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The effect of liposomes' surface electric potential on the uptake of hematoporphyrin

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ABSTRACT

Hematoporphyrin is being used as a photosensitizer in photodynamic therapy of tumors, as well as of other clinical cases. Many classes of tetrapyrroles, including hematoporphyrin, are partitioning quite easily into the external cytoplasmic membrane as the mechanism of cellular uptake. Several chemical and physical parameters of the membrane were studied for their effect on the extent of porphyrins' partitioning. In this manuscript we report, for the first time, a quantitative analysis of the effect of the membrane's surface electric potential on the partitioning. We prepared liposomes, as membrane models, composed on zwitterionic DMPC lipid, as well as DMPC liposomes that contain a small, varying fraction of negatively charged DMPS and positively charged DOTAP. We found that indeed the surface potential had a very strong effect on the binding constant of HP, which is negatively charged at the physiological pH that was used. The trend in the apparent binding constant can be formulated and fitted with the Gouy-Chapman model of surface potential. We found that the average concentration of HP within the aqueous shell that has a thickness of the Debye layer around the liposome is determining the extent of binding in the law of mass action.

1. Introduction

Natural and synthetic porphyrins are being used as photosensitizers in photodynamic therapy (PDT) of malignancies and other diseases. The two important attributes of photosensitizers are their preferential uptake by malignant cells in tissues and their efficient generation of singlet oxygen upon their illumination. Molecules of the different classes of tetrapyrroles exhibit these properties and are employed for cancer therapy [1–5], anti-microbial activity and virus purging [6–8]. An essential factor in choosing a sensitizer for PDT is its ability to incorporate into the cell. This is especially crucial with amphiphilic and hydrophobic compounds, which are capable of passively partitioning into the cytoplasmic membrane. In the case of anti-bacterial activity the partitioning of the sensitizer, hematoporphyrin (HP), into the cytoplasmic membrane was shown to be a prerequisite for its cellular uptake [9].

Over the last 3 decades an intensive effort was placed in studying the effect of various chemical, physical and environmental parameters on the uptake of various sensitizers by cells and artificial liposomes, which serve as membrane models. Artificial liposomes are very often employed in general investigations of drug uptake and especially in the study of photosensitizers for use in PDT. Thus, for example, the composition of lipids in the membrane [10–12], the temperature and the phase of the lipid bilayer [13,14], its viscosity [15], the existence of a cross-membrane Nernst potential [16,17] and other parameters, were evaluated.

In this manuscript we report, for the first time, the major effect that the surface potential, also known as the Gouy-Chapman potential [18,19], has on the binding of the common photosensitizer HP. About 10-20% of the membrane components, depending on the type of cell or sub-cellular organelle [20], are negatively charged at physiological pH and therefore they contribute to the electric surface potential. We have prepared several types of liposomes that were composed of mostly zwitterionic dimyristoyl-phosphatidylcholine lipids, in which we incorporated a small fraction, usually much less than 20%, of charged lipids. We have observed a dramatic change in the extent of binding of HP, which at the physiological pH that we have used carries 2 negative charges as a result of deprotonation of the two carboxylic acid residues. The direction of change in the binding of HP was indeed found to be governed by the sign of the membrane's surface charges and is influenced by the concentration of electrolytes in solution. We show that the Gouy-Chapman theory can be used as a good model for this effect. We also show that one has to consider the average concentration of the binding molecules, HP, within the Debye layer near the membrane's surface, and not the bulk concentration, as the correct value to be used in the equation of mass-action for the binding equilibrium. This gives the Debye parameter a practical and intuitive meaning.

2. Materials and Methods

2.1. Chemicals

Hematoporphyrin IX (HP) was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solutions of 2 mM HP was prepared in N,N-dimethylformamide (Frutarom Ltd., Haifa, Israel), from which

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aliquots were taken for the experiments in this study. The final volume fraction of DMF in the studied samples was usually ~0.1-0.2%. Diethyl ether (>99.8%) was from Fluka Chemie (Buchs, Switzerland). 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)[1], 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL).

2.2. Preparation of liposomes

10 milligrams of lipids, which were dissolved in chloroform: methanol, were taken into a vial and the solvent was evaporated under nitrogen. The lipid was re-dissolved in diethyl ether that was then re-evaporated thoroughly under nitrogen to complete dryness. Buffer was added to form a lipid concentration of 5 mg/ml and the sample was vortexed for 2 minutes and then sonicated for 10 or 20 minutes at 4 °C with a probe sonicator (Sanyo-MSE Soniprep 150, Crawley UK). To eliminate the Ti particles from the sonicator's probe, the solution was centrifuged for 15 min. The liposomes that are formed under these conditions have a diameter of ~150-400 nm.

2.3. Spectroscopic measurements

Absorption spectra were recorded on a Shimadzu UV-2501 UV-visible computer-controlled spectrophotometer (Kyoto, Japan). Fluorescence excitation and emission spectra were measured on a Perkin-Elmer digital fluorimeter (Norwalk, CT, model LS-50B).

2.4. Determination of liposome binding constants

A spectroscopic titration technique was used to determine the binding constant (K_b) of the dyes to lipid vesicles. Details of this technique were described previously [21,22]. Briefly, the fluorescence intensity of the dye was monitored as a function of added lipid concentration. In all the studied cases the intensity of the fluorescence increased at 622 nm, the location of the membrane-bound HP, and decreased at 613 nm, the emission peak of water-solubilized HP. We have established in each case that the system has achieved equilibrium after just 2–3 minutes, after which no additional change in the spectrum was observed. The set of emission spectra of HP, with increasing concentrations of lipid, is thus composed of linear combinations of the basic spectra of the two species of HP. They were resolved globally by Principal Component Analysis (PCA) using the Matlab platform (The MathWorks, Natick, MA). After the two components were resolved, the fluorescence intensity at 622 of the resolved spectrum of the membrane-bound HP nm was plotted vs. lipid concentration and fitted to Eq. (1), by a nonlinear regression routine in Origin (Microcal Software, Northampton, MA).

$$F = \frac{F_{\text{init}} + F_{\text{comp}} K_b[\text{L}]}{1 + K_b[\text{L}]} \tag{1}$$

Where F_{init}, F and F_{comp} are the fluorescence intensity of the dye at 622 nm that is measured without lipid, with lipid at concentration [L] and the value obtained asymptotically at complete binding, respectively. Each set of binding constants was repeated at least 3 times and the average value, $\overline{K_b}$, of several measurements, each yielding a result, x_i, and a measurement error, σ_i, were calculated by Eq. (2)[23]:

$$K_b \cong \frac{\sum\left(x_i/\sigma_i^2\right)}{\sum\left(1/\sigma_i^2\right)} \quad \sigma_K^2 \cong \frac{1}{\sum\left(1/\sigma_i^2\right)} \tag{2}$$

[1] DMPC - 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DMPS - 1,2-dimyristoyl-sn-glycero-3-phospho- L-serine; DOTAP - 1,2-dioleoyl-3-trimethylammonium-propane.

3. Results and Discussion

Fig. 1A shows the changes that were observed in the fluorescence spectrum of HP when the concentration of DMPC liposomes increased from 0 to 0.86 mg/ml. One observes here the well-established behavior of the emission band's peak shifting from 613 nm to 622 nm upon the transfer of HP from the aqueous to the membranal medium. The existence of only these two species of HP is also indicated by the presence of isosbestic points at 617 and 683 nm. Fig. 1B shows a plot of the fluorescence intensity at 622 nm after the emission spectra were resolved so that the spectra of the free and membrane-bound HP were delineated. The fit to Eq. (1) yielded a binding constant of 4.41 ± 0.43 ml/mg. As said above, such measurements were repeated and averaged.

When the measurement of binding constants was done with liposomes composed of DMPC:DMPS lipid mixtures, with the weight fraction of DMPS varying from 0 to 25%, the results of K_b that were obtained are shown in Table 1 and relative to the K_b for pure DMPC liposomes in Fig. 2. As can be seen, the binding constant decreases by more than a factor of 10 along this set of experiments.

We do not imply by these data that the thermodynamic binding, or partitioning, constant is changing as a result of electric charges at the membrane's surface. K_b only appears to decrease in this set of experiments as a result of the fact that the concentration of HP near the surface is lower than in the bulk, due to the negative surface potential which repels the negatively-charged HP. Thus, the K_b that is calculated using the total concentration of HP that was placed in the sample appears lower, as a result of the fact that the local concentration near the membrane is smaller than the bulk concentration. One has to know the local concentration that has to be used in the law of mass action, or inversely, use the K_b to calculate the local effective concentration. This will be discussed later.

In order to accommodate the K_b results with the effect that the surface electric potential exerts, one has to consider the central equation of the Gouy-Chapman model [18], Eq. (3).

$$\frac{A\sigma}{\sqrt{C}} = \sinh\left(\frac{ze\psi_o}{2kT}\right) \quad A = \left(8N\in_r\in_o kT\right)^{-1/2} \tag{3}$$

σ is the density of charges at the membrane's surface, in unit charges per Å^2, C is the concentration of electrolyte, having a valence of z, in the solution, and ψ_0 is the resultant electric potential at the surface. This potential decays as a function of x, the distance from the surface, according to Eq. (4) and is thus defined as zero at large distance.

$$\psi(x) = \frac{2kT}{ze}\ln\left(\frac{1+\alpha\cdot e^{-Bx}}{1-\alpha\cdot e^{-Bx}}\right); \quad \alpha = \frac{\exp\left(\frac{ze\psi_o}{2kT}\right)-1}{\exp\left(\frac{ze\psi_o}{2kT}\right)+1}; \quad B = \left(\frac{2e^2\cdot n(\infty)z^2}{\in_r\in_o kT}\right)^{0.5} \tag{4}$$

B^{-1} is termed the Debye length and is seen to be defined by common parameters and by the concentration of electrolyte at large distance away from the membrane's surface, n(∞). For small surface potentials, one obtains that $\psi(x) = \psi_o e^{-Bx}$. B can be represented by $\frac{\sqrt{C}}{0.304}$ for a membrane in an aqueous solution at 25 °C and in the presence of a 1:1 electrolyte, $\frac{\sqrt{C}}{0.152}$ for a 2:2 electrolyte and $\frac{\sqrt{C}}{0.176}$ for a 1:2 electrolyte [24].

As a consequence of the existence of the surface potential a gradient is formed in the concentration of ions near the surface, as given by Eq. (5), which shows that anionic species are present at

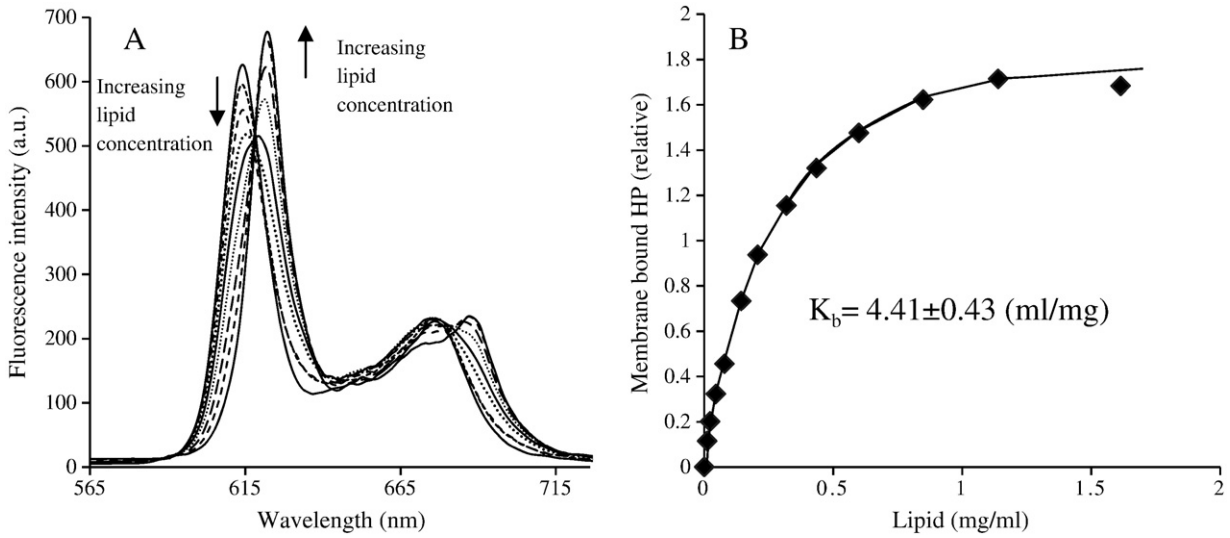

Fig. 1. A: Changes in the fluorescence spectrum of HP upon titration with DMPC liposomes at the following lipid concentration: 0, 0.01, 0.02, 0.12, 0.19, 0.30, 0.44, 0.62 and 0.86 mg/ml. B: Change in fluorescence intensity at 622 nm of the resolved emission spectra of the membrane-bound HP.

lower concentration in layers that are close to a negatively charged membrane, and oppositely for cations.

$$n(x) = n(\infty)\cdot\ exp-\left[\frac{ze\psi(x)}{kT}\right] \tag{5}$$

We took the cross-sectional area of a phospholipid molecule at the surface as 62 Å^2 [24], and we calculated the surface potentials, ψ_0, for each of the lipid mixtures that we used to make liposomes. The concentration of electrolyte in this case was the concentration of the buffer itself, 5 mM, since the concentration of the HP is negligible compared to this value. We used Eq. (4) to calculate the electric potential at each distance x from the membrane's surface and Eq. (5) was used to calculate the concentration of HP at that distance. We thus obtained the distribution of concentrations of the double negatively charged molecules of HP near the liposomes' membranes. For example, when the mole fraction of DMPS is 20% Eq. (5) predicts that the local concentration of HP at a distance of 1 nm from the surface is just 7.5% of its concentration in the bulk. We thus give in Table 1 the calculated average concentration of HP within the whole Debye layer, whose width was calculated in our case, by Eq. (4), as 2.49 nm. The average concentration within the Debye layer is seen to drop drastically from its value in the bulk, as the relative concentration of DMPS increases. Only at distances greater than about five Debye lengths, namely around 12.5 nm in this case, does the local concentration of HP approach its bulk value to within 1% or less.

Table 1
Measured binding constants of HP, surface potentials and the average concentration of HP in the Debye layer relative to that in the bulk, for liposomes composed of DMPC and DMPS.

DMPS weight%	K_b	$\frac{K_{b,i}}{K_{b,\%DMPS}}$	ψ_0 (mV)	$\frac{[HP]_{Debye\ layer}}{[HP]_{bulk}}$
0	3.97 ± 0.13	1.00	0	1.000
2	1.19 ± 0.12	0.300 ± 0.040	-29.0	0.509
4	0.96 ± 0.08	0.242 ± 0.028	-51.7	0.320
5	0.80 ± 0.16	0.201 ± 0.047	-60.8	0.271
6	1.63 ± 0.11	0.411 ± 0.041	-68.8	0.237
8	0.76 ± 0.16	0.191 ± 0.047	-82.0	0.195
10	0.31 ± 0.13	0.078 ± 0.030	-92.6	0.169
15	0.66 ± 0.12	0.166 ± 0.036	-112.6	0.137
18	0.09 ± 0.08	0.023 ± 0.021	-121.7	0.127
20	0.82 ± 0.24	0.207 ± 0.067	-127.0	0.122
25	0.50 ± 0.21	0.126 ± 0.057	-138.3	0.113

Fig. 2 shows a plot of the calculated binding constant of HP at given percentages of DMPS in the membrane, relative to the binding constant to pure DMPC liposomes, as a function of the fraction of DMPS in the lipid. This figure also shows the normalized average concentration of HP within the Debye layer, as calculated from Eq. (5). This decreasing local concentration of HP when more DMPS is included in the membrane is responsible for the appearance of a decreasing binding constant. One can see that this average concentration can indeed differ strongly from the nominal bulk concentration of HP.

We also calculated the local concentration of HP when it was averaged over other layer thicknesses: a quarter of the Debye layer, or one half layer, or two times the Debye layer, in addition to one Debye layer. In all these cases the agreements to the measured data were much worse than when a single Debye length was considered. The R^2 parameters for averaging over these layer thicknesses were: 0.6107, 0.7185, 0.4836 or 0.8424, respectively. Thus the thickness of a Debye layer appears to be best suited as the micro-medium in which the average concentration of the HP is to be considered for the law of mass action for the binding process. Put in other words, if the average concentration in the Debye layer is taken for the law of mass action, the same binding constant, K_b will result, as one would expect for this thermodynamic equilibrium parameter.

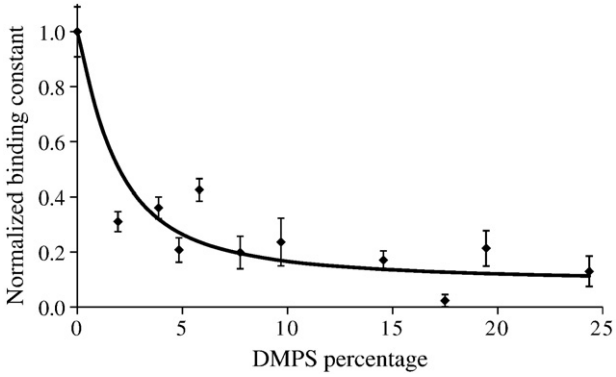

Fig. 2. Relative change in the binding constant of HP to liposomes composed of varying ratios of DMPC:DMPS (dots) and the theoretical line derived from the Gouy-Chapman theory.

It should be pointed out that in our studies the ratio between the concentration of DMPS and HP was 35 at the lowest DMPS concentration that was used and reached 875 at the highest. Thus the binding of HP to the membrane does not alter the charge density at the membrane's surface, σ, to any significant extent.

Next we measured the binding of HP to liposomes that contained the positively charged lipid, DOTAP. Positively charged lipids are very rare in natural membranes and this set of experiments was done as a control test. The results of measurement and calculations are given in Table 2. The opposite trend to the one that was seen with negatively charged liposomes is evident. An increasing fraction of DOTAP in the membrane raises the positive surface potential and this in turn is seen to increase the apparent binding constant of negatively charged HP. This arises from the increase in HP's local concentration near the membrane's surface. Fig. 3 depicts the measured binding constant of HP at given percentages of DOTAP in the membrane, relative to the binding constant to pure DMPC liposomes, as a function of the fraction of DOTAP in the membrane. The solid curve gives the calculated average concentration of HP within the Debye layer, relative to the concentration in the distant bulk, and it is seen to increase as the concentration of DOTAP increases. It is this increase in the local concentration which causes the observed increase of the binding constants of HP. An order-of-magnitude change is observed in K_b, as well as in the calculated local concentration, upon increasing the DOTAP content from 0 to 15% of weight. In this case, too, the best fit to the measured K_b was obtained when the concentration of HP was averaged over one Debye layer. The concentration of HP in the Debye layer appears therefore to be the determining parameter for the extent of partitioning of HP between the aqueous phase and the membrane. Here too, the vast difference between the large concentrations of DOTAP and the low concentration of HP leads to minimal alteration of the charge densities on the membrane's surface and of the Stern layer.

In both cases, namely binding of HP to DMPC liposomes that contain DMPS or DOTAP, we tested the effect of added electrolyte. Such addition is predicted by the Gouy-Chapman model, Eq. (3), to affect the uptake, by screening the surface potential. In the presence of increasing electrolyte concentrations the binding constants of porphyrins to liposomes with neutral lipids increases as a result of a "salting out" effect, and a correlation was observed with the Hofmeister series [25]. Therefore, to delineate and isolate the effect of surface potential we measured the binding of HP to liposomes made of DMPC only or of DMPC and containing DMPS or DOTAP, under identical conditions, and with increasing concentrations of $MgSO_4$. While K_b increased slightly when 100 mM $MgSO_4$ was introduced to DMPC liposomes due to salting-out, it increased drastically when the liposomes were made of a 85:15 mixture of DMPC:DMPS. The effect of 100 mM $MgSO_4$ on the mixed liposomes relative to DMPC liposomes was an increase of 80%. This reflects a screening of the surface potential, which in the case of these mixed liposomes was responsible for the reduction of the binding of HP.

Table 2
Apparent binding constants of HP, surface potentials and the average concentration of HP in the Debye layer relative to that in the bulk, for liposomes composed of DMPC and DOTAP.

DOTAP weight%	K_b	$\frac{K_{b,i}}{K_{b,\%DOTAP}}$	ψ_0 (mV)	$\frac{[HP]_{Debye\ layer}}{[HP]_{bulk}}$
0	3.63 ± 0.07	1.00	0	1.000
2	7.70 ± 0.52	2.121 ± 0.184	29.1	2.055
4	8.98 ± 0.52	2.474 ± 0.191	51.9	3.603
5	23.76 ± 1.80	6.545 ± 0.621	61.1	4.484
6	14.85 ± 1.75	4.091 ± 0.560	69.0	5.407
8	14.97 ± 1.82	4.124 ± 0.580	82.2	7.323
10	26.68 ± 1.65	7.350 ± 0.595	92.9	9.280
15	38.51 ± 2.29	10.609 ± 0.834	112.8	14.165

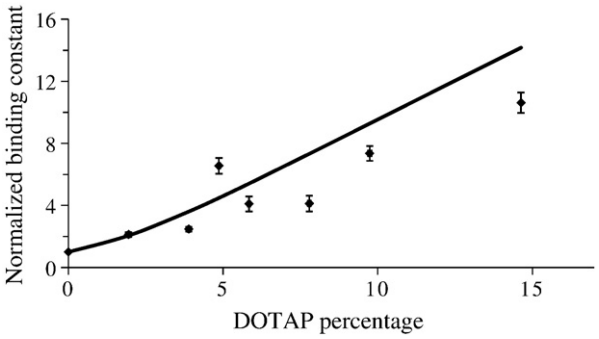

Fig. 3. Relative change in the binding constant of HP to liposomes composed of varying ratios of DMPC:DOTAP (dots) and the theoretical line derived from the Gouy-Chapman theory.

When the effect of $MgSO_4$ was checked with DMPC:DOTAP liposomes, the binding constant decreased, relative to that with liposomes of only DMPC, by 40%, again reflecting a diminishing effect of electrolyte on the otherwise increased binding of HP to the positively charged liposomes due to the inclusion of DOTAP. Additional consequences could emerge from the increased concentrations of $MgSO_4$, such as aggregation and fusion of liposomes. However, taking the ratio between the observed effects on K_b for liposomes of mixed lipids and of pure DMPC, as we did here, effects that are not directly connected to the surface charges should be diminished. Our interest was to demonstrate that the screening effect of an electrolyte does indeed exert its effect and this trend is evident.

As another control experiment we measured the effect of surface potential on the binding of neutral HP, namely at pH = 4.4. We preferred this choice rather than using esterified HP or other neutral molecule, in order to retain basic structural similarity. The ionization constants of the two propionic acid groups in HP were determined by Brault *et al.* and the two pK_a values were reported as 5.0 and 5.4 [26]. Barret *et al.* reported the slightly different values of 6.0 and 6.8 [27]. We have measured the two pK_a of ZnHP as 5.7 and 6.9 [28]. In this last molecule the inner pyrrolic nitrogens are complexed by zinc and do not exhibit acid-base properties. At pH = 4.4 we are 0.6 to 1.6 pH units below the lowest pK_a and thus the large majority of the HP molecules have protonated carboxyl groups, and are thus electrically neutral. We found, indeed, that when the content of DMPS increased to 5% and to 10%, the binding constant, at pH 4.4, remained constant within the error range of about 20%, while at pH = 7.5 it decreased by about a factor of 5. The binding constant to liposomes that contained 5% and 10% DOTAP increased by 40% and 90%, respectively. This may reflect the effect of surface potential on the small fraction of HP that is still monoanionic even at pH = 4.4. At pH = 7.5 K_b increased by a factor of 7 and 25, respectively. It is thus clear that the effect of surface potential can be almost completely cancelled when the large majority of the binding HP molecules are neutralized.

As an indication for the practical consequences of surface potential on the uptake of HP by liposomal membranes, we compared the efficiency of photochemically-induced damage to a singlet oxygen target. We measured the kinetics of destruction of membrane-localized 9,10-dimethylanthracene, a molecule which is oxidized by singlet oxygen in a well-established mechanism. In all samples the total lipid concentration was 0.17 mg/ml and HP was added at a concentration of 2 μM. The binding constants of HP to DMPC liposomes, DMPC:DMPS (9:1) liposomes and DMPC:DOTAP (9:1) liposomes are 3.97 ± 0.13, 0.31 ± 0.13 and 26.68 ± 1.65 $(mg/ml)^{-1}$, respectively. The law of mass action yields the concentration of HP that is membrane-bound under these conditions, of the total concentration of 2 μM: 0.81 ± 0.03, 0.10 ± 0.05 and 1.64 ± 0.10 μM, respectively. The relative rates of singlet-oxygen induced photodamage to 9,10-dimethylanthracene, as measured by the kinetics of disappearance of its fluorescence intensity, were: 1, 0.61 and 1.70,

respectively. The rate of this photodamage does not depend only on the concentration of HP but is affected also by the rate of escape of singlet oxygen from the membrane's phase. However, the observed trend does reflect the fact that increased uptake causes increased photodamage.

The practical biological implication of our study is in two directions. When studying the uptake of charged photosensitizers by cells one must take into consideration the possible effect of surface potential. As seen in this study, this effect can be dramatic and it may thus interfere with other parameters that exist in the studied system. As we indicate here, calculating the average concentration of the binding molecule within the Debye layer may point to the extent of increase, or decrease, of the binding that is caused by the surface potential. An option for diminishing the effect of surface potential is by using electrolytes. As the screening effect depends on the ion's charge, z, trivalent electrolytes might abolish surface potential efficiently in some cases [29], if they do not affect adversely the system. In addition, when electrophysiological studies are carried out, such as the study of the influence of membrane potential on porphyrin uptake by cells, it is imperative that one separates the two major components of the electric profile of a membrane: the cross-membrane, bulk-to-bulk Nernst potential, which depends on the difference in the concentration of diffusible ions on the two sides of the membrane, and the surface potential which depends on surface charges. Together, these two parameters generate a convoluted electric field across the membrane which may also be non-symmetrical on the two sides, because of different charge densities on the two leaflets of the membrane [30]. A quantitative assessment of the effect of surface potential on porphyrins' binding to cells and the involvement of attributes such as surface charge density and electrolyte concentration is required. This methodology was suggested in this manuscript.

4. Conclusions

We have shown in this study the big effect that the membrane's surface potential has on the uptake of HP, when it is negatively charged at physiological pH. Liposomes composed of neutral DMPC and containing a small fraction of negatively charged DMPS or positively charged DOTAP phospholipids exhibited a decreased, or respectively increased, uptake of HP as the additive's content increased. We show that the trend can be fitted very nicely with the Gouy-Chapman model of surface potential. Moreover, the results indicate that the average concentration of HP within an aqueous Debye layer near the membrane's surface is the value to be considered for calculation of the effect of the surface potential on the membrane-binding of HP, or possibly of other membrane-intercalating molecules as well.

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