



Supporting Information

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Enhanced Two-Photon Fluorescence Imaging and Therapy of
Cancer Cells via Gold@Bridged Silsesquioxane Nanoparticles

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I. GENERAL PROCEDURES

Materials. THF, diisopropylamine, bis(triphenylphosphine) dichloro-palladium, copper iodide tetraethoxysilane, cetyltrimethylammonium bromide, sodium hydroxide, dimethylsulfoxide, sodium azide potassium tert-butoxide, and chloropropyltriethoxysilane were purchased from Sigma-Aldrich. Absolute ethanol was purchased from Fisher Chemicals. Tin chloride, 4-nitrocinnamadehyde, (4-nitrobenzyl)triphenylphosphonium bromide, iodine, sodium thiosulfate, were purchased from Alfa Aesar. Hydrochloric acid was purchased from Anal. R. Norma Pure. (3-azidopropyl)trimethoxysilane was obtained from a reported procedure by M. Ortega-Muñoz et al (*Adv. Synth. Catal.* **2006**, 348, 2410). THF and diisopropylamine were dried over sodium/benzophenone and CaH₂ respectively.

Methods. ¹H and ¹³C NMR spectra were recorded with a Bruker AC 400 spectrometer. Chemical shifts (in δ units, ppm) are referenced to TMS using CHCl₃ (δ = 7.26 ppm) and CDCl₃ (δ = 77.0 ppm) as the internal standards, respectively, for ¹H and ¹³C NMR spectra. IR spectra were recorded on a Perkin-Elmer 100 FT spectrophotometer. Absorption spectra were recorded on a Hewlett-Packard 8453 spectrophotometer and fluorescence data were collected on a Perkin-Elmer LS55 fluorimeter. Mass spectrometry was carried out at the Laboratoire de Spectrométrie de Masse (Lyon, France) with a Thermo-Finnigan MAT95 apparatus in electrospray ionization mode. Dynamic light scattering analyses were performed using a Cordouan Technologies DL 135 Particle size analyzer instrument. ²⁹Si and ¹³C CPMAS solid state NMR sequences were recorded with a VARIAN VNMR300, using Q8MH8 and adamantane references respectively. TEM images were recorded with a JEOL instrument. SEM images were recorded with a FEI instrument. Energy dispersive spectroscopy was performed *via* an FEI scanning electron microscope.

II. SYNTHESIS AND CHARACTERIZATION OF THE TWO-PHOTON PRECURSOR

2PS synthesis. A general scheme of this multistep synthesis is presented in figure S1, each step is numbered and described as follows.

1) Wittig reaction: To a mixture composed of absolute ethanol (100 mL), 4-nitrocinnamadehyde (9.35 g, 52.7 mmol), and (4-nitrobenzyl)triphenylphosphonium bromide (25.35 g, 52.7 mmol) stirred in a 1 L round-bottom flask, was added a solution of potassium tert-butoxide (17.1 g, 152.4 mmol) in absolute ethanol (300 mL) via cannula. The reaction mixture was stirred for 24 h at room temperature. Afterwards, deionized water (250 mL) was added to precipitate the product, which was collected through filtration and washed with an ethanol/water mixture (60:40 v:v).

2) Photoisomerization: The crude product was dissolved in anhydrous tetrahydrofuran (THF, 300 mL) in a 1 L round-bottom flask to which an iodine (I₂, 40 mg) solution in THF (30 mL) was added. 365 nm irradiation (75 W) was applied for 42 h. A saturated aqueous solution of sodium thiosulfate (100 mL, 275 g) was then poured to induce the precipitation. The resulting mixture was stirred for 40 minutes, and filtered. The precipitate was dried under vacuum. The overall yield of these two reactions was of 99%, leading to (*E,E*)-bis(4-nitrophenyl)butadiene (15.4 g, 52 mmol).

3) Reduction of the nitro groups: The resulting (*E,E*)-photoisomerized compound (15.4 g, 52.0 mmol) was stirred overnight at 73 °C with tin chloride (93.1 g, 491 mmol) in absolute ethanol (200 mL) in a two neck 500 mL round-bottom flask previously flushed with argon. Then, an aqueous solution of sodium hydroxide (NaOH, 10 g, 250 mmol, H₂O, 160 mL) was slowly added until the solution reached a pH of 8, which induced the precipitation of the tin by-products. The compound was mixed with ethyl acetate (600 mL) in a 1 L round-bottom flask, and vigorously stirred for 12 h under reflux. Finally, the resulting mixture was filtered, and the solid was subjected again to a 12 h extraction at reflux. The operation was repeated several times until complete extraction. The yield of the reaction was of 92%, leading to the bis(4-aminophenyl)butadiene (11.3 g, 47.8 mmol).

4) Propargylation: To the as-prepared diamine (3.0 g, 12.7 mmol) dissolved in an ethanol/water mixture (80:20 v:v, 100 mL), was added potassium carbonate (12 g, 86.8 mmol), and the solution was stirred vigorously under reflux. Propargyl bromide (80% in toluene, 10 mL, 89.8 mmol) was added through micropipette, and the reaction mixture was refluxed for 3 days. The product was purified via chromatographic separation using a mixture of dichloromethane/pentane (80/20, v:v) as eluent. The yield of the reaction was of 75% (down to 55% when the reaction was scaled-up by 4), leading to the tetrapropargylyed compound (3.7 g, 9.5 mmol). ^1H NMR (200 MHz, CDCl_3): δ 7.42 (d, $^3\text{J} = 8.75$ Hz, 4H, $\underline{\text{H}}_{\text{Ph-butadiene}}$), 6.97 (d, $^3\text{J} = 8.78$ Hz, 4H, $\underline{\text{H}}_{\text{Ph-amine}}$), 6.82 (dd, $^3\text{J} = 12.17, 2.89$ Hz, 2H vinyl), 6.57 (dd, $^3\text{J} = 11.9, 2.73$ Hz, 2H, vinyl central), 4.17 (d, $^4\text{J} = 2.27$ Hz, 8H, N- $\underline{\text{CH}}_2$), 2.30 (t, $^4\text{J} = 2.2$ Hz, 4H, $\text{CH}_2\text{-C}\equiv\text{C}\underline{\text{H}}$). ^{13}C NMR (400 MHz, CDCl_3): δ 131.2, 129.4, 129.1, 127.3, 127.2, 115.6, 79.1, 72.8, 40.4. IR (neat KBr) $\nu_{\text{max}}/\text{cm}^{-1} = 3257, 3014, 2827, 2105, 1636, 1601, 1560, 1541, 1511, 1457, 1418, 1365, 1346, 1323, 1292, 1256, 1237, 1215, 1185, 1145, 1117, 916, 894, 847, 804, 751, 700, 684, 597, 513, 450, 423$. UV/Vis λ_{max} (EtOH): 368 nm. MS (ESI+) m/z (%): 350.2 (100) [trialkyl fragment], 388.2 (19) [$\text{M}^{\bullet+}$], 389.2 (17) [MH^+], 390.2 [$\text{MH}^+ \text{ }^{13}\text{C}$]. HRMS (ESI+): m/z calcd for $\text{C}_{28}\text{H}_{24}\text{N}_2$: 388.1939, found 388.1936. The rare phenomenon of the formation of the [$\text{M}^{\bullet+}$] does occur in this ESI+ analysis due to the conjugated structure of the molecule (see also 2PS Ref). This is consistent with the intensities of the peaks of the [$\text{M}^{\bullet+}$] (19), and [MH^+] (17) which do not correspond to the isotopic proportions.

5) CuAAC coupling: To a mixture of the tetrapropargylyed precursor (200 mg, $5.15 \cdot 10^{-1}$ mmol) and [$\text{CuBr}(\text{PPh}_3)_3$] (15 mg, $1.6 \cdot 10^{-2}$ mmol) in anhydrous tetrahydrofuran (THF, 2.5 mL) placed in a 10 mL microwave sealable reactor was added (3-azidopropyl)triethoxysilane (510 mg, 2.06 mmol). The tube was flushed with argon, then microwave irradiation (max power 200 W, 100 °C, 15 min) was applied. After evaporation of the solvents, extraction with pentane and concentration, the 2PS was quantitatively obtained as a slightly brown solid (710 mg, $5.15 \cdot 10^{-1}$ mmol). ^1H NMR (200 MHz, CDCl_3): δ 7.48 (d, $^3\text{J} = 6.0$ Hz, 4H, $\underline{\text{H}}_{\text{Ph-butadiene}}$), 7.30 (s, 4H, triazole), 6.89 (d, $^3\text{J} = 6.5$ Hz, 4H, $\underline{\text{H}}_{\text{Ph-amine}}$), 6.78 (d, $^3\text{J} = 14.4$ Hz, 2H, vinyl), 6.53 (d, $^3\text{J} = 12.2$ Hz, 2H, vinyl central), 4.71 (s, 8H, N- $\underline{\text{CH}}_2$ -triazol), 4.31 (t, $^3\text{J} = 7.0$ Hz, 8H, Triazol- $\underline{\text{CH}}_2$), 3.80 (q, $^3\text{J} = 6.9$ Hz, 24H, O- $\underline{\text{CH}}_2$ - CH_3), 1.99 (t, $^3\text{J} = 7.8$ Hz, 8H, Triazol- CH_2 - $\underline{\text{CH}}_2$), 1.21 (t, $^3\text{J} = 6.8$ Hz, 36H, , O- CH_2 - $\underline{\text{CH}}_3$), 0.57 (t, $^3\text{J} = 7.9$ Hz, 8H, $\underline{\text{CH}}_2$ -Si). ^{13}C NMR (400 MHz, CDCl_3): δ 147.3, 145.2, 130.9, 127.7, 127.5, 126.6, 122.1, 113.7, 58.6, 52.6, 47.0, 24.3, 18.4, 8.1. ^{29}Si NMR (400 MHz, CDCl_3): δ -46.9. IR (neat KBr) $\nu_{\text{max}}/\text{cm}^{-1} = 3117, 3068, 3013, 2950, 2929, 2857, 1604, 1562, 1521, 1457, 1374, 1337, 1299, 1261, 1213, 1186, 1102, 1053, 1025, 982, 941, 835, 808, 793, 577, 518$. UV/Vis λ_{max} (EtOH): 374 nm. Emission (EtOH): $\lambda_{\text{max}} = 416, 438$ nm ($\lambda_{\text{excitation}} = 388$ nm). MS (ESI+) m/z (%): 279.0 (100) [OPPh_3],

689.2 (7) $[MH_2^{2+}]$, 1378.74 (1) $[MH^+]$. HRMS (ESI+): m/z calcd for $C_{64}H_{109}N_{14}O_{12}Si_4$: 1377.7427, found 1377.7426.

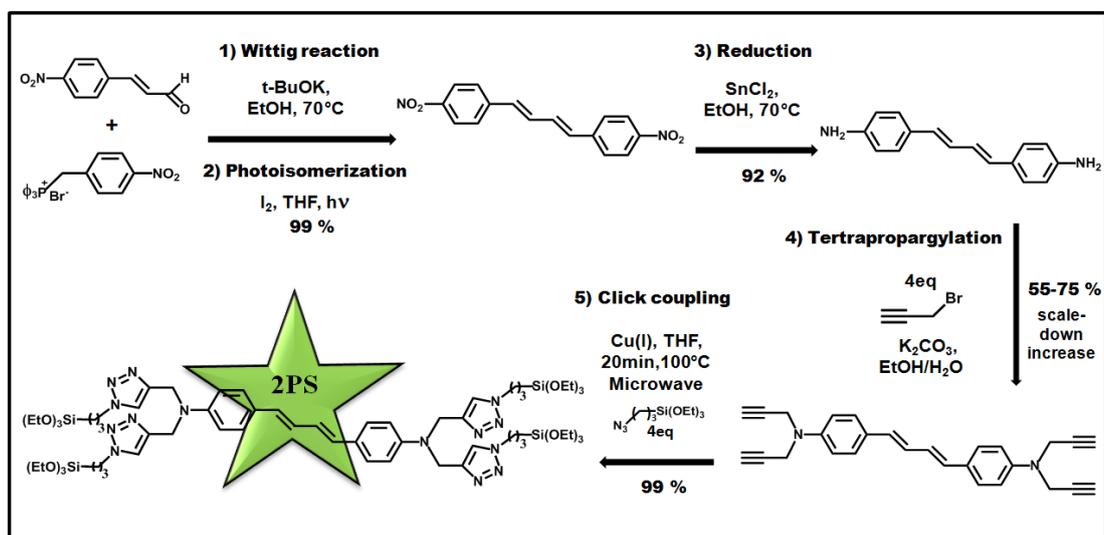


Figure S1. Multistep synthetic pathway to design the two-photon photosensitizer (2PS).

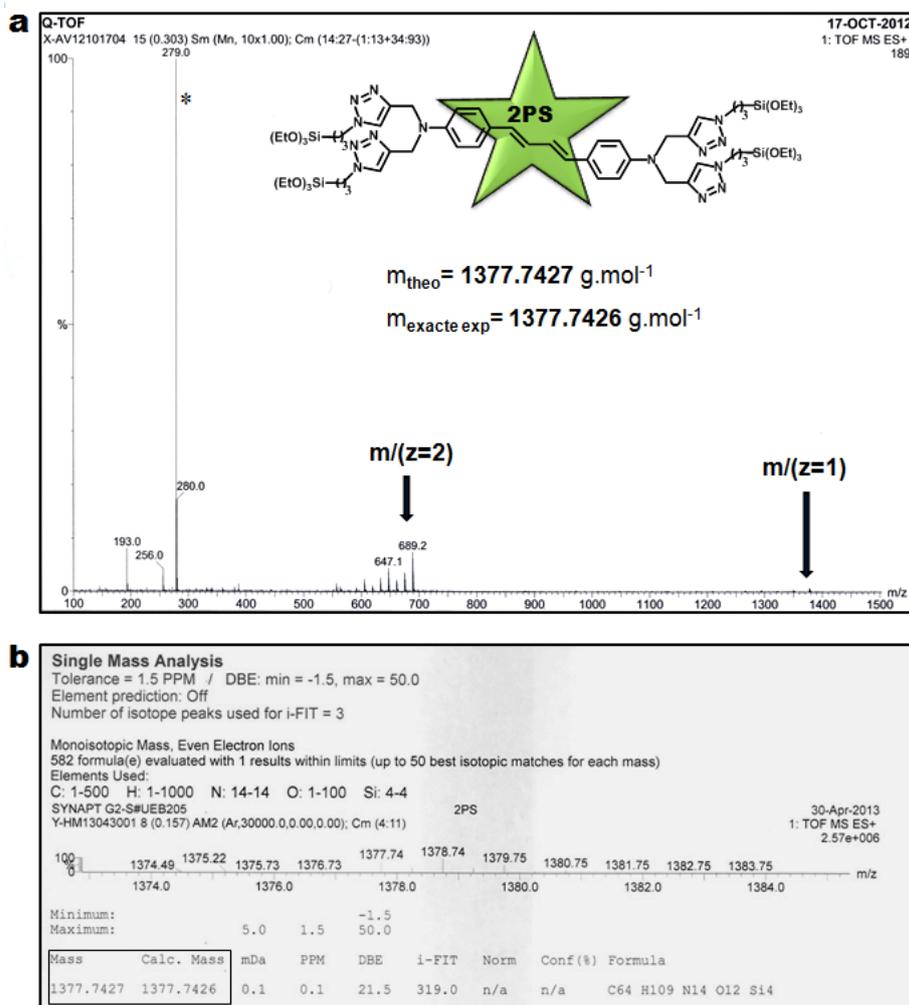


Figure S2. Q-TOF high resolution mass spectrum of the 2PS (a), and the high resolution single mass analysis (b). *Triphenylphosphine oxide.

III. SYNTHESIS AND CHARACTERIZATION OF THE TWO-PHOTON REFERENCE

2PS ref synthesis. The synthesis of the spectroscopic reference was done by modifying the fifth step of the 2PS procedure using hexylazide (262 mg, 2.06 mmol) as click partner through microwave irradiation (max power = 200 W, 100 °C, 14 min) (see Figure S3 below). The reaction was also quantitative (463 mg, $5.15 \cdot 10^{-1}$ mmol). ^1H NMR (200 MHz, CDCl_3): δ 7.49 (d, $^3J = 6.5$ Hz, 4H, $\underline{\text{H}}_{\text{ph-butadiene}}$), 7.33 (s, 4H, triazol), 6.94 (d, $^3J = 7.4$ Hz, 4H, $\underline{\text{H}}_{\text{ph-amine}}$), 6.78 (d, $^3J = 14.2$ Hz,

2H, vinyl), 6.51 (d, $^3J = 13.8$ Hz, 2H, vinyl central), 4.73 (s, 8H, N-CH₂), 4.31 (t, $^3J = 7.2$ Hz, 8H, Triazol-CH₂), 1.88 (m, 8H, Triazol-CH₂-CH₂), 1.30 (m, 24H, CH₂ alkyl), 0.90 (t, $^3J = 7.9$ Hz, 12H, CH₃ alkyl). ¹³C NMR (300 MHz, CDCl₃): δ 147.4, 145.5, 131.2, 130.8, 127.7, 127.0, 122.3, 114.2, 50.8, 47.4, 31.5, 30.6, 26.5, 22.8, 14.3. IR (neat KBr) $\nu_{\max}/\text{cm}^{-1} = 3119, 3016, 2957, 2929, 2857, 1605, 1514, 1503, 1462, 1371, 1333, 1296, 1201, 1186, 1142, 1101, 1054, 1026, 981, 941, 835, 793, 512, 476$. UV/Vis λ_{\max} (EtOH): 378 nm. Emission (EtOH): $\lambda_{\max} = 442$ nm ($\lambda_{\text{excitation}} = 388$ nm). MS (ESI+) m/z (%): 279.1 (6) [OPPh₃], 449.3 (100) [MH₂²⁺], 732.5 (8) [trialkyl fragment], 897.7 (27) [M^{•+}], 898.7 (15) [MH⁺]. HRMS (ESI+): m/z calcd for C₅₂H₇₇N₁₄: 897.6457, found 897.6456.

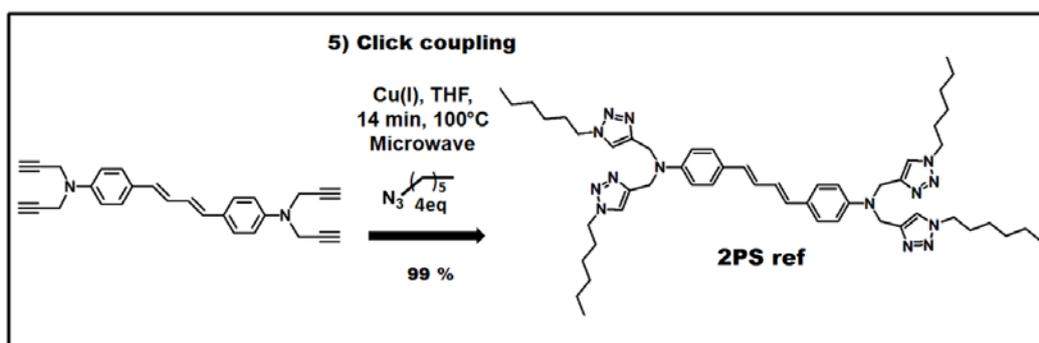


Figure S3. Modified CuAAC reaction to design the two-photon photosensitizer reference (2PS ref).

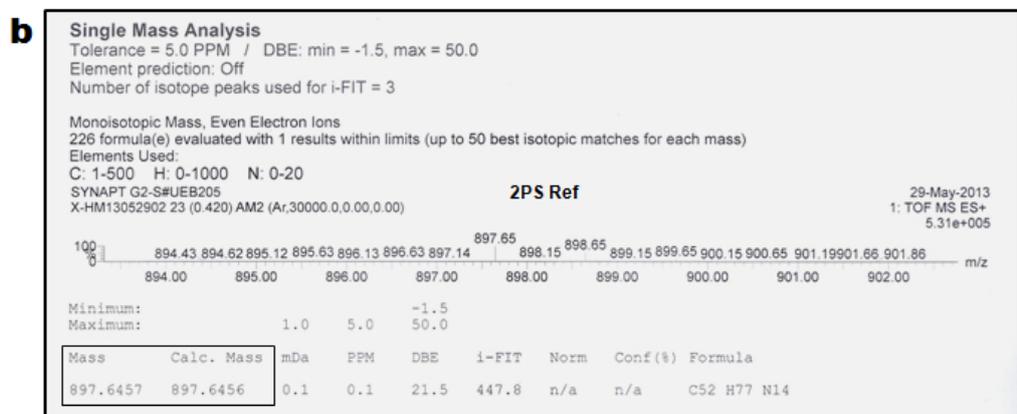
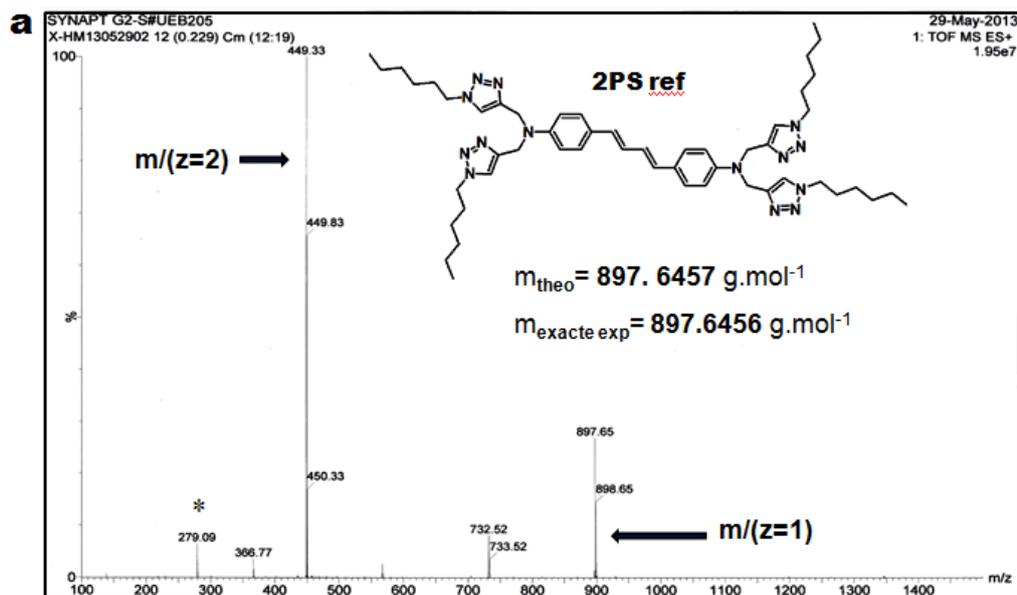


Figure S4. Q-TOF high resolution mass spectrum of the 2PS ref (a), and the high resolution single mass analysis (b). *Triphenylphosphine oxide.

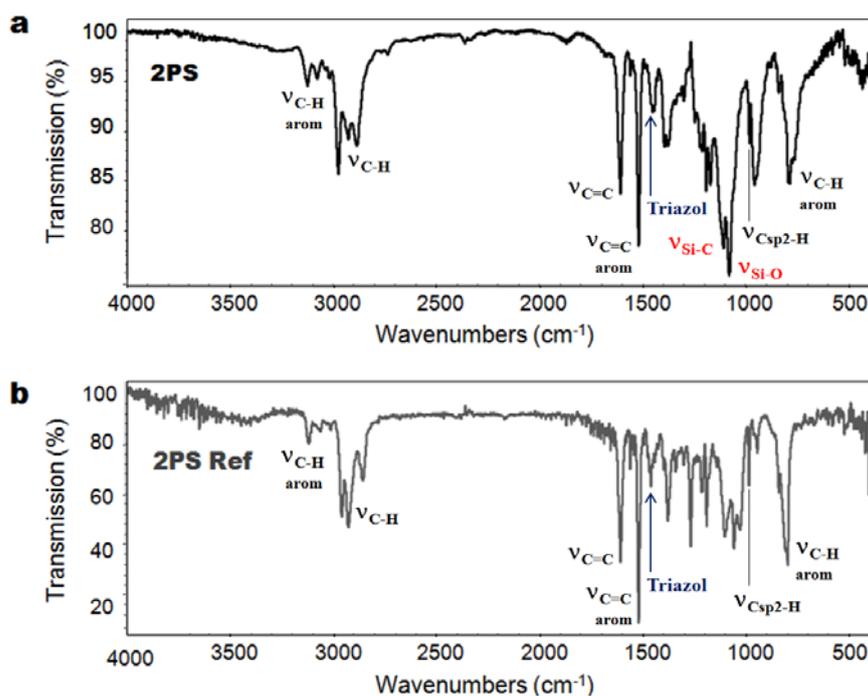


Figure S5. FTIR spectra comparison of the 2PS and the 2PS ref compounds (a and b respectively), validating the absence of the Si-O bonds ($1000\text{-}1100\text{ cm}^{-1}$) in the 2PS ref, while retaining the C=C conjugated structure ($1100\text{-}1650\text{ cm}^{-1}$).

IV. SYNTHESIS AND CHARACTERIZATION OF THE BRIDGED SILSESQUIOXANE NPs

BSNPs (with Et_3N catalyst). A mixture composed of water (100 mL), ethanol (40 mL), cetyltrimethylammonium bromide (0.640 g, 1.76 mmol), and triethylamine (15 μL) was stirred at 80 °C in a 250 mL round-bottom flask. Then a solution of the 2PS precursor in anhydrous ethanol (2PS: 88.8 mg, $5.4 \cdot 10^{-2}$ mmol; EtOH: 1000 μL) was quickly injected. The reaction was conducted for 2 minutes at 80 °C, the mixture was cooled down for ten minutes, and the nanomaterial was collected through centrifugation at 21000 rpm for 10 minutes. At this point, the NPs could be either washed and dried as described for BSNPs with NaOH catalyst, or mixed with gold nanospheres as follows.

Au@BSNPs. A mixture of water (50 mL), ethanol (20 mL), and cetyltrimethylammonium bromide (0.320 g, $8.80 \cdot 10^{-1}$ mmol) in a 100 mL three neck round-bottom flask was stirred at 70 °C. Then an aqueous solution of potassium tetrachloroaurate (KAuCl₄, 26 mg, $6.90 \cdot 10^{-2}$ mmol; H₂O, 2 mL) was injected. The mixture was stirred for 5 minutes. Sodium hydroxide (NaOH, 200 μL (2 M)) was injected to induce the instantaneous nucleation of the nanoparticles. The nanoparticles growth was conducted for 20 minutes under a 600 rpm stirring. Afterwards, tetraethylorthosilicate (25 μL, $1.12 \cdot 10^{-4}$ mol) and sodium hydroxide (NaOH, 200 μL (2 M)) were added dropwise in order to stabilize the gold nanocrystals with a thin silica shell. After five minutes of stirring, a solution of the 2PS precursor in anhydrous ethanol (2PS: 88 mg, $6.39 \cdot 10^{-2}$ mmol; EtOH: 1 mL) was quickly injected. The reaction mixture was stirred for 10 minutes, and the solution was neutralized by adding hydrochloric acid solution (0.2 M, *ca* 1.38 mL), and cooled to room temperature while stirring. The solid was gathered through centrifugation at 7830 rpm for 15 minutes. Each fraction was washed with ethanol (40 mL per fraction) and centrifuged for 10 minutes. This operation was repeated twice, and the compound was dried under vacuum for few hours.

BS@AuNPs. A mixture of water (25 mL), ethanol (10 mL), and cetyltrimethylammonium bromide (0.160 g, $4.40 \cdot 10^{-1}$ mmol) in a 100 mL three neck round-bottom flask was stirred at 70 °C. Then an aqueous solution of potassium tetrachloroaurate (KAuCl₄, 13 mg, $3.45 \cdot 10^{-2}$ mmol; H₂O, 1 mL) was injected. The mixture was stirred for 5 minutes. Sodium hydroxide (NaOH, 100 μL (2 M)) was injected to induce the instantaneous nucleation of the nanoparticles. After 30 seconds, hydrochloric acid (HCl, 18 μL (2M)) was added, and the nanoparticles growth was conducted for 12 minutes under a 600 rpm stirring, and the temperature was set at 80 °C. Secondly, freshly prepared BSNPS were redispersed in deionized water (25 mL) and added to the previous solution. The reaction was left for 10 minutes, then the mixture was cooled down to 40 °C and centrifuged at 21000 rpm for 10 minutes. The nanomaterial was washed with a water:ethanol mixture (5:2, v:v) and centrifuged for 10 minutes. This operation was repeated twice, and the compound was dried under vacuum for few hours.

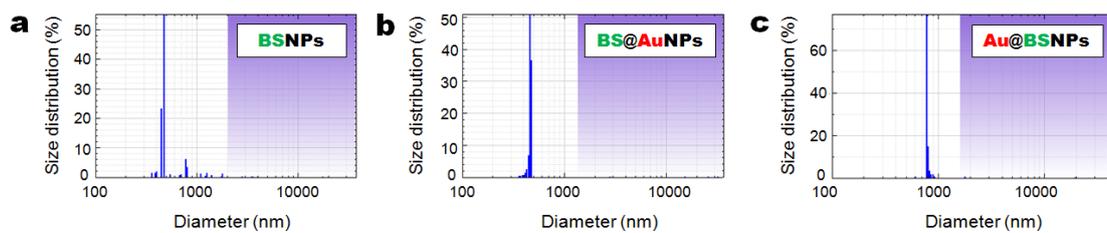


Figure S6. DLS size distributions of BSNPs (a), BS@AuNPs (b), and Au@BSNPs (c).

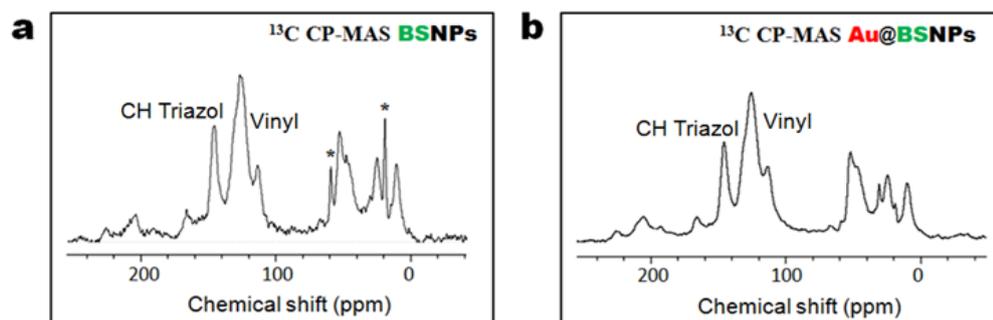


Figure S7. Solid state ^{13}C CP-MAS NMR spectra of BSNPs (a) and Au@BSNPs (b). *Si-O-CH₂CH₃.

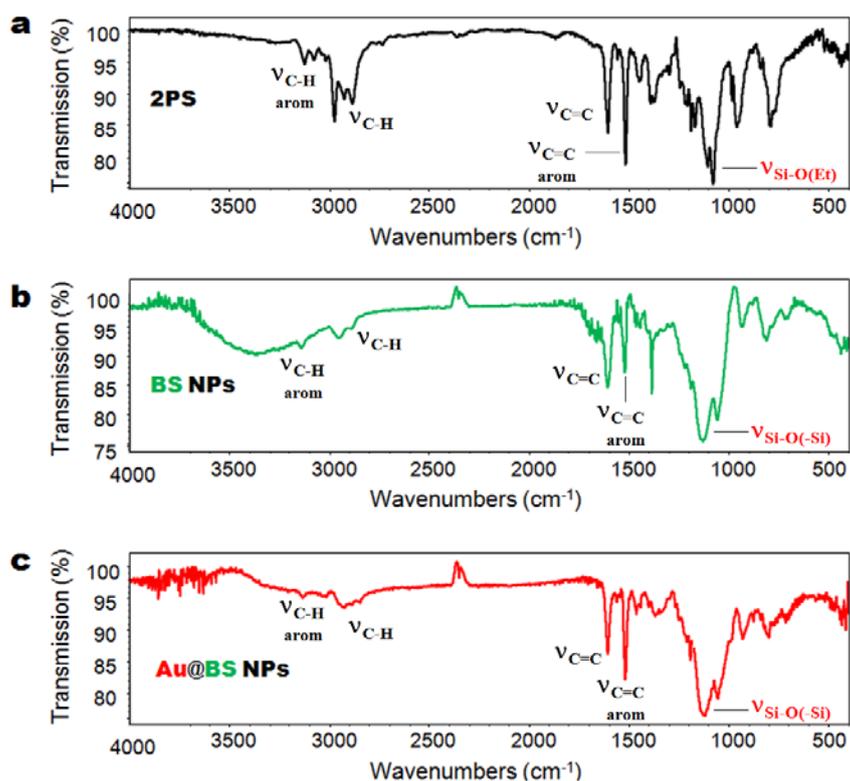


Figure S8. FTIR spectra comparison of the 2PS precursor (a), BS NPs (b), and Au@BS NPs confirming the sol-gel condensation and preservation of the organic structure of the photosensitizer.

Sample	9.0 wt% Au Control ^[a]	Au@BS NPs	BS@Au NPs
Gold wt %	9.76	24.12	25.10 ^[b]

Table S1. Induced-coupled plasma-atomic emission spectroscopy (ICP-AES) determination of the gold weight percent (wt %) in Au@BS NPs. [a] ICP-AES gold control: bare gold nanoparticles, made from the same procedure (but without 2PS), were mixed with a mesoporous silica powder (theoretical ratio of 9.0 wt% in gold) to determine the accuracy of the ICP calibration. [b] Energy dispersive spectroscopy gold weight percent measurement of BS@Au NPs.

V. IN-VITRO TWO-PHOTON STUDIES

Two-photon fluorescence imaging. The day prior to the experiment, MCF7 cells were seeded onto bottom glass dishes (World Precision Instrument, Stevenage, UK) at a density of 10^6 cells.cm⁻². Adherent cells were then washed once and incubated in 1 mL culture medium containing nanoparticles at a concentration of 40 $\mu\text{g.mL}^{-1}$ for 20 h. Fifteen minutes before the end of incubation, cells were loaded with Cell Mask (Invitrogen, Cergy Pontoise, France) for membrane staining at a final concentration of 5 $\mu\text{g.mL}^{-1}$. 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 760 nm with a slice depth (Z stack) of 0.62 μm .

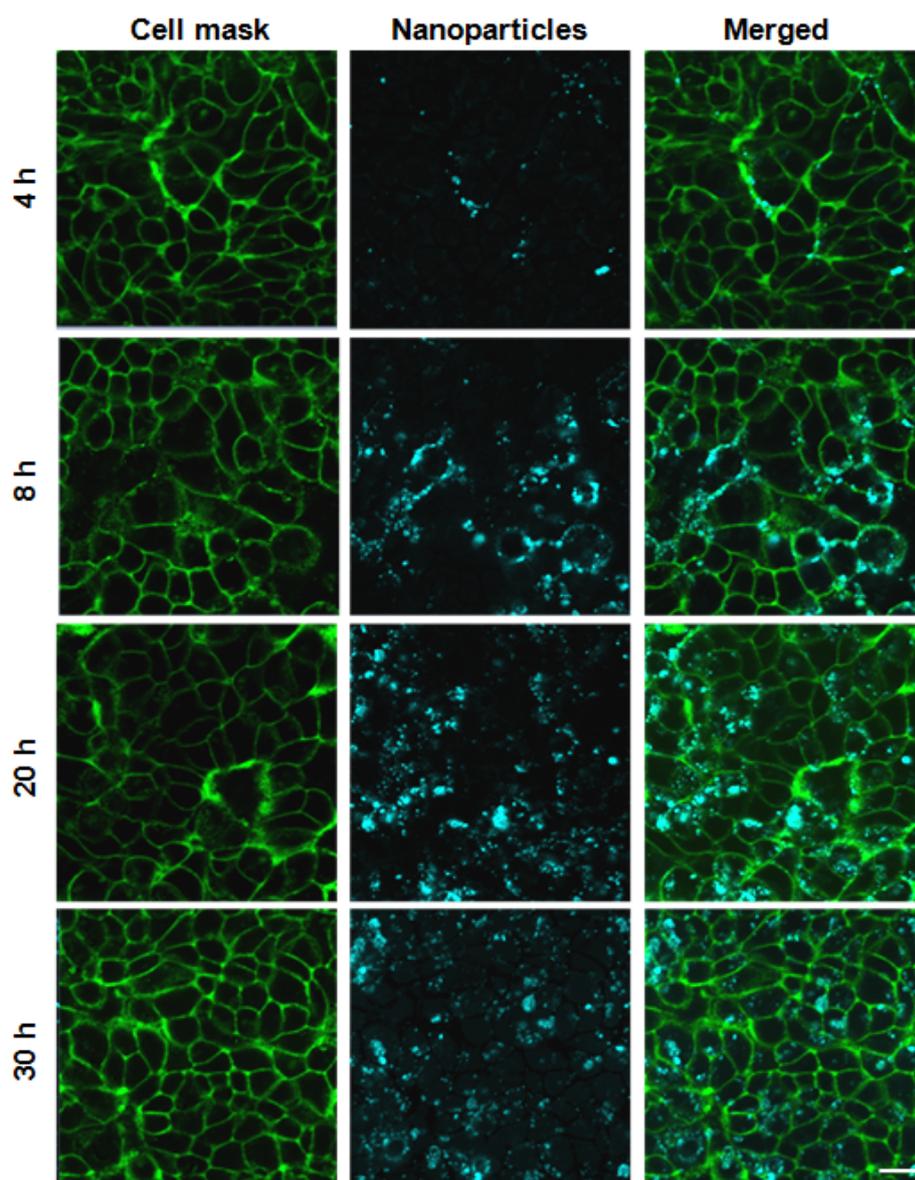


Figure S9. Time course of two-photon imaging on MCF-7 cells treated with $40 \mu\text{g.mL}^{-1}$ of BS@AuNPs. Scale bar $5 \mu\text{m}$.

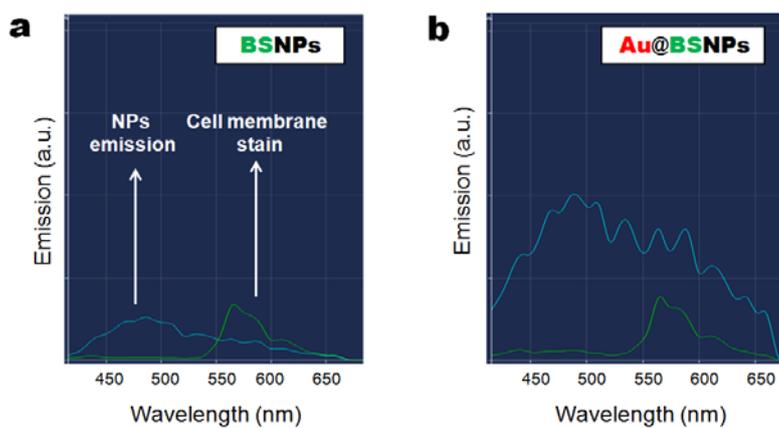


Figure S10. Fluorescence emission spectra of BSNPs and Au@BSNPs *in-vitro* (a and b respectively).

Comment [x1]: Integrated on all the image ???

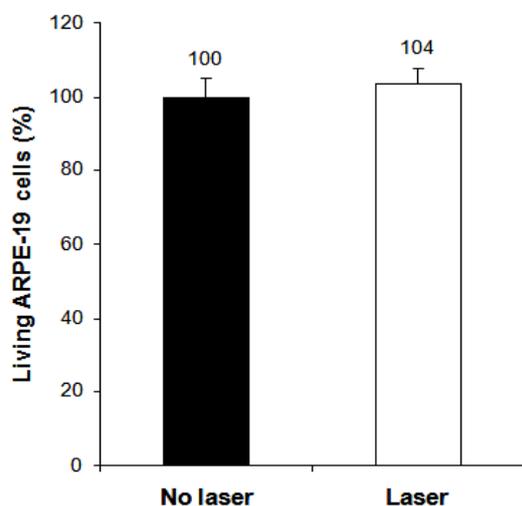


Figure S11. Impact of the laser irradiation on normal epithelial cells. Bar graphs demonstrate the absence of irradiation-induced cytotoxicity on the ARPE-19 growth. Values represent the mean \pm SD of two independent experiments realized in triplicate.