Synthesis and Cellular Studies of Nonaggregated Water-Soluble Phthalocyanines

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Water-soluble phthalocyanines are promising photosensitizers for application in cancer therapy and in the photoinactivation of viruses. The water-soluble zinc(II) phthalocyanines **5** and **6** were synthesized by converting the corresponding ester derivative **4** into the sodium carboxylate and carboxylic acid species. Compound **5** can be solubilized in water as a monomeric species, as demonstrated by UV/vis and fluorescence spectroscopy. These compounds were characterized by analytical and spectroscopic methods and, in the case of **4**, by X-ray crystallography. The water-soluble phthalocyanines were found to have low dark cytotoxicity toward V79 hamster fibroblasts and human HEp2 cells, to be phototoxic at low light and drug doses, to be taken up by cells in culture, and to localize intracellularly, mainly in the cell lysosomes. Conjugation of the anionic phthalocyanines with positively charged LipoGen liposomes resulted in effective delivery of these compounds into the nuclei of cells. It is concluded that these highly water-soluble phthalocyanines are promising sensitizers for the photodynamic therapy of tumors.

Introduction

Photodynamic therapy (PDT) is a special form of phototherapy, a treatment that uses light to induce chemical reactions in patients, and that can potentially destroy harmful tissue while sparing normal tissue.[1] In the absence of light, the photosensitizer is harmless and has no effect on either healthy or abnormal tissue. However, when light is directed onto drug-localized tissue, the drug becomes activated and the tissue is rapidly destroyed. Thus, by careful application of the light beam, the technique can selectively target and destroy abnormal tissue in the presence of normal tissue. Recently, the use of PDT for the treatment of selected solid tumors has been studied by investigators with the objective of enhancing its efficacy and broadening its applications.[2] Since the preparation of a purified form of hematoporphyrin derivative (HpD) in 1983, a considerable amount of research has centered on the development of new porphyrin-based drugs, with improved tumor-selectivity and photosensitizing properties over the first generation compounds.[3−7] However, most porphyrin-based drugs are excited clinically with 630 nm light, although they absorb only weakly in the red region of the spectrum where light penetrates deeper through tissue, and are thus unsuitable for the treatment of deep-seated tumors. Therefore, a single pure compound with a strong long-wavelength absorption in the red region, low dark toxicity, and an effective generator of cytotoxic oxygen species in the proximity of the tumor cell nucleus is the ideal drug for PDT investigations.

Phthalocyanines are tetrabenzo[5,10,15,20]tetraazaporphyrins that have traditionally found applications as dyes and photoconducting agents in photocopying devices,[8] chemical sensors,[9] electrochromism agents,[10] molecular metals,[11] and liquid crystals.[12] In addition to their traditional uses, the importance of phthalocyanines is rapidly growing in many other fields. One of their most important applications is as photosensitizers in PDT.[1b,13] Due to their intense absorption in the red region of the optical spectrum (ca. 700 nm) and superior photophysical properties, phthalocyanines have been used in the PDT treatment of various cancers and for the photoinactivation of viruses.[1b,14] Among these compounds, water-soluble phthalocyanines are among the most promising photosensitizers.[15,16] However, aggregation (especially in aqueous media) is a very common phenomenon in this family of compounds due to their large π-conjugated systems; this drastically decreases their fluorescence quantum yields, shortens their triplet state lifetime, and reduces their photosensitizing efficiency.[17−21] Hydrophilic and nonaggregated phthalocyanines are therefore important and potentially useful materials. To date, a number of phthalocyanines bearing hydrophilic moieties, such as carboxylates,[21,22] sulfonates,[19,23−26] glucose,[27] phosphonates,[28,29] polyoxyethylene,[30−32] amino,[33] and carboranyl[34,35] groups, have been reported. Most of these compounds are highly aggregated in aqueous media, this phenomenon being most-easily recognized by severe broadening of the absorption bands in their optical spectra. To reduce the extent of aggregation, several methods have been employed to promote disaggregation of phthalocyanines into individual molecules in water; these include (1) using hydrophilic groups as axial ligands coordinated to a central metal such as Ru or Si;[36,37] (2) using surfactants to create a micro-heterogeneous environment;[21,38−40] and (3) introducing dendrimers as substituents on the phthalocyanine macrocycle, which sterically inhibit molecular aggregation and enhance solubility.[41] Although all the above methods can to some extent improve the solubility and decrease aggregation of phthalocyanine systems, to the best of our knowledge no phthalocyanine has ever been reported to be a

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monomeric species in water in the absence of surfactants or other disaggregating agents.

We report herein the synthesis and characterization of highly water-soluble phthalocyanines with terminal carboxylate functionalities.[42] These new compounds exhibit the characteristic monomeric UV−vis absorption spectrum of phthalocyanine in water and clearly well-defined ^{1}H NMR spectra in D_2O. These water-soluble compounds were found to have low dark cytotoxicity toward V79 hamster lung fibroblasts and human HEp2 cells, to be phototoxic at low light dose (1 J/cm^2), to be taken up by these cells in vitro, and to localize intracellularly. Our work suggests that these highly water-soluble phthalocyanines are promising sensitizers for the PDT of tumors.

Experimental Section

1. Chemistry. All reactions were carried out under an argon atmosphere in degassed dried solvents. Commercially available starting compounds were purchased from Sigma-Aldrich and used directly without further purification. Silica gel 60 (70−230 mesh, Merck) was used for column chromatography. Analytical thin-layer chromatography (TLC) was performed using Merck 60 F254 silica gel (precoated sheets, 0.2 mm thick). ^{1}H NMR spectra were obtained using a Bruker DPX 250 MHz spectrometer; chemical shifts are expressed in ppm relative to DDS (0 ppm), or chloroform (7.26 ppm). Electronic absorption spectra were measured on a Perkin-Elmer Lambda 35 UV−vis spectrophotometer and fluorescence spectra were measured on a Perkin-Elmer LS55 spectrometer. Mass spectra were obtained on a Bruker Prolix III MALDI-TOF mass spectrometer. Melting points were measured on a MELT-TEMP apparatus. FT-IR spectra were obtained on a Bruker Tensor 27 with a a Pike single-bounce ATR cell with ZnSe windows.

4,5-[(3,5-Bismethoxycarbonyl)phenoxy]phthalonitrile (3). A mixture of 4,5-dichlorophthalonitrile **1** (1.0 g, 10.15 mmol), 5-hydroxyisophthalate **2** (4.28 g, 40.60 mmol), and K_2CO_3 (16.6 g, 0.12 mol) in DMF (60 mL) was stirred at 65 °C for 24 h. The reaction mixture was poured into ice−water to give a white-brown precipitate, which was extracted with $CHCl_3$ (5 × 100 mL). The organic extracts were dried over anhydrous $MgSO_4$ and concentrated under vacuum to give a yellow oil. After recrystallization from methanol, the title compound **3** was obtained as a white solid (2.35 g, 85%), mp 191−192 °C. ^{1}H NMR ($CDCl_3$): δ 8.56 (s, 2H, Ar−H), 7.83 (s, 4H, Ar−H), 7.32 (s, 2H, Ar−H), 3.97 (s, 12H, CH_3). FTIR (solid) 1727.1 (C=O), 2235.1 (CN) cm^{-1}. MS (MALDI) *m/z* 544.13 (M+H$^+$). Anal. Calcd. for $C_{28}H_{20}N_2O_{10}$: C 61.77, H 3.70, N 5.15. Found: C 61.46, H 3.76, N 4.99.

Zinc(II) 2,3,9,10,16,17,23,24-Octa[(3,5-bispentyloxycarbonyl)phenoxy]phthalocyanine (4). A mixture of dinitrile **3** (400 mg, 0.81 mmol), $Zn(OAc)_2{\cdot}2H_2O$ (65 mg, 0.30 mmol) and a few drops of DBU in *n*-pentanol (10 mL) was heated overnight at 140 °C. The volatiles were removed under reduced pressure to give a greenish blue solid, which was purified by column chromatography using dichloromethane/ethyl acetate (20:1) for elution. The crude product was recrystallized from THF/MeOH to give the title compound **4** as a green solid (265 mg, 46%), mp 158−160 °C; ^{1}H NMR ($CDCl_3$): δ 9.11 (s, 8H, Ar−H), 8.42 (s, 8H, Ar−H), 7.88 (d, J = 1.3 Hz, 16H, Ar−H), 4.22 (t, J = 6.7 Hz, 32H, 16 OCH_2), 1.65−1.55 (m, 32H, 16 CH_2), 1.28−1.07 (m, 64H, 16 CH_2CH_2), 0.81−0.76 (t, J = 7.0 Hz, 48H, 16 CH_3); ^{1}H NMR ($CDCl_3$ with one drop of pyridine-d_5): 8.62 (s, 8H, Ar−H), 8.18 (s, 8H, Ar−H), 7.95 (d, J = 1.1 Hz, 16H, Ar−H), 4.10 (t, J = 6.8 Hz, 32H, 16 OCH_2), 1.65−1.55 (m, 32H, 16 CH_2), 1.28−1.07 (m, 64H, 16 CH_2CH_2), 0.73−0.68 (t, J = 7.0 Hz, 48H, 16 CH_3). UV−vis ($CHCl_3$): λ_{max} (log ϵ) 676 (5.56), 646 (4.68), 610 (4.74), 357 (5.11) nm. FTIR (solid) 1725.8 (C=O) cm^{-1}. MS (MALDI) *m/z* 3141.99 (M$^+$). Anal. Calcd. for $C_{176}H_{208}N_8O_{40}Zn$: C 67.30, H 6.67, N 3.57; Found: C 66.77, H 7.05, N 3.34.

Crystal data for **4**·MeOH: $C_{177}H_{212}N_8O_{41}Zn$, M_r = 3172.9, triclinic space group P-1, a = 25.671(6), b = 26.458(5), c = 28.530(8) Å, α = 64.196(7), β = 73.220(7), γ = 84.283(18)°, V = 16 695(7) Å^3, Z = 4, ρ_{calcd} = 1.262 g cm^{-3}, Mo Kα radiation (λ= 0.71073 Å; μ = 0.228 mm^{-1}), T = 102 K. Dark blue blade crystal, size 0.10 × 0.20 × 0.37 mm. 85 616 data by Nonius KappaCCD, R = 0.21 ($F^2 > 2\sigma$), Rw = 0.382 (all F^2) for 30367 unique (11066 observed) data having θ < 20.0° Not all of the pentyl ester C atoms could be located, and the crystals, which were grown from THF/MeOH, contain solvent of unknown identity and quantity.

Zinc(II) 2,3,9,10,16,17,23,24-Octa[(3,5-biscarboxylate)-phenoxy]phthalocyanine (5 and 6). Compound **4** (200 mg) was dissolved in THF (5 mL) and added slowly to a saturated NaOH solution in water/methanol (1:5) (100 mL). The mixture was stirred at 40 °C for 4 h, and the resulting precipitate was filtered and washed repeatedly with MeOH and $CHCl_3$. The crude product was dissolved in water and neutralized using 1 M HCl until pH 7. Compound **5** precipitated upon addition of ethanol to yield a green solid (148 mg, 98%). ^{1}H NMR (D_2O, pH 8): δ 9.33 (s, 8H, Ar−H), 8.08 (s, 8H, Ar−H), 7.70 (s, 16H, Ar−H); (D_2O, pH 13): δ 8.91 (s, 8H, Ar−H), 7.91 (s, 8H, Ar−H), 7.66 (s, 16H, Ar−H). UV−vis (H_2O, pH = 8): λ_{max} (log ϵ) 679 (5.19), 650 (4.44) 613 (4.40), 355 (4.79) nm. FTIR (solid) 1554.2 (C=O) cm^{-1}. HRMS (MALDI-TOF) *m/z* 2019.1491 (calculated for $C_{96}H_{49}N_8O_{40}Zn$ 2019.1357). Anal. Calcd for $C_{96}H_{32}N_8O_{40}ZnNa_{16}{\cdot}16H_2O$: C 43.37, H 2.43, N 4.22; Found: C 43.39, H 2.94, N 3.82.

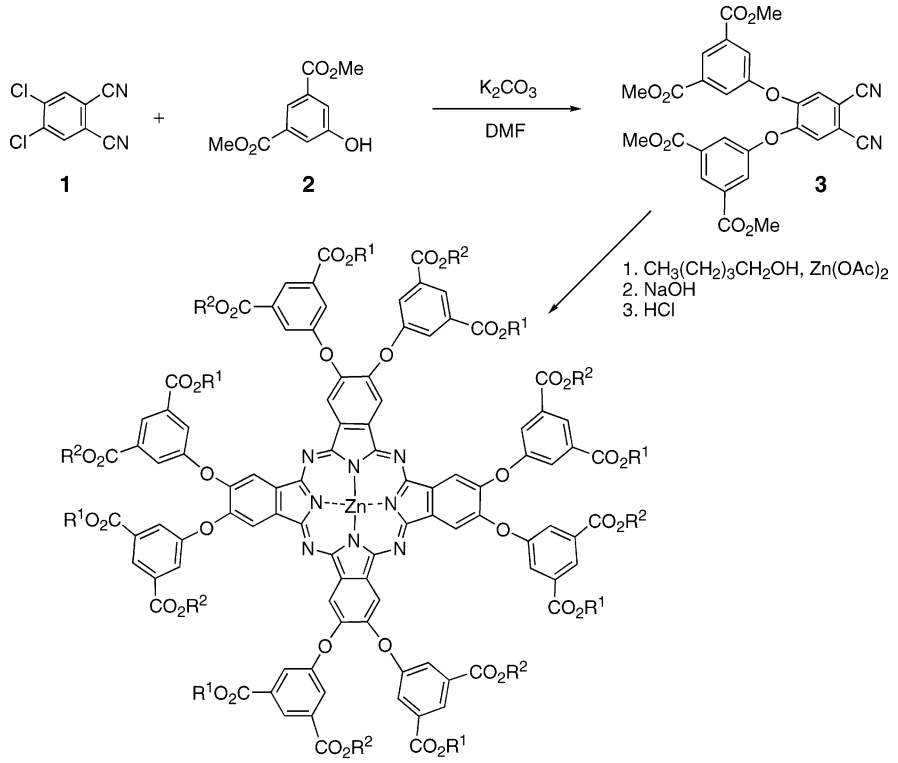
Compound **5** (200 mg) was dissolved in water and 1 M HCl was added until pH 2. The resulting green precipitate was filtered and washed with water and MeOH. Compound **6** was obtained in quantitative yield. For phthalocyanine **6**: UV−vis (DMSO): λ_{max} (log ϵ) 677 (5.14), 650 (4.40), 613 (4.40), 364 (4.74) nm. ^{1}H NMR (DMSO-d_6): δ 12.96 (br, 16H), 8.81 (s, 8H, Ar−H), 7.96 (s, 8H, Ar−H), 7.79 (s, 16H, Ar−H). FTIR (solid) 1695.4 (C=O) cm^{-1}. HRMS (MALDI-TOF) *m/z* 2019.1446 (calculated for $C_{96}H_{49}N_8O_{40}Zn$ 2019.1357). Anal. Calcd. for $C_{96}H_{48}N_8O_{40}Zn{\cdot}6H_2O$: C 54.23, H 2.85, N 5.27; Found: C 54.79, H 3.42, N 5.29.

2. Cell Culture. Hamster V79 cells and human HEp2 cells were obtained from ATCC. V79 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HEp2 cells were maintained in a 50:50 mixture of MEM:Advanced MEM (both from Gibco) supplemented with 5%FBS. Phosphate-buffered saline (PBS), FBS, and trypsin were purchased from Gibco. CyQUANT cell proliferation assay kit was purchased from Molecular Probes and CellTiter 96 nonradioactive cell proliferation kit from Promega. LipoGen cationic liposomes were purchased from InvivoGen. Microscopy was performed using a Zeiss Axiovert 200M inverted fluorescent microscope fitted with a standard Texas Red filter set (Chroma Technology Corp.). Compound **5** was completely soluble in medium. Compound **6** required the preparation of a concentrated DMSO stock solution, which was then diluted in medium; the final DMSO concentration never exceeded 1% in medium. Medium solutions were filter sterilized (0.22 μm pore size) prior to use.

2.1. Cytotoxicity. To assess the cytotoxic effect of the phthalocyanines, V79 hamster fibroblasts and human HEp2 cells were seeded onto 96-well plates at 1000 and 5000 cells per well, respectively. The cells were allowed to settle and attached for 24 h. For dark cytotoxicity experiments, phthalocyanines **5** and **6** were added to triplicate wells in 2-fold serial dilutions from 400 μM to 50 μM and incubated for 24 h. The compounds were then removed, fresh drug-free medium was added, and the cells were incubated for an additional 24 h. V79 cell proliferation was quantified using the CyQUANT cell proliferation assay kit and HEp2 cell proliferation using the MTT based CellTiter 96 nonradioactive cell proliferation kit, both as per manufacturers instructions.

Phototoxicity experiments were performed on human HEp2 cells in 96-well plates as above, with phthalocyanine concentrations of 10, 5, 2.5, 1.25, 0.625, and 0 μM. After 24 h incubation, the compounds were removed, the cells washed

Scheme 1. Synthetic Route to Phthalocyanines **4**−**6**



4: $R^1 = R^2 = {}^nC_5H_{11}$
5: **(a)** R^1 = Na, R^2 = H; **(b)** $R^1 = R^2$ = Na
6: $R^1 = R^2$ = H

with medium and then exposed to light from a 100 W halogen lamp equipped with a 610 nm cutoff filter for 10 min. During irradiation water was used as an IR blocking filter, and the cells were cooled on ice. The total light dose was 1 J/cm^2. The cell survival was assessed 24 h after irradiation using the MTT assay as above.

2.2. Time-Dependent Cellular Uptake. HEp2 cells were seeded on 96-well plates at 20 000 cells per well and exposed to 10 μM concentrations of phthalocyanines in medium from 10 min to 24 h to probe time-dependent drug accumulation. At the end of the incubation interval, the cells were washed with PBS and solubilized in 100 μL of 0.25% Triton X-100 in PBS. The retention of cell-associated phthalocyanine was measured by determining the fluorescence emission of the accumulated phthalocyanine with a BMG FluoStar Optima plate reader using excitation/emission wavelengths of 610 and 685 nm, respectively.

2.3. Intracellular Localization. Cells were grown in a Lab-Tek II chamber coverglass system with standard culture medium. The phthalocyanines were added to reach final concentrations of 10 μM. The cells were incubated in the dark for 6 and 24 h and then washed with drug-free medium three times to remove unbound phthalocyanines. Finally, fresh medium containing 50 mM HEPES pH 7.2 was added to cells and these examined immediately by fluorescence microscopy.

To facilitate drug entry into cells, LipoGen cationic liposomes were also used as a delivery vehicle. Liposome/drug complexes were prepared by mixing 10 μL of stock liposomes with 90 μL of medium and by incubating 10 min at room temperature. This liposome mixture was then added to 100 μL of medium containing phthalocyanine and incubated an additional 20 min. The liposome/drug complex was then added to the cells on chamber slides containing 1 mL of medium and incubated for 24 h. The final phthalocyanine concentration was 8.3 μM. After incubation, the cells were washed with fresh medium and fresh HEPES-containing medium was added as above, followed by fluorescence microscopy.

Results and Discussion

1. Synthesis and Characterization. Scheme 1 shows the synthetic route used to the targeted octasubstituted zinc(II) phthalocyanines. While this manuscript was being considered for publication, a communication appeared reporting an alternate synthesis of compound **6**.[43] Commercially available 4,5-dichlorophthalonitrile **1** and hydroxyisophthalate **2** in DMF were converted into the diether **3** in the presence of K_2CO_3, in 85% yield. Heating phthalonitrile **3** in *n*-pentanol with zinc(II) acetate and a catalytic amount of DBU led to phthalocyanine **4** with concomitant transesterification of the ester functions to give the corresponding pentyl esters; this has also been previously observed by others.[41,44] After chromatographic purification using dichloromethane/ethyl acetate (20:1) and recrystallization from THF/MeOH, phthalocyanine **4** was obtained in 46% yield. Two views of the crystal structure of **4**·MeOH are shown in Figure 1. Two independent molecules of the Zn complex exist in the crystal, each having an axially coordinated methanol molecule. In each, the 24-atom phthalocyanine core is planar to within 0.08(4) Å, and the square-planar Zn atom lies 0.35 Å out of this plane. The mean Zn−N distance is 1.993 Å, and the mean Zn−O distance is 2.144 Å. The two molecules form a stacked dimer in the crystal, with parallel phthalocyanine planes and a Zn···Zn distance of 4.115(6) Å. The two phthalocyanines are rotated with respect to each other by 20(1)° about the Zn···Zn vector. The bis-pentyl isophthalate groups of the two molecules interdigitate, as shown in Figure 1b, with the pentyl groups of each molecule wrapping around the other molecule.

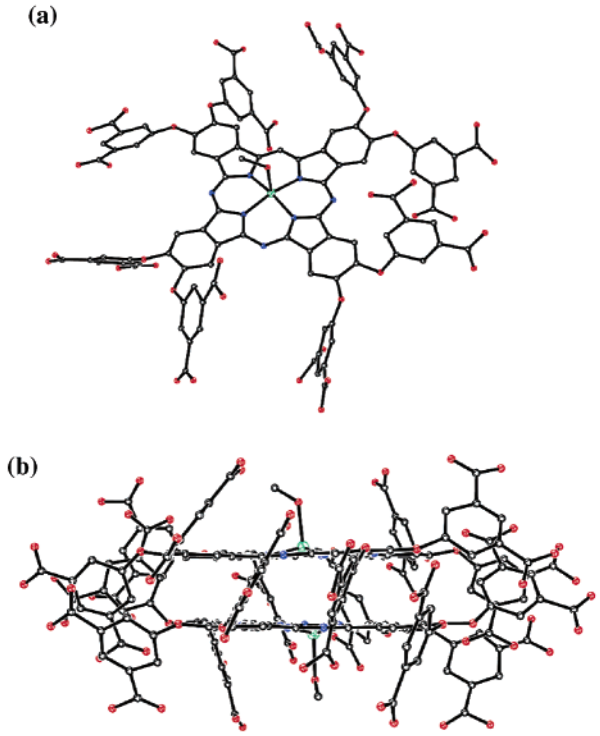


Figure 1. Crystal structure of **4**·MeOH: (a) one of the two independent molecules, with pentyl groups omitted for clarity; (b) side view of the two stacked molecules, also without pentyl groups.

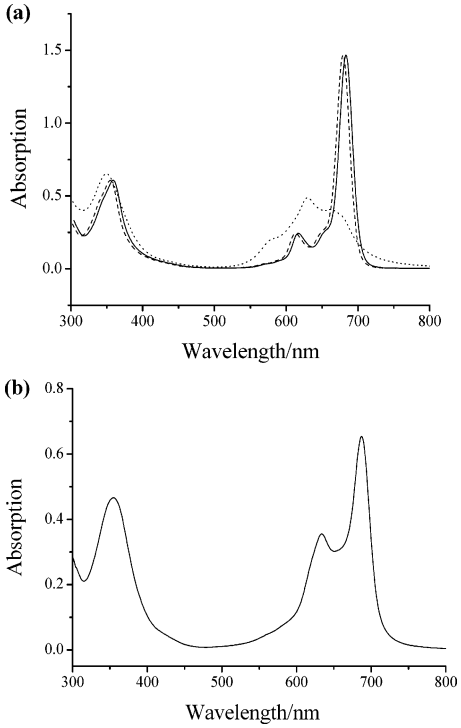


Figure 2. UV−vis spectra of phthalocyanine **5** in (a) distilled water, pH = 5.5 (·····), pH = 8 (- - -) and pH = 13 (—); (b) in medium containing 10% FBS.

Hydrolysis of the ester groups of **4** using NaOH in a mixed solvent system (THF/MeOH/H_2O), according to the method described by Ng,[41] gave the highly water-soluble phthalocyanine **5b** bearing sixteen sodium carboxylate groups. Upon addition of 1 M HCl to an aqueous solution of **5** until pH 2, a green precipitate of **6** was obtained in quantitative yield.

Compound **5** is highly soluble in water; the carboxylate groups in **5** lead to an increased inter-ring distance between two neighboring macrocycles, which significantly lowers its tendency to form stacked aggregates and increases its solubility. Furthermore, the nonplanarity of the adjacent carboxyphenyl groups, as can be seen in Figure 1, probably also contributes to decreased aggregation. This behavior is indicated by the UV−vis spectrum in water at pH > 7, which shows a highly resolved Q-band, centered at 679 nm, assigned to the monomeric species, accompanied by two transitions into excited vibration states at 613 and 650 nm (Figure 2a). In addition, the ^{1}H NMR spectrum of **5** in D_2O at pH > 7 shows clearly resolved peaks for all the macrocyclic protons. Furthermore, phthalocyanine **5** displays a fluorescent emission band in water at 685 nm (excitation at 350 or 610 nm), although fluorescence has been rarely observed for phthalocyanines in aqueous media.[20b,45] Phthalocyanine **6** on the other hand is poorly soluble in both polar and nonpolar solvents, such as $CHCl_3$, THF, benzene, acetone, and water, due to its higher tendency for aggregation. It is however soluble in DMF and DMSO and slightly soluble in MeOH. The UV−vis spectrum of **6** in DMSO exhibits a typical B-band at 364 nm and a Q-band at 677 nm along with weaker absorptions at 613 and 650 nm. Similarly to compound **5** in water, phthalocyanine **6** in DMSO displays a fluorescence emission band at 685 nm upon excitation at 350 or 610 nm.

UV−vis and ^{1}H NMR studies indicate that compound **5** dissolved in water or D_2O exists mainly as two species, **5a** and **5b**. The ratio of the two main components is pH-dependent, as illustrated by the ^{1}H NMR spectra in D_2O at different pH values (Figures 3a−f). In pure D_2O, the ^{1}H NMR spectrum exhibits two broad peaks, at ~8.7 and ~8.0 ppm in a 1:3 ratio, assigned to the α-protons on the phthalocyanine ring and the ortho- and para-protons on the phenoxy substituents, respectively (Figure 3a). Increasing the pH value to 8 upon addition of NaOH resulted in remarkable changes (Figure 3b). Three clear resolved peaks appeared at 9.33, 8.08, and 7.70 ppm in a 1:1:2 ratio, which are assigned to the α-, para-, and ortho-protons of phthalocyanine **5a**, respectively. Further increase in the pH value resulted in the appearance of three new peaks at 8.91, 7.91, and 7.66 ppm, which are attributed to phthalocyanine **5b** (Figures 3c−e). At pH 13 only the signals corresponding to **5b** are seen in the ^{1}H NMR spectrum (Figure 3f). These results indicate that phthalocyanine **5** exists mainly as two species in aqueous solution, **5a** and **5b**; at weakly basic pH values (pH = 8) **5a** is the main component, whereas at pH = 13 **5b** is the main species in solution. Therefore, we believe that when compound **5b** is initially dissolved in distilled water, partial hydrolysis of the sodium carboxylates occurs to give a bufferlike system containing aggregated species, as indicated by the poorly resolved ^{1}H NMR spectrum (Figure 3a) and the UV−vis in distilled water (Figure 2a). At slightly basic pH, deprotonation of one of the carboxylic acid groups from each phenoxy substituent occurs to give **5a** as the predominant species (Figure 3b). The ratio of **5b**:**5a** increases with the pH (Figures 3c−e) until at pH 13 all of **5a** is converted into **5b** (Figure 3f). In agree-

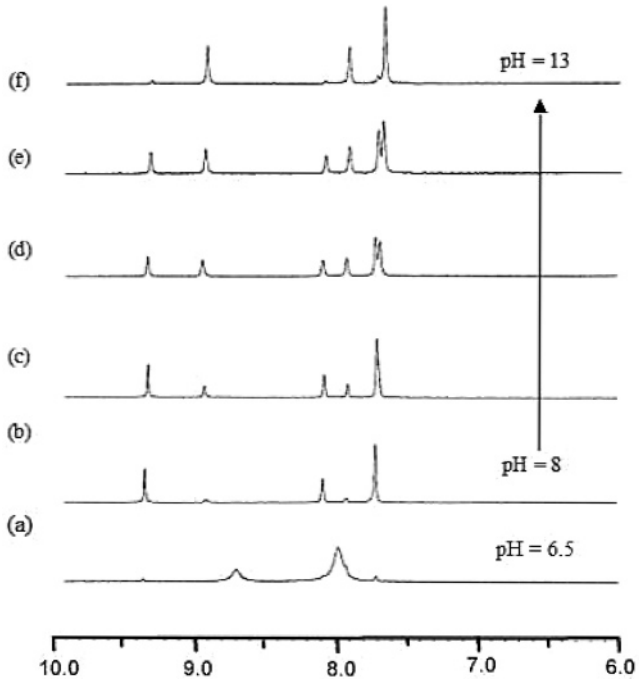


Figure 3. ^{1}H NMR spectra of phthalocyanine **5** in D_2O at different pH values.

ment with these observations, a sharp absorption band is seen in the UV−vis spectrum of **5** at pH 8 in water, likely corresponding to the monomeric species **5a** (Figure 2a). A similar spectrum was obtained for phthalocyanine **5** in medium containing 10% FBS (Figure 2b). The conversion of **5a** to **5b** was also observed by UV−vis spectrophotometry; the Q-band slightly red-shifts from 679 nm for **5a** (pH = 8) to 682 nm for **5b** (pH = 13). In contrast, in distilled water the most intense absorption band at 630 nm is assigned to aggregated species (also present in medium), whereas the Q-band corresponding to the monomeric phthalocyanine appears as a shoulder (Figures 2a,b). These results indicate that at physiological pH, phthalocyanine **5a** predominates over **5b**.

2. Cell Culture Studies. 2.1. Cytotoxicity. The concentration-dependent dark cytotoxicity was investigated in both V79 and HEp2 cells exposed to increased concentrations of each phthalocyanine (up to 400 μM) for 24 h in the dark. Only the cells exposed to the higher concentration of the phthalocyanines showed slight growth inhibition. The dose−response curves found for compounds **5** and **6** were nearly identical, indicating low dark toxicity toward both V79 and HEp2 cells, even at concentrations as high as 400 μM (Figure 4). The slight decrease in absolute signal observed for compound **6** is probably due to the effect of 1% DMSO present in the medium, as seen in the DMSO control curves (Figure 5), or to the higher cellular uptake of **6** compared with **5** (Figure 7).

On the other hand, phthalocyanines **5** and **6** were found to be phototoxic toward human HEp2 cells, upon exposure to low light dose (1 J/cm^2), as shown in Figure 6. Concentrations of phthalocyanines higher than 1.25 μM were found to have a pronounced effect on cell survival and the IC_{50} (light treatment that resulted in a 50% inhibition of cell proliferation compared to controls) estimated from Figure 6 are 4.5 μM for both phthalocyanines.

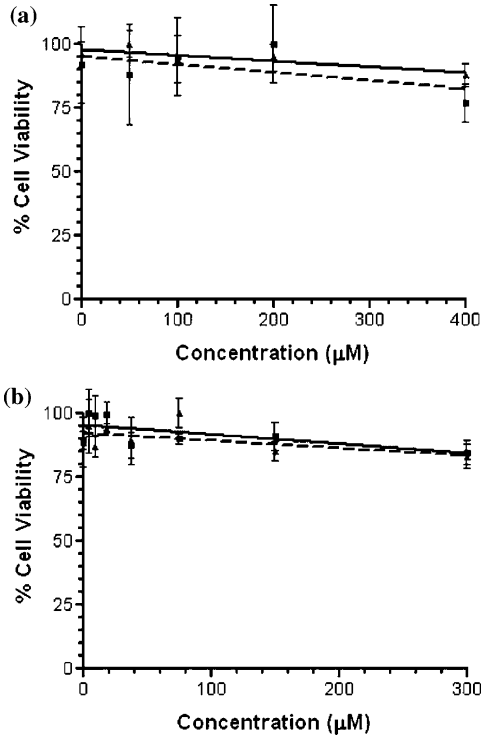


Figure 4. Cytotoxicity of phthalocyanines **5** (full line) and **6** (dash line) toward (a) V79 cells using the CyQuant assay and (b) HEp2 cells using the MTT based CellTiter96 assay.

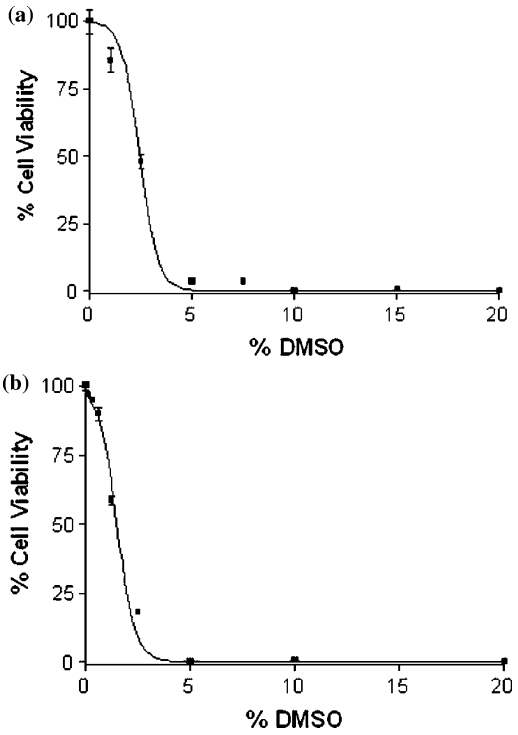


Figure 5. Cytotoxicity of DMSO determined by (a) the CyQuant assay using V79 cells and (b) the MTT assay using HEp2 cells.

Our results are in agreement with published works that show that phthalocyanines are inherently less cytotoxic in the dark and more phototoxic to cells in culture compared with HpD.[46,47] In fact, even at 400 μM concentrations in the dark we found more than 80% cell viability for both compounds **5** and **6** (Figure 4), whereas upon exposure to light (1 J/cm^2), IC_{50} = 4.5 μM were determined.

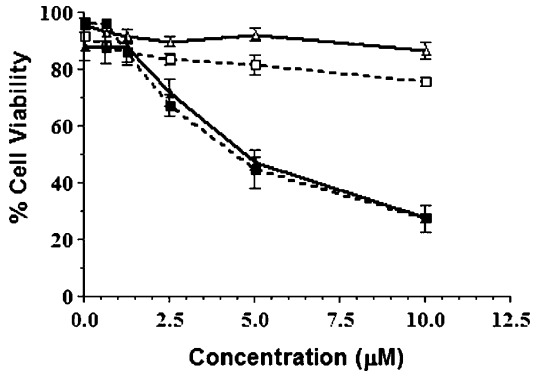


Figure 6. Phototoxicity of **5** (full lines, triangles) and **6** (dash lines, squares) toward HEp2 cells in the absence (hollow symbols) and presence (solid symbols) of 1 J/cm^2 dose light using the MTT assay.

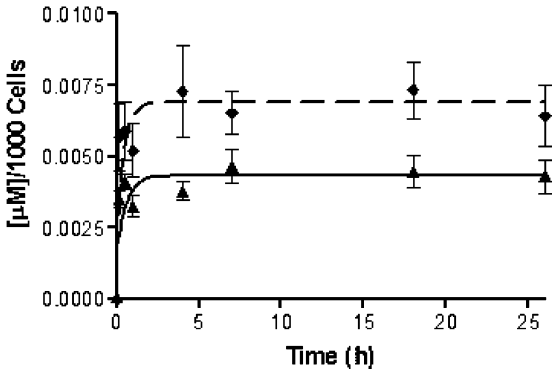


Figure 7. Time-dependent uptake of phthalocyanines **5** (full line) and **6** (dash line) at 10 μM by HEp2 cells.

2.2. Time-Dependent Cellular Uptake. The time-dependent uptake was investigated in HEp2 cells exposed to 10 μM of phthalocyanine solutions for different time periods. The uptake of both compounds occurred rapidly in the first 2 h, after which it reached a plateau (Figure 7). Under similar conditions, phthalocyanine **6** was taken up by the HEp2 cells to a significantly higher extent than phthalocyanine **5**. This suggests that these compounds exist as different species in solution, and that compound **6** is taken up to a higher extent probably as a result of the higher hydrophobicity of its species in solution compared with those of phthalocyanine **5**. The relative uptake of phthalocyanine compounds in cultured cells has been shown to be significantly influenced by the polarity of the macrocyclic substitutents, the nature of the centrally chelated metal ion, and associated axial ligand(s).[48] In a study conducted using aluminum(III) phthalocyanines sulfonated to different degrees (AlPcS$_n$), the mono- and disulfonated compounds were taken up to a greater extent and at a faster rate compared with the higher sulfonated molecules, by dolo26 carcinoma cells in culture.[49,50] In an animal study, water-soluble zinc(II) phthalocyanines were shown to be rapidly taken up by RIF-1 mouse murine fibrosarcomas, and the delay in tumor regrowth was greater after light irradiation at 1 h postadministration rather than at 24 h.[51]

We believe that in medium at pH = 7.4 compounds **5** and **6** exist as different species, phthalocyanine **5** mainly as monomer **5a** bearing eight negative charges, and phthalocyanine **6** as the more hydrophobic, less charged aggregated species. The latter are more readily taken up by cells in culture than the more charged, mainly monomeric species.

2.3. Intracellular Localization. Fluorescence microscopy was used to examine the intracellular localization of phthalocyanines in both V79 and HEp2 cells. The cells were exposed to 10 μM concentrations of phthalocyanines **5** and **6** for 6 and 24 h and examined for intracellular fluorescence. Both compounds showed similar intracellular fluorescent patterns at both time points; the punctated fluorescence was predominately perinuclear, and the cell lysosomes appear to be the major site of localization for these phthalocyanines (Figures 8 and 9). During our experiments we observed redistribution of the phthalocyanines upon brief light exposure, possibly due to light-induced permeabilization of the lysosomes. In agreement with previous reports, we observed that this redistribution is accompanied by a significant increase in fluorescence of the phthalocya-

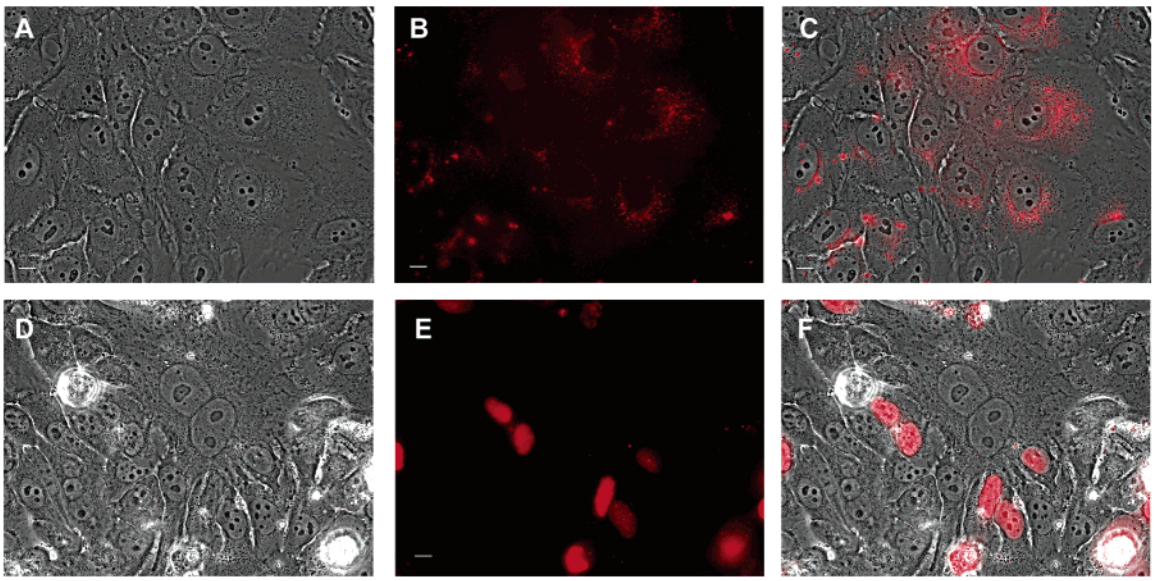


Figure 8. Intracellular localization of **5** in HEp2 cells. A–C: 10 μM of **5** for 24 h; D, E: 8.3 μM of **5**/LipoGen conjugate for 24 h; A, D: phase contrast; B, E: **5** fluorescence; C, F: overlay. Scale bar: 10 μm.

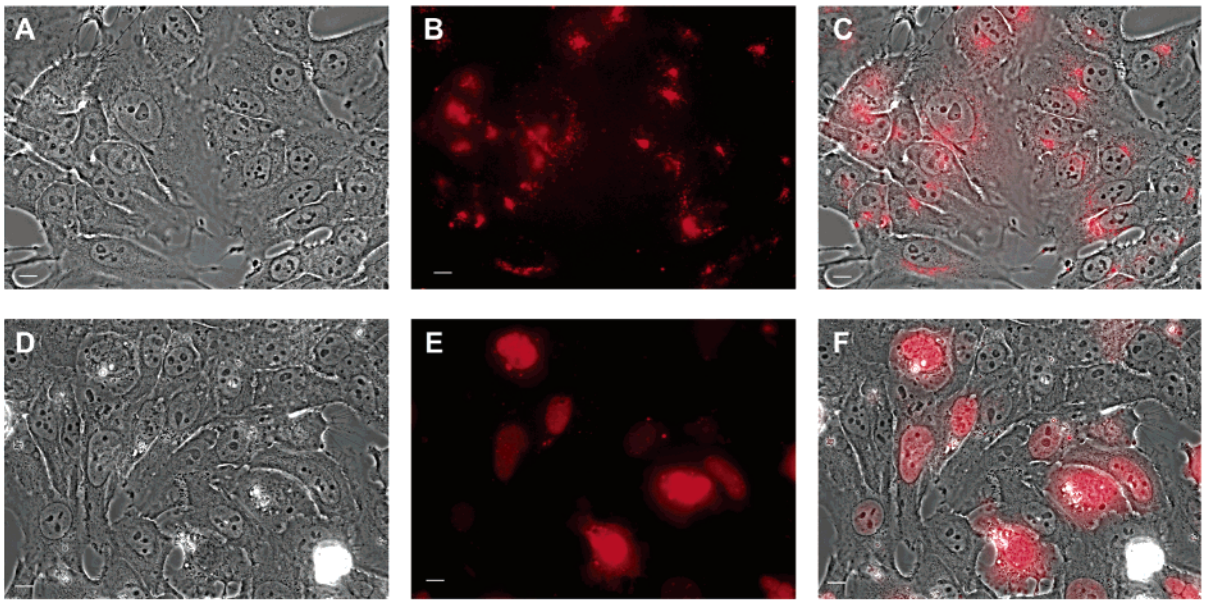


Figure 9. Intracellular localization of **6** in HEp2 cells. A−C: 10 μM of **6** for 24 h; D, E: 8.3 μM of **6**/LipoGen conjugate for 24 h; A, D: phase contrast; B, E: **6** fluorescence; C, F: overlay. Scale bar: 10 μm.

nine, presumably due to monomerization of aggregated dye species.[52] Since the preferential subcellular sites of localization of phthalocyanines **5** and **6** seem to be lysosome-like vesicles in close proximity to the cell nucleus, effective tumor cell destruction could potentially be achieved in vivo upon light activation of these compounds.

Furthermore, we observed that the delivery of phthalocyanines **5** and **6** by LipoGen liposomes resulted in the specific nuclei localization of these compounds, in both V79 and HEp2 cell lines (Figures 8 and 9). In contrast to the experiment above, no redistribution was observed for compounds **5** and **6** after their localization in the nuclei of both V79 and HEp2 cells. In both cell lines phthalocyanines **5** and **6** display similar localization behavior in the presence or absence of LipoGen liposomes. While endosomal uptake in the absence of liposomes might sequester the phthalocyanines in intercellular vesicles where they remain trapped, LipoGen-mediated uptake delivers the phthalocyanines directly into the cytoplasm and subsequently to the cell nuclei. Clearly our studies indicate, as previously observed, that the use of liposomes, or other delivery vehicles, profoundly affects the uptake and biodistribution of this type of compounds.[53−55] Liposomes of various compositions are often used for the selective delivery of highly hydrophobic photosensitizers, such as Zn(II)-phthalocyanine, to animal tumors; the most common are liposomes of dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylethanolamine (DPPE), and dioleoylphosphatidylcholine (DOPC).[55] For example, Zn(II)-phthalocyanine and a ^{131}I-labeled derivative were selectively delivered in DPPC liposomes to MS-2 fibrosarcoma tumors in mice.[56] In our studies, the positively charged LipoGen liposomes were found to effectively deliver our water-soluble anionic phthalocyanines to cell nuclei, which could potentially increase the biological effectiveness of these photosensitizers. Other liposome-mediated uptake of a zinc(II) phthalocyanine in NHIK 3025 human carcinoma cells is reported to deliver the dye mainly to the Golgi apparatus and the mitochondria,[54] which indicates that the choice of liposome formulation can modulate the biodistribution of this type of compound and consequently deeply affect its biological activity.

Conclusions

Zinc(II) phthalocyanines **5** and **6**, bearing multiple carboxylate groups, were synthesized and evaluated in cells in culture, using V79 hamster fibroblasts and human HEp2 cells. Both phthalocyanines display several in vitro characteristics that make them highly suitable for continued evaluation as PDT agents, namely low dark toxicity, phototoxicity at low light dose (1 J/cm^2), substantial uptake by cells, and favorable intracellular sites of localization. The negatively charged phthalocyanine **5** is highly water-soluble and at physiological pH and above, it exists mainly as a monomeric species. In contrast phthalocyanine **6** is poorly soluble in water. Despite its hydrophilic character, **5** still possesses remarkable cell membrane penetration capabilities, although under similar conditions **6** was taken up to a higher extent by cells in culture compared with **5**. The preferential subcellular sites of localization of phthalocyanines **5** and **6** were found to be the cell lysosomes. Specific nuclear localization was achieved with phthalocyanine/LipoGen liposome conjugates, which indicates that effective nucleus-targeting and subsequent cell destruction upon dye activation could be achieved using these compounds.

The molecular structure of precursor **4** to both phthalocyanines **5** and **6** was determined. In the crystal, these molecules were found stacked so as to form unusual dimeric arrangements, with parallel phthalocyanine macrocycles rotated relative to each other by about 20°, and with the pentyl groups of one molecule wrapped around the other molecule.

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Supporting Information Available: IR spectra of compounds **3**−**6**, HRMS spectra for compounds **5** and **6**, and typical ^{1}H NMR spectrum for compound **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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