Supplementary Information for

"Anionic porphyrin-grafted porous silicon nanoparticles for photodynamic therapy"

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Experimental details

Preparation of pSiNP

Wafers of p^{++} type, boron doped, (100)-oriented crystalline silicon with 0.0008-0.0012 Ω.cm resistitvity (Sitronix, Inc.) were electrochemically etched in a 3:1 volume ratio solution of 48% aqueous hydrofluoric acid (HF) and ethanol (Sigma-Aldrich Chemicals, inc.). Etching was performed in a Teflon cell with a platinum ring counter electrode. A constant current of 200 $mA.cm^{-2}$ was applied for 150 s, after which the cell was rinsed 3 times with ethanol. The porous layer was then electropolished in a 3.3% HF solution in water:ethanol with a constant current of 4 $mA{\cdot}cm^{-2}$ for 250 s. 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 pSi film was fractured by ultrasonicaton for >16 hr. The largest particles were then removed by centrifugation at 2700 g for 2 min. The supernatant was then filtered through a 0.2 µm nylon filter membrane (Carl Roth). In order to remove the smallest particles, the solution was finally centrifuged at 22,000 g for 30 min. The pellet of pSiNP was then redispersed in absolute ethanol.

Characterization of pSiNP

Transmission electron microscope (TEM) images were obtained using a Jeol 1200 EX II microscope. Dynamic light scattering (DLS) measurements were obtained on a Malvern 4800 instrument. Nitrogen adsorption-desorption volumetry experiments were performed at -196 °C using a Micromeretics ASAP2020 instrument. Prior to the analysis, pSiNP were outgassed overnight in situ at 30 °C. The pore diameter was determined using the BdB (Broekhoff and de Boer) method [1,2] whilst the samples' surface area was determined from the BET (Brunnauer-Emmett-Teller) theory.[3,4] ATR-FTIR spectra were recorded using a Bruker Vector 22 equipped with a diamond ATR crystal. UV-Vis absorption measurements were performed using a Lambda 35 de Perkin Elmer spectrometer. Prior to the measurement a known quantity of pSiNP-Porph-NH_2 was dissolved in a 1M potassium hydroxide solution, and the absorbance of the solution was recorded between 350 and 550 nm. Calibration curves are also recorded in potassium hydroxide solution, in order to determine the molar absorption coefficient under the same conditions.

Functionalization of the pSiNP with allylisocyanate

PSiNP dispersed in ethanol were first centrifuged at 22,000 G for 30 min, then rinsed with diethylether and dried under a flux of nitrogen. PSiNP were dispersed in pure allylisocyanate (2mL) and the hydrosilylation reaction was performed at 90°C for 3 h under reflux. The reaction was performed in an inert (dry N_2) atmosphere. The pSiNP were then rinsed twice with tetrahydrofurane (THF).

Coupling of Porph-NH_2 with the isocyanate-functionalized pSiNP

Immediately after the hydrosilylation reaction, the pSiNP were re-dispersed in THF (4 mL), and a solution (500 µL) of diisopropylethylamine-activated Porph-NH_2 in DMF (dimethyformamide) (1 mg. ml^{-1}) was added. Ethanol (200 µL) was added and the reaction was performed at reflux (80°C) for 18 hours, under N_2 and in the dark. The functionalized pSiNP were extensively rinsed with absolute ethanol (2x) and then distilled water (6x) until the yellow coloration was no longer observed in the supernatant, and then the nanoparticles were rinsed in absolute ethanol (2x) and diethylether (2x). The chemically modified PSiNPs were then dried under flowing N_2.

Here, we used the absorption cross section of the porph-NH_2 indicated in the reference [5].

Cell culture

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% foetal bovine serum and 50 µg mL^{-1} gentamycin. Cells were allowed to grow in a humidified atmosphere at 37°C under 5% CO_2.

Live cell imaging

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 10^6 cells cm^{-2}. 24 h later, the cells were washed (once) and incubated in 1 mL medium containing the test compounds at a concentration of 20 µg mL^{-1} nanoparticle (or the equivalence for porphyrin or vehicle) for 5 h. 15 min before the end of incubation, cells were loaded with Cell Mask Orange (Invitrogen, Cergy Pontoise, France) for membrane staining at a final concentration of 5 µg 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 633 nm with a slice depth (Z stack) of 0.62 µm.

Quantification of cell internalization

After 5 h internalization, cells were harvested and lysed in buffer containing 50 mM Hepes, 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 2.5 mM EGTA, and protease inhibitors (Complete, Roche Diagnostics, USA) with three freeze-thaw cycles. Cell extracts were then evaluated for fluorescence using a Cary Eclipse Fluorescence Spectrophotometer (Agilent, France). Known quantities of pSiNP-Porph-NH_2 or free porphyrin were directly dissolved in the same buffer and the fluorescence of the solution was recorded at 415 nm for standard curves. Complete dissolution of the porous silicon nanoparticles occured after 6 days.

Semi-quantification of nanoparticle internalization in living cells

Semi-quantitative analysis was performed using ImageJ software. Delimited-cell sections were drawn with the particle analysis tool and then merged with the original compound acquisition. Intensities of the signals inside the areas were then quantified.

PDT and cell death measurement

MCF-7 cells were seeded into 96-well plates at 2 10^4 cells per well in 100 µL culture medium and allowed to grow for 24 h. Then cells were incubated for 5 h with free porphyrin (0.25 µg mL^{-1}) or pSiNP-Porph-NH_2 (20 µg mL^{-1}). After incubation, the medium was removed and cells were washed with PBS and maintained in 100 µL fresh culture medium. Finally, the cells were subjected to laser irradiation for 40 min (650 nm, 6-7 mW cm^{-2}, 14 J cm^{-2}). For the control (no laser) experiments, the cells were maintained in the dark for 40 min.

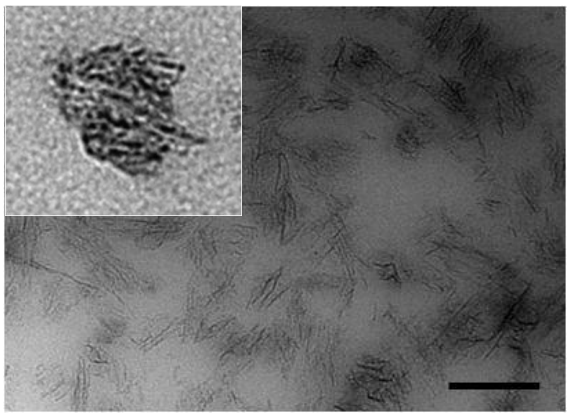
Two days post-irradiation, a MTT assay was performed to evaluate the phototoxicity. Briefly, cells were incubated for 4 h with 0.5 mg mL^{-1} of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Promega) in media. The MTT/media solution was then removed and the precipitated crystals were dissolved in EtOH/DMSO (1:1). The solution absorbance was read at 540 nm.

Release kinetics of Porph-NH_2

About 500µg of pSiNP-Porph-NH_2 were dispersed in 3mL of phosphate buffer saline (PBS, pH=7.4) and incubated at 37°C on a stirring plate at 100 rpm. At given times, aliquots of 500µL were taken and centrifuged at 22000g for 30 min. 500µL of PBS were added to the original solution to maintain a constant volume. The supernatant aliquots were stored in dark at 4°C until analysis. The nanoparticles in the sample aliquots were redispersed prior to analysis by UV-Vis absorption spectroscopy. The amount of porphyrin present was determined by comparison with calibration curves.

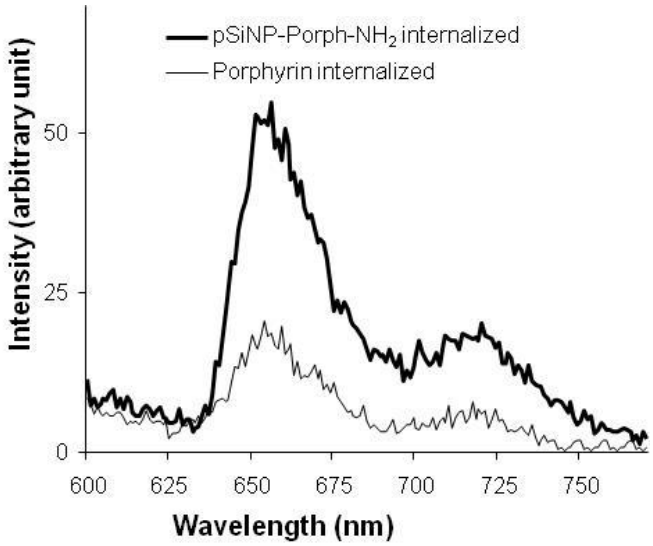
Detection of singlet oxygen from the pSiNP-Porph-NH_2 nanoparticles

2 mL of a solution containing 0.06 mM of diphenylisobenzofuran (DPBF) and the functionalized pSiNP-Porph-NH_2 were placed in a quartz cuvette closed with a cap. The solution was agitated and illuminated with a halogen lamp. The light was filtered through a bandpass filter centered at 650nm (bandwidth 10 nm). The sample was irradiated for 40 min, and its absorbance at 411 nm was measured every 10 min with a Lambda 35 Perkin-Elmer UV-visible spectrometer.

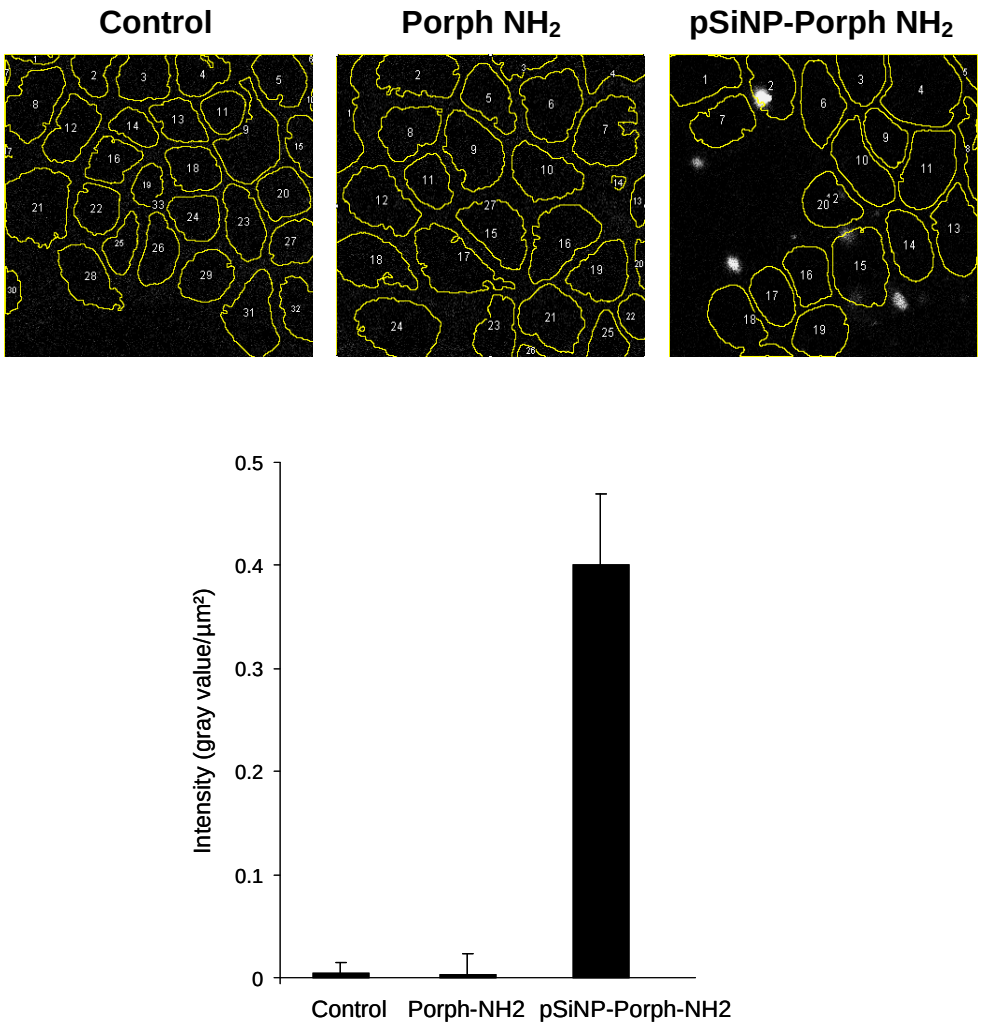


Supplementary Figure S1. TEM image of a population of pSiNPs. Scale bar is 200 nm. Insert: Close-up view of a single pSiNP. Frame is ~150x150 nm.

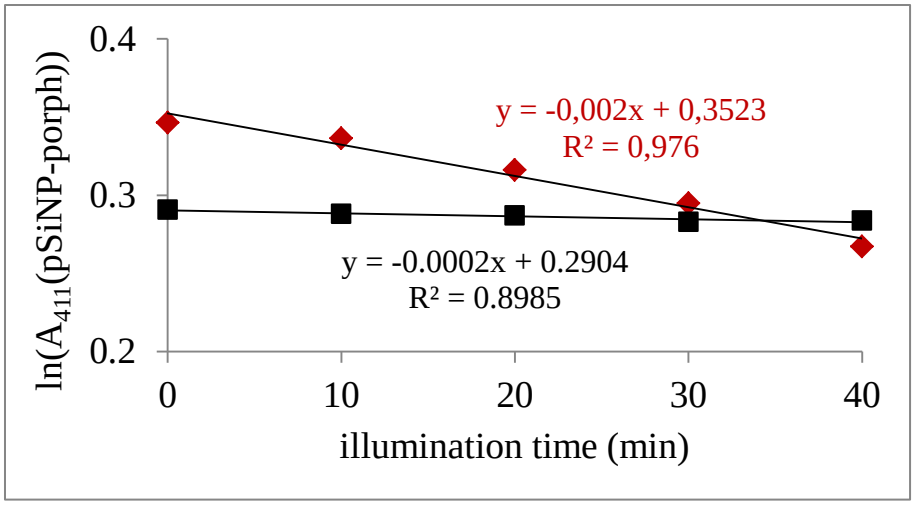
Supplementary Figure S2. ATR-FTIR spectrum of pSiNP-Porph-NH_2. The attenuated total reflectance Fourier-transform infrared spectrum of the Porph-NH_2-grafted pSiNP displays absorption bands characteristic of the urea bond, with vibrations assigned to the C=O stretching and to the NH- stretching modes at 1620 cm^{-1} and 1542 cm^{-1} respectively, indicating successful coupling between the isocyanate-functionalized pSiNP and the Porph-NH_2 species. In addition, the spectral region between 2858 cm^{-1} and 2957 cm^{-1} display characteristic bands of aliphatic and aromatic C-H bonds present in the porphyrin and in the linker, while a vibrational band of the C=O bond from the isocyanate group at 2251 cm^{-1} is no longer observed.



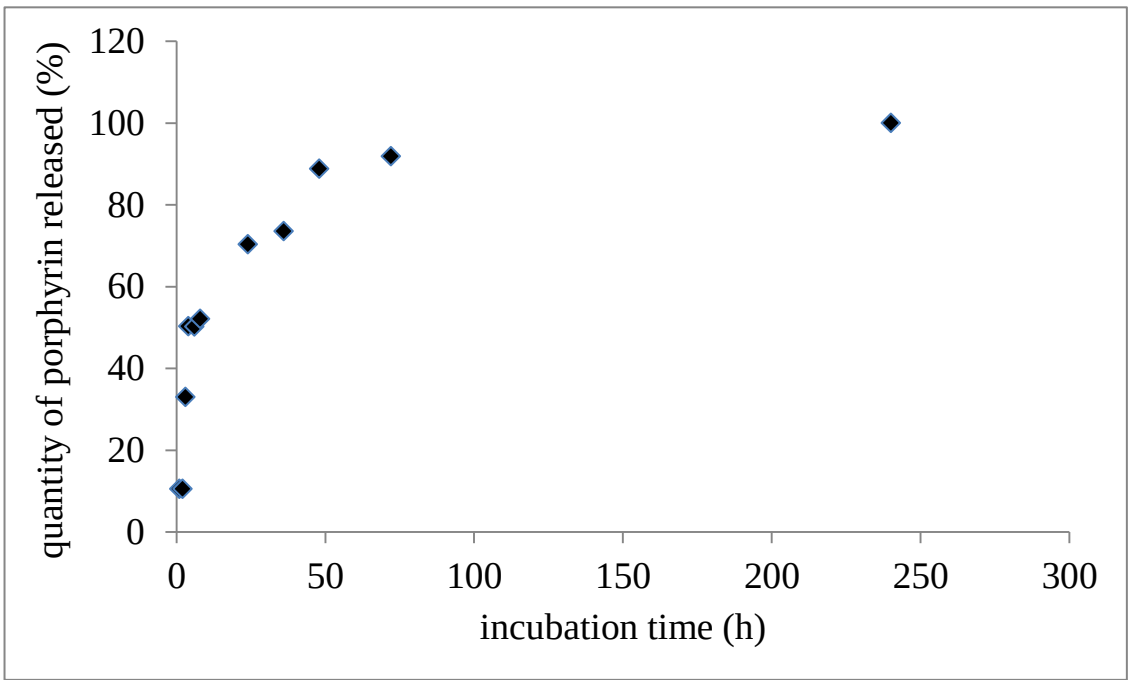
Supplementary Figure S3. Quantification of pSiNP-Porph-NH_2 nanoparticles and free porphyrin internalized in living MCF-7 breast cancer cells after 5 h incubation. The internalization was monitored with a spectroscopic determination of the amount of porphyrin in cell lysates and compare to dilution series. The quantities of porphyrin internalized from nanoparticles is in the range of 105 ± 5 $ng.ml^{-1}$ of porphyrins while the internalized free porphyrin correspond to 35 ± 5 $ng.ml^{-1}$.



Supplementary Figure S4. Semi-quantitative analysis of pSiNP-Porph-NH_2 nanoparticles and free-porphyrin (Porph-NH_2) internalized in living MCF-7 breast cancer cells after 5 h incubation. A mean intensity inside the cells was determined from five distinct areas and expressed as gray value by µm². The internalized nanoparticles were found to represent 45% of the total detected nanoparticles.



Supplementary Figure S5. Trace of the natural logarithm of absorbance at 411 nm as a function of illumination time for DPBF alone (black squares), and for pSiNP-Porph-NH_2 + DPBF (red diamonds). The decrease observed for the logarithm of the absorbance for the pSiNP-Porph-NH_2 nanoparticles in the presence of DPBF indicates the generation of singlet oxygen by the nanoparticles. Interference from scattering of the pSi nanoparticles and absorbance of the porphyrin precludes quantitative determination of quantum yield for singlet oxygen in these experiments.



Supplementary Figure S6. Release kinetics of porph-NH_2 from the pSiNP-Porph-NH_2 nanoparticles, incubated at 37°C in PBS (pH=7.4). The stability of the pSiNP-Porph-NH_2 nanoparticles in PBS was monitored by spectroscopic determination of the amount of porphyrin appearing in solution as a function of time. The data indicate that 50% of the porphyrin was released after 5 h, and that 89% of the porphyrin was released after 48h of incubation. Under the experimental conditions used in this study, this degradation time is compatible with the use of the pSiNP-Porph-NH_2 for imaging and PDT.

Cited References

[1] J. C. P. Broekhoff, J. H. de Boer, *J. Catal.* **1968**, 10, 377-390.
[2] A. Galarneau, H. Cambon, F. Di Renzo, R. Ryoo, M. Choi, F. Fajula, *New J. Chem.* **2003**, 27, 73-79.
[3] S. Brunauer, P. H. Emmett, E. Teller, *J. Am. Chem. Soc.* **1938**, 60, 309-319.
[4] S. J. S. Gregg, *Adsorption, Surface Area and Porosity, 2nd ed*. Academic Press Inc.: London, GB **1982**.
[5] W. J. Kruper, T. A. Chamberlin, M. Kochany. J. Org. Chem. 1989, 54, 2753-2756.