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Synthesis and Photosensitizing Properties of an Activatable Phthalocyanine-Subphthalocyanine Triad

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In this article, we describe a photosensitizer (PS) whose ability to generate singlet oxygen ($^1O_2$) and fluorescence emission has been designed as tumor responsive. More specifically, a PS consists of a silicon phthalocyanine (SiPc) core, axially substituted with two subphthalocyanine (SubPc) units, covalently linked by a disulfide linker, which is cleavable in the presence of a strong reducing agent. Initially, the SubPc units quench the SiPc fluorescent and $^1O_2$-generating properties, most probably by electronic energy transfer. Upon entering a reducing environment, *e.g.*, like those in tumor cells, the disulfide linker would be cleaved and the quenching would be undone, resulting in a sudden generation of $^1O_2$ and a strong fluorescence of both the SiPc and SubPc units. Herein, this behavior has been probed in solution by using DTT as a reducing agent that induces the PS activation.

Keywords: Phthalocyanine, Subphthalocyanine, Triad, Photosensitizer, Theranostics

INTRODUCTION

The integration of therapeutics and diagnostics, also called theranostics, is currently a hot topic in medicine and, accordingly, the development of new theranostic agents is of utmost importance. Ideally, the use of theranostic agents comprising both the therapeutic agent and a diagnostic probe, within a single molecular framework, results in more site-specific, effective and personalized healthcare [1,2]. Theranostic agents have also made their entry in photodynamic therapy (PDT), which is a minimally invasive medical technology that makes use of photoinduced singlet oxygen ($^1O_2$) to treat cancer and other diseased tissues [3-5]. The therapeutical agent in PDT is a photosensitizer (PS), which is responsible for the generation of the cytotoxic $^1O_2$ upon photoactivation. Combining this PS with a fluorophore as diagnostic probe could aid in its visualization, and shall contribute to the damaging of only unhealthy tissue. To this end, a major challenge is to improve the current low target tissue-to-background signal ratios and poor imaging resolution of therapeutic agents, resulting from the fact that most theranostic probes are unresponsive to their environment and thus, are permanently in an "on" state. Ideally, the theranostic probe should be a "smart" probe which remains in an "off" state until is activated selectively by external stimuli characteristic of the unhealthy tissues [6-9]. In this way, only the unhealthy target tissue is visualized, while the healthy tissue is not, increasing the signal-to noise-ratio and the imaging resolution tremendously. Furthermore, if not only the diagnostic probe but also the therapeutic agent could be in an "off" state, until they are activated by the characteristic unhealthy tissue stimuli, the PDT therapy would even be more effective, reducing the treatment side effects and resulting in lower doses of therapeutic agent required to be applied. However, to date, few theranostic systems present a simultaneously activatable therapeutic function [10-12].

In the field of PDT, next to porphyrins, phthalocyanines (Pcs) are among the most promising PS [13,14]. This can be explained by the several advantages they display, such as their strong absorption at the near-infrared (NIR) spectral

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region (*ca*. 670-700 nm), high molar extinction coefficients, and long-lived fluorescence [15,16]. Furthermore, upon photoactivation of Pcs, their singlet excited state can undergo intersystem crossing to the excited triplet state, which can in turn react with the triplet ground state of molecular oxygen, resulting in the formation of $^1O_2$ in a very effective way. Advantageously, within silicon Pcs (SiPcs), this process can be quenched if photo- or electroactive units are connected to the axial positions of the central silicon atom. Ng and co-workers have exploited this behavior to develop pH- and redox-responsive activatable PS [12,17-19], and similar approaches have been followed employing bodipy dyes by Akkaya and co-workers [20-23] . More recently, we reported a SiPc derivative bearing a pyrene unit as activatable fluorophore [24]. This PS presented three output signals (fluorescence at 377 and 683 nm, and $^1O_2$ production), potentially enabling multifunctional theranostics. However, the fluorescence and $^1O_2$ generation quenching before the PS activation was only moderate, mainly due to inefficient electronic energy transfer from the pyrene unit to the SiPc core. Its practical application was therefore limited by the small enhancement of $^1O_2$ formation upon the activation process.

To overcome such limitations, herein we report a new SiPc derivative (1) bearing two SubPc units as additional fluorophores at the axial positions of the central silicon atom (Fig. 1). SubPcs are lower homologues of Pcs, known to display significantly higher fluorescence quantum yields. Importantly, their absorption bands appear at regions of low SiPc absorption (*ca*. around 550 nm), enabling to get a panchromatic photosensitization that should in principle maximize $^1O_2$ generation. Moreover, SubPcs are also able to generate $^1O_2$, and having two SubPc units on the SiPc can contribute to the overall $^1O_2$ quantum yield of this PS system upon activation. For this purpose, the SubPc units are connected to the SiPc core by a cleavable disulfide linker, which can be easily and selectively cleaved by a strong reducing agent [25]. Presenting a reducing environment is a characteristic that distinguishes tumor tissues from healthy tissues, since the intracellular glutathione (GHS) level in tumor cells is a lot more elevated than that in healthy cells or in the extracellular matrix [26-28]. Therefore, in our design, the fluorescence of both the SiPc and SubPc units would be quenched outside of tumor cells, as will be their $^1O_2$ generation, as a result of energy transfer between the SubPc and SiPc moieties [29]. As a consequence, only upon entering the tumor cell, the disulfide bridges in 1 would be cleaved, by action of the reducing agent, disconnecting the SubPc units from the SiPc, and recovering in this way the fluorescence of both kinds of chromophores and their capacity for $^1O_2$ production (Fig. 1). Herein, this behavior has been probed in solution by means of using DTT as the reducing agent that induces the PS activation.

EXPERIMENTAL

Materials and Methods

Chemicals were used as purchased from commercial sources without further purification. Solvents were dried using standard techniques prior to use. All reactions were performed in standard glassware under inert argon atmosphere. Reactions were monitored by thin-layer chromatography (TLC) using TLC plates pre-coated with silica gel 60-F254 (Merck). Column chromatography was carried out on Merck silica gel 60, 40-63 μm (230-400 mesh). Gel permeation chromatography (GPC) was performed using Biorad Biobeads SX-1 (200-400 mesh). Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AC-300 instrument. UV-Vis and fluorescence spectra were recorded with a JASCO V-660 and a JACSO FP-8600 spectrophotometer, respectively. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) MS and high-resolution (HR-MS) mass spectra were recorded with a Bruker Ultrareflex III spectrometer. Fast atom bombardment (FAB) mass spectra were recorded with a VG AutoSpec de Waters instrument. IR spectra were recorded on a FTIR Bruker IFS66v spectrophotometer using KBr disks. Melting points were measured in a Buchi B-540 apparatus.

Subphthalocyanine 3. Boron subphthalocyanine chloride [30] (2) (0.080 g, 0.190 mmol) and silver trifluoromethanesulfonate (0.060 g, 0.230 mmol) were dissolved in dry toluene (3 ml) and the mixture was stirred at room temperature under argon atmosphere until the SubPcBCl reagent was consumed (*ca*. 40 min). Once the SubPc triflate was generated, 2-hydroxyethyl disulfide (0.058 g, 0.380 mmol) and N,N-diisopropylethylamine

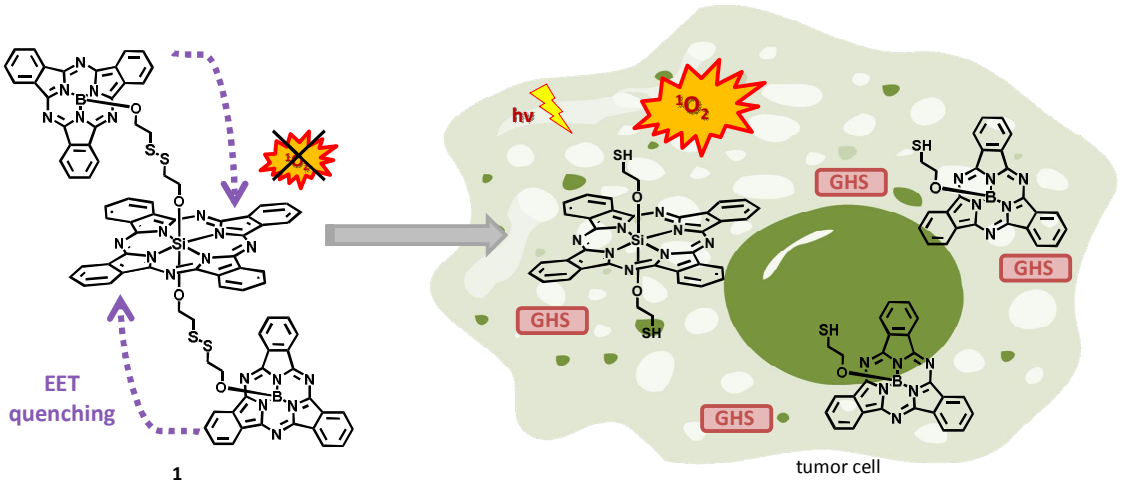


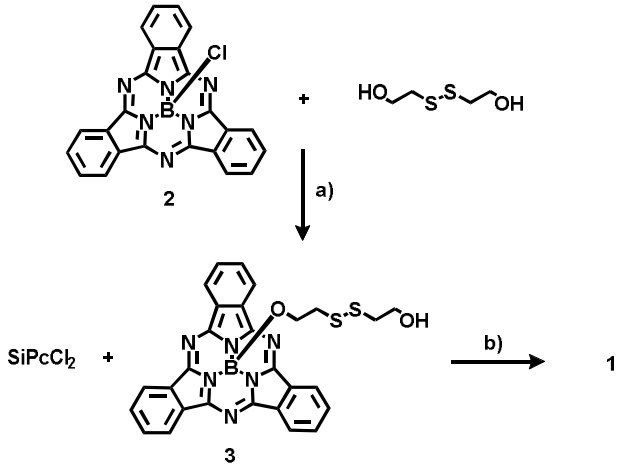
Fig. 1. Chemical structure and schematic representation of the possible mechanism of action of PS 1 upon entering a tumor cell. GHS means glutathione, and represents the characteristic reducing environment of tumor cells, which is responsible for the cleavage of the disulfide linkers of 1 and the subsequent activation of its $^1O_2$ generation and fluorescence response.

(DIPEA) (40 µl, 0.230 mmol) were added. The mixture was stirred at 40 ºC until the reaction was completed (6h). The solvent was removed by evaporation under reduced pressure and the product was directly purified by chromatography on silica gel, using a toluene/THF gradient (from 30:1 to 10:1) as the eluent. After trituration in water, compound 3 was obtained as a dark pink solid (0.063 g, 56%). ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 8.90-8.78 (m, 6H), 7.96-7.81 (m, 6H), 3.62 (t, J = 5.8 Hz, 2H), 2.43 (t, J = 5.8 Hz, 2H), 1.76 (dt, J = 9.8, 4.9 Hz, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ(ppm) 151.61, 131.11, 129.96, 122.29, 60.22, 58.04, 41.50, 39.92. MS (MALDI-TOF, DCTB): m/z = 564.2 (5), 548.2 [M^+] (100), 498.2 (5), 471.2 [M-$(CH_2)_2OH]^+$ (15), 412.2 [M-L_{axial}+OH]$^+$ (10), 242.3 (20) HR-MS (MALDI-TOF, DCTB): Calcd. for $C_{28}H_{21}BN_6O_2S_2$: 548.1260; Found: 548.1261.

SiPc-SubPc triad 1. Silicon phthalocyanine dichloride ($SiPcCl_2$, obtained from Sigma Aldrich) (0.027 g, 0.044 mmol), the SubPc 3 (0.121 g, 0.222 mmol) and DIPEA (0.081 ml) were heated in dry toluene (4.1 ml) under reflux and argon atmosphere for 18 h. The solvent was removed under reduced pressure, and the crude was purified by size exclusion chromatography (SEC), with Bio-Beads as the stationary phase and CH_3Cl_3 as the eluent, to yield 1 as a dark purple solid (0.008 g, estimated yield, 11% [31]). Importantly, due to the lability of the axial groups in triad 1 (see below), it was not possible to detect the molecular ion by mass spectrometry, yet NMR characterization is conclusive about the purity and identity of the product (see Fig. S5).^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 9.52 (dd, J = 5.8, 3.0 Hz, 8H), 8.75 (dd, J = 5.9, 3.1 Hz, 12H), 8.23 (dd, J = 5.7, 3.0 Hz, 8H), 7.81 (dd, J = 5.9, 3.1 Hz, 12H), 0.98-0.69 (m, 4H), 0.42 (t, J = 6.6 Hz, 4H), -0.97 (t, J = 6.6 Hz, 4H), -2.16 (t, J = 6.7 Hz, 4H).^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 151.37, 149.27, 136.01, 130.97, 129.75, 123.75, 122.13, 57.24, 53.57, 29.86, 22.85.

RESULTS AND DISCUSSION

The synthesis of the new SiPc derivative 1 is described in Scheme 1. The SubPc 2 was prepared according to standard procedures reported in literature [30], and its axial chloride substituent replaced by 2-hydroxyethyl disulfide following an axial substitution methodology developed in our group [32], obtaining in this way the SubPc 3. The next step involved the nucleophilic displacement of the chlorine atoms in $SiPcCl_2$, which is commercially available (Sigma Aldrich), with the primary alcohol 3. As a result, the SiPc-



Scheme 1. Synthesis of the SiPc-SubPc triad 1. a) AgOTf, toluene, rt, 6h, 56%, b) DIPEA, toluene, reflux, 18h

Table 1. Fluorescence and $^1O_2$ Quantum Yield Values for Compounds 1, 3 and 4

	1	3	4
Φ_Δ[a]	0.11	0.49	0.36
Φ_F (Sub)[b]	0.02	0.38	-[d]
Φ_F (Pc)[c]	0.05	-[d]	0.37

[a]Using ZnPc in DMF as the reference ($\Phi_\Delta = 0.56$). [b]Excited at 520 nm, using $ClSubPcF_{12}$ in benzonitrile as the reference ($\Phi_F = 0.58$). [c]Excited at 610 nm, using ZnPc in DMF as the reference ($\Phi_F = 0.28$). [d]Not applicable.

SubPc triad 1 was obtained, where the SubPc units are connected to the SiPc through a disulfide linker that can be selectively cleaved under reducing conditions (*e.g.*, DTT, in the present case).

The absorption and photochemical properties of the SiPc-SubPc triad 1, the SubPc 3, and a reference SiPc (4, see in the SI its molecular structure, Fig. S1) were measured in DMF (Table 1). The UV-Vis spectra of the SubPc 3 and the SiPc 4 show their typical broad Soret bands (at 300-400 nm) and sharp Q bands (at 562 and 675 nm, respectively). Accordingly, the UV-Vis spectrum of the SiPc-SubPc triad 1 shows a broad Soret band at 300-400 nm and two Q-bands; the first one originating from the two axial SubPc units (at 562 nm) and the second one from the SiPc unit (at 675 nm), in an expected 2:1 stoichiometrical ratio (Figure S2). Fluorescence quantum yields (Φ_F) were measured by exciting the compounds at 520 nm (for SubPcs) or 610 nm (for Pcs). $^1O_2$ quantum yields (Φ_Δ), in turn, were measured by the relative method, using 1,3-diphenylisobenzofuran (DPBF) as chemical scavenger and excitation with visible light filtered below 455 nm ($h\nu > 455$ nm -- see SI). As expected, compared to the reference SiPc 4 and the SubPc 3, the SiPc-SubPc triad 1 shows a significant deactivation of its fluorescence and $^1O_2$ generation capacity, probably as a result of an electronic energy transfer process in which the SubPc emission band and the SiPc Q-band partially overlap [28].

To investigate the effect of reducing environment (as in tumor cells) on the capacity of triad 1 for $^1O_2$ generation in a physiologically relevant medium, $^1O_2$ studies were performed in phosphate buffered saline (PBS) solutions with varying 1,4-dithiothreitol (DTT) concentrations, as the reducing environment simulation agent. Adequate solubility in PBS was achieved by adding 0.5% Cremophor EL (v/v), a solubilizing agent commonly employed in the pharmaceutical industry. In these studies, the rate of DPBF photodegradation upon irradiation of the SiPc-SubPc triad 1, which is proportional to the amount of $^1O_2$ formation, is measured in combination with 0 μM, 2 μM, 5 mM, 20 mM and 50 mM of DTT. Figure 2 shows the scavenger absorption decay at 414 nm, sensitized by 1 upon $h\nu > 455$ nm illumination, for different DTT concentrations. The slope of the resulting straight lines indicates the relative capacity of $^1O_2$ generation for each of the compounds; the steeper the decrease, the higher the amount of $^1O_2$ generated by the compound in the respective conditions. A clear trend can be deduced from these data, demonstrating that more efficient $^1O_2$ generation results from samples exposed to a higher concentration of DTT. Thus, the degree of disulfide cleavage seems to be directly correlated to the $^1O_2$ generation efficiency of 1. In the absence of DTT or in the presence of only 2 μM of DTT (corresponding to the extracellular glutathione concentration) there is no enhancement of the $^1O_2$ generation, which reflects the need of a strong reducing environment to promote such activation

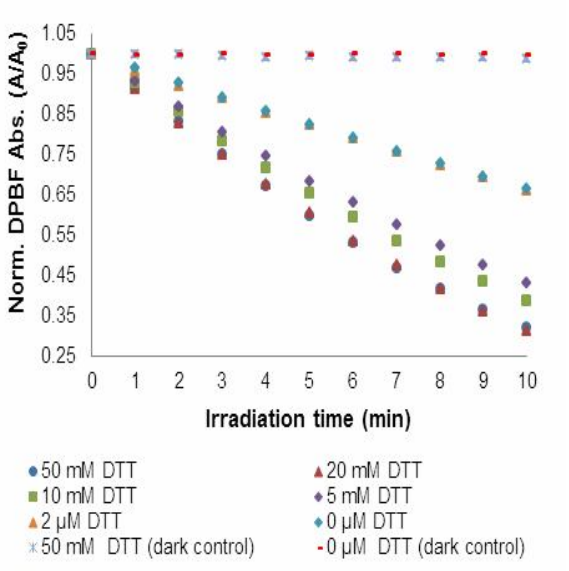


Fig. 2. Effect of DTT on the capacity of 1 for $^1O_2$ generation: normalized absorption (A/A_0) of DPBF at various concentrations of DTT over time, showing its photodegradation rate sensitized by 1 upon excitation with light above 455 nm.

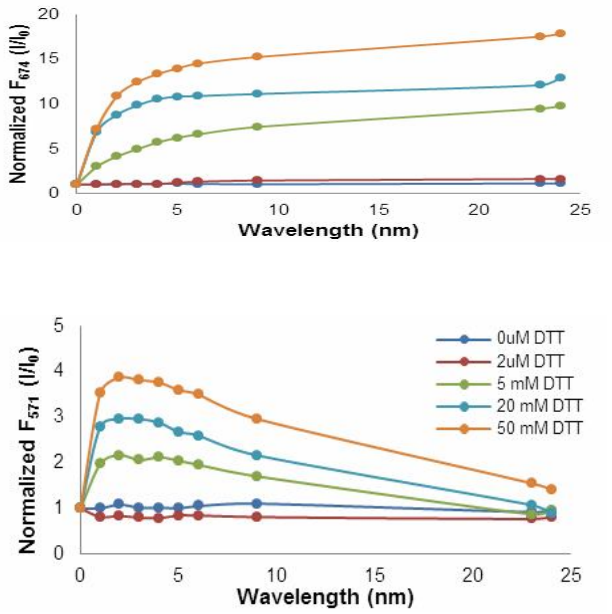


Fig. 3. Effect of DTT on the fluorescence emission of 1: normalized emission (I/I_0) over time of a) the SiPc unit (at 674 nm) and b) the SubPc units (at 571 nm) at various concentrations of DTT.

via disulfide cleavage. Moreover, dark control experiments confirm that, under all conditions, compound 1 is unable to produce $^1O_2$ without hν > 455 nm illumination.

To further investigate the influence of intratumor-like disulfide cleavage on the fluorescence properties of 1, its emission spectra were monitored for 24 h in the same buffer solutions with varying DTT concentrations (Fig. S3). As can be seen in Fig. 3a, the fluorescence intensity of the SiPc unit (at 674 nm -- λ_{ex} = 345 nm) exhibits approximately a 9-, 13- and 18-fold increment in the presence of 5, 20 and 50 mM of DTT respectively, while in the absence of DTT, or in the presence of only 2 µM of DTT, there is no SiPc fluorescence enhancement. This increase in fluorescence intensity indicates that the SiPc emission is recovered upon disulfide cleavage. On the other hand, the fluorescence intensity of the SubPc unit (at 571 nm -- λ_{ex} = 345 nm) displays a similar initial recovery, and reaches a maximum recovery around 2 h, after which the fluorescence intensity starts decreasing again (Fig. 3b).

To explain this last unexpected result, the influence of DTT on the absorption of 1 was studied (Fig. S4). From such experiment, it became clear that 1 was unstable to the strong reducing environment, probably due to the lability of the SubPc axial moieties, as indicated by a decrease of the SubPc absorbance at 562 nm in function of time, the extent of the decrease being greater at higher concentrations of DTT. However, this instability, although unexpected and in principle undesired, does not necessarily urge to discard the use of 1 as an activatable tumor-responsive and theranostic PS for PDT. The reason is that the outcome caused by the actual decomposition of the SubPc unit, or by the foreseen cleavage of the disulfide bridge, remains intact with respect to both the capacity for $^1O_2$ generation and the fluorescence response of the SiPc unit. Indeed, both outcomes would be activated once 1 enters the strong reducing environment of a tumor cell, just as they are in PBS solution.

CONCLUSIONS

In sum, we have developed a PS with the ability to generate $^1O_2$, the main reactive species in PDT, in a

substantially higher amount once entered in a reducing environment simulating those of tumor cells. To this end, we connected a photoactive quencher to the SiPc core through a disulfide linker, which can be selectively cleaved in the presence of a reducing agent (*e.g.*, DTT). As quencher, two fluorescent SubPc units have been used, resulting in a simultaneous increase of the SiPc and SubPc emission upon activation with DTT in PBS buffer. These results open the door to the possibility of employing multifunctional PS in therapeutic treatments with orthogonal imaging protocols. However, the SubPc units of this particular PS are not completely stable towards the employed reducing conditions. Our next steps will therefore be to implement analogous PS systems with alternative fluorescent agents. Furthermore, an increased array of stimuli-responsive groups could be used and the PS could be incorporated into biocompatible carrier platforms.

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