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A Galactose-Dendritic Silicon(IV) Phthalocyanine as a Photosensitizing Agent in Cancer Photodynamic Therapy

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Abstract: Two protected galacto-dendritic units have been axially coordinated to the central ion of a silicon(IV) phthalocyanine to afford **SiPcPGal$_4$** containing four units of galactose per macrocycle. These biological moieties provided better solubility in aqueous medium and a sensitizer with higher absorption peaks at 680–690 nm. The photodynamic activity of **SiPcPGal$_4$** was evaluated against UM-UC-3 human bladder cancer cell line and the results were compared with the activity of the reported **SiPcPGal$_2$** and **SiPc(OH)$_2$**. **SiPcPGal$_4$** had a better uptake and it was a better toxicity inducer than **SiPcPGal$_2$** and **SiPc(OH)$_2$** owing to its four galactose units, protected by isopropylidene groups, that can act as targeted micelles.

Introduction

Photodynamic therapy (PDT) is a treatment modality with clinical applications for both neoplastic and nonneoplastic diseases[1]. This therapeutic approach combines a photosensitizer (PS) with light of appropriate wavelength and molecular oxygen (O_2). Individually, none of those is toxic, but when combined, they initiate a cascade of reactions to generate reactive oxygen species responsible for the selective destruction of the biological target.[2]

Silicon phthalocyanines (SiPcs) exhibit interesting photophysical properties. In particular, they have strong absorption peaks in the red region of the spectrum of light and generate relatively long-lived triplet states, which allows a deeper tissue penetration of light and high quantum yields for singlet oxygen, respectively.[2] In addition, their four-coordinate central atom provides manifold structural possibilities and thus, a flexibility of the synthetic design, such as the incorporation of different modulators to the axial ligands.[3] The first SiPc approved for clinical trials was Pc4.[4] This PS showed excellent PDT activity for the treatment of cutaneous neoplasms.[5] Preclinical studies on this Pc have shown that this drug is able to bind to cytosolic membranes, the membranous portions of the mitochondria, Golgi apparatus and endoplasmic reticulum.[6]

Other related examples include SiPcs conjugated with BSA[7] and carbohydrates (protected glucose and galactose molecules)[8] to promote a specific accumulation in human hepatocarcinoma cancer cells; with monoclonal antibodies[9] to target cells overexpressing human epidermal growth factor receptor 1 and 2; and with low-density lipoproteins (LDL) to provide selective targeting of HepG2 cancer cells overexpressing LDL receptors.[10]

Despite their ideal suitability as PS for PDT[11], phthalocyanines (Pcs) have some adversities in their characteristics (planar structure and hydrophobicity) that lead to the formation of aggregates in aqueous medium.[12] These could be overcome by associating the PS in different ways with specialized carriers (nanoparticles, liposomes, quantum dots, PEGylation) by encapsulation[13]; or by conjugating dendrimers to enhance the PSs solubility in physiological medium. [12] Besides improving the biocompatibility and the photochemical properties of PSs, dendrimers also create multivalent interactions with more than one receptor binding site, contributing to an active cancer cell targeting and improving cellular recognition.[12] Several porphyrins, Pcs and chlorins conjugated with glycodendrimers have been reported in literature[14] as promising drugs for bladder cancer (BC) treatment. Galactose molecules can be recognized by galactose-binding proteins overexpressed in cancer cells.[15] Ng *et al* reported the synthesis of a SiPc conjugated with two protected galactose molecules and their *in vitro* results using a Cremophor EL emulsion against human hepatocellular carcinoma HEPG2 were very promising.[8a] This prompted us in designing a novel SiPc axially conjugated with two dendritic units of protected-galactose (Scheme 1), with the aim of obtaining more aqueous solubility due to the dendritic unit

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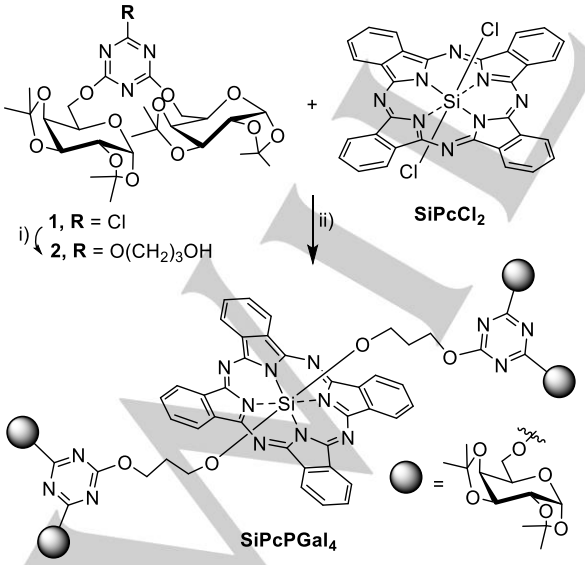
and therefore, efficient photodynamic activity against a BC cell line.

Results and Discussion

Synthesis and characterization of SiPcPGal$_4$

The synthesis of a SiPc containing dendritic units of galactose was initially attempted using a well-stablished synthetic strategy.[14b] The attachment of galactose dendrimers through thiolate functions was essayed by treating $SiPcCl_2$ with the thiol-containing galactose-dendritic unit **3** (see Figure S1 in supporting material), DMF at 120 ºC for 24 h. Under these conditions, the desired Pc was not obtained. Therefore, **SiPcPGal$_4$**, containing a similar dendritic motif but attached through a diol linker was designed (Scheme 1). Treatment of **1** with 1,3-bispropanediol in toluene, in the presence of *N,N*-diisopropylethylamine (DIPEA), afforded dendrimer unit **2** in a 61% yield. The preparation of **SiPcPGal$_4$** was carried out in a 12% yield by replacement of the chlorine atoms in SiPcdichloride ($SiPcCl_2$) with the alcoxy ligand **2** in toluene, in the presence of pyridine. The resulting phthalocyanine was highly soluble in a wide range of organic solvents and was readily purified by column chromatography. **SiPcPGal$_4$** was characterized by ^{1}H NMR and UV–vis spectroscopy, as well as mass spectrometry (see Figures S2 to S6 in Supporting Material).

The ^{1}H NMR spectrum of **SiPcPGal$_4$** showed well-defined peaks indicating lack of aggregation in $CDCl_3$ solution. In particular, two multiplets at δ 8.29-8.31 and 9.55-9.57 ppm were assigned to the Pc-Hα and Pc-Hβ protons, respectively. The most informative signals were those corresponding to the propanediol linker, which are influenced by the Pc aromatic ring current, falling in the shielding cone. The effect is stronger for protons closer to the macrocycle. Therefore, the protons assigned to the propanediol linker appear upfield shifted by 5.75 and ~3ppm, respectively, related to the free ligand **2**.



Scheme 1. Synthesis of a silicon phthalocyanine axial conjugated with a galactose-dendritic unit. i) 1,3-dipropanediol, DIPEA, toluene, 80 °C, 72 h; ii) pyridine, toluene reflux, overnight.

The former is also influenced by its direct attachment to a silicon atom. All the signals for the ketal-protected galactoses appeared between 4.22 and 5.64 ppm. From these, only the closer protons attached to C-6 showed its resonance influenced by the Pc diatropicity, appearing by 0.12 ppm shielded, related to the corresponding signals in **2**. Mass spectrometry showed a peak at *m/*z = 1903.6789, assigned to $[M + Na]^+$ which confirmed the structure of **SiPcPGal$_4$**. Deprotection of the galactose units was attempted by two different approaches: i) with TFA/H_2O (9:1), at room temperature (RT), during 5 h; and ii) with bromotrimethylsilane (TMSBr), using dichloromethane as solvent, at RT, during 24 h. However, both attempts led to the loss of the dendritic units, thus producing SiPcdihydroxide (**SiPc(OH)$_2$**). Therefore, the solketal-protected **SiPcPGal$_4$** was used for the biological studies.

Photophysical and photochemical properties

The electronic absorption spectra of **SiPcPGal$_4$** were acquired in DMSO, DMF and PBS, and its extinction coefficients (ε) in these solvents are summarized in Table 1. In the organic solvents, **SiPcPGal$_4$** (10 µM) gave typical UV/Vis spectra for a non-aggregated Pc, showing an intense and sharp Q band in the red region at 679 nm (DMF) and 685 nm (DMSO) (see Figure 1). In PBS containing 3% DMSO, the absorption features of **SiPcPGal$_4$** somewhat broaden (Figure 1), although the Q/B band ratio is essentially maintained, supporting the little aggregation of the PS also in aqueous medium. This point is a critical parameter for the application of a photosensitizer in PDT, since a high aggregation tendency produces a decrease of efficacy as a PDT agent.[11]

The steady-state fluorescence emission spectrum of **SiPcPGal$_4$** was analyzed in DMF and the obtained fluorescence quantum yield (φ_F) was 0.38. This study showed fluorescence emission bands in the red region of the spectrum (approximately between 680 and 750 nm), which can be used to determine the concentration of these PSs inside the cells or tissues. Moreover, a calibration slope of **SiPcPGal$_4$** fluorescence was performed in 1% (m/v) SDS solution in PBS to validate the PS's ability to fluoresce in aqueous media for further studies *in vitro* (see Figure S7 in Supporting Material).

In order to study the maximum concentration of **SiPcPGal$_4$** that can be used in cells, UV/Vis studies with increasing concentrations of the compounds (0-10 µM) in PBS solution (with DMSO ≤ 3%) were determined (see Figure S8 in Supporting Material). Data showed that **SiPcPGal$_4$** strictly follows the Beer-Lambert law at the studied concentrations enabling its use at concentrations below 10 µM. However, since cell viability is influenced by DMSO concentrations higher than 0.5%[14a], only concentrations of **SiPcPGal$_4$** below 1.7 µM in PBS (% DMSO ≤ 0.5) were tested in the *in vitro* assays using UM-UC-3 human bladder cancer cells.

Table 1. Photophysical data of the SiPcPGal$_4$ in PBS, DMSO and DMF

Solvent	λ_{max} [nm] (log ε)[a]
PBS	343 (4.31), 613 (4.00), 690 (4.50)
DMSO	348 (4.00), 608 (3.95), 646 (3.84), 685 (4.26)
DMF	369 (2.65), 607 (2.88), 646 (2.71), 679 (4.21)

[a] log ε M^{-1} cm^{-1}

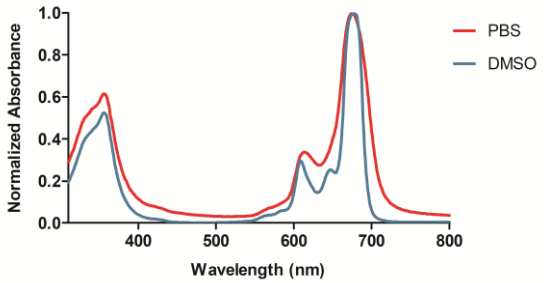


Figure 1. Normalized UV/Vis absorption spectra of **SiPcPGal$_4$** at 10 µM in PBS (3% DMSO) and DMSO.

The ability of **SiPcPGal$_4$** to produce singlet oxygen was indirectly evaluated in DMF, using 1,3-diphenylisobenzofuran (DPBF) as $^1O_2$ scavenger.[16] A comparative study was also carried out on two reported Pcs, namely a SiPc axial conjugated with two directly attached, protected galactose molecules (**SiPcPGal$_2$**, see Figure S9 in Supporting Material)[8a], and the commercially available **SiPc(OH)$_2$**, lacking galactose units (see Figure S9 in Supporting Material). **SiPcPGal$_4$**, **SiPcPGal$_2$** and **SiPc(OH)$_2$** were able to photo-oxidize DPBF (at 0.3 µM; Figure 2). Under the conditions of the assay, **SiPcPGal$_2$** demonstrated higher ability to photo-oxidize DPBF in comparison to **SiPcPGal$_4$** and **SiPc(OH)$_2$**. Nevertheless, further *in vitro* studies will give more clear results about the PS's ability to destroy the targeting cells.

SiPcPGal$_4$, **SiPcPGal$_2$** and **SiPc(OH)$_2$** demonstrated to be photostable when exposed to 150 mW.cm^{-2} light power over the irradiation period of 30 min (see Table S1 in Supporting Material).

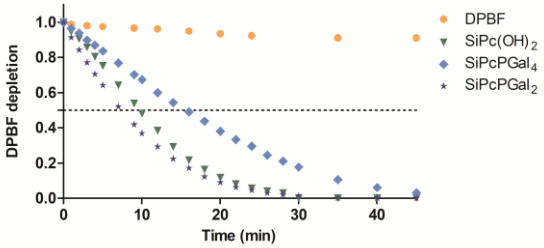


Figure 2. Photo-oxidation of DPBF (30 µM) in DMF with or without **SiPcPGal$_4$**, **SiPcPGal$_2$** and **SiPc(OH)$_2$** (0.3 µM), after irradiation with red light emitted by a LEDs array (λ > 600 nm, 5 mW.cm^{-2}). The DPBF absorbance was recorded at 415 nm. Data are the mean value ± SEM of two independent experiments.

SiPcPGal$_4$ accumulates in bladder cancer cells and is non-toxic in the absence of light

Most of the PDT studies *in vitro* using galactose-PSs involve conjugates containing unprotected sugars to allow sugar interaction with carbohydrate recognition domain (CRD) in the galactose-binding protein.[17] Interestingly, Vedachalam *et al*. have reported that conjugates containing galactose residues protected by isopropylidene groups have improved metabolic stability and facilitated cancer cells internalization.[18] It was suggested that the protective groups are cleaved in the acidic medium within cancer cells, being then accumulated intracellularly in the lysosomes and thereafter induce caspase-dependent apoptotic pathway after PDT.[19]

The photodynamic activity of **SiPcPGal$_4$** was evaluated against UM-UC-3 human BC cell line.[14] Comparative studies were also carried out on **SiPcPGal$_2$** and **SiPc(OH)$_2$**. BC UM-UC-3 cells were incubated with 1.70 µM of the three PSs, under dark conditions, for 3 h in PBS containing 0.5% of DMSO. The fluorescence properties of these compounds allowed the evaluation of their intracellular accumulation by quantitative spectrofluorimetry and fluorescence microscopy. Spectrofluorimetry data showed that **SiPc(OH)$_2$** is more readily taken up into cells than **SiPcPGal$_4$** or **SiPcPGal$_2$**. Moreover, **SiPcPGal$_4$** had an improved uptake over **SiPcPGal$_2$** (Figure 3a), which can be justified by the number of galactose molecules. Here, the solketal group was thought to act as a targeted micelle, which results in a higher intracellular concentration of the PS and an increase in photodynamic effect.[18,20]

The intracellular accumulation of PSs by UM-UC-3 cancer cells was confirmed by fluorescence confocal microscopy. Cells were incubated with 1.70 µM of **SiPcPGal$_4$**, **SiPcPGal$_2$** and **SiPc(OH)$_2$** for 3 h (in darkness), and cell nuclei were stained with DAPI. PSs exhibited strong fluorescence with occasional bright spots in the perinuclear region. **SiPc(OH)$_2$** had the higher accumulation, probably due to its high amphiphilicity relatively to the other two PSs (Figure 3b).

After confirming the three PSs uptake by UM-UC-3 cancer cells, their cytotoxicity in darkness was determined. UM-UC-3 cells were incubated with increasing concentrations of **SiPcPGal$_4$**, **SiPcPGal$_2$** and **SiPc(OH)$_2$** (up to 1.70 µM) in PBS (DMSO ≤ 0.5%) under darkness conditions for 3 h. Cell viability was determined 24 h after treatment by MTT colorimetric assay, where the yellow-colored MTT is reduced by mitochondrial dehydrogenases in living cells to a formazan precipitate, which absorbance is directly correlated with the metabolic activity of living cells. The Pcs at the studied concentrations did not induce significant dark toxicity (see Figure S10 in Supporting Material).

SiPcPGal$_4$ induces cytotoxicity after photoactivation

To test the effect of light irradiation (red light at potency of 2.5 mW.cm^{-2}) for 40 min after PS uptake on cell viability, MTT study was performed 24 h after treatment. No cytotoxicity was observed in sham irradiated cells or untreated cells in the presence of 0.5% DMSO (data not shown). However, when cells were incubated with PS and then irradiated, there was an increase of the phototoxicity dependent on the PS concentration (Figure 4a). Between 0.05 and 0.80 µM there was a proportional decrease in the percentage of reduced MTT with the concentration of **SiPcPGal4**. Moreover, at the highest concentration (1.70 µM), both **SiPcPGal$_4$** and **SiPcPGal$_2$** had the same percentage of toxicity (approximately 20.4%). Nevertheless, at lower concentrations, **SiPcPGal$_4$** demonstrated to be a better phototoxicity inducer with ~9 % more toxicity when compared to **SiPcPGal$_2$** (Figure 4a). **SiPc(OH)$_2$** did not show significant ability to induce cell death upon light activation, even though it was able to have a higher cellular uptake by UM-UC-3 cells, highlighting therefore the importance of the axial or peripheral conjugation of biomolecules to the Pcs for the improvement of their photophysical and photochemical properties.

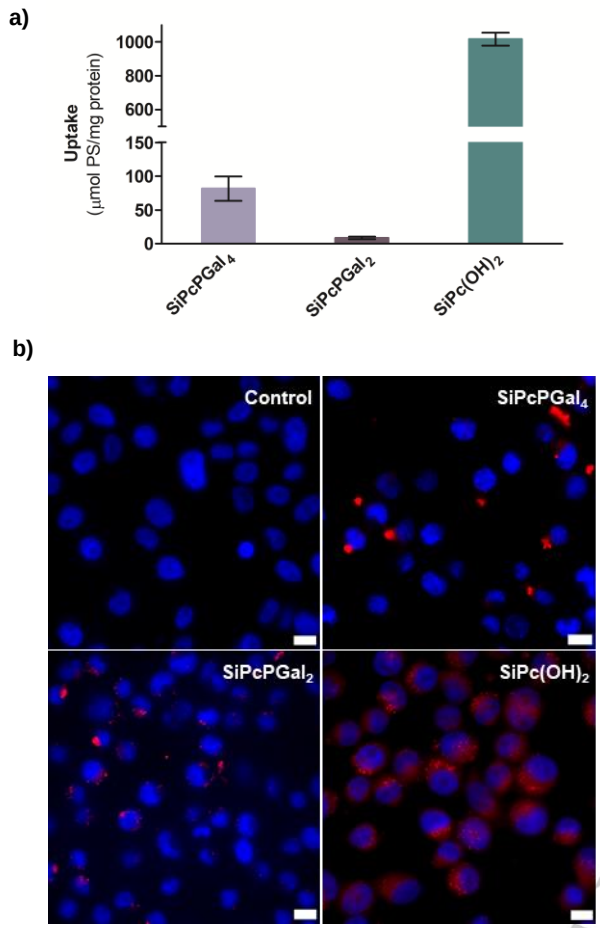


Figure 3. SiPcPGal$_4$, SiPcPGal$_2$ and **SiPc(OH)$_2$** accumulates in UM-UC-3 human bladder cancer cells. **a**) Intracellular uptake of **SiPcPGal$_4$**, **SiPcPGal$_2$** and **SiPc(OH)$_2$** by UM-UC-3 cells. Cells were incubated with (1.70 µM) PS (in PBS containing 0.5% DMSO) for 3 h. The concentration of PS was assessed by fluorescence spectroscopy and the results were normalized to protein quantity. Data are the mean ± SEM of two independent experiments performed in triplicates. **b**) Representative fluorescence images of UM-UC-3 cells incubated with 1.70 µM of **SiPcPGal$_4$**, **SiPcPGal$_2$** and **SiPc(OH)$_2$** for 3 h in darkness (red) with nucleus stained with DAPI (blue). Scale bars, 20 µm, are indicated on images. Original magnification: 60x.

To determine whether generation of ROS contribute to the phototoxic effects, intracellular ROS levels were evaluated, immediately after PDT (40 min of irradiation) in cells previously incubated with 1.70 µM of the three PSs for 3 h. Three different ROS-sensitive probes were used – i.e. DHE, H_2DCFDA and MitoPy1.[21] Interestingly, mitochondrial H_2O_2 levels, detected using MitoPy probe, were higher in PDT with **SiPcPGal$_4$** and **SiPcPGal$_2$** when compared with **SiPc(OH)$_2$** (Figure 4b), suggesting that **SiPcPGal$_4$** and **SiPcPGal$_2$** target the mitochondria. However, further studies are needed to clarify the preferential localization of these PSs in the mitochondria. In the case of their accumulation in mitochondria, phototoxicity could be enhanced, by damage of the mitochondrial outer membrane and cytochrome c release, triggering apoptosis.[22,23]

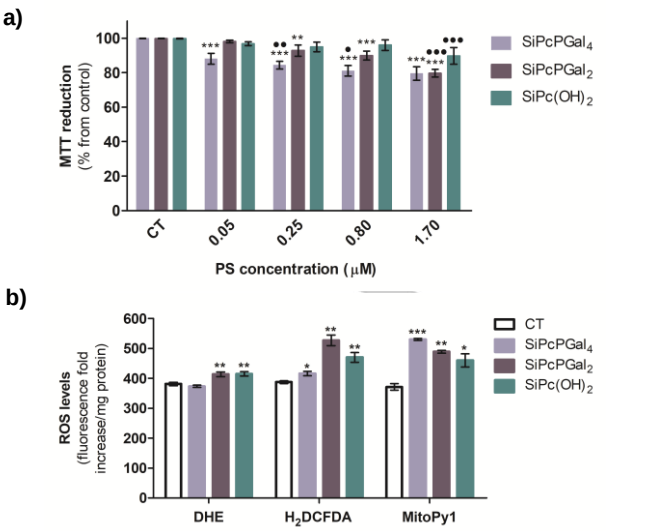


Figure 4. Photocytotoxic effect of SiPcs in UM-UC-3 cells was evaluated 24 h after PDT. **a)** UM-UC-3 BC cells were incubated with increasing concentrations (up to 1.70 µM) of **SiPcPGal$_4$**, **SiPcPGal$_2$** and **SiPc(OH)$_2$** for 3 h after 40 min of irradiation with red light at a fluence rate of 2.5 mW.cm^{-2}. Cytotoxicity was assessed using MTT colorimetric assay 24 h after treatment. The percentage of cytotoxicity was calculated relatively to control cells (CT, cells incubated with PBS (DMSO ≤ 0.5%) for 3 h in darkness and then irradiated). **b**) Quantification of DHE, H_2DCFDA and MitoPy1 fluorescence increase (as a measure of ROS production) after PDT by fluorescent spectroscopy. Data are the mean value ± SEM of two independent experiments performed in triplicates. *($p < 0.05$), **($p < 0.001$), ***($p < 0.0001$) significantly different from CT; •($p < 0.05$), ••($p < 0.001$), •••($p < 0.0001$) significantly different from the previous concentration (**figure a**).

Conclusions

In summary, we have successfully developed a galacto-dendritic silicon phthalocyanine that exhibits interesting properties as a PDT agent: (i) no aggregation in aqueous medium; (ii) ability to generate $^1O_2$; (iii) photostability and (v) high fluorescence. *In vitro*, at 1.70 µM, **SiPcPGal$_4$** is taken up by UM-UC-3 bladder cancer cells, however inducing low toxicity upon light activation. Altogether, these data suggest that **SiPcs** containing several units of galactose at their axial positions can be good platforms to be used in the design of new phthalocyanine-based photosensitizers, although it seems that they should be deprotected for a high cell toxicity.

Experimental Section

SiPcCl$_2$ was purchased from Sigma-Aldrich and used without further purification.

2-chloro-4,6-bis(1,2:3,4-di-*O*-isopropylidene-α-D-galactopyran-6-yl)-1,3,5-triazine (compound 1, Scheme 1) and 3-[(4,6-bis(1,2:3,4-di-*O*-isopropylidene-α-D-galactopyran-6-yl)-1,3,5-triazin-2-yl)thio]propane-1-thiol (compound 3, Figure S1, see Supporting Material) were synthesized as previously reported.[14b]

Synthesis of galacto-dendritic unit 2: 3-[(4,6-bis(1,2:3,4-di-O-isopropylidene-α-D-galactopyran-6-yl)-1,3,5-triazin-2-yl)oxi]propane-1-ol**:** Compound **1** (720 mg, 1.14 mmol) was dissolved in dry toluene (9.0 mL) under Ar, and DIPEA (0.55 mL, 5.72 mmol) and 1,3-propanediol (0.46 mL, 5.72 mmol) were added. The mixture was stirred at 80 ºC during 72 h. After rotary evaporation, the residue was purified by column chromatography on silica gel (Hept/AcOEt 6:4 to 1:1 v/v) to afford

compound **2** (471 mg, 61%) as a gummy yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.31, 1.32, 1.44 and 1.49 (4s, 4 x 6H, CH_3), 1.99 (m, 2H, b-CH_2), 3.76 (m, 2H, c-CH_2), 4.18 (ddd, 2H, $J_{4'}$= 1.8, $J_{5\text{-}6a}$ = 6.5 and $J_{5\text{-}6b}$ = 6.6 Hz, H-5'), 4.30-4.35 (m, 4H, H-2 and H-4), 4.51 (m, 4H, H-6a and H-6b), 4.54 (m, 2H, a-CH_2), 4.61 (dd, 4H, $J_{2\text{-}3}$ = 8.4, $J_{3\text{-}4}$ = 2.4 Hz, H-3) and 5.52 (d, 2H, $J_{1\text{-}2}$ = 5.0 Hz, H-1) ppm. HRMS-ESI (MeOH + 0.1% formic acid): calcd. for $C_{30}H_{46}N_3O_{14}$ $[M+H]^+$ 672.2974, found 672.3000; and $C_{30}H_{45}N_3O_{14}Na_1$ $[M+Na]^+$ 694.2793, found 694.2819.

Synthesis of SiPcPGal$_4$: SiPcCl$_2$ (67 mg, 0.0934 mmol), compound **2** (300 mg, 0.447 mmol) and a large excess of pyridine (0.2 mL) were dissolved in 12 mL of toluene and the mixture refluxed overnight. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel (Hex/AcOEt 3:2 to 1:1 v/v) affording **SiPcPGal$_4$** as a blue solid (30 mg, 12%). ^{1}H NMR (300 MHz, $CDCl_3$): δ −1.99 (t, 4H, *J* = 6.5 Hz, c-CH_2), −1.08 to −1.03 (m, 4H, b-CH_2), 1.38, 1.41, 1.54 and 1.55 (4s, 4 x 12, CH_3), 4.22 (ddd, 4H, 8H, $J_{4\text{-}5}$= 1.0, $J_{5\text{-}6a}$ = 4.0, $J_{5\text{-}6b}$ = 4.0 Hz, H-5), 4.30-4.34 (m, 4H, H-4), 4.38-4.44 (m, 12H, H-2 and H-6), 4.69 (dd, 4H, $J_{2\text{-}3}$ = 4.7, $J_{3\text{-}4}$ = 1.4 Hz, H-3), 5.64 (d, 4H, $J_{1\text{-}2}$ = 2.9 Hz, H-1), 8.29-8.31 (m, 8H, Pc–H$_\beta$) and 9.55-9.57 (m, 8H, Pc–H$_\alpha$) ppm. ^{13}C NMR ($CDCl_3$): δ 24.5, 25.0, 26.1 and 26.2 (CH_3), 27.8 (b-CH_2), 52.6 (a or c-CH_2), 65.5 (a or c-CH_2), 96.4, 107.9, 108.8, 109.5, 114.4, 123.6, 129.7, 130.9, 131.0, 135.8, 137.7 (Pc-C$_\beta$), 139.1, 139.5, 140.1, 149.2 (Pc-C$_\alpha$), 165.3 (C-4, C-6, TCT) and 172.2 (C-2, TCT) ppm. HRMS MALDI-TOF (DCTB): calcd. for $C_{92}H_{104}N_{14}Na_1O_{28}Si_1$ $[M+Na]^+$ 1903.6706, found 1903.6789.

SiPcPGal$_4$ photochemical and photophysical characterization: The PS solutions in PBS buffer, dimethylformamide (DMF) and dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO) were prepared by diluting the stock solutions previously made in DMSO (concentration of DMSO inferior to 3% (v/v)) and then their absorbance was measured in a UVIKON 922 spectrophotometer (Biotek instruments).

PS fluorescence emission spectrum was measured in DMF on a computer controlled Horiba JobinYvon FluoroMax-3 spectrofluorimeter, with wavelengths between 620 to 800 nm, after excitation at 601 nm. The excitation and emission slits width were set at 2.0 nm.

Solutions of PSs at 10 µM were prepared in PBS buffer (3% (v/v) DMSO) and its photostability was determined by monitoring the decrease of the Q bands absorbance between 640 and 730 nm, after different times of irradiation with a halogen/quartz 250 W lamp coupled to one optic fiber probe (400-800 nm) from the Light Source Model LC-122 illumination system (LumaCare). The fluence rate was 150 mW.cm^{-2} and was determined with the energy meter Coherent FieldMaxII-Top with a Coherent PowerSens PS19Q energy sensor.

Singlet oxygen study was accessed through an indirect chemical method using 1,3-diphenylisobenzofuran (DPBF) as $^1O_2$ scavenger.[14] Solutions containing DPBF (30 µM) with or without the PSs (0.30 µM) were prepared in DMF and then irradiated at room temperature (RT) with a Light Emitting Diode (LED) array composed by a matrix of 6x8 LEDs that emits red light (λ = 600 ± 20 nm). The fluence rate was 5 mW.cm^{-2} and it was regulated by a Plug-in adaptor with LED indicator 800 mA (Mean Well). The results were expressed by plotting the DPBF depletion against the irradiation time.

Cells culture and treatments: Human bladder cancer cell line UM-UC-3 (ATCC® Number: CRL-1749™) derived from a transitional carcinoma isolated from an urinary bladder of a male Caucasian, and was cultured in Roswell Park Memorial Institute medium (RPMI, Sigma) supplemented with 2 g·L^{-1} sodium bicarbonate (Sigma), 2 mM L-glutamine (Sigma), 10% (v/v) of heat-inactivated Fetal Bovine Serum (FBS; Life Technologies, Carlsbad, CA, USA) and antibiotic/antimycotic containing 100 units.mL^{-1} penicillin, 100 µg.mL^{-1} streptomycin and 0.25 µg.mL^{-1} amphotericin B (Sigma). UM-UC-3 cells were seeded into cell culture plates of 24 or 96-wells at a density of 18 x 10^4 and 3 x 10^4 cells.cm^{-2} respectively, and after incubation overnight, they were treated with desired concentrations of sterile solutions of **SiPcPGal$_4$** in the dark, **SiPcPGal$_2$** (synthesis previously reported[8a]) and **SiPc(OH)$_2$** (Sigma-Aldrich).

SiPcPGal$_4$ uptake in UM-UC-3 cells: After PS incubation in the dark, UM-UC-3 cells were washed twice with PBS and then mechanically scrapped in 1% (m/v) SDS solution (Sigma-Aldrich) in PBS (pH 7.0). Intracellular fluorescence of the PSs was determined by fluorometric measurement using Typhoon FLA 9000 imager (GE Healthcare). The results were normalized for protein concentration (determined by bicinchoninic acid reagent – Pierce, Rockford, IL, USA). To evaluate the PS intracellular fluorescence by microscopy, UM-UC-3 were grown in coverslips coated with poly-L-lysine (Sigma), for 24 h and then incubated with 1.7 µM of PSs for 3 h. After PS uptake and washing, cells were fixed with 4% paraformaldehyde (PFA; Merck, Darmstadt, Germany) for 10 min at RT. The samples were then mounted using Vectashield mounting medium containing DAPI (Vector Laboratories, CA, Burlingame) for visualization under the fluorescence microscope Leica DFC350 FX (Leica Microsystems, Bannockburn, IL, USA).

Cell metabolic activity: After PSs incubation during 3 h, with fresh RPMI medium, irradiation with red light (620-750 nm) was delivered by an illumination system (LC-122 LumaCare, London) for 40 min with a potency of 2.5 mW.cm^{-2} as measured with an energy meter (Coherent FieldMaxII-Top) combined with a Coherent PowerSens PS19Q energy sensor. During the same period of time, cells incubated with the three PSs were kept under dark conditions. In all treatments, triplicate wells were established under each experimental condition, and each experiment was repeated twice. Cell metabolic activity of PSs after 24 h of photodynamic treatment, was determined with MTT assay, in which it is measured the ability of BC cells to reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) to a colored formazan crystals, using a microplate reader spectrophotometer (Synergy™ HT, Biotek instruments) at 570 nm (using 620 nm as the background wavelength). The percentage of absorbance for each treated sample was normalized to each untreated CT.

Detection of intracellular reactive oxygen species generation: After photodynamic treatment and washing, UM-UC-3 cells were incubated with 5 µM of 2',7'-dichlorohydrofluorescin (H_2DCFDA; Life sciences), dihydroethidium (DHE; Life Sciences) and mitochondria peroxy yellow 1 (MitoPy1; Sigma-Aldrich) probes, under dark conditions, for 1 h at 37 ºC. After incubation, cells were washed with PBS and mechanically scrapped in 1% (m/v) SDS solution in PBS (pH 7.0). Then, the intracellular fluorescence was measured using a microtiter plate reader (Synergy HT) with excitation and emission filters of 485/20 and 590/35 nm for H_2DCFDA, 485/20 and 528/20 nm for DHE and MitoPy1. Protein concentration was determined using the Pierce® BCA Protein Assay Kit.

Statistical Analysis: The results are presented as mean ± standard error of the mean (S.E.M.) with *n* indicating the number of experiments. Statistical significance among two conditions was assessed using the nonparametric Mann-Whitney test. P-value was considered at the 5% level of significance to deduce inference of the significance of the data.

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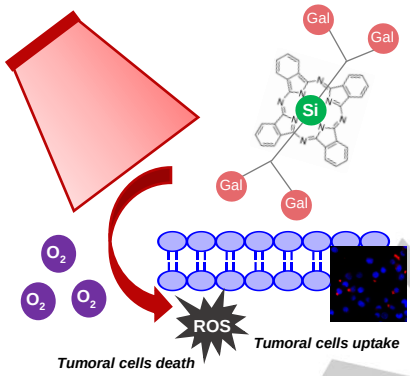
FULL PAPER

Solketal-protected galactose-dendritic units have been attached to the axial position of a silicon phthalocyanine and the **SiPcPGal$_4$** dye has been tested as photosensitizer for PDT in UM-UC-3 bladder cancer cells. The observed toxicity of **SiPcPGal$_4$** upon light activation arises in part from its enhanced cellular uptake comparing to **SiPcPGal$_2$**.



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A Galactose-Dendritic Silicon(IV) Phthalocyanine as a Photosensitizing Agent in Cancer Photodynamic Therapy