Journal of Porphyrins and Phthalocyanines
J. Porphyrins Phthalocyanines **2**, 191–199 (1998)

Sulfonated Phthalocyanines: Photophysical Properties, *in vitro* Cell Uptake and Structure–activity Relationships

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Received 27 May 1997
Accepted 9 September 1997

ABSTRACT: Aluminium phthalocyanines sulfonated to a different degree ($AlPcS_n$) and consisting of various isomeric species were studied by spectroscopic techniques to determine their tendencies to form dimers and aggregates. These characteristics were compared with the cell-penetrating properties of the species, using the Ehrlich ascites mouse tumor cell line, to arrive at structure–activity relationships. $AlPcS_n$ preparations consisting of the least number of isomeric species exhibited the highest tendency to form dimers and aggregates, whereas the more complex preparations, consisting of many isomeric products, showed more consistent monomeric features in aqueous environments. Uptake in cells was shown to correlate well with the overall hydrophobicity of the preparation and inversely with its degree of aggregation in the extracellular environment. Among the purified, single isomeric $AlPcS_n$ the amphiphilic disulfonated $AlPcS_{2a}$, enriched in positional isomers featuring sulfonate groups on adjacent phthalic subunits, showed the best membrane-penetrating properties. Even higher cell uptake was observed for the $AlPcS_{2mix}$ reflecting a combination of optimal lipophilicity and a low degree of aggregation. Similarly, in the case of $AlPcS_4$, the pure isomeric compound showed less cell uptake than the mixed isomeric preparation of similar hydrophobicity, reflecting the higher degree of aggregation invoked by its symmetrical structure. Our data indicate that mixed sulfonated phthalocyanine preparations may exert higher photodynamic efficacy in biological applications as compared to the pure isomeric constituents. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: sulfophthalocyanines; absorption and fluorescence spectra; aggregation and cell uptake

INTRODUCTION

There has been considerable interest in phthalocyanines (Pc) for use in photodynamic therapy (PDT), mainly because of their high absorption coefficient (at 650–680 nm) where penetration of light into tissue is optimal [1–3]. Water-soluble, sulfonated metallophthalocyanines ($MPcS_n$) are particularly attractive PDT agents; they are void of local and systemic toxicity and their photosensitizing properties have been evaluated extensively, both in cell cultures and under *in vivo* conditions [1–3]. It is of particular interest to note that a mixture of differently sulfonated AlPcS (Photosens®) has been used routinely in Russia since 1994 for both diagnosis and clinical PDT of various cancers, including small lung tumors [4, 5]. The degree of sulfonation, the isomeric composition and the nature of the central metal ion affect the solubility and the extent of aggregation, which in turn affect the cell uptake and intracellular distribution pattern, as well as *in vivo* pharmacokinetics [6]. Uptake *in vitro* decreases with the degree of sulfonation [7, 8]; however, phototoxicity does not parallel uptake, reflecting differences in the intracellular distribution pattern among the sulfonated compounds [9]. Furthermore, in contrast with the *in vitro* behavior, tumor retention *in vivo* appears to increase with sulfonation [10, 11]. Such discrepancies indicate complex relationships between the structure and composition of $MPcS_n$ preparations and their biological behavior. In this paper, we evaluate physical-chemical characteristics of purified and mixed isomeric aluminum sulfophthalocyanine preparations ($AlPcS_n$, where $n = 0$, 2, 3 and 4) with regard to

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CCC 1088–4246/98/030191–09 $17.50

lipophilicity, molecular symmetry and tendencies to aggregate; we correlate such parameters with cell uptake and intracellular localization pattern.

EXPERIMENTAL

Starting Materials

Aluminum Sulfophthalocyanines. Sensitizers used in this work were obtained from different sources and were prepared by different methods. Commercial samples were used without further purification and included $AlPcS_{2mix}$ and $AlPcS_{4mix}$ from Porphyrin Products (Logan, UT, USA) and $AlPcS_{3mix}$ from MidCentury (Posen, IL, USA). Reverse-phase, high-pressure liquid chromatography (HPLC) revealed that these materials consisted of complex mixtures, characteristic of those prepared by direct sulfonation of AlPc [12]. Non-sulfonated AlPcCl was obtained from Aldrich (Milwaukee, WI, USA) and formulated as a water–oil emulsion. In-house prepared compounds included $AlPcS_{2a}$ (sulfonated on adjacent phthalic subunits), $AlPcS_{2o}$ (sulfonated on opposite phthalic subunits) and $AlPcS_4$. These were synthesized by a condensation method, in which ammonium molybdate, urea, NH_4Cl, phthalic acid, 4-sulfophthalic acid and $AlCl_3$ were pulverized to give a homogeneous powder, and heated to 180 °C until the urea decomposed to give ammonia. To complete the reaction, the temperature was raised to 200 °C for 4 h. The sulfonation degree was selected by adjusting the ratio between phthalic acid and sulfophthalic acid; the final products were purified by reverse-phase HPLC [12].

For $AlPcS_n$ we distinguish between 'compounds' (i.e. different n), 'geometrical' isomers (e.g. adjacent or opposite) and 'regioisomers' (adjacent 4,4′; 4,5′; etc.). Relative retention times (Rt) are a measure of hydrophobicity. In the case of differently sulfonated AlPcS, Rt varies from 1.0 for $AlPcS_4$ (taken as unity) to 2.4 for the hydrophobic $AlPcS_1$. $AlPcS_3$ isomers elute around Rt = 1.3, whereas the disulfonated fraction elutes from Rt = 1.6 for $AlPcS_{2o}$ to Rt = 1.9 for the amphiphilic $AlPcS_{2a}$ [12]. HPLC analysis of $AlPcS_{4mix}$ showed a mixture of tri- and tetrasulfonated products, whereas $AlPcS_{3mix}$ contained a mixture of di-, tri- and tetrasulfonated products.

For the commercial preparations $AlPcS_{2mix}$, $AlPcS_{3mix}$ and $AlPcS_{4mix}$, millimolar stock solutions were obtained by dissolving the material in phosphate-buffered saline (PBS). The purified $AlPcS_n$ (n = 2a, 2o, 4) are less soluble in water and were formulated by first dissolving in dimethyl sulfoxide (DMSO) and then diluting with PBS to give 20% DMSO stock solutions. Hydrophobic AlPc was dissolved in dimethyl formamide (DMF) which was evaporated while adding PBS/cremophor/1,2-propanediol (87:10:3). Stock solutions were kept at 10 °C in the dark; just before incubation, aliquots were diluted tenfold with medium containing 2% serum.

Methods

Cell Cultivation. Ehrlich ascites cells (mouse carcinoma) were grown in suspension culture in RPMI medium 1640, supplemented with 10% heat-inactivated new-born calf serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin, 2.5 μg mL^{-1} amphotericin B and 10 mM HEPES buffer, all obtained from Biological Industries (Beth HaEmek, Israel). The cell line was maintained by seeding 10^5 cells mL^{-1} every three days. Twenty-four hours after seeding, the cells were reseeded in the incubation medium containing 2% serum, 2% DMSO and 10^{-4} M $AlPcS_n$, which was previously sterilized by filtering through a 0.2 μm pore size syringe filter. We established that 10^{-4} M $AlPcS_n$ is an excess concentration, i.e. increasing (by up to five times) cell density N_{cell} (cells L^{-1}) (as measured with a Coulter counter) after processing to make a single-cell suspension, did not affect intracellular uptake though it increased the optical density (OD) of the cell suspension proportionally. Moreover, cell survival was not affected at that sensitizer concentration (apart from $AlPcS_{2mix}$, which will be discussed below). In contrast, when reducing the sensitizer concentration to 10^{-5} M, the OD for the same cell density decreased sublinearly. After 24 h incubation, i.e. when the logarithmic phase of cell growth was reached, the incubation medium was disposed off by gentle centrifugation; cells were washed with 5 mL PBS and resuspended in fresh PBS. To measure cell efflux, NaOH (0.1 N) was added to the washed suspension (3:1 by vol.).

Spectroscopic Studies. Absorbance and fluorescence measurements of $AlPcS_n$ in solutions and in cell suspensions were carried out using a diode array spectrophotometer (HP 845A2) and a luminescence spectrometer (LS-50 Perkin Elmer), respectively. For absorption measurements of sensitizer-loaded cell suspensions, a cuvette containing PBS served as a blank (rather than a suspension of non-incubated cells)

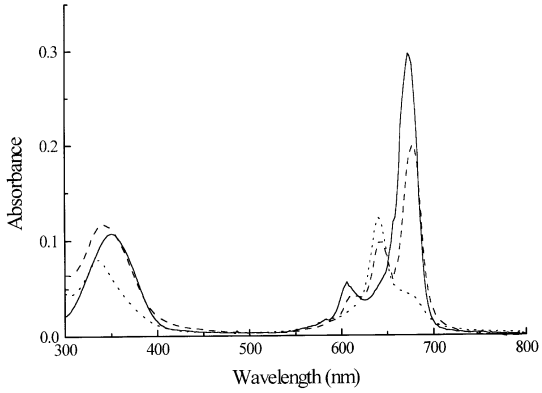

Fig. 1. Absorbance spectrum of (—) $AlPcS_{4mix}$, (- - -) purified $AlPcS_4$ and (...) $AlPcS_{2a}$; 10^{-6} M in PBS.

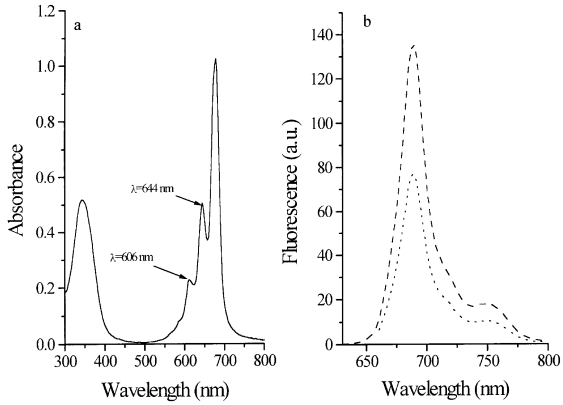

Fig. 2. (a) Absorbance and (b) fluorescence spectrum of purified $AlPcS_4$, 6×10^{-6} M in PBS; (- - -) $\lambda_{ex} = 606$ nm, (...) $\lambda_{ex} = 644$ nm.

since it proved to be impossible to prepare the blank at an identical value of N_{cell}. Qualitative uptake measurements were performed with a fluorescence microscope (Axioskop MC 100 Zeiss, Germany) by visual inspection of the cells adhered to a glass plate. The excitation filter transmitted in the range $\lambda = 350$–450 nm, and the emission filter transmitted at $\lambda > 550$ nm.

RESULTS

Physical and Chemical Properties

All sensitizers have a similar absorbance spectrum in DMSO or DMF and exhibit a strong absorption band at 674 nm, $\varepsilon_{674} \cong 2.5 \times 10^5\ cm^{-1}\ M^{-1}$ and a weak Q band at 606 nm. $AlPcS_{3mix}$ and $AlPcS_{4mix}$ dissolve easily in PBS and show Q bands at 606 nm and 672 nm, similar to that in DMSO. Under the same conditions, $AlPcS_{2mix}$ showed an additional Q band at 644 nm. Purified $AlPcS_n$ ($n = 0$, 2a, 2o) exhibited a main band at 644 nm in PBS, whereas $AlPcS_4$ showed a DMSO-type spectrum with a minor band at 644 nm (Fig. 1). Exciting at $\lambda_{ex} = 644$ nm gave a lower fluorescence yield than exciting at $\lambda_{ex} = 606$ nm (Fig. 2), even though the absorbance A is higher ($A_{644} > A_{606}$). For $AlPcS_4$, the 644 nm absorbance decreased with concentration relative to the 672 nm band (A_{644}/A_{672} decreased, see Fig. 3) and the emission band at 680 nm was red-shifted (Fig. 4). Adding Triton-X (1–2% by volume) to $AlPcS_{2o}$ lowered the ratio A_{644}/A_{672} in the absorbance spectrum and increased the fluorescence intensity ($\lambda_{ex} = 606$ nm); for both spectra, a bathochromic shift was observed (data not shown). For each sensitizer the degree of aggregation was defined as:

$$\text{Aggregation (\%)} = (A_{672}(\text{DMSO}) - A_{672}(\text{PBS})) \times 100/A_{672}(\text{DMSO})$$

where A_{672}(DMSO) and A_{672}(PBS) denote, respectively, the absorbances of 5×10^{-7} M $AlPcS_n$ solutions in DMSO and in PBS (Table 1). Clearly, pure isomeric compounds show a higher degree of aggregation than the more complex commercial preparations.

For each sensitizer, calibration curves of absorbance and fluorescence in PBS and in NaOH were obtained by diluting a reference 10^{-4} M stock solution (in DMSO), with either DMSO, PBS or NaOH (0.075 M) to 1×10^{-7}–5×10^{-7} M (which represents the

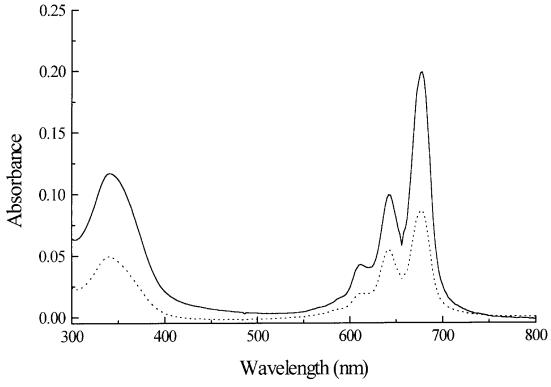

Fig. 3. Absorbance spectrum of purified $AlPcS_4$ in PBS; (—) 1×10^{-6}M, (...) 0.5×10^{-6} M.

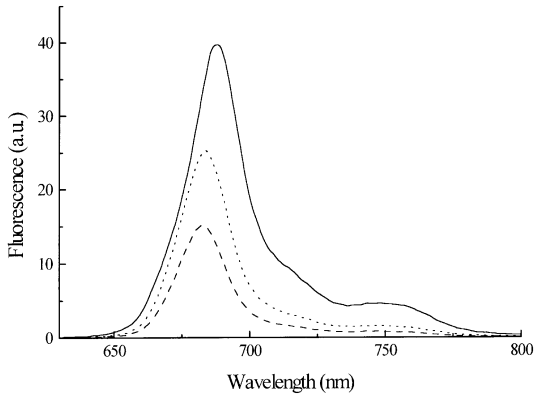

Fig. 4. Fluorescence spectrum of purified $AlPcS_4$ in PBS; (—) 8×10^{-7} M, (....) 4×10^{-7} M, (- - -) 2×10^{-7} M.

measured range of cell uptake values). The reference DMSO solution served to specify the exact concentration, based on the molar extinction coefficient $\varepsilon_{674} \cong 2.5 \times 10^5\ cm^{-1}\ M^{-1}$. Table 2 summarizes the 'effective' absorption coefficient of the monomer band, $\varepsilon_{eff,m}(\lambda)$, and of the aggregate band, $\varepsilon_{eff,a}(\lambda)$, in PBS and in NaOH. Some sensitizers dissolve as aggregates in aqueous solutions, therefore the corresponding values of $\varepsilon_{eff,m}(\lambda)$ presented in Table 2 appear to be lower than the value $\varepsilon_{674} \cong 2.5 \times 10^5\ cm^{-1}\ M^{-1}$. The degree of aggregation presented in Table 1 correlated with $1-\varepsilon_{eff,m}(\lambda)/\varepsilon_{674}$. Fluorescence intensities of these solutions (normalized to that of $AlPcS_{4mix}$ in PBS) were obtained from the calibration curves and are presented in Table 3.

Table 1. Aggregation degree of $AlPcS_n$

Sensitizer	Aggregation degree (%)
AlPc	85 ± 5
$AlPcS_{2a}$	85 ± 5
$AlPcS_{2o}$	75 ± 5
$AlPcS_4$	37 ± 5
$AlPcS_{2mix}$	11 ± 5
$AlPcS_{4mix}$	12 ± 5
$AlPcS_{3mix}$	0

Uptake

Twenty-four hours after incubation (i.e. 48 h after seeding), cells were washed and resuspended in PBS. Visual inspection of centrifuged cell suspensions provided a qualitative measure of uptake. The blue color of the cells was more intense as the uptake values were higher. For $AlPcS_{4mix}$ loaded cells, fluorescence microscopy (λ_{ex} = 350–450 nm; $\lambda_{fluor} > 550$ nm) performed by cover-slipping cells from the suspension, revealed diffuse fluorescence which emanated from the cell but was absent from some organelles, such as the nucleus. This is in partial agreement with reports in the literature that $AlPcS_4$ and $AlPcS_3$ localize in lysosomes, whereas $AlPcS_2$ and $AlPcS_1$ distribute diffusely throughout the cytoplasm [13, 14]. The situation for $AlPcS_2$ is confused by conflicting reports of lysosomal localization [15–17] and diffuse dis-

Table 2. Effective absorption coefficient of the monomer band, $\varepsilon_{eff.m}(\lambda)$, and aggregate band, $\varepsilon_{eff.a}(\lambda)$, of $AlPcS_n$ in PBS and NaOH solutions

Sensitizer	Effective absorption coefficient ($cm^{-1}M^{-1}$)			
	in PBS		in NaOH	
	$\varepsilon_{eff,m}(\lambda)$	$\varepsilon_{eff,a}(\lambda)$	$\varepsilon_{eff,m}(\lambda)$	$\varepsilon_{eff,a}(\lambda)$
AlPc	$\sim 3.3 \times 10^4$ (678)	$(3.8 \pm 0.2) \times 10^4$ (644)	$(7.6 \pm 0.1) \times 10^4$ (678)	$(5.1 \pm 0.2) \times 10^4$ (642)
$AlPcS_{2a}$	$(4.0 \pm 0.1) \times 10^4$ (672)	$(9.8 \pm 0.2) \times 10^4$ (640)	$(1.4 \pm 0.05) \times 10^5$ (672)	$(7.1 \pm 0.2) \times 10^4$ (640)
$AlPcS_{2o}$	$(6.1 \pm 0.3) \times 10^4$ (680)	$(1.4 \pm 0.1) \times 10^5$ (646)	$(2.1 \pm 0.1) \times 10^5$ (680)	$(7.0 \pm 0.1) \times 10^4$ (664)
$AlPcS_4$	$(1.6 \pm 0.05) \times 10^5$ (679)	$(9.2 \pm 0.3) \times 10^4$ (642)	$(2.0 \pm 0.05) \times 10^5$ (672)	$(5.4 \pm 0.3) \times 10^4$ (642)
$AlPcS_{2mix}$	$(2.0 \pm 0.05) \times 10^5$ (672)	$(4.8 \pm 0.1) \times 10^4$ (644)	$(2.1 \pm 0.05) \times 10^5$ (672)	$(5.1 \pm 0.1) \times 10^4$ (642)
$AlPcS_{4mix}$	$(2.6 \pm 0.05) \times 10^5$ (672)	–	$(2.4 \pm 0.05) \times 10^5$ (668)	–
$AlPcS_{3mix}$	$(2.5 \pm 0.1) \times 10^5$ (672)	–	$(2.6 \pm 0.05) \times 10^5$ (668)	–

Table 3. Relative fluorescence intensity

Sensitizer	In PBS	In NaOH
AlPc	3.3 ± 0.3	100.7 ± 2.2
$AlPcS_{2a}$	7.8 ± 0.2	63.8 ± 4.8
$AlPcS_{2o}$	11.2 ± 0.4	65.4 ± 2.7
$AlPcS_4$	62.2 ± 1.1	111.6 ± 1.4
$AlPcS_{2mix}$	74.4 ± 4.7	100.5 ± 19.3
$AlPcS_{4mix}$	100.0 ± 1.7	113.4 ± 2.7
$AlPcS_{3mix}$	96.4 ± 2.3	103.0 ± 2.2

tribution throughout the cytoplasm [13]. Injured cells, as identified visually by light microscopy, showed higher fluorescence intensity, indicating larger uptake for dead cells, probably because membrane damage reduced the natural self-defense. In a sense, this is similar to the reported [14, 18–20] fluorescence increase following irradiation, due to photodynamically induced redistribution of sensitizer into more lipophilic subcellular compartments.

Uptake was also determined quantitatively (in molar units) by dividing the measured monomer absorbance A_m of a cell suspension (at density N_{cell}) by the corresponding value $\varepsilon_{eff,m}$ (λ) in PBS (from Table 2). After multiplying $A_m/\varepsilon_{eff,m}$ (λ) by N_{Avog}/N_{cell}, uptake is expressed in units 'molecules/cell'. Results based on fluorescence were obtained from Table 3. Total sensitizer concentration was determined in the same way after efflux of cells with NaOH; effluxing with 100% DMSO gave similar uptake values (data not shown). The amount of sensitizer in suspension increased with N_{cell}; however, the number of sensitizer molecules per cell remained constant (data not shown). Averages of intracellular uptake in PBS and in NaOH solutions are given in Table 4. For comparison, the reported [11] mean cellular $AlPcS_{2a}$ uptake, though for a different cell line and incubation conditions, was approximately 10^7 molecules per cell, which is about 60 times lower than measured in the present study.

Incubation with 10% serum, instead of 2% serum (both containing 2% DMSO), lowered $AlPcS_3$ uptake by 30%. Overnight incubation in $AlPcS_3$ with 10% serum is counter productive: molecules are bound to serum which prevents diffusion and limits cell penetration mechanisms to endocytosis. Ethylenediamine tetraacetic (EDTA) solution, which was used for cell removal when needed, did not affect spectroscopy behavior nor *in vitro* cell uptake.

DISCUSSION

Physical and Chemical Properties

$AlPcS_n$ (n = 0, 2a, 2o, 4) and $AlPcS_{2mix}$ show a band at 644 nm when diluted in PBS, which indicates the presence of dimers even at 10^{-7}M. These sensitizers are less soluble in PBS than non-aggregated $AlPcS_{3mix}$ and $AlPcS_{4mix}$, which do not show the 644 nm dimer band. The increase of the 644 nm band is at the expense of the monomer bands at 672 nm and 606 nm (Fig. 1) and becomes more pronounced with aggregation (Table 1): $AlPcS_4$ has a DMSO-type spectrum, with a weak band at 644 nm (also observed in NaOH 0.1 M solution but not interpreted [10]), whereas for

Table 4. Intracellular uptake of different senzitizers, based on absorbance and fluorescence measurements

Sensitizer	Intracellular uptake (10^8 molecules/cell)			
	Natural cells (PBS)		Effluxed cells (NaOH)	
	Absorption	Fluorescence	Absorption	Fluorescence
AlPc	–	0.03 ± 0.01	–	0.03 ± 0.01
$AlPcS_{2a}$	2.4 ± 0.4	2.4 ± 0.9	8.7 ± 0.5	5.9 ± 1.8
$AlPcS_{2o}$	0.9 ± 0.06	1.5 ± 0.4	1.7 ± 0.2	1.9 ± 0.3
$AlPcS_4$	0.7 ± 0.1	0.4 ± 0.2	1.4 ± 0.3	1.2 ± 0.6
$AlPcS_{2mix}$	11.1 ± 3.5	5.6 ± 3.2	58.7 ± 18.1	30.1 ± 8.8
$AlPcS_{4mix}$	1.4 ± 0.3	1.1 ± 0.2	1.9 ± 0.2	3.8 ± 0.4
$AlPcS_{3mix}$	1.3 ± 0.2	0.7 ± 0.1	2.4 ± 0.3	4.3 ± 0.9

compounds $AlPcS_n$ ($n = 0$, 2a, 2o) the 644 nm band becomes the main feature. Dimer formation is corroborated by the lower fluorescence measured at $\lambda_{ex} = 644$ nm, compared to $\lambda_{ex} = 606$ nm (see Fig. 2) in spite of the fact that $A_{644} > A_{606}$. Since monomer fluorescence is more intense than that of dimers, and aggregates do not fluoresce at all [21, 22], we assign the 644 nm band to dimers. For $AlPcS_{4mix}$ no evidence of dimerization in PBS was observed (this study and others [22]), though at a higher concentration, 1×10^{-5} M in 90% EtOH/H_2O, a band at 685 nm has been ascribed to dimers [22]. For $AlPcS_4$ (prepared by condensation [23]), at 1.1×10^{-6} M in 66% EtOH/H_2O, a broad band at 750–800 nm was attributed [23] to the formation of dimers. These extra bands [22, 23] may be due to the presence of alcohol, which changes the nature of hydrogen bonding (essential for dimer formation [24]), and are not relevant to physiologic solutions. $AlPcS_{2mix}$, synthesized by direct sulfonation [25, 26], could be resolved in at least eight fractions by HPLC, one of which showed absorption at 630 nm in aqueous solution; this band was not observed in fluorescence excitation and therefore was assigned to dimers [25]. In MeOH at concentrations up to 6.4×10^{-5} M, there was no spectral evidence for dimerization [23]. Non-sulfonated AlPc, does not undergo monomerization, even in vesicles, since it exhibits a band at 640 nm, attributed to dimers [26].

The conformation of the dimers may also be deduced from spectral observations. The Soret bands of $AlPcS_n$ ($n =$ 2a, 2o, 4) were blue-shifted (1500 cm^{-1}) and broadened compared to the Soret bands of (non-aggregated) $AlPcS_{4mix}$ or $AlPcS_{3mix}$ (Fig. 1), whereas the Q bands showed a bathochromic shift (100 cm^{-1}). For porphyrins, spectral shifts and broadening similar to those described above have been attributed to 'sandwich'-type dimer formation [27, 28]. We adopt this concept for phthalocyanines in PBS, and conclude that the purified $AlPcS_n$ ($n =$ 2a, 2o, 4) form 'sandwich'-type dimers. Such dimers are face-to-face complexes where the axial chlorides or hydroxyl groups of monomers are in opposite (outward) directions. We propose that because of steric hindrance between SO_3^- groups, the phthalocyanine skeletons are staggered, i.e. rotated by 180, 90 and 45° for, respectively, $n =$ 2a, 2o and 4. Aggregation of monomers and 'sandwich'-type dimers can occur for pure, symmetrical regioisomers where building blocks can be repeated with minimal steric perturbations. The decrease of the ratio A_{644}/A_{672} with concentration for $AlPcS_4$ (Fig. 3), indicates the formation of oligomers. Moreover, the bathochromic shift of the Soret band with increasing concentration indicates branched-chain formation [27, 29]. The mixed isomeric preparations $AlPcS_{4mix}$ and $AlPcS_{3mix}$ do not form dimers and aggregates but, as expected, $AlPcS_{2mix}$ does form sandwich-type dimers.

For $AlPcS_4$ solutions in PBS, dimers persisted even at 10^{-7} M. Adding detergent (Triton-X) dissociated dimers into monomers: the ratio A_{644}/A_{672} decreased and the fluorescence intensity ($\lambda_{ex} = 606$ nm) increased, accompanied by a bathochromic shift. In contrast, when adding Triton-X to solutions of non-aggregated $AlPcS_{4mix}$ preparations, no spectral changes were observed. Therefore, it can be concluded that bathochromic shifts result from dissociation of aggregates and dimers. The bathochromic shift and the non-linear dependence of fluorescence intensity at 680 nm, upon increasing the $AlPcS_4$ concentration from 2×10^{-7} to 8×10^{-7} M (Fig. 4), may be due to reabsorbance of emitted photons by the band at 672 nm.

For $AlPcS_n$ ($n =$ 2a, 2o, 4) the degree of aggregation in PBS increased with lipophilicity, as expected (see Table 1). The effective absorption coefficients, ε_{eff} (λ), measured in this work (Table 2) can be understood from the lipophilicity–symmetry relationship. Pure compounds $AlPcS_n$ showed $\varepsilon_{eff,m}$ (λ) which increased with hydrophilicity; mixed isomeric compounds generally had higher $\varepsilon_{eff,m}$ (λ) because their low symmetry prevented aggregation. Values of ε_{672} reported earlier [30, 31] for $AlPcS_n$ ($n =$ 3, 4) are about 25% lower than ours.

For $AlPcS_{2a}$ and $AlPcS_{2o}$ the situation is complex. $AlPcS_{2a}$ is more aggregated than $AlPcS_{2o}$ (Table 1), yet its value $\varepsilon_{eff,a}$ (λ) is lower (Table 2). This indicates that amphiphilic $AlPcS_{2a}$ aggregates to higher oligomers, whereas $AlPcS_{2o}$ creates mostly dimers. $AlPcS_{2mix}$, obtained by direct sulfonation, contains various regioisomers within each geometrical isomer, $AlPcS_{2a}$ or $AlPcS_{2o}$, which prevents dimerization. In general, in NaOH solutions, $\varepsilon_{eff,m}$ (λ) was higher than in PBS and $\varepsilon_{eff,a}$ (λ) was lower (Table 2). The high sensitivity of fluorescence to aggregation is shown in Table 3. For example, the absorbance of $AlPcS_{2a}$ increased 3.5-fold due to the dissociation of aggregates in NaOH, whereas the fluorescence intensity increased eight-fold.

Uptake

Cellular uptake of porphyrins and phthalocyanines has been reported to increase with lipophilicity [7, 8, 32].

This was explained by a reduction in the membrane permeability barrier towards lipophilic compounds. In this study, we present measurements of intracellular concentrations of $AlPcS_n$ and relate them to solubility data in the incubation medium which, in turn, depend on lipophilicity and to a lesser degree on molecular symmetry.

For each sensitizer, the number of molecules taken up per cell was independent of N_{cell} because sensitizer concentration in the incubation medium was in excess (1×10^{-4} M). As can be seen from Table 4, uptake of $AlPcS_n$ decreased with the increase of the degree of sulfonation (except for AlPc) because of the inability of hydrophilic sensitizers to diffuse through the lipid bilayer of membranes. Owing to its amphiphilicity, $AlPcS_{2a}$ uptake was significantly higher than that of $AlPcS_{2o}$. The amphiphilic isomer is capable of interacting with hydrophobic membrane lipids and, at the same time, to bind to a membrane-transport protein which facilitates cell penetration [32, 33]. In addition, stereochemical factors may play a role [34]. For two compounds with the same hydro/lipophilicity, intracellular uptake is inversely proportional to the degree of aggregation in the incubation medium, which increases with the symmetry of the molecule. For example, $AlPcS_{4mix}$ showed a higher uptake due to its lower degree of aggregation (Table 1). The high aggregation of AlPc counteracts its lipophilicity advantage, so much so that AlPc exhibited the lowest uptake. Because of its chemical characteristics [32–34], $AlPcS_{2mix}$ is easily internalized and exhibits five times higher uptake than other sensitizers (Table 4) since both factors that promote uptake are optimal: lipophilicity and absence of aggregation. At these high uptake values dark toxicity should be considered. Indeed, after 24 h incubation with $AlPcS_{2mix}$, cells revealed different properties: their growth rate was about a third of the usual growth rate and after centrifugation they were less adhered to each other, compared to centrifuged cells incubated with other sensitizers. These unusual cell properties suggest that at high intracellular concentrations, $AlPcS_{2mix}$ is toxic and can overcome natural cell defenses even at no/low light exposures.

After uptake, sensitizer molecules are localized in cells (or are membrane bound) since washing, which was part of the procedure, removed non-bound sensitizer. Intracellular concentrations (C_{in}) are given by:

$$C_{in} = IU/(N_{Avog} \times V_{cell})$$

where IU is total intracellular uptake (Table 4, column 4) and V_{cell} is the average volume of Ehrlich ascites cells; $V_{cell} = 1.0 \pm 0.02$ pL cell^{-1} (under isotonic conditions) [35]. For $AlPcS_{4mix}$ this yields $C_{in} = 3.2 \times 10^{-4}$ M and for $AlPcS_{2a}$ C_{in}=14.5×10^{-4} M, and so on for the other sensitizers. Since these intracellular concentrations are higher than in the incubation medium (1×10^{-4} M), uptake must have involved endocytosis or a continuous diffusion process in conjunction with sensitizer-cell binding which shifted the equilibrium towards more uptake. Such an 'active' mechanism was also suggested for $AlPcS_2$ in tumor cells [16]. Mathematical modeling [36] of the accumulation kinetics of hydrophobic sensitizers in cells yields for porphyrins, C_{in}/C_{ex} = 1–5 (depending on the molecule, cell line, incubation protocol and pH), where C_{ex} is the extracellular concentration. Our experimental results for $AlPcS_{4mix}$ and $AlPcS_{2a}$ yield C_{in}/C_{ex} = 3.2 and 14.5, respectively.

When considering sensitizers for PDT, it should be noted that monomers constitute the more photoactive fraction. For all sensitizers, free monomer concentration when measured in intact cell suspensions (without NaOH), appeared lower than when measured in lysed cell suspensions treated with NaOH (Table 4). After effluxing sensitizer-loaded cells, the fluorescence intensity increases as a consequence of sensitizer release from intracellular granulars such as lysosomes. This is due to NaOH-induced dissociation of the aggregates and to absence of a high local concentration which causes self-quenching. As a sensitizer becomes more aggregated, or more concentrated inside cellular organelles, the difference between monomer and total concentration is higher. For example, absorbance measurements yielded about the same number of monomers per cell for $AlPcS_{4mix}$ and $AlPcS_{3mix}$ (Table 4, column 2). However, the total number of $AlPcS_{4mix}$ molecules was 1.3 times larger, whereas for $AlPcS_{3mix}$ it was twice as large. This is due to internalization in the form of aggregates, or bound to biomolecules, which are less amenable to detection by spectroscopic means.

Sensitizer uptake, as measured by absorbance, parallels that measured by fluorescence (Table 4), except for $AlPcS_{2o}$ for which monomer uptake appears to be higher than that of $AlPcS_{4mix}$ and $AlPcS_{3mix}$ when based on fluorescence, but lower when based on absorbance. This singularity may be explained by the ability of $AlPcS_{2o}$ to undergo intracellular monomerization which strongly affects fluorescence. In general, more detailed information about monomerization can be obtained from fluorescence, which is more sensitive to the microenvironment than is absorbance (Table 4).

For $AlPcS_{3mix}$ this effect was more pronounced, presumably since $AlPcS_{3mix}$ tends to localize more in cellular compartments where the fluorescence yield is low, leading to seemingly lower monomer concentrations than for $AlPcS_{4mix}$. That is to say, dissimilarities between monomer and total concentration must be related to a difference in local sensitizer concentrations in intracellular organelles. After efflux of cells with NaOH, the microenvironment for $AlPcS_{3mix}$ and $AlPcS_{4mix}$ equalizes and so does the fluorescence yield.

Generally, two opposing influences play a role in the spectroscopy of a natural cell suspension: light scattering due to the presence of cells in suspension, which affects mainly absorbance, and aggregation or high local concentration, which affects mainly fluorescence. Spectra taken after cell efflux with NaOH (when there are no cells left to scatter the light and NaOH has dissociated the sensitizer aggregates), give more accurate values for the total uptake in cells.

Intracellular Monomerization

Sensitizers undergo monomerization in an intracellular environment. The degree of monomerization depends on the physical–chemical characteristics of the sensitizer molecules. The monomer fraction (*MFc*) in cells is defined as:

$$MFc = C_m/C_t$$

where C_m denotes intracellular monomers in a natural cell suspension (in PBS) and C_t the total molecules/cell (measured after addition of NaOH). In order to determine the cellular contribution to the monomerization process, the monomer fraction in PBS (*MFp*) was determined as:

$$MFp = F_m/F_t$$

where F_m and F_t denote fluorescence of $AlPcS_n$ in PBS and NaOH, respectively. For all sensitizers, *MFp* was measured at 1×10^{-7} M (Table 5, column 3).

Monomerization of a sensitizer, due to the cellular microenvironment, is presented as 'localization effect' (*LE*) (Table 5, column 4).

$$LE = MFc/MFp$$

For $AlPcS_4$, $AlPcS_{4mix}$, $AlPcS_{3mix}$ and $AlPcS_{2mix}$, values of *LE* less than one indicate a decrease in monomer concentration after internalization due to aggregation and quenching at the high concentrations achieved in certain cell compartments and to sensitizer–protein complexation. The combined effects of local concentration and sensitizer lipophilicity will determine the effective monomerization. For example, due to its higher uptake, $AlPcS_{3mix}$ displayed a more pronounced decrease in monomerization than did $AlPcS_4$. This also explains the difference between $AlPcS_{2a}$ ($LE = 3$) and $AlPcS_{2o}$ ($LE = 4.5$). AlPc showed the highest intracellular monomerization ($LE = 100$) due to the combined effects of high lipophilicity and low uptake.

CONCLUSIONS

Comparing photophysical and chemical properties of a series of structurally related photosensitizers in solution provides important information on their potential photodynamic applications. In this work we

Table 5. Intracellular monomerization and localization effect, based on fluorescence measurements

Sensitizer	Monomer fraction		*LE*
	In cell (*MFc*)	In PBS (*MFp*)	
AlPc	1.0 ± 0.13	0.01	100
$AlPcS_{2a}$	0.37 ± 0.02	0.12	3
$AlPcS_{2o}$	0.77 ± 0.14	0.17	4.5
$AlPcS_4$	0.38 ± 0.05	0.57	0.65
$AlPcS_{2mix}$	0.26 ± 0.06	0.74	0.35
$AlPcS_{4mix}$	0.29 ± 0.03	0.88	0.35
$AlPcS_{3mix}$	0.17 ± 0.03	0.93	0.2

have shown that cell uptake of the monomeric (photoactive) form is optimal for preparations consisting of various regioisomers or of several differently substituted compounds. Although single compounds are preferred as pharmaceuticals, our data indicate that mixed isomeric preparations may be more efficient as photodynamic agents than isomerically pure compounds and that the former are likely to be more efficient drugs for biomedical applications. This may be one of the reasons for the relatively high PDT efficacy of HpD, Photofrin® and Photosens®.

Acknowledgments

This work was supported by the US–Israel Binational Science Foundation under grant no. 93-00154 and by the Medical Research Council of Canada. R. E. gratefully acknowledges Prof. G. Neufeld at Technion for professional advice in biology related matters and for permission to use the facilities at the Department of Biology.

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