Interactions of Porphyrin Covalently Attached to Poly(methacrylic acid) with Liposomal Membranes

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The interactions between the 5-(4-acryloyloxyphenyl)-10,15,20-tritolylporphyrin covalently attached to poly(methacrylic acid) chain (PMA-Po) and phosphatidylcholine liposomes in aqueous solution at different pH values were studied. The binding constants (K_b) for the liposome− PMA-Po in solutions in the pH range from 6.5 to 9.2 were determined using fluorescence spectroscopy. The binding was found to be efficient. The acid−base properties of the porphyrin chromophores were also studied. Both pK values associated with imine-N protonation of the porphyrin core were found to be 6.4. The quantum yield (Φ_Δ) of singlet oxygen production by Po in the lipid−PMA-Po system was found to be high (0.88 ± 0.05).

Introduction

Porphyrins and their analogues are widely used as photosensitizers in the photodynamic therapy (PDT) of malignancies and other diseases.[1] It is generally recognized that the molecules of these dyes are preferentially retained in solid tumor tissues when introduced to the body. Irradiating them with visible light leads to the generation of highly reactive species, mostly singlet oxygen. These reactive oxygen species have the ability to damage various cell membranes including plasma, mitochondria, lysosomal and nuclear membranes, and protein modifications.[2] Thus, the ability of these dyes to be incorporated into the cells' membranes is an important factor for efficient PDT.

Liposomes are widely used as models for cell membranes, especially in general studies on drug uptake.[3] They are spherical vesicles formed by some amphiphilic compounds, e.g., phosphatidylcholines and ceramides. The amphiphilic bilayer surrounds the aqueous phase; therefore the liposomes may solubilize both hydrophilic and hydrophobic substances.

Tetraarylporphyrins (TArPs) are the most commonly encountered derivatives of porphyrin. Since the parent compound, tetraphenylporphyrin (TPP), is insoluble in water, many approaches were undertaken to overcome that problem. The ionic water-soluble derivatives of TArPs have been developed by sulfonation, by carboxylation, or by alkylation of *N*-pyridyl-substituted porphyrin.[4] The nonionic water-soluble TArPs have been synthesized, e.g., glucose- or galactose-residue-bearing tetraphenylporphyrins.[5,6] Another approach is encapsulating of TArPs inside dendrimers with hydrophilic outer layers.[7,8] However, using these methods it is difficult to avoid some other problems with the system such as for example modification of photophysical and photochemical properties of porphyrin.

In this paper, an alternative solution to the solubility problem is suggested. The porphyrin molecules, 5-(4-acryloyloxyphenyl)-10,15,20-tritolylporphyrin, were covalently attached to the end of chains of water-soluble poly(methacrylic acid) (PMA). In this way, the sensitizer gains an amphiphilic character which has been shown to be essential for incorporation into liposomes.[9] The results of model studies on interactions between PMA-Po and liposomes are presented.

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Materials and Methods

Chemicals and Sample Preparation. *meso*-Tetratolylporphyrin (TTP, 99%), hematoporphyrin IX dihydrochloride (HP), and 9,10-dimethylanthracene (DMA, 99%) were received from Aldrich Chemical Co. (Milwaukee, WI). L-α-Phosphatidylcholine (PC) type XIII-E from egg yolk (99%, solution of 100 mg/mL in ethanol) was obtained from Sigma Chemical Co. (St. Louis, MO). It was a mixture of lipids with the following fatty acid makeup: 33% C16:0 (palmitic), 13% C18:0 (stearic), 31% C18:1(oleic), and 15% C18:2 (linoleic) (other fatty acids being minor contributors), which gives an average molecular weight of approximately 768 g. Pyrene (Aldrich, Milwaukee, WI; 99+%) was recrystallized twice from ethanol.

Polymer Synthesis and Characterization. Poly(methacrylic acid)−5-(4-acryloyloxyphenyl)-10,15,20-tritolylporphyrin (PMA-Po) with no more than one porphyrin chromophore at the end of the polymeric chain was synthesized by anionic polymerization, as reported previously.[10] The PMA-Po was characterized by UV and ^{1}H NMR spectroscopy. The number-average molecular weight, $\bar{M}_n$, was determined by NMR to be 8400 g/mol. The porphyrin was successfully attached to 8% of the polymer chains.

Preparation of Liposomes. Phospholipid vesicles were prepared by extrusion. The lipid solution in ethanol (25 μL) was placed in a volumetric flask, and the ethanol was evaporated under flow of nitrogen. The lipid film was next dissolved in diethyl ether (0.8 mL) and reevaporated under nitrogen to complete dryness. Then, 10 mM phosphate buffer was added (2 mL) and the sample was sonicated for 5 min at 20 °C with a BRONSONIC ultrasonic cleaner. The resulting suspension was then extruded using the PPH MARKER manual extruder by means of the 100 nm Nucleopore Track-Etch Membrane Whatman filters. For each pH value, a separate stock solution of liposomes was prepared.

Determination of Liposome-Binding Constants. A spectroscopic titration technique was used to determine the binding constants (K_b) of the chromophores to lipid vesicles. Details of

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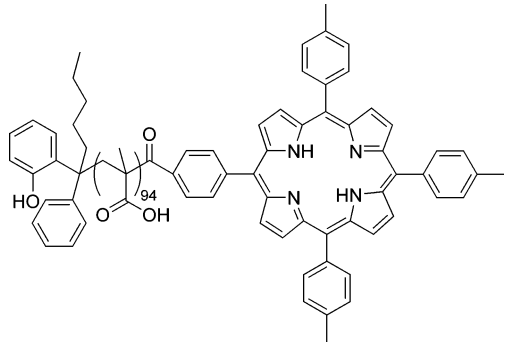

Figure 1. Chemical structure of the PMA-Po polymer.

this technique were described previously.[9,11] K_b is given in units of $(mg/mL)^{-1}$ throughout this study.

Measurements of UV−Vis Spectra. UV−vis absorption spectra at 25 °C of the samples were measured using a Hewlett-Packard 8452A diode-array spectrophotometer equipped with a HP 89090A Peltier temperature control accessory.

Fluorescence Spectra Measurements. Steady-state fluorescence spectra of the samples were recorded on an SLM-AMINCO 8100 Instruments spectrofluorimeter at room temperature. Emission spectra were corrected for the wavelength dependence of the detector response by using an internal correction function provided by the manufacturer.

Irradiation. The light source used to irradiate samples was a xenon short arc lamp (type UXL450S-0; Ushio Inc., Japan) coupled with a monochromator with a bandwidth of 10 nm.

Results and Discussion

Properties of PMA-Po. The chemical structure of the polymer used in these studies (PMA-Po), which is a homopolymer of methacrylic acid with covalently attached 5-(4′-acryloyloxyphenyl)-10,15,20-tri(*p*-tolyl)porphyrin (Po), is shown in Figure 1. PMA-Po is well-soluble in water and in polar organic solvents such as methanol and DMF. The polymer absorbs light in the visible spectral region. Its absorption spectrum is strongly pH dependent (see below). The fluorescence quantum yields for the polymer were determined using TPP as a standard ($\varphi_f = 0.11$ in benzene)[12] and found to be equal to 0.1 and 0.062 in DMF and in aqueous solution at pH 9.1, respectively.

Conformation of the PMA-Po Polymer Chain in Aqueous Solution. The conformation of PMA is known to be strongly pH dependent.[13−15] At low pH values, the carboxylic acid groups of PMA are protonated and the polymer chain adopts a tightly coiled conformation. With increasing pH, the carboxylic groups become ionized and the chain expands due to the repulsion of the charged species along the chain. The experimental relationship between the degree of dissociation of the carboxylic groups on the polymer chain, α, and the pH of the salt-free aqueous solution of PMA was found to be[15]

$$\mathrm{pH} = 6.98 - 1.98 \log[(1 - \alpha)/\alpha] \qquad (1)$$

Because the values of pH and α vary with the concentrations of polymer and added salt and are dependent on the types of counterions, eq 1 can be used for rough estimation of α. In our case, we have used it to estimate α of PMA-Po in solution containing 0.1 M KCl (see Table 1).

To follow the conformational changes for our polymer induced by the changes of pH and ionic strength of the aqueous solution, pyrene was used as a molecular probe. It is known that the ratio of the intensities of the third and the first vibrational bands, I_3/I_1, in the fluorescence emission spectrum of pyrene

TABLE 1: Experimentally Measured Effective Binding Constants (K_b) and Calculated Values of the Degree of Dissociation (α)

pH	α (%)[a]	K_b $(mg/mL)^{-1}$	pH	α (%)[a]	K_b $(mg/mL)^{-1}$
6.51	36.67	111 ± 14	7.95	75.63	79.8 ± 19
6.93	48.55	126 ± 13	8.42	84.14	62.9 ± 9.6
7.24	57.62	128 ± 15	9.21	93.04	62.4 ± 8.6
7.51	64.83	105.1 ± 7.4			

[a] α was calculated from the equation pH = 6.98 − 1.98 log[(1 − α)/α] (ref 15).

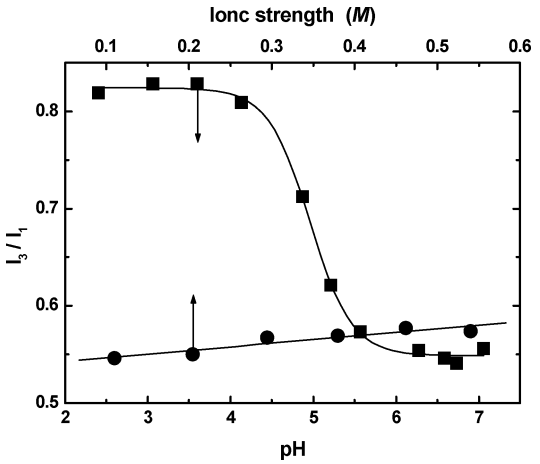

Figure 2. Dependence of the I_3/I_1 ratio for pyrene (concentration = 5×10^{-5} M, $\lambda_{exc} = 334$ nm) solubilized in aqueous PMA-Po solution on pH (■) and on the ionic strength (●).

can serve as a measure for the polarity of the microdomain's interior. Namely, I_3/I_1 is low in polar media and high in hydrophobic environments.[16] Thus, the conformation of the PMA chain in our polymer was monitored by recording fluorescence spectra of pyrene solubilized in the polymer solution. The plot of I_3/I_1 ratio vs pH is given in Figure 2. The sigmoidal shape of the plot is similar to that found by other authors.[13,14] At pH lower than 4, the I_3/I_1 ratio reaches the value of 0.83. The previous studies indicated that the maximal value of I_3/I_1 for PMA, characteristic of the most compact conformation of the PMA chain, is dependent on the polyelectrolyte molecular weight and rises with the increase of the molecular weight. The I_3/I_1 ratio of approximately 0.95,[13] 1.05, and 1.1[14] was reported for PMA with molecular weight of 10 000, 120 000, and 250 000 g/mol, respectively.

In the pH range between 4.5 and 6.0, one can observe a drastic reduction of the I_3/I_1 ratio to the value of about 0.56, which is comparable to that characteristic of pyrene in water. That observation suggests that at pH above 6 the polymer chain adopts a more expanded conformation. As a consequence, pyrene is exposed to the aqueous environment. That observation is in agreement with the results of studies on PMA conformational behavior published earlier by Liu et al.[15] They have calculated the end-to-end distance in PMA chains in aqueous solution and found it to be a strong function of pH.

The effect of the ionic strength of the surrounding medium on the conformation of PMA-Po was also studied. Figure 2 shows that for the solution at pH 6.42 the change in the concentration of KCl from 0.1 to 0.5 M causes only a slight increase in the value of I_3/I_1. That indicates that the addition of salt within this concentration range has only a limited effect on the conformation of PMA-Po at the slightly acidic pH.

Acid−Base Equilibrium of Po Chromophores in the Aqueous Solutions of PMA-Po. Figure 3a shows the absorption spectra of PMA-Po in the Soret band spectral region of the

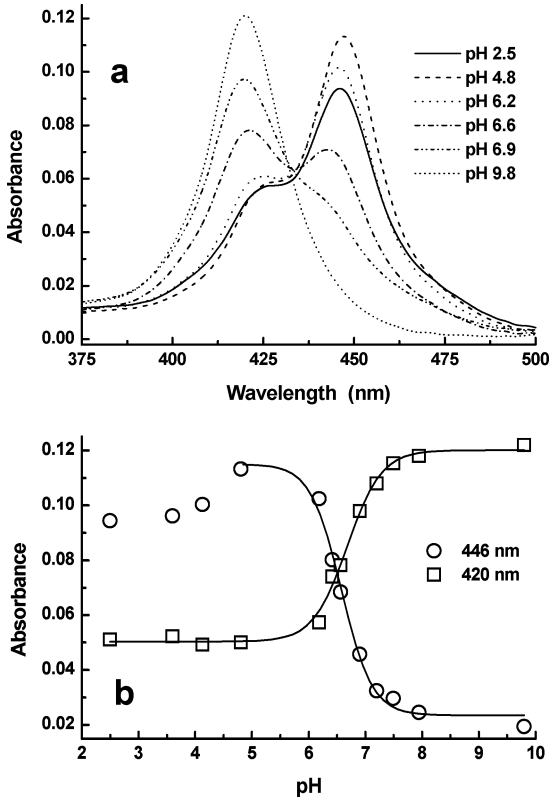

Figure 3. (a) Changes in the absorption spectra of PMA-Po (c_{Po} = 0.4 μM, 0.1 M KCl) as a function of pH. (b) Dependence of the absorbance at 420 nm (□) and at 446 nm (○) on pH.

aqueous solution containing 0.1 M KCl upon acid−base titration. Based on these data, one can conclude that with the lowering of pH the intensity of the band centered at 420 nm decreases with the concomitant appearance of the band at 446 nm (see also Figure 3b). The changes in the spectra are a result of the acid−base equilibria inside the porphyrin chromophore. Two imine nitrogen atoms of a free-base porphyrin are able to attach protons forming mono- and dications. Using the generally accepted convention that constants K_3 and K_4 are associated with imine-N protonation, while the constants K_1 and K_2 correspond to the deprotonation of amine groups,[17] the equilibria can be represented by the following expression:

$$H_2Po^{2+} \overset{pK_4}{\rightleftharpoons} HPo^{+} \overset{pK_3}{\rightleftharpoons} Po \qquad (2)$$

where H_2Po^{2+} and HPo^{+} denote the dicationic porphyrin species (two protons attached to the imino nitrogen atoms in the acidic medium) and the monocationic one, respectively.

To extract information on the pK values from the absorption spectra, an evolutionary factor analysis[18] with the following mathematical model was applied. The association constants are defined as

$$K_3 = \frac{[Po][H^+]}{[HPo^+]} \qquad K_4 = \frac{[HPo^+][H^+]}{[H_2Po^{2+}]} \qquad (3)$$

The total concentration of all the species is

$$c = [Po] + [HPo^+] + [H_2Po^{2+}] \qquad (4)$$

By simple mathematical operations, we obtained the following expressions for the species concentrations as a function of pH:

$$[Po] = \frac{c}{\beta} \qquad \beta = 1 + 10^{(pK_3 - pH)} + 10^{(pK_3 + pK_4 - 2pH)} \qquad (5)$$

$$[HPo^+] = \frac{c}{\chi} \qquad \chi = 1 + 10^{(pH - pK_3)} + 10^{(pK_4 - pH)} \qquad (6)$$

$$[H_2Po^{2+}] = \frac{c}{\gamma} \qquad \gamma = 1 + 10^{(pH - pK_4)} + 10^{(2pH - pK_3 - pK_4)} \qquad (7)$$

The measured absorption spectra were fitted to the above model. The best fit was obtained for pK_3 = pK_4 = 6.4. The extracted absorption spectra of dicationic, monocationic, and neutral forms are shown in Figure 4.

The values of pK for several water-soluble TArPs have been determined, and the influence of the peripheral charges surrounding the protonation sites was observed. *meso*-Tetrakis(4-*N*-methyl-pyridiniumyl)porphyrin (TMPyP) with positively charged groups has pK values of about 0.9,[19] whereas porphyrins with four negatively charged peripheral substituents possess pK about 4 pH units higher. For instance, pK values of *meso*-tetrakis(4-sulfonatophenyl)porphyrin ($TPPS_4$) were determined to be 4.6 ± 0.01 and 5.17 ± 0.01,[20] respectively. The pK_3 of *meso*-tetracarboxyphenylporphyrin (TCPP) has been reported to have the value of 5.5, while the second constant, pK_4, could not be measured due to the aggregation.[17] The increase in pK values of porphyrins with negatively charged peripheral groups is due to the electrostatic stabilization of dications. This effect was widely investigated by Finikova et al.[8] They studied water-soluble dendrimeric tetraarylporphyrins. For Gen 4 polyglutamic porphyrin dendrimer (H_2P-Glu^4OH), Gen 2 dendrimer (H_2P-Glu^2OH), and uncharged dendrimer (H_2P-$Glu^2OPEG350$) (received from H_2P-Glu^2OH by blocking the charges with neutral methoxypoly(ethylene glycol) fragments), they reported the apparent pK values of 6.4, 5.4, and 2.5, respectively. The power of the stabilization effect seems to decrease rapidly with increasing distance separating these charges from the core. The difference between the pK of H_2P-Glu^4OH (with 64 peripheral carboxylate groups) and that of H_2P-Glu^2OH (with 16 peripheral carboxylate groups) is only 1 pH unit. The same difference between the pK values of H_2P-Glu^2OH and H_2P-$Glu^2OPEG350$ (bearing no charges) is about 3 pH units.

The results for PMA-Po are very similar to those obtained for H_2P-Glu^4OH. This suggests a through-space electrostatic shielding effect of the charged carboxyl groups on the protonation sites of the porphyrin. To confirm that effect, we studied the influence of the ionic strength on the acid−base equilibrium of Po chromophores. Changes of the absorption spectra in the Soret band region recorded for PMA-Po in aqueous solution at pH = 6.42 upon the addition of KCl are shown in Figure 5a. Contrary to the rather weak effect of the ionic strength on the conformation of the PMA-Po polymer chain, the effect of ionic strength on the acid−base equilibrium of Po chromophores is much more pronounced. The increase in the ionic strength leads to the disappearance of the absorption band at 446 nm, characteristic of H_2Po^{2+}, and to the increase in the intensity of the absorption band at 420 nm (see also Figure 5b). Rough calculations (based on spectra shown in Figures 4 and 5a) revealed that the increase of KCl concentration from 0.1 to 0.53 M shifted pK_3 from 6.4 to 5.8. That can be explained assuming that the addition of salt reduces the shielding effect due to "screening" of the charged carboxylic groups on the polymer chain by the potassium ions.

Aggregation of Po Chromophores in the Aqueous Solutions of PMA-Po. Aggregation has been considered to be an important factor in lowering the efficiency of the porphyrin

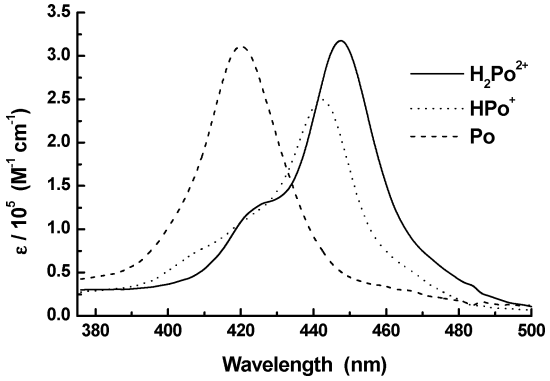

Figure 4. Calculated absorption spectra of the dicationic (H_2Po^{2+}), monocationic (HPo^+), and neutral form (Po) of the porphyrin chromophore.

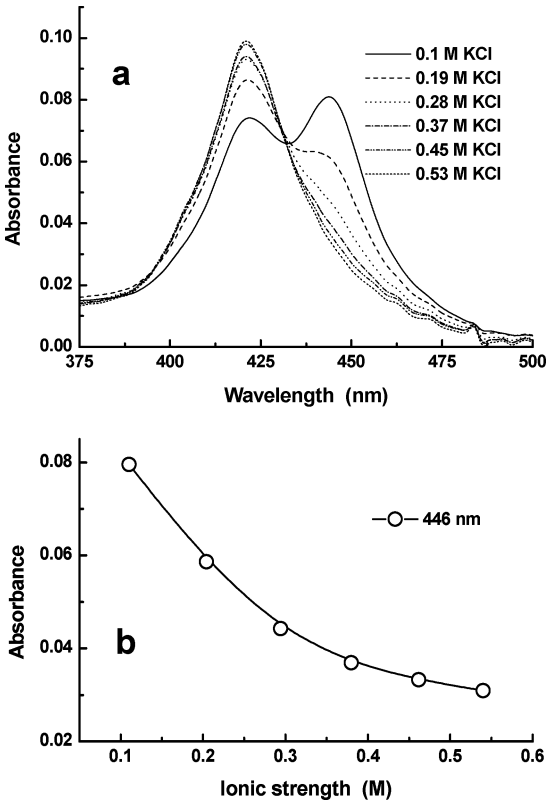

Figure 5. (a) Changes in the absorption spectra of PMA-Po (c_{Po} = 0.4 μM in buffered solution at pH 6.42) as a function of KCl concentration. (b) The absorbance of the PMA-Po solution at 446 nm (○) as a function of ionic strength (c_{Po} = 0.4 μM in buffered solutions at pH 6.42).

photosensitizer. For this reason, we decided to study aggregation of PMA-Po in acidic and basic aqueous solutions. It was found that at pH = 5 about 96.5% of porphyrin chromophores are present in solution as H_2Po^{2+} and the degree of dissociation of the carboxylic groups on the polymer chain is 9.1%. At that pH, there was no spectroscopic evidence for the occurrence of the aggregation in the solution at the concentration range used in the experiments (up to 80 μM). Based on the studies presented above on the conformation of the PMA chain, one can assume that under these experimental conditions the porphyrin molecules are surrounded by the polymer chain. That prevents them from direct interactions and π-stacking leading to the aggregation. Beer's law was obeyed in the whole range of concentration studied. The molar absorption coefficient at the wavelength characteristic of the Soret band of H_2Po^{2+} was determined to be log ϵ_{446} = 5.5.

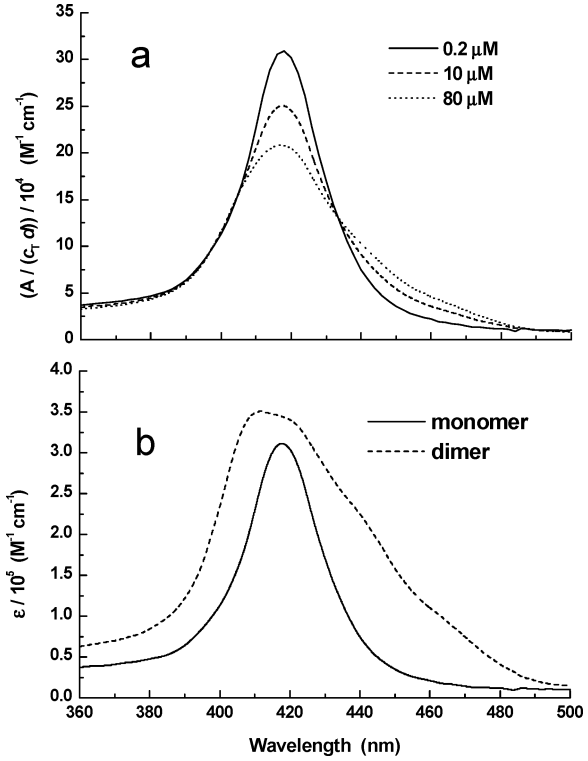

Figure 6. (a) Concentration-normalized absorption spectra of PMA-Po in the concentration range 0.2−80 μM (in buffered solutions at pH 8). (b) Calculated absorption spectrum of Po monomer and dimer.

To avoid the strong repulsive effect resulting from the interaction between negatively charged polymer chains occurring at high pH values, on one hand, and to bring the porphyrin chromophores into the neutral form, which also occurs at high pH, on the other hand, a compromise value of pH = 8 was chosen to study the aggregation phenomenon of the neutral form. At this pH, about 76.5% of the carboxylic groups are dissociated and 97% of porphyrin chromophores are present in solution in the neutral form. In contrast to the spectra of H_2Po^{2+}, the spectra of Po are concentration dependent in the range of 0.2−80 μM. As can be seen in Figure 6, an increase in the concentration produces the broadening of the spectra. In the whole range of concentration investigated, isosbestic points can be observed at 397 and 433 nm, suggesting the existence of the equilibrium between monomeric (Po) and dimeric (D) species. The absorption spectra have been analyzed using the above-mentioned evolutionary factor analysis with the monomer−dimer model:

$$2\mathrm{Po} = \mathrm{D} \qquad K_D = [\mathrm{D}]/[\mathrm{Po}]^2 \tag{8}$$

$$c = [\mathrm{Po}] + 2[\mathrm{D}] \tag{9}$$

Fitting our model to the sets of recorded spectra yielded the absorption spectrum of the monomer and dimer and the equilibrium constant for dimerization, $K_D = 6.9 \times 10^4$ M^{-1}.

Csík et al.[5] have investigated nonionic water-soluble TArPs, namely, glucosylated derivatives of tetraphenyl porphyrins. For porphyrin with four symmetrically placed glucosyl groups, 5,10,15,20-tetrakis(4-β-D-glucosylphenyl)porphyrin [TP(4-OGluOH)$_4$P], they found the dimerization constant of 3.5×10^4 M^{-1}. Replacing of one glucosylphenyl group with a phenyl group in 5,10,15-(4-β-D-glucosylphenyl)-20-phenylporphyrin [TP(4-OGluOH)$_3$P] causes a 10-fold increase in K_D to the value of 3×10^5 M^{-1}. The aggregation processes of ionic water-soluble TArPs have been investigated by Pasternack et al.[4] They

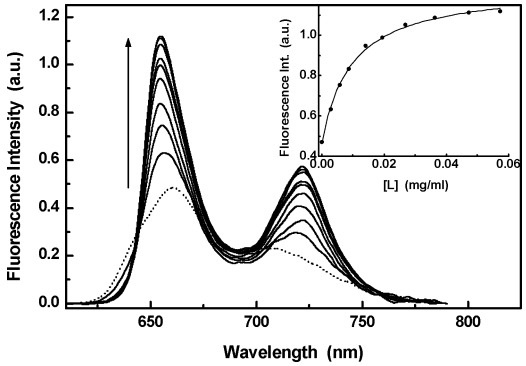

Figure 7. Fluorescence spectra of PMA-Po ($c_{Po} = 0.2$ μM, $\lambda_{ex} = 422$ nm) in the buffer (pH 7.5, dotted line) in the presence of increasing concentration of lipid (solid lines). The insert shows the peak (653 nm) fluorescence intensity as a function of added lipid concentration, as well as the line fitted to eq 10.

showed that TMPyP containing positive groups on its periphery exists in aqueous solution as a monomer throughout a wide pH and concentration range. On the contrary, tetraphenylporphyrin trisulfonate ($TPPS_3$) and TCPP, both having negatively charged groups on the periphery of their molecules, dimerize in water. The equilibrium constants for dimerization in 0.1 M solution of KNO_3 at pH 7.5 were determined to be 4.82×10^4 M^{-1} and 4.55×10^4 M^{-1}, respectively, for $TPPS_3$ and TCPP.

The value of K_D for PMA-Po obtained in this study is very close to these for TArPs with negative periphery groups. In the case of the Po chromophores attached to PMA, the negative charges are located on the polymer chain at one side of the molecule, whereas in the latter case the charges are distributed symmetrically around the pyrrole ring. Such location of the charges is of great importance for interaction with lipid vesicles.

Nakajima et al.[21] have investigated 5-(*p*-aminophenyl)-10,15,20-tri(*p*-tolyl)porphyrin coupled with dextran, a nonionic natural polymer. On the basis of Beer's law experiments, they concluded that the porphyrin chromophore exists in a monomeric state up to the concentration of about 60 μM, but their experiments were carried out in the presence of 10 mM Tris−HCl buffer (pH 7.5) containing 50% DMSO.

Interaction of PMA-Po with Liposomes. The partitioning of PMA-Po between the lipid vesicles and aqueous phase was examined as a function of pH in the range from 6.5 to 9.2 using the spectroscopic titration technique developed earlier.[9,11] At a pH lower than 6.5 we were not able to obtain any reasonable results, since the addition of the liposome suspension to an acidic solution of PMA-Po makes that solution cloudy and a precipitate appears with time. Interaction of PMA with liposomes was studied previously by Oto et al.[22] They observed PMA-induced flocculation of lipid vesicles dispersed in acidic solution.

The sets of steady-state emission spectra for PMA-Po aqueous buffered solution have been measured with the lipid concentrations [L] varying from 0 to 0.06 mg/mL. Spectral changes were observed upon the addition of lipid vesicles to polymer solution. Figure 7 presents a typical set of spectra obtained at pH equal to 7.5 in the presence of various lipid concentrations. Similar changes in the fluorescence spectra were observed for the polymer solutions of different pHs.

The effective binding constant, K_b, was determined by fitting the experimental data to the following formula:[9]

$$F = \frac{F_{init} + F_{comp} K_b[L]}{1 + K_b[L]} \qquad (10)$$

where F_{init}, F, and F_{comp} are the fluorescence intensity of the dye that is measured in solutions in the absence of the lipid and in the presence of the lipid at concentration [L] and the asymptotic value of the intensity at complete binding, respectively. To obtain the K_b for each pH, F versus [L] data were plotted and fitted to eq 10 by a nonlinear regression routine. (The insert in Figure 7 shows the fluorescence intensity at 653 nm versus [L] and the fitted line.) The calculated binding constants for the different pH values are listed in Table 1. They can be compared to the values found for TMPyP ($K_b = 0.76 \pm 0.53$ $(mg/mL)^{-1}$), *meso*-tetra(*m*-hydroxyphenyl)chlorine (THPC) ($K_b = 136 \pm 21$ $(mg/mL)^{-1}$), and tetrabenzoporphyrin (TBP) ($K_b = 89 \pm 25$ $(mg/mL)^{-1}$).[9] Interestingly, TCPP and coproporphyrin I (CP) were not incorporated into the lipid phase at pH 7.3.[9] Under these conditions, they have four negatively charged substituents distributed on each side of the tetrapyrrole ring. Additional data for the comparison with values of K_b determined for PMA-Po can be found for the glucosylated derivatives of tetraphenyl porphyrins.[6] $TP(4\text{-}OGluOH)_4P$ is not able to bind to L-α-phosphatidylcholine dimyristoyl liposomes. However, $TP(4\text{-}OGluOH)_3P$] partitions between lipidic and aqueous phase with the very high K_b of 147.5 $(mg/mL)^{-1}$ (in the original paper the value is given as 1×10^5 M^{-1}).

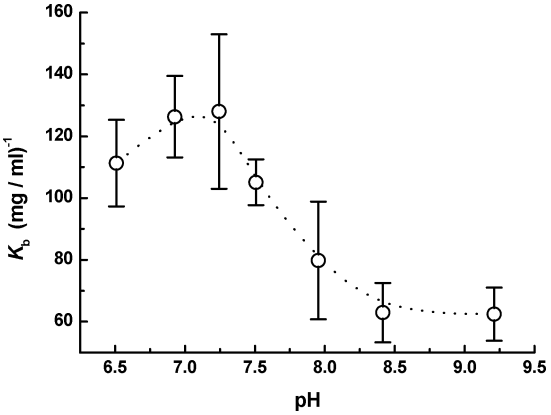

Figure 8. Dependence of the binding constants on pH.

Figure 8 shows the dependence of K_b on the pH. A similar bell-shaped curve was reported previously for dicarboxylic porphyrins.[23] The K_b of PMA-Po in the pH range from 7.0 to 7.3 is very close to those estimated for nonionic TArPs, i.e., THPC or $TP(4\text{-}OGluOH)_3P$]. For lower pH, the value of K_b decreases due to the appearance of the protonated forms of Po. Monocationic and dicationic forms of porphyrins have been shown to possess no affinity for lipid membranes.[23] The reduction of K_b with increasing pH can be attributed to the ionization of the polymeric chain. The different affinities of the Po chromophores for the lipid bilayer result from the various dissociation degrees of the carboxylic groups, especially those which are located close to the dye. In the previous study on dicarboxylic metalloporphyrins, it was shown[24] that the dye with different ionization degrees of propionic acid moieties has a different binding constant.

However, although the values of K_b decrease with increasing pH of the solution, they are considerably higher than those found for the commonly used photosensitizers: e.g., hematoporphyrin derivative (12.2 ± 0.3 $(mg/mL)^{-1}$ [25]) or Photofrin II (9.2 ± 0.8 $(mg/mL)^{-1}$ [25]).

Singlet Oxygen Formation by Po in Methanol and in the Liposome−PMA-Po System. The quantum yield of singlet oxygen formation (Φ_Δ) was determined according to the procedure described in the literature.[26] The procedure is based on the determination of the rates of oxidation of singlet oxygen

acceptor photosensitized by a standard molecule (of known quantum yield of singlet oxygen formation) and by the molecule studied. DMA was applied as a chemical trap for singlet oxygen, and HP served as the standard sensitizer. The value of Φ_Δ for HP in methanol solution is equal to 0.52,[27] and in liposomes $\Phi_\Delta = 0.77$.[28] Both systems, containing photosensitizer (HP or PMA-Po) and DMA at the identical concentration ($c = 5\ \mu$M), were irradiated under identical conditions with light absorbed only by the photosensitizer ($\lambda_{exc} = 516$ nm). In the case of liposome vesicle solution, DMA was introduced (pH = 7.21) and the system was allowed to equilibrate for 30 min. Then, PMA-Po was added and the system was equilibrated in the dark. The kinetics of the photooxidation of DMA was followed by the measurement of fluorescence intensity decay. The quantum yield of singlet oxygen formation can be calculated using the following expression:

$$\frac{V_{DMA}(PMA-Po)}{V_{DMA}(HP)} = \frac{I_{abs}(PMA-Po)\Phi_\Delta(PMA-Po)}{I_{abs}(HP)\Phi_\Delta(HP)} \quad (11)$$

where V_{DMA} is the rate of acceptor oxidation. The mean intensity of the light absorbed by the samples (I_{abs}) was calculated according to eq 12:

$$I_{abs} = I_{516}\int F(\lambda)(1-10^{-A(\lambda)})\,d\lambda \quad (12)$$

where $A(\lambda)$ is the absorbance of the sensitizer and $F(\lambda)$ is the spectral distribution of the light emitted by the lamp given as $I(\lambda) = I_{516}(\lambda)$.

The quantum yield of singlet oxygen formation by PMA-Po solution was found to be equal to 0.70 ± 0.03 and 0.88 ± 0.05 in methanol and in liposome dispersion, respectively. The quantum yields are high. It is known that tetraarylporphyrins produce singlet oxygen with high quantum yields in organic solvents and liposomes. For instance, the Φ_Δ values of TPP in benzene, 5,10,15,20-tetrakis(3,4,5-trimethoxyphenyl)porphyrin (TTMPP) in ethanol, and TMPyP in a D_2O/dimyristoylphosphatidylcholine system are 0.66, 0.67,[29] and 0.85,[30] respectively. The value of Φ_Δ for PMA-Po in methanol is comparable to those determined for other TArPs in organic solvents.

The measurement of Φ_Δ in microheterogenic systems such as a lipid bilayer, in contrast to that in the homogeneous solution, is more complicated, and the results may depend on the method used. The comparison of these values with those obtained with the sensitization method that we had used in our study can produce misleading results due to different localization of the sensitizers in vesicles. The comparison is fully justified only if both sensitizers reside at the same depth in the bilayer.[31] When $^1O_2$ is produced, it diffuses freely between the lipidic and aqueous phases. It is obvious that the efficiency of the singlet oxygen escape from the membrane is higher when the sensitizer is located closer to the water interface. Thus, the efficiency of the oxidation of the target molecule (and thereby the measured quantum yield) is a function of the localization of the dye in the membrane.

Conclusions

Our results show that anchoring hydrophobic tetraarylporphyrin to the PMA chain can improve its properties as a photosensitizer. In this way, the sensitizer gains an amphiphilic character. PMA-Po is very well soluble in water, and it binds effectively to the liposomes. Although the values of K_b decrease with increasing pH of the solution, they reach the maximum around the neutral pH. In the whole pH range, they are considerably higher than those found for the commonly used photosensitizers. The lowering of K_b in basic solutions is mostly due to the ionization of the polymeric chain. The ionization of PMA results in formation of polyanions, which do not have affinity to the negatively charged liposome vesicle. It is important, however, that the value of K_b is still quite high even at pH = 9, when more than 90% of the carboxylic groups on the polymer chain are ionized. In addition, there is no noticeable aggregation of Po chromophores in acidic solution. The aggregation observed in basic solution is no higher than that observed for negatively charged TArPs. The anchoring of the dye to the polymer chain has no effect on the quantum yield of the singlet oxygen formation, so the porphyrin chromophore attached to the PMA chain can act as a very efficient photosensitizer in oxidation of organic compounds solubilized in liposomes.

References and Notes

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