateral resolution. In fact, as shown in Figure S12, we would have two different resolutions in a sample: the resolution of the multiphoton excitation of the dye and the resolution of the UCNP emission.

Figure S13. Schematic representation to scale of pixel size (white dashed squares, 0.5x0.5 µm\textsuperscript{2}) and diffraction-limited 975 nm laser spot (red circle, \textphi = 1.13 µm) over a SEM image of the nanoparticles sample. Yellow ellipse has been zoomed in to show a set of close-lying UCNPs.

References: