Controlled Multiple Functionalization of Mesoporous Silica Nanoparticles:
Homogeneous Implementation of pairs of Functionalities Communicating through
Energy or Proton Transfers.

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Supporting Information
Experimental section:

Materials:

α-cyclodextrin, 6-hydroxyquinoline, cetyltrimethylammonium bromide (CTAB), sodium ascorbate, rhodamine B and doxorubicin were purchased from Sigma Aldrich. Perfluorooctanoic acid (PFOA), copper sulphate pentahydrate, sodium N,N-diethylthiocarbamate trihydrate from Alfa Aesar. (3-Azidopropyl)triethoxysilane (AzPTES), N-propargyl bis(triethoxysilylpropyl)amine (Prec-Alk), N-propargyl 4-aminonaphthalimide, 1-azidomethylpyrene, N,N-(bispropargyl)anisidine were synthesized according to literature.

Synthesis of the clickable photoacid N-(2-azidoethyl)-6-hydroxyquinolinium iodide (AHQI):

In a two-necked round bottom flask equipped with a condenser, 6-hydroxyquinoline (1.0 g, 6.9 mmol) was suspended in 10 mL acetonitrile. 1-Azido 2-iodoethane (3.0 g, 15 mmol) was then added; the mixture was then stirred at 80 °C for 4 days (The reaction progress was monitored by $^1$H NMR). The acetonitrile was removed under reduced pressure and the product was washed with diethyl ether. After filtration and drying, AHQI was obtained as a dark green solid in 95 % yield. $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$ = 11.2 (s,1H, OH), 9.25 (d, 5.4 Hz, 1H), 9.11 (d, 8.5 Hz, 1H), 8.53 (d, 8.5 Hz, 1H), 8.08 (m, 1H), 7.75 (dd, 8.5 Hz and 1.5 Hz; 1H), 7.63 (d, 1.5 Hz, 1H), 5.20 (br, 2H), 4.06 (br, 2H). $^{13}$C NMR (101 MHz, DMSO-d$_6$) $\delta$ = 158.0, 146.7, 145.7, 132.2, 131.9, 127.6, 121.8, 120.9, 110.7, 56.0, 49.4. HRMS: calculated for C$_{11}$H$_4$N$_4$O$^+$: 215.0933 found 215.0935

Synthesis of bis(clickable) mesoporous silica nanoparticles (MSN):

To a solution of sodium hydroxide (2 M, 1.0 mL, 2.0 mmol) in distilled water (80 mL, 4.4 mol), was added CTAB (0.30 g, 0.80 mmol) (in the case of nanorods, PFOA was added at this step with 6 weight % relative to CTAB, 0.04 mmol). The mixture was heated to 80 °C and stirred vigorously (750 rpm) until the surfactant was completely dissolved. The silica source tetraethylorthosilicate (TEOS) ((1-2x) × 9 mmol), together with the clickable organosilanes...
AzPTES (x × 9 mmol) and Prec-Alk (x × 9 mmol) (x = 1, 2 or 5%) were added dropwise over 2 min. A white precipitate appeared and the reaction mixture was stirred at 80 °C for 2 h. The precipitate was then filtered, washed copiously with water and methanol and dried at RT. The molar ratio of the mixture was: Si/ CTAB/ PFOA/ NaOH/ Water: 1/ 0.09/ 0.004/ 0.22/ 490. The surfactant extraction was achieved by Soxhlet extraction (24 h) of the material with a mixture of 200 mL ethanol and 10 mL HCl 12 M. After filtration, the powder was washed with methanol and then dried at 70 °C for 6 h.

Procedure for successive CuAAC click reactions:

CuAAC 1: Bis(clickable) nanoparticles ((1+10 x) × 90 mg, 1.5 mmol) with x azide were firstly incubated with 3 eq of the propargylated partner; the energy acceptor (A) N-propargyl 4-aminonapthalimide (x × 1.12 g, x × 4.5 mmol) or the stalk (ST) N,N-bispropargylanisidine, (x × 0.89 g, x × 4.5 mmol) in the presence of copper sulphate pentahydrate (x × 13 mg, x × 0.3 mmol , 0.2 eq of N₃) and sodium ascorbate (x × 26 mg , x × 0.6 mmol, 0.4 eq of N₃) in 4 mL water/t-butanol mixture (v/v = 1). After sonicating for 5 min, the mixture was stirred vigorously at RT for 24 h. The nanoparticles were recovered by centrifugation (8000 rpm, 10 min) and washed with water, sodium N,N-diethylthiocarbamate (0.1 M in methanol, 10 mL), methanol (10 mL) and acetone (10 mL) until the clearness of the supernatant (4 ~ 6 times). The resulting material was dried at 70 °C for 6 h.

CuAAC 2: The resulting material with reactive propargyl groups, was incubated with 3 eq of the azide-clickable partner: the energy donor (D) azidomethylpyrene (x × 1.15 g, x × 4.5 mmol) or the photoacid generator (PAG) AHQI (x × 1.54 g, x × 4.5 mmol) under the same conditions as for the first CuAAC reaction. The afforded bisclicked nanoparticles are denoted NSₙ-DA (D and A are the donor and the acceptor) or NRₙ-PAG-ST (PAG and ST are the photoacid generator and the stalk respectively) while x = 1%, 2% or 5%.
Characterization:
The SEM images were obtained with a Hitachi S-4800 apparatus after platinum metallization. TEM micrographs were obtained using a JEOL 1200 EX2 apparatus equipped with a SIS Olympus Quemesa 11 Mpixel camera. FTIR spectra were recorded using a Perkin100 spectrometer equipped with a mono internal reflection ATR module. Raman spectra were recorded with a LabRAM ARAMIS (Horiba) spectrometer using a HeNe laser (633 nm). Absorption spectra were recorded using an Agilent 8453 UV-visible Spectroscopy System. X-ray diffraction was performed on XPERT Pro MPD PAnalytical with a power of 45 kV x 20 mA and a radiation of CuKα 1.5418 Å, data acquisition was realized in Bragg Brentano mode with slots of divergence 1/16 and anti-diffusion of 1/32°. N2 adsorption–desorption isotherms were obtained using a Micromeritics ASAP 2020 apparatus after outgassing the samples for 18 h at 40 °C. The specific surface areas were determined from the linear part of the BET transform of the adsorption isotherms in the range of 0.05 <p/p°< 0.2. NMR spectra were recorded with a 400 MHz Bruker spectrometer in dry CDCl3 at 298 K. 1H and 13C chemical shifts are reported in ppm relative to Me4Si. Mass spectrometry was carried out in the platform of physico-chemical analyses of the IBMM, University of Montpellier.

Loading and release procedures:

In order to monitor the ability of the designed system to induce light-controlled activation, the nanoparticles were loaded with a fluorescent dye (rhodamine B). In vitro tests were then carried out on the nanoparticles loaded by doxorubicin. These tests were performed with nanorods because of their higher loading capacity.

Loading: A solution of rhodamine B (5 mM) or doxorubicin (3 mM) in water (1 mL) was added to 3 mg of functionalized nanoparticles. The mixture was sonicated for 10 min and stirred vigorously at room temperature for 24 h in the dark. After 2 washings, α-cyclodextrin (6 mg) was added in water (1 mL) and the medium was sonicated for 5 min and stirred in the dark for 48 h. After successive washings with water (about 10 times centrifugation 22000 rpm, 5 min) to eliminate the dye which is not retained in the pores, the particles were kept overnight at RT for drying.
**Release procedure**: The nanoparticles loaded by rhodamine B were placed in a *cuvette* and slightly ground with the spatula to improve the diffusion of the dye. Distilled water was carefully added to ensure that no particles are floating into the aqueous phase. After the baseline recording, the *cuvette* was placed under 365 nm irradiation (See details in the Supporting Information, Figure S6) and UV-vis spectra of the solution were regularly recorded at different time intervals to track the released rhodamine B at 553 nm.

**In vitro experiments on breast cancer cells, imaging, cytotoxicity and drug delivery:**

Human breast cancer cells (MCF-7) were purchased from ATCC (American Type Culture Collection, Manassas, VA) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM-F12) supplemented with 10% fetal bovine serum and 50 μg mL⁻¹ gentamycin. Cells were allowed to grow in a humidified atmosphere at 37°C under 5% CO₂.

The day prior to the experiment, MCF7 cells were seeded onto the bottom of sterile glass dishes (World Precision Instrument, Stevenage, UK) at a density of 10⁶ cells cm⁻². After 24 h, the cells were washed (once) and incubated in 1 mL medium containing the nanorods loaded with rhodamine B at a concentration of 50 μg mL⁻¹ for 20 h. Before visualization, cells were washed gently with phenol red-free DMEM. Cells were then scanned with a LSM 780 LIVE confocal microscope (Carl Zeiss, Le Pecq, France), at 405 nm with a slice depth (Z stack) of 0.62 μm.

For photo-induced drug release, MCF-7 cells were seeded into 384-well plates at 10³ cells per well in 100 μL culture medium and allowed to grow for 24 h. Then cells were incubated for 20 h with nanorods at a concentration of 50 μg mL⁻¹. After incubation, the cells were submitted to laser irradiation for 15 sec (405 nm, 18 mW cm⁻², 9 J cm⁻²). After two days, a cell proliferation assay was performed (MTS) to quantify the cell death.
Figure S1: FTIR and Raman spectra of parent bisclickable (left) nanorods and (right) nanospheres.
Figure S2: Images obtained by (left) SEM and (right) TEM of a) NR2 b) NS2 and c) NS5 SEM shows nanospheres with narrow sizes (100-150 nm). TEM shows ordered pore channels and the inset in b) is the FFT of the selected part of the image indicating a 2D-hexagonal structure.
Figure S3: (left) X-ray diffractograms and (right) N\textsubscript{2} physisorption of parent bisclickable nanospheres. (inset: BJH plot)

Figure S4: Spectral overlap between the emission of the donor and the excitation of the acceptor

Figure S5: SEM of NS2-DA
In order to confirm that the energy transfer is intraparticular (interaction of donor and acceptor within the same nanoparticles) instead of interparticle interaction at high concentration, one of the samples (NS1-DA) was diluted from 10 to 50 times (from 300 to 30, 15, 10 and 6 mg/L) and the emission spectra were recorded for the samples. The ratio “r” of emission intensities maxima between pyrene (395 nm) and naphthalimide (525 nm) does not decrease with the concentration confirming that an intraparticular FRET is occurring.

**Figure S6**: Emission spectra of NS1-DA recorded after several dilutions. (Excitation wavelength = 343 nm)
Figure S7: (Above) Molecular structure of the photoacid and the stalk and (below) light excitation setup.

Figure S8: Absorption spectra of (red) NR5-PAG-ST and (black) NR2-PAG-ST. Dashed spectra are (orange) the corresponding anisidine monoclicked nanoparticles NR2-ST, (green) PAG in ethanol and (brown) ST in ethanol.
Figure S9: (left) FTIR spectra of bisclicked nanoparticles: (blue) 2% and (orange) 5% nanospheres with (red) 2% and (green) 5% nanorods. Dashed lines correspond to the (green) AHQI (PAG) and (brown) Alk-anisidine (ST) precursors. Inset: zoom on N\textsubscript{3} peak in the zone 2050-2150 cm\textsuperscript{-1} (right) UV-vis spectra of the clicked nanospheres.

Figure S10: N\textsubscript{2} sorption isotherms of the (black) parent and (red) 2% (left) and 5% (right) bisclicked nanospheres
Figure S11: Evolution of the (left) specific surface area and (right) total pore volume in (blue cones) parent and (red cones) bisclicked nanoparticles.

Figure S12: Absorption spectra of the photoacid PAG and the stalk ST with the used wavelength corresponding to release experiments with rhodamine B and in vitro tests with doxorubicin.
**Figure S13**: Release profiles of rhodamine B: (a) in pure water with bulk acidification for control NR2-ST; sequential on/off cycles (b) in pure water and (c) in Tris buffer. Red, black and green traces correspond to NR2-PAG-ST, NR5-PAG-ST and NR2-ST, respectively.
**Figure S14**: Cytotoxicity of nanorods loaded with doxorubicin, without photo-activation. MCF-7 cells were treated with NR2-PAG-ST (blue) and NR5-PAG-ST (pink) with a concentration range of 1 to 100 µg.mL⁻¹. Three days after treatment, a cell proliferation assay was performed (MTS).

**Scheme S1**: Synthetic route for obtaining bifunctional nanorods from original precursors *via* CuAAC click reaction.
Table S1: textural parameters of parent bisclickable nanospheres

<table>
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<tr>
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<th>d_{10}</th>
<th>BET surface area (m²/g)</th>
<th>Uptake at saturation (cm³/g)</th>
<th>BJH most probable pore diameter (Å)</th>
<th>Total pore volume (cm³/g)</th>
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<tr>
<td>NS2</td>
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<tr>
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Table S2: Concentrations of clicked photoacid and stalk on nanoparticles and the corresponding functionalization rate.

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<tr>
<td>ε (M cm⁻¹)</td>
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<td>4800 (λ = 261 nm)</td>
<td>13200 (λ = 265 nm)</td>
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<td>x</td>
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<table>
<thead>
<tr>
<th></th>
<th>NR x-PAG-ST</th>
<th>NS x-PAG-ST</th>
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<tbody>
<tr>
<td>ε (M cm⁻¹)</td>
<td>0.16 (35%)</td>
<td>0.34 (29%)</td>
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<tr>
<td>x</td>
<td>0.05 (16%)</td>
<td>0.11 (13%)</td>
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<td>0.29 (65%)</td>
<td>0.41 (38%)</td>
<td>0.10 (30%)</td>
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References:


