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Preparation and in vitro photodynamic activities of novel axially substituted silicon (IV) phthalocyanines and their bovine serum albumin conjugates

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Abstract—Two novel axially substituted phthalocyanines, namely bis(4-(4-acetylpiperazine)phenoxy)phthalocyaninatosilicon (IV) (**1**) and its *N*-methylated derivative **2**, have been synthesized. The dicationic phthalocyanine **2** is non-aggregated in water and exhibits good photophysical properties. The non-covalent BSA conjugates of these compounds have also been prepared. Compound **2** and the conjugate **2**-BSA show extremely high photodynamic activities toward B16 melanoma cancer cell lines. The corresponding 50% growth-inhibitory (IC_{50}) ratios are 33 and 38 nM, respectively.

Phthalocyanines represent a unique class of macrocyclic compounds which have been extensively studied as functional materials for various applications.[1,2] One of their most important applications is functioning as photosensitizers for photodynamic therapy (PDT).[3,4] Due to the intense absorption in the red visible region, high efficiency to generate reactive oxygen species (such as singlet oxygen), and low dark toxicity, phthalocyanines have been used in this avenue for the treatment of various cancers and photoinactivation of viruses.[5] It has been established that non-aggregated and hydrophilic phthalocyanines are extremely important and potentially useful for this application.[6] Molecular aggregation of phthalocyanines, which is an intrinsic property of these large π-conjugated systems, provides an efficient non-radiative energy relaxation pathway, thereby shortening the excited state lifetimes and greatly reducing the photosensitizing efficiency.[7] Although considerable efforts have been devoted to improve the water-solubility and avoid aggregation of these macrocycles, only a few phthalocyanines have been reported so far which exist mainly in monomeric form in aqueous media in the absence of surfactants or other disaggregating agents.[5c,6,8–10] We report herein the preparation and in vitro photodynamic activities of two novel silicon (IV) phthalocyanines **1** and **2**, which are axially linked to the 4-(4-acetylpiperazine)phenoxy group or its N-methylated derivative, and their bovine serum albumin (BSA) conjugates. By introducing two piperazine-containing substituents at the axial positions of a silicon phthalocyanine, we hope to increase the hydrophilicity and inhibit the self-aggregation of these compounds. Silicon (IV) phthalocyanines are promising photosensitizers for PDT.[8–10] Apart from their desirable photophysical properties, these compounds can have axial substituents to prevent aggregation and impact desirable characterizations to the molecules. Piperazine has been used as an active group of many kinds of drugs.[11] It would also be interesting to evaluate whether this functionality has beneficial effects on the photodynamic process.

The synthesis of compounds **1** and **2** is shown in Scheme 1. Treatment of the readily available silicon (IV) phthalocyanine dichloride with 1-acetyl-4-(4-hydroxyphenyl)piperazine in the presence of NaH in toluene led

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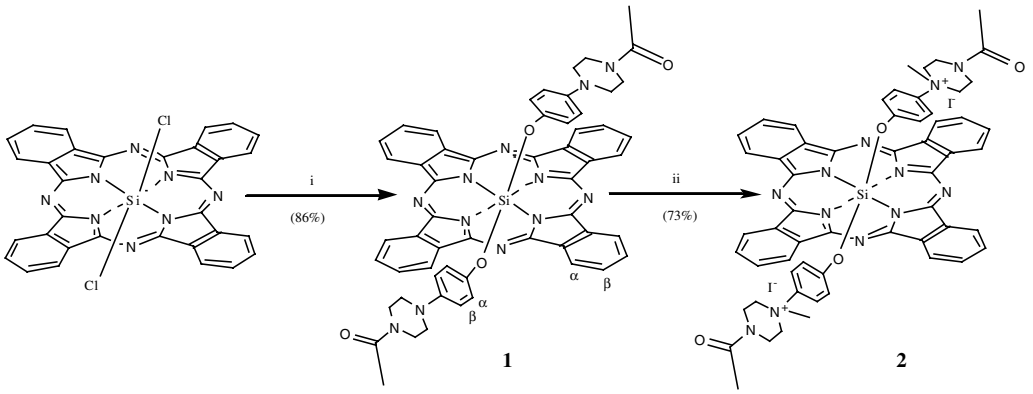

Scheme 1. Synthesis of **1** and **2**. Reagents and conditions: (i) 1-acetyl-4-(4-hydroxyphenyl)piperazine, NaH, toluene, reflux 12 h. (ii) CH_3I, $CHCl_3$, reflux 2 h followed by stirring at rt for 12 h.

to the formation of the disubstituted product **1** in 86% yield. Reaction of **1** with a large excess of iodomethane in $CHCl_3$ gave the dicationic compound **2** in 73% yield. The new compounds **1** and **2** were fully characterized by ^{1}H NMR, MS, and FT-IR spectroscopic method together with elemental analysis.[12]

The electronic absorption spectra of **1** and **2** in *N*,*N*-dimethylformamide (DMF) were typical for non-aggregated phthalocyanines, showing a Q-band at 680 nm (for **1**) (Fig. 1) or 685 nm (for **2**) (Fig. 2), both with a molar extinction coefficient of 2.3×10^5 M^{-1} cm^{-1}. With the addition of 0.5% DMF, **1** could become soluble in water. As shown in Figure 1, the absorption spectrum in water shows a broad signal peaking at ca. 710 nm, indicating that it is highly aggregated in this system. The dicationic analogue **2** was readily soluble in water and exhibited a strong and sharp Q-band at 690 nm (Fig. 2). This absorption obeyed the Lambert–Beer law (inset of Fig. 2) showing that the compound is essentially free from aggregation in water. The axial cationic groups are therefore very effective to inhibit aggregation of the macrocycle.

Upon excitation at 610 nm, compound **1**, either in DMF or water (with 0.5% DMF), showed a weak fluorescence emission at 681–683 nm with a fluorescence quantum

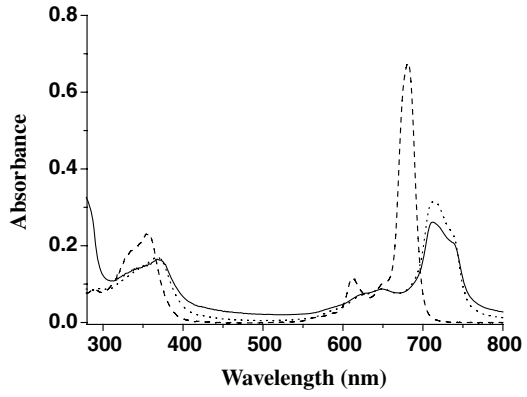

Figure 1. Electronic absorption spectra of 3 μM of **1** (---) in DMF, **1**-BSA (—) in water, and **1** (· · · · · ·) in water with 0.5% DMF.

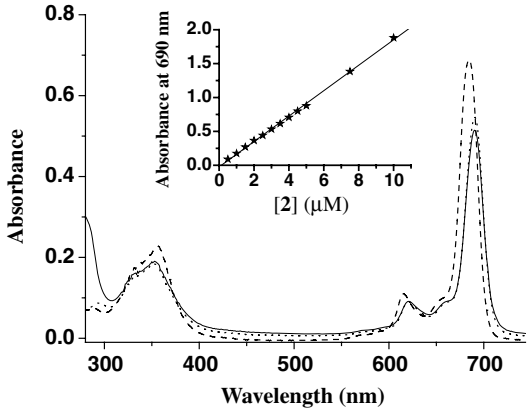

Figure 2. Electronic absorption spectra of 3 μM of **2** (---) in DMF, **2**-BSA (—), and **2** (· · · · · ·) in water. The inset shows the plot of the Q-band absorbance against the concentration for **2** in water.

yield (Φ_F) of 0.01–0.02. By contrast, the dicationic derivative **2** gave a strong fluorescence emission either in DMF ($\Phi_F = 0.22$) or in H_2O ($\Phi_F = 0.28$). The data are summarized in Table 1. To evaluate the photosensitizing efficiency of these phthalocyanines, their singlet oxygen quantum yields (Φ_Δ) were also determined by a steady–state method with 1,3-diphenylisobenzofuran as the scavenger.[13] It was found that **2** is an excellent singlet-oxygen generator with a Φ_Δ value of 0.49, which is about three times higher than that of **1**. It is likely that the amino groups in compound **1** reductively quench the singlet excited state, resulting in a weaker fluorescence emission and lower efficiency to generate singlet oxygen,[14] while N-methylation removes the electron-donating centers, leading to higher values of Φ_F and Φ_Δ.

With the goal of enhancing the biocompatibility and selectivity, compounds **1** and **2** were complexed with BSA. BSA is a common protein carrier for anticancer drugs to improve their passive targeting properties,[15] but its use for targeted delivery of phthalocyanine-based photosensitizers remains little studied.[16,17] We first investigated the interactions of **1** and **2** with BSA by a fluorescence quenching method.[8] Figure 3 shows the change in the fluorescence spectrum of BSA in a Tris–HCl buffer solution (pH 7.4) upon titration with **2**.[18]

Table 1. Photophysical data for **1**, **2**, **1**-BSA, and **2**-BSA

Compound	Solvent	λ_{max} (nm)	ε ($\times 10^5$ M^{-1} cm^{-1})	λ_{em} (nm)[a]	Φ_F[b]	Φ_Δ[c]
1	DMF	680	2.3	681	0.02	0.15
2	DMF	685	2.3	690	0.22	0.49
1	Water[d]	680–750 (br)	—	692	0.01	—
2	Water	690	1.9	696	0.28	—
1-BSA	Water	680–750 (br)	—	691	0.01	—
2-BSA	Water	690	1.8	691	0.30	—

[a] Excited at 610 nm.
[b] Using unsubstituted zinc(II) phthalocyanine (ZnPc) in DMF as the reference ($\Phi_F = 0.28$).[5f]
[c] Using ZnPc in DMF as the reference ($\Phi_\Delta = 0.56$).[13]
[d] Contained 0.5% (v/v) DMF.

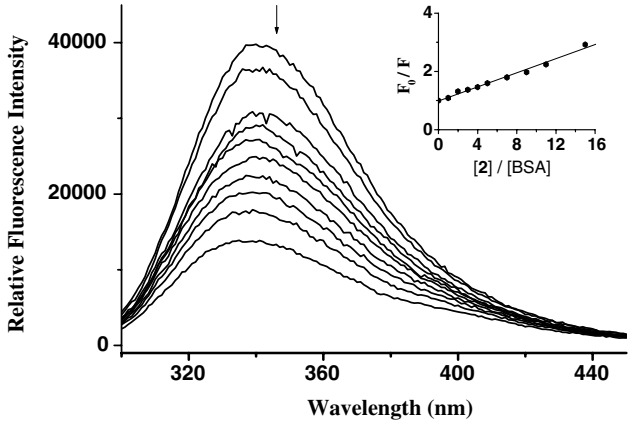

Figure 3. Change in fluorescence spectrum of BSA (2 μM, excited at 280 nm) in a Tris–HCl buffer upon titration **2.** The inset shows the corresponding Stern–Volmer plot.

The emission band at 340 nm decreases in intensity and shifts gradually to 338 nm. The quenching data follow the Stern–Volmer equation as shown in the inset of Figure 3, giving a quenching constant K_{SV} of $1.2 \times 10^5 M^{-1}$. Compound **1** behaved similarly and the corresponding K_{SV} value was found to be 1.6×10^5 M^{-1}. The high K_{SV} values suggest that there are strong interactions between the two phthalocyanines and the protein.

In view of the strong interactions between these phthalocyanines and BSA, attempts were made to prepare their non-covalent conjugates according to the literature procedure.[8] The conjugates were obtained by stirring a mixture of **1** (or **2**) and BSA (with a molar ratio of 6) in a Tris–HCl buffer at ambient temperature overnight, followed by gel chromatography on a G-100 Sephadex column using a 20 mM aqueous NH_4HCO_3 (pH 8.3) as eluent. The conjugates collected as the first blue fraction were then lyophilized to remove NH_4HCO_3 and water. The protein content in the conjugates was determined with the Bio-Rad protein assay kit using BSA as standard, while the phthalocyanine concentration was calculated from the Q-band absorbance in a diluted DMF solution with reference to the corresponding molar absorptivity (Table 1). The molar ratio of phthalocyanine to BSA was found to be 2:1 for both **1**-BSA and **2**-BSA conjugates. The corresponding ratio by weight was found to be 2.9% for **1**-BSA and 3.8% for **2**-BSA, respectively.

These two conjugates were readily soluble in water. Their spectroscopic properties in water were similar to those of the corresponding non-complexed counterparts (Table 1). As shown in Figures 1 and 2, the absorption spectra of **1** and **1**-BSA, as well as for **2** and **2**-BSA, are similar except for the stronger absorption at ca. 280 nm in the spectra of the conjugates, which is the typical absorption of BSA. In both conjugates, one BSA molecule binds to two phthalocyanine molecules. It is likely that the two phthalocyanine molecules of **1** are quite close to each other in the position cavity. The aggregation leads to a rather broad Q-band, peaking at ca. 710 nm. By contrast, the two phthalocyanine molecules of **2** bind to BSA in different sites. Each of them is encapsulated in the site giving a sharp Q-band typical for non-aggregated phthalocyanines.

The photodynamic activities of phthalocyanines **1** and **2**, and their albumin conjugates were investigated against B16 melanoma cell line using the procedure described earlier.[19] Figure 4 shows the dose-dependent survival curves for **2** and **2**-BSA. It can be seen that both systems are not cytotoxic in the absence of light, but exhibit a very high photocytotoxicity; about 0.1 μM photosensitizer is sufficient to kill virtually all the cells. The IC_{50} values, defined as the photosensitizer concentration required to kill 50% of the cells, were calculated to be 33 nM for **2** and 38 nm for **2**-BSA, respectively. The comparable

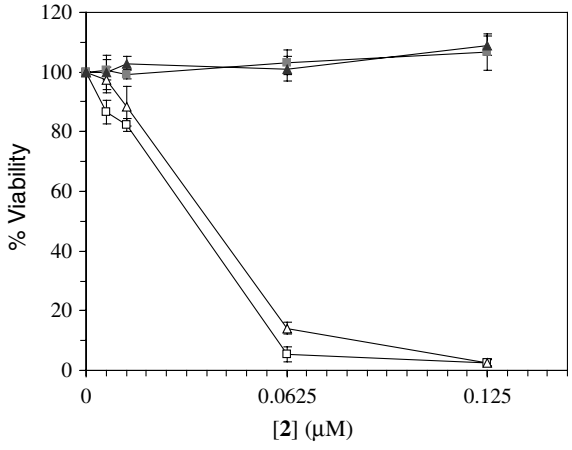

Figure 4. Effects of **2** (squares) and **2**-BSA (triangles) on B16 cells in the absence (closed symbols) and presence (open symbols) of light. For the latter, the cells were illuminated with a red light ($\lambda > 610$ nm, 50 mW cm^{-2}, 60 J cm^{-2}). Date are expressed as means ± SD ($n = 3$).

IC_{50} values suggest that conjugation with BSA still retains the high in vitro photodynamic activity of **2**.

By contrast, **1** and **1**-BSA conjugate did not show significant photocytotoxicity (up to 2.5 μM of **1**) toward B16 cells. When 2.5 μM of phthalocyanine was used, the cell viability was found to be 95.8 ± 2.8% for **1** and 90.0 ± 5.6% for **1**-BSA, respectively. This might be related to their higher aggregation tendency and lower efficiency to generate singlet oxygen.

In summary, we have prepared and characterized two new axially substituted silicon (IV) phthalocyanines and their BSA conjugates. The water-soluble dicationic compound **2** has a well-defined structure, good photophysical properties, a non-aggregated nature in water, and a very high photodynamic activity, and is therefore a highly promising new photosensitizer for PDT. Its BSA conjugate also exhibits a high in vitro potency and may serve as a good candidate for targeted PDT for cancer cells with macrophage origin, such as mammary carcinoma, which have a high affinity for albumin by receptor-mediated endocytosis.[20] Further studies of these systems are in progress.

Acknowledgments

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12. Characterization data for **1**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.59–9.62 (m, 8 H, Pc-H_α), 8.34–8.36 (m, 8 H, Pc-H_β), 5.18 (d, J = 8.1 Hz, 4 H, Ph-H_β), 3.35–3.36 (m, 4 H, CH_2), 3.17–3.18 (m, 4 H, CH_2), 2.38 (d, J = 8.1 Hz, 4H, Ph-H_α), 2.14–2.22 (m, 8 H, CH_2), 1.96 (s, 6 H, CH_3). IR (KBr) (ν_{max}, cm^{-1}): 3005 (–C═C–H), 2922 (–CH_2 and –CH_3), 1651 (–C═O), 1505, 1440 (–CH_2), 1336 (–C–N–), 1291, 1081 (–Si–O), 3054, 1431, 830 (–C_6H_4–). MS (ESI): *m/z* 980.8 $(M+H)^+$. Anal. Calcd for $C_{56}H_{46}N_{12}O_4Si$: C, 68.69; H, 4.74; N, 17.17. Found: C, 68.43; H, 4.99; N, 16.86. Characterization data for **2**: ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.64–9.67 (m, 8 H, Pc-H_α), 8.50–8.53 (m, 8 H, Pc-H_β), 6.25 (d, J = 9.3 Hz, 4 H, Ph-H_β), 3.27–3.29 (m, 8 H, CH_2), 2.59 (d, J = 9.3 Hz, 4 H, Ph-H_α), 2.56 (s, 6 H, NCH_3), 2.48–2.50 (m, 8 H, CH_2), 2.17 (s, 6 H, CH_3). IR (KBr) (ν_{max}, cm^{-1}): 3009 (–C═C–H), 2925 (–CH_2 and –CH_3), 1642 (–C═O), 1507, 1466 (–CH_2), 1336 (–C–N–), 1293, 1083 (–Si–O), 3060, 1430, 845 (–C_6H_4–). MS (ESI): *m/z* 504.8 $(M-2I)^{2+}$. Anal. Calcd for $C_{58}H_{52}I_2N_{12}O_4Si$: C, 55.16; H, 4.15; N, 13.31. Found: C, 55.40; H, 4.50; N, 13.02.
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18. Tris–HCl buffer aqueous solution contained 50 mM Tris(hydroxymethyl)amino-methane and 0.1 M NaCl, which was adjusted to pH 7.4 with HCl(aq).
19. For in vitro studies, compound **2** (or **1**-BSA and **2**-BSA) was dissolved in water to form a 10 μM stock solution, while **1** was dissolved in 0.5% DMF in water to form a 25 μM stock solution. The stock solution was further diluted with the medium to different phthalocyanine concentrations. About 1×10^4 B16 cells per well in 100 μL RPMI medium 1640 supplemented with 10% fetal calf serum were incubated in 96-multiwell plates overnight at 37 °C under 5% CO_2. The cells were rinsed with phosphate-buffered saline (PBS) and incubated with the above phthalocyanine solution (100 μL) for 2 h under the same conditions. The cells were then rinsed again with PBS and re-fed with the growth medium (100 μL) before being illuminated (λ > 610 nm, 50 mW cm^{-2}, 60 J cm^{-2}) at ambient temperature. Cell viability was determined after 24 h by the colorimetric MTT assay. The detailed experimental procedures were described previously.[8]
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