Supporting information

Mesoporous silicon nanoparticles for targeted two-photon theranostic of prostate cancer

I. Experimental section

Preparation of pSiNPs

Boron-doped p^{++}-type Si (0.8-1.2 mΩ.cm resistivity, <100> orientation) from Siltronix (France) was electrochemically etched in a 3:1 (v:v) solution of aqueous 48% hydrofluoric acid (HF):absolute ethanol (Sigma-Aldrich). Etching was performed in a Teflon cell with a platinum ring counter electrode. A constant current of 170 $mA.cm^{-2}$ was applied for 150 s, and the sample was rinsed 3 times with ethanol. The porous layer was then removed from the substrate by application of a constant current of 4 $mA.cm^{-2}$ for 250 s in an electrolyte solution containing 1:20 (v:v) aqueous 48% hydrofluoric acid:absolute ethanol. After 3 rinses with ethanol, the porous layer was placed in ethanol in a glass vial. After degassing the sample for 20 min under a nitrogen stream, the porous silicon film was fractured by ultrasonication during 16 h. The largest particles were removed by spinning them down by centrifugation at 3,000 rpm for 2 min (Minispin, Eppendorf). In order to remove the smallest particles, the solution was finally centrifuged at 14,000 rpm for 30 min (centrifuge Eppendorf 5804). The pellet was then redispersed in absolute ethanol.

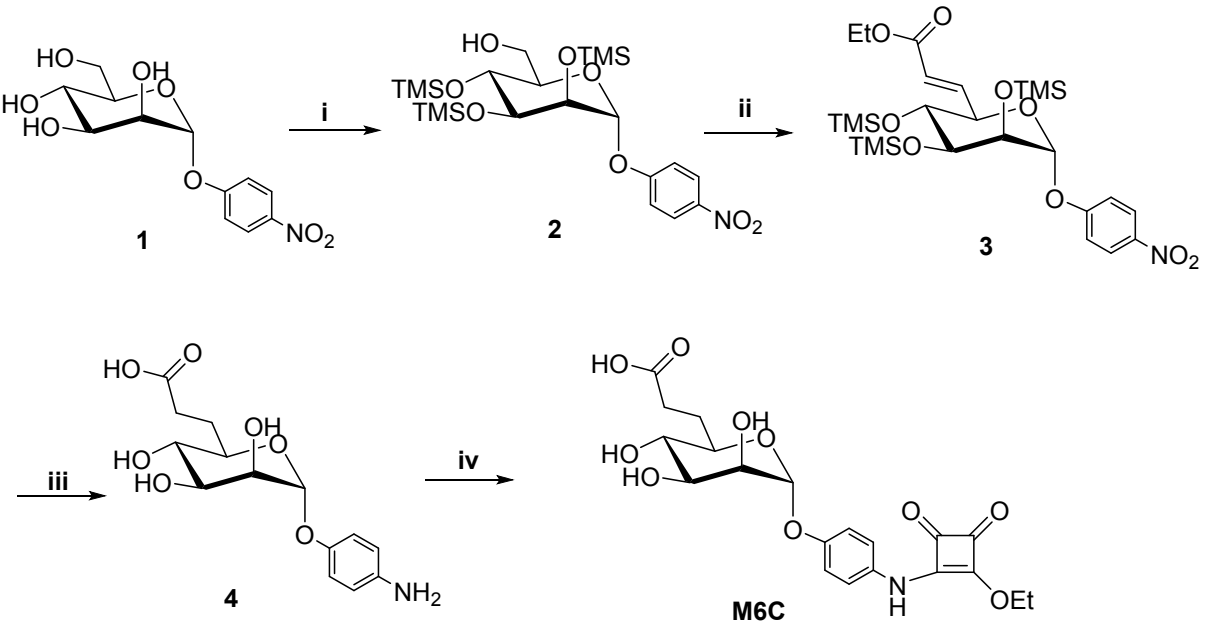
Synthesis of the porphyrin

The water-soluble 5-(4-iso-thiocyanatophenyl)-10,15,20-tris(4-*N*-1-methyl-4-pyridinio) porphyrin trichloride was prepared according method described by J. M. Sutton *et al.*[1] Succinctly, 5-(4-acetamidophenyl)-10,15,20-tri-(4-pyridyl)porphyrin was obtained by Adler condensation. 5-(4-aminophenyl)-10,15,20-tri-(4-pyridyl)porphyrin prepared from by 5-(4-acetamidophenyl)-10,15,20-tri-(4-pyridyl)porphyrin by acid hydrolysis (HCl 5 N), was converted to 5-(4-iso-thiocyanatophenyl)-10,15,20-tri-(4-pyridyl)-porphyrin then methylated by methyl iodide.

Synthesis of the analogue M6C

The M6C was prepared as previously described.[2] Briefly, the commercially available 4-nitrophenyl α-D-mannopyranoside **1** was persilylated then selectively deprotected at position 6 (Scheme S1). The alcohol **2** thus obtained was oxidized by Swern reaction and the aldehyde function subjected to a HWE reaction with the anion of the triethyl phosphonoacetate to afford **3**. To lead to **4**, the following steps were the reduction of both the double bond and the nitro function under H_2 atmosphere and

then the saponification of the ester. The final step was the introduction of the squarate moiety leading to the M6C derivative. All compounds were characterized by ^{1}H, ^{13}C NMR and mass spectrometry.



Scheme S1. i) 1. TMSCl, CH_2Cl_2, Et_3N, RT, 21 h, 95%; 2. K_2CO_3, MeOH, 0°C, 15 min, 50%; ii) 1. DMSO, $(COCl)_2$, Et_3N, THF, -78°C; 2. triethyl phosphonoacetate, NaH, THF, RT, 90 min, 73% over 2 steps; iii) 1. HCl 0.5N, THF, RT, 10 min, 91%; 2. H_2, Pd/C, ethanol/H_2O (4:1) RT, overnight, 98%; 3. NaOH 0.1N, THF, RT, 6 h, 91%; iv) diethyl squarate, EtOH/H_2O (2:1.5), RT, 4 h, 56%. DMSO=dimethylsulfoxide, THF=tetrahydrofuran, TMS=trimethylsilyl.

Chemical functionalization of the pSiNP

pSiNP grafting with allylamine by hydrosilylation

Freshly prepared pSiNP were centrifuged at 14 000 rpm for 30 minutes in ethanol, then centrifuged once with allylamine. 30 mg of pSiNP were redispersed in 8 mL of allylamine and reacted for 2 h at 70°C under nitrogen. After the reaction, the aminated pSiNP (pSiNP-NH_2) were centrifuged at 14 000 rpm and washed 5 times with absolute ethanol to remove the allylamine adsorbed onto their surface. The pSiNP-NH_2 were finally redispersed in 2 mL ethanol.

Coupling of the pSiNP-NH_2 with the porphyrin

The pSiNP-NH_2 nanoparticles were redispersed in a mixture containing 2 mL of absolute ethanol, 500 µL of a solution of the porphyrin at 1 mg.mL^{-1} in ethanol, and 2.5 mL of ethanol/water (1:1 volume

ratio) solution. A fraction of 250 µL of triethylamine was added to the suspension of pSiNP-NH_2. The reaction was performed under stirring during 18 h at room temperature. The obtained nanoparticles were rinsed and centrifuged twice with ethanol, twice with deionized water, twice with ethanol and twice with diethylether, and then redispersed in 2 mL of absolute ethanol.

Coupling of the pSiNP-NH_2 with mannose M6C

The pSiNP-NH_2 nanoparticles were redispersed in a mixture containing 2 mL of absolute ethanol, 2.5 mL of mannose M6C solution at 16 mM in a 1:1 (v:v) ethanol/water solution, and 250 µL triethylamine were added to the suspension of pSiNP-NH_2. The reaction was performed under stirring during 18 h at room temperature. The obtained nanoparticles were rinsed and centrifuged twice with ethanol, twice with deionized water, twice with ethanol and twice with diethylether, and then redispersed in 2 mL of absolute ethanol.

Coupling of the pSiNP-NH_2 with the porphyrin and with mannose M6C

For the simultaneous grafting of the porphyrin and of mannose M6C, 2.5 mL of a solution of mannose M6C at 16 mM in 1:1 (v:v) ethanol/water mixture, 500 µL of a solution of the porphyrin at 1 $mg.mL^{-1}$ in ethanol, and 250 µL of triethylamine were added to the suspension of the pSiNP-NH_2. The reaction was performed under stirring during 18 h at room temperature. The obtained nanoparticles were rinsed and centrifuged twice with ethanol, twice with deionized water, twice with ethanol and twice with diethylether, and then redispersed in 2 mL of absolute ethanol.

II. Characterizations of pSiNP

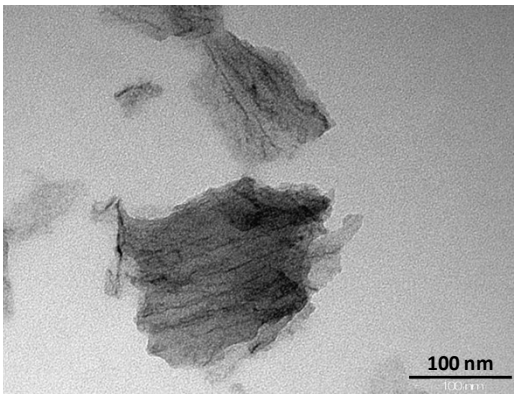


Figure S1. TEM image of the pSiNP.

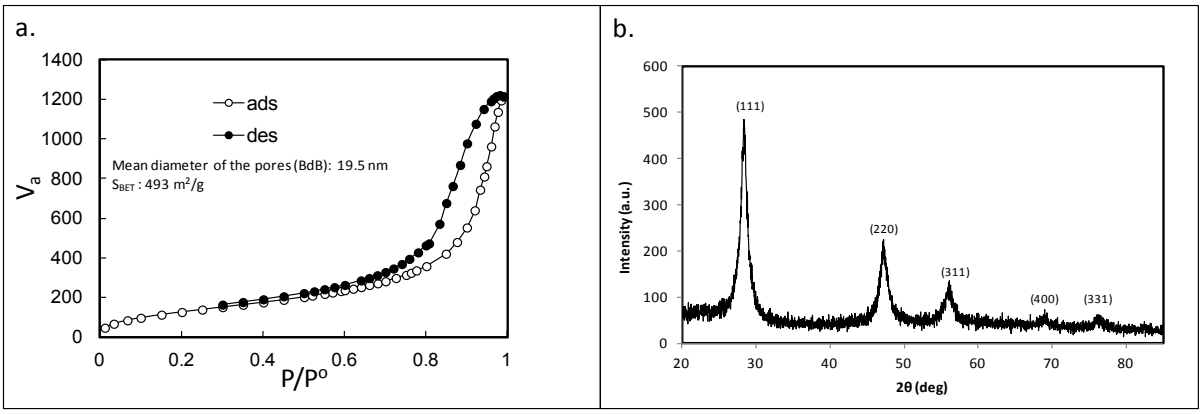


Figure S2. (a) N_2 Adsorption/desorption isotherm of pSiNP, and (b) XRD of pSiNP.

Transmission Electron Microscope (TEM) image was performed with a Jeol 1200 EX II microscope. For the nitrogen adsorption/desorption analysis, the pSiNP samples were outgassed overnight in situ at 303 K. The nitrogen adsorption/desorption isotherms were recorded at 77 k using a micromeritics ASAP 2020 volumetric apparatus. The pSiNP powder was analysed by X-ray diffraction with a bruker D8 Advance diffractometer (weighted mean CuKα radiation at λ = 1.541838 Å) with a Bragg-Brentano geometry and equipped with Bruker Lynx Eye detector. The data were recorded in the range 0.5-6 ° and 20-85° 2Θ with an angular step size of 0.020°.

Note: The large nanoparticle size distribution is due to the preparation method using ultrasonic fracture, filtration and centrifugation of freestanding porous silicon films. Nanoparticles can be easily prepared uniformely using photolithography or pre-perforation techniques.[3] Therefore this point does not constitute a scientific lock for application in nanomedecine.

III. Characterization of the functionalized pSiNP

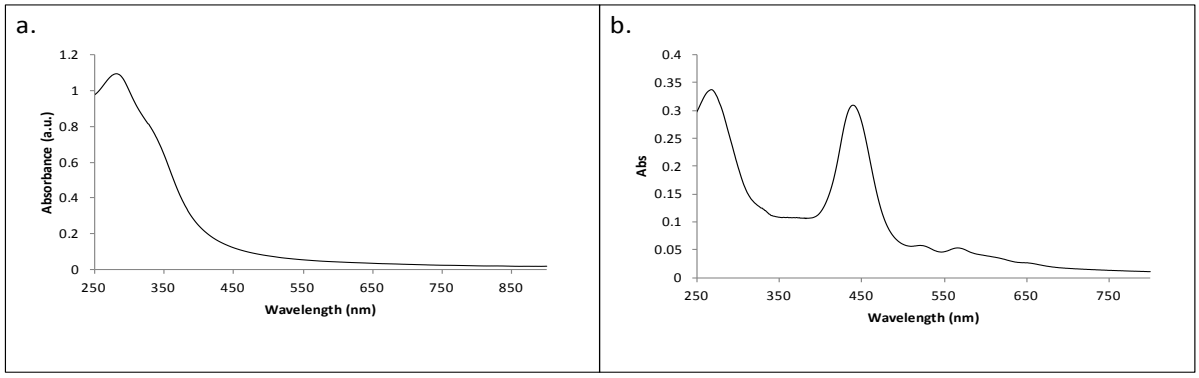


Figure S3. UV-vis spectroscopy. (a) Absorbance spectrum of pSiNP, (b) absorbance spectrum of pSiNP-Porph. The soret band at 449 nm and the Q bands between 500 nm and 750 nm correspond to the porphyrin. UV-vis absorption measurements were performed using a lambda 35 Perkin Elmer spectrometer.

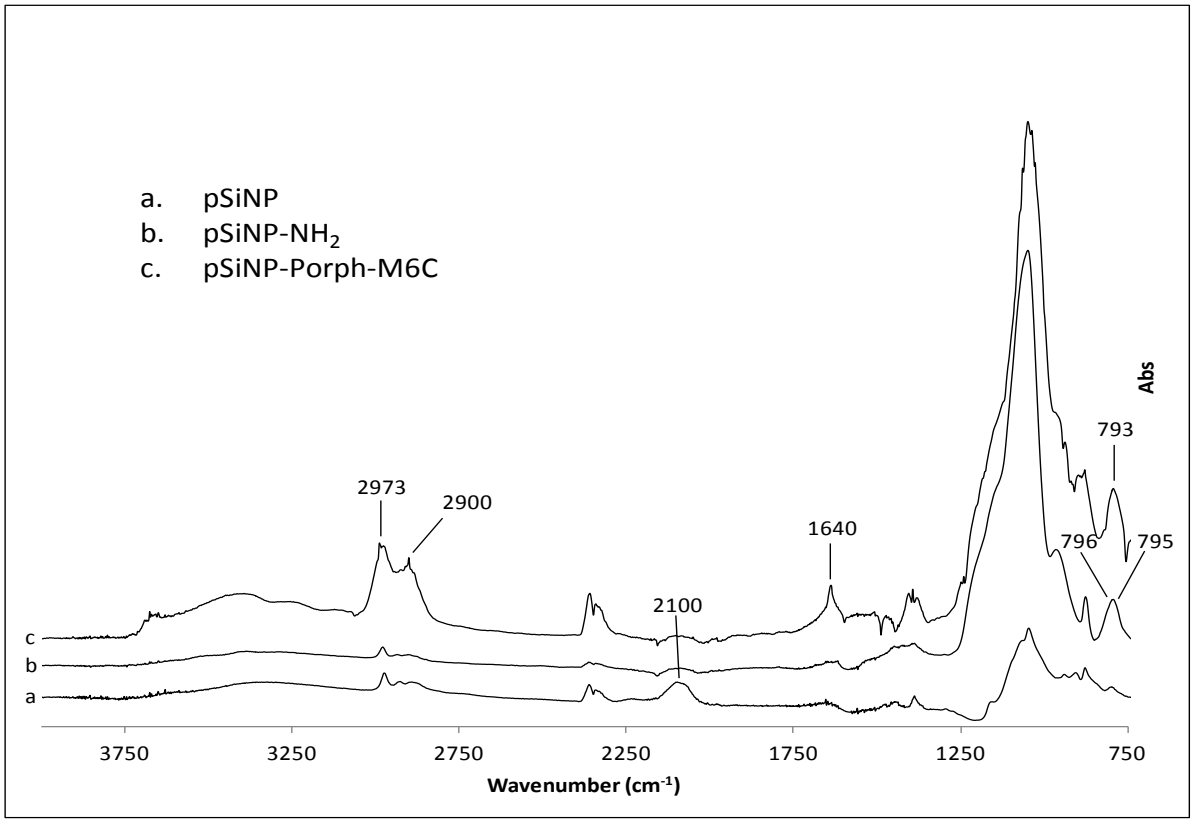


Figure S4. DRIFT spectra of (a) freshly prepared pSiNP, (b) pSiNP after hydrosilylation with allylamine (pSiNP-NH_2), and (c) pSiNP-NH_2 after coupling with the porphyrin and with mannose M6C (pSiNP-Porph-M6C) (c).

After the hydrosilylation of freshly etched pSiNP with allylamine, the band observed at 2100 cm^{-1} corresponding Si-H stretching vibration disappears (trace a-b). The high intensity band observed at 795 cm^{-1} is attributed to the streching vibration of the Si-C bond. This band confirmed the covalent linkage between the alkene of allylamine and silicon hydride (trace b). In addition, the high intensity band observed at 1640 cm^{-1} corresponds to the angular deformation of N-H bond. After the functionalization of pSiNP-NH_2 with porphyrin-NCS and M6C (trace c), three intense bands, corresponding to the stretching vibration of C-H bonds present in the porphyrin and mannose, were observed between 2900 cm^{-1} and 2973 cm^{-1}. Moreover, between 1400 cm^{-1} and 1600 cm^{-1} (trace c), we note the presence of aromatic C=C bonds of the porphyrin and phenyl mannose squarate. The intense band at 1640 cm^{-1} corresponds to the thiourea group indicating the covalent grafting of the porphyrin. Finally, the broad and intense band observed at 1053 cm^{-1} may possibly mask other band such as C-O and C-N stretching vibrations.

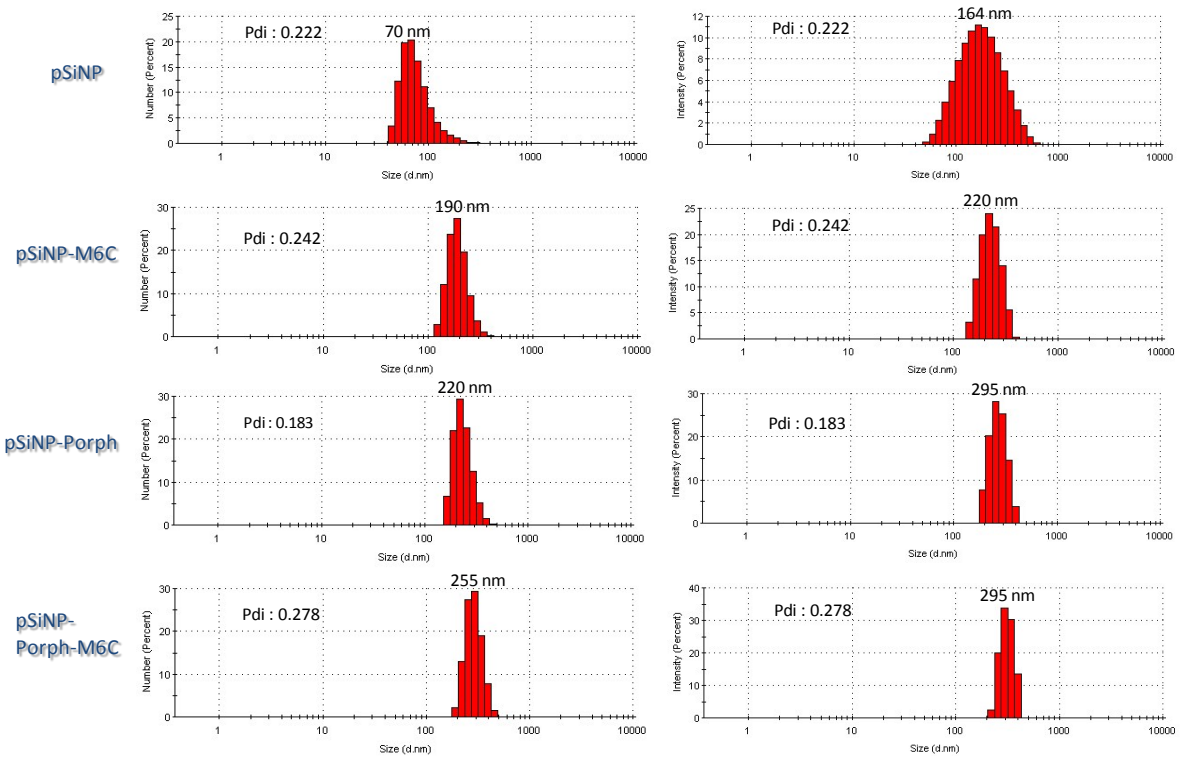


Figure S5. Dynamic light scattering (DLS) in number and intensity of pSiNP, pSiNP-M6C, pSiNP-Porph and pSiNP-Porph-M6C.

DLS measurements of the formulations

The hydrodynamic diameter measured for the pSiNP was 70 nm in number and 164 nm in intensity. After grafting the mannose and the porphyrin to the pSiNP, the hydrodynamic diameter of pSiNP-M6C, pSiNP-Porph and pSiNP-Porph-M6C increased up to 190-220-255 nm in number and to 220-295-295 nm in intensity. The DLS measurements were performed on a Malvern nanozetasizer instrument.

Samples	Zeta Potential in (mV)
pSiNP	-37.3 ± 2.08 mV
pSiNP-M6C	-31.5 ± 5.47 mV
pSiNP-Porph	18.9 ± 0.586 mV
pSiNP-Porph-M6C	12.3 ± 0.569 mV

Figure S6. Zeta potential values for pSiNP, pSiNP-M6C, pSiNP-Porph and pSiNP-Porph-M6C.

Zeta potential measurements of the formulations

The negative charge of the pSiNP is due to the partial oxidation of their surface. After grafting the cationic porphyrin, the zeta potential value increased up to 18.9 ± 0.586 mV in the case of pSiNP-Porph and 12.3 ± 0.569 mV in the case of pSiNP-Porph-M6C. The Zeta potential measurements were performed on a Malvern nanozetasizer instrument.

Samples	Porph amount (µg per mg of pSiNP)	Porph amount (mmol per g of pSiNP)	Mannose M6C amount (µg per mg of pSiNP)	Mannose M6C amount (mmol per g of pSiNP)
pSiNP-M6C			35	0.194
pSiNP-Porph	25.1	0.035		
pSiNP-Porph-M6C	20	0.028	70	0.389

Table S1. Amounts of porphyrin and mannose M6C grafted on the pSiNP.

Quantifications of the porphyrin and of the mannose M6C grafted on the pSiNP

UV-vis absorption measurements were performed using a lambda 35 de Perkin Elmer spectrometer for dosing the porphyrin and mannose. The amounts of porphyrin grafted on the pSiNP was determined using a known quantity of nanoparticles functionalized with porphyrin (pSiNP-Porph and pSiNP-Porph-Man) and dissolved in 1 M solution of potassium hydroxyde (KOH). The absorbance of the solution was recorded between 350 and 550 nm. Calibration curves were also recorded in KOH, in order to determine the molar absorption coefficient of the porphyrin under the same condition (ε = 62996 $L.mol^{-1}.cm^{-1}$ at 450 nm). The amounts of porphyrin load for pSiNP-Porph and pSiNP-Porph-Man are 25.1 µg (0.035) and 20 µg (0.028) per mg of pSiNP respectively.

The amount of mannose M6C grafted on the pSiNP was determined by specific colorimetric reaction. The molar absorption coefficient of the mannose was determined using a solution of mannose in hydrated sulfuric acid and resorcinol (1,3-dihydroxybenzene) at different concentrations.[4] Mannose amounts ranging from 1 to 6 mg were dissolved in 20 mL of acetic acid (0.01 M). Then, 200 µL of each solution was added to the solution containing 1 mL of 75 % sulfuric acid and 200 µL of resorcinol. The samples were vortexed for 5 min and heated at 90 °C during 30 min. The samples were stored in dark, at 6°C for 30 min. The absorbance of the solutions was recorded between 350 and 600 nm in order to determine the molar absorption coefficient of the mannose (ε_{422} = 11651 $L.mol^{-1}.cm^{-1}$). The 75 % sulfuric acid (1 ml), resorcinol (200 µL) and acetic acid (200 µL) solution was used as a reference solution. The dosing of the mannose M6C grafted on the pSiNP followed the procedure described above. Here, the mannose solution was replaced by the formulations grafted with M6C mannose (pSiNP-M6C and pSiNP-Porph-M6C). The amounts of M6C mannose are 35 (0.194 mmol) and 70 (0.389 mmol) µg of mannose per mg of pSiNP respectively.

Cell culture

Cell lines were purchased from ATCC (American Type Culture Collection). Human normal fibroblasts were routinely maintained in DMEM supplemented with 10% FBS and 50 µg ml^{-1} gentamicin. LNCaP prostate cancer cells were cultured in RPMI-1640 medium supplemented with 10 % FBS, 100 IU ml^{-1} penicillin and 100 µg mL^{-1} streptomycin, 1% sodium pyruvate, 1% HEPES and 1% glucose. Cell lines were allowed to grow at 37°C in an atmosphere containing humidified air with 5% CO_2.

TPE- Imaging

One day prior the experiment, LNCaP cells were plated 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 pSiNP-Porph-M6C at a concentration of 80 μg.mL^{-1} for 5 h. Fifteen min before the end of incubation, cells were loaded with CellMask™ plasma membrane stain (Invitrogen, Cergy Pontoise, France) at a final concentration of 5 μg.mL^{-1}. Prior the observation, cells were washed gently with phenol red-free DMEM. Cells were then visualized with a LSM 780 LIVE confocal microscope (Carl Zeiss, Le Pecq, France), at 800 nm using a high magnification (63x/1.4 OIL DIC Plan-Apo).

TPE-PDT

For *in vitro* phototoxicity, 1000 cells by well were plated in a 384 multiwell glass-bottomed plate (thickness 0.17 mm), in 50 μL of culture medium. Twenty four hours after seeding, dispersed pSiNP-Porph-M6C were added on cells at a concentration of 80 μg.mL^{-1} for 5 h. After this incubation, cells were submitted (or not) to laser irradiation; with the Carl Zeiss Microscope LSM 780 LIVE confocal microscope (laser power input 3W). Four different areas of the well were irradiated at 800 nm by three scans of 1.57 s duration. Thus, half of the well was irradiated. The laser beam was focused by a microscope objective lens (Carl Zeiss 10x , NA 0.4). Two days after irradiation, cell viability assay was performed with CellTiter 96® (also called MTS) as described by manufacturer and results were corrected according to the following formula AbsControl-2(AbsControl-AbsNPs) where Abs is absorption reads at 492 nm.

The reason for using 5h comes from the fact that pSiNP are bioresorbable and degrade quite quickly in aquous media. This time of incubation was optimized for pSiNP in a previous study[5].

Cytotoxic study of NP

LNCaP cells were seeded into 96-well plates in 200 μL culture medium and allowed to grow for 24 h. Increasing concentrations of pSiNPs were incubated in culture medium of LNCaP cells during 72 h. Then, a MTT assay was performed to evaluate the toxicity. Briefly, cells were incubated for 4 h with 0.5 mg.mL^{-1} of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Promega) in media. The MTT/media solution was then removed and the precipitated crystals were dissolved in EtOH/DMSO (1:1). The solution absorbance was read at 540 nm.

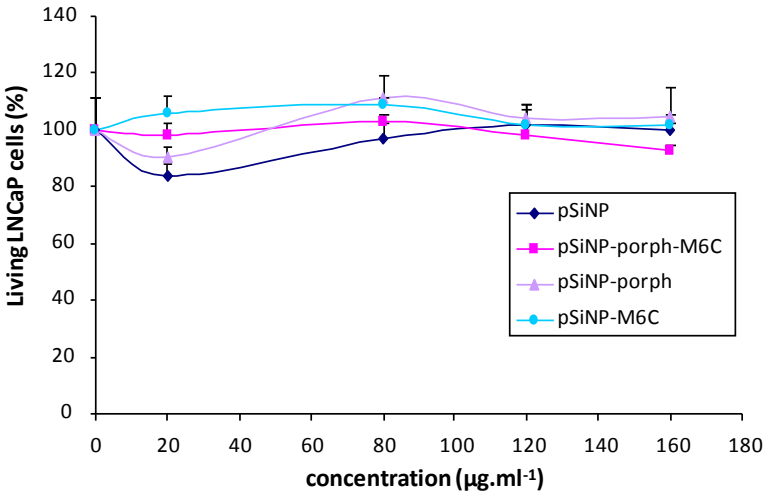


Figure S7. Cytotoxic study of nanomaterials on LNCaP cells. Data are mean ± standard deviation of three experiments.

This experiment shows that no significant cell death was observed in the absence of appropriate stimulus.

Detection of intracellular ROS by DCFDA assay in LNCaP cancer cells

Oxidative stress was measured in LNCaP cells using DCFDA which fluoresces when exposed to intracellular peroxides.

Control cells (A) show very low level of fluorescence when stained with DCFDA compared to nanoparticles treated cells. No difference in fluorescence was observed with pSi nanoparticles before and after TPE at 800 nm, indicating no ROS production. Increase in fluorescence observed with pSi-porph, pSi-M6C and pSi-porph-M6C nanoparticles after a TPE at 800 nm clearly indicated ROS production (C, D and E).
Functionalized PSiNP induce oxidative stress generation in LNCaP cells.

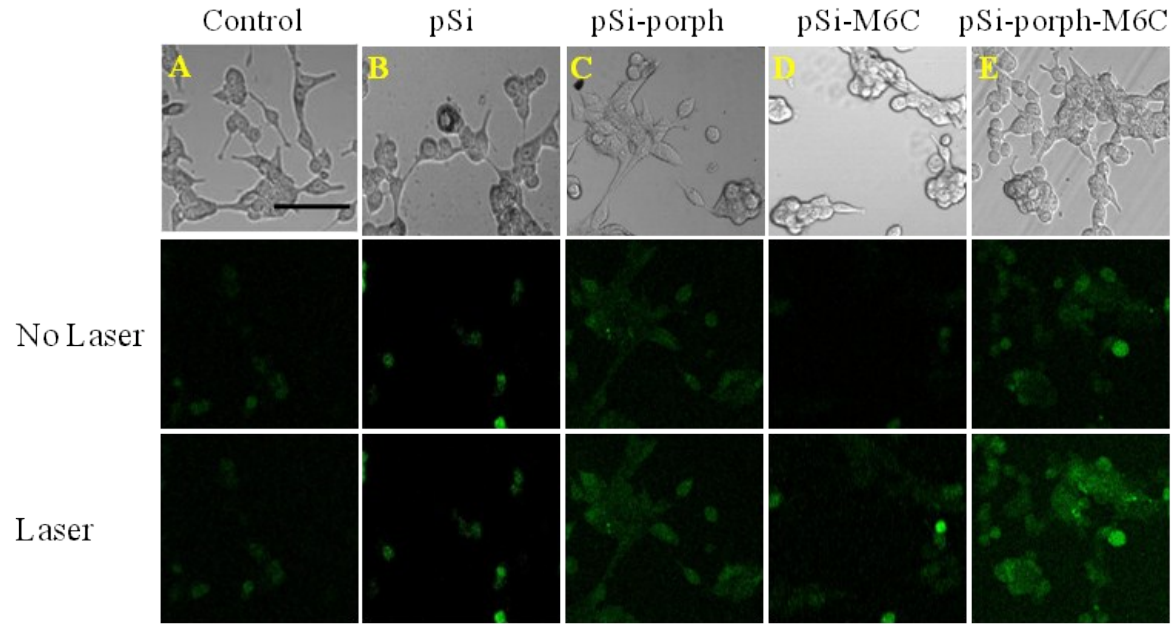


Figure S8. Confocal microscopy images before and after TPE at 800 nm of **(A)** Control cells with DCFDA, **(B)** pSi nanoparticles with DCFDA**, (C)** pSi-porph nanoparticles with DCFDA**, (D)** pSi-M6C nanoparticles with DCFDA, and **(E)** pSi-porph-M6C nanoparticles with DCFDA. Scale bar represents 50 µm for all images.

Reactive oxygen species (ROS) measurements

The LNcaP cells were seeded into 384 multi-well glass bottom (thickness 0.17 mm), with a black polystyrene frame, 2000 cells per well in 50 µL culture medium and allowed to grow for 48 h. The day of the experiment, the cells were successively incubated with 80 µg mL^{-1} of nanoparticles for 5 hours and for 45 min with 20 µM 2', 7'-dichlorofluorescin diacetate (abcam). Finally the cells were washed two times for 3 minutes with cells media and submitted to laser irradiation.
Two-photon irradiation was performed on the confocal microscope used for TPE-PDT. The cells were submitted to one scan irradiation at 800 nm for 1.57 s. Confocal fluorescence photos were performed before and after laser irradiation, under a 535 nm wavelength excitation.

1. J. M. Sutton, O. J. Clarke, N. Fernandez and R. W. Boyle, *Bioconjugate Chem*, 2002, **13**, 249-263.

2. O. Vaillant, K. El Cheikh, D. Warther, D. Brevet, M. Maynadier, E. Bouffard, F. Salgues, A. Jeanjean, P. Puche, C. Mazerolles, P. Maillard, O. Mongin, M. Blanchard-Desce, L. Raehm, X. Rebillard, J. O. Durand, M. Gary-Bobo, A. Morere and M. Garcia, *Angew Chem Int Edit*, 2015, **54**, 5952-5956.

3. M. J. Sailor, *Porous silicon in practice : preparation, characterization and applications*, Wiley-VCH, Weinheim, 2012.

4. M. Monsigny, C. Petit and A. C. Roche, *Anal Biochem*, 1988, **175**, 525-530.

5. E. Secret, M. Maynadier, A. Gallud, M. Gary-Bobo, A. Chaix, E. Belamie, P. Maillard, M. J. Sailor, M. Garcia, J. O. Durand and F. Cunin , *Chem. Commun.,* 2013, **49**, 4202--4204