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The partition and distribution of porphyrins in liposomal membranes. A spectroscopic study

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Spectroscopic techniques were employed to establish the first comparative evaluation of the partition of the photosensitizers hematoporphyrin derivative (HPD) and photofrin II (PF-II) into phosphatidylcholine (PC) and PC/cholesterol liposomes. The fluorescence of the dyes was monitored while they were titrated with liposomes, yielding the dyes' effective binding constants to the membranes. The binding constants of HPD and PF-II to PC liposomes are 12.2 ± 0.3 $(mg/ml)^{-1}$ and 9.2 ± 0.8 $(mg/ml)^{-1}$ and to PC/cholesterol (50% w/w) liposomes they are 6.7 ± 0.9 $(mg/ml)^{-1}$ and 8.0 ± 0.8 $(mg/ml)^{-1}$, respectively. The vertical distribution of the dyes in the bilayer was determined by quenching their fluorescence with spin-labeled stearic acids. PF-II was found to reside deeper in the membrane than HPD. Cholesterol was found to modulate the distribution of the two dyes to a greater extent then DPPC and DMPC. The modulation mechanism is discussed.

Hematoporphyrin derivative (HPD) has been used in recent years as a photosensitizing agent of cancer and bacterial cells in vitro and in cancer photodynamic therapy [1–4]. It is composed of a mixture of porphyrins with various side-chain groups as well as polymerized molecules of such components [5]. PF-II is the high-molecular weight fraction of HPD, which is believed to have a higher photoactivity in cells [6]. Their utilization as sensitizers relies on their selective uptake and retention by cancerous cells [7,8], and light-activated cell damage is caused by either collisional energy transfer from the excited porphyrin molecule to cell components, or through a singlet oxygen mechanism [9]. The relevance of studying their membrane-association properties lies in the cell's membranes being a major site for the photosensitized reactions in vivo [1,10].

In previous studies we showed that increasing the cholesterol content in PC liposomes' membranes caused a decrease in both HPD and PF-II association [11,12]. In a collisional fluorescence quenching study of the porphyrins by iodide ions we alluded to the possibility that PF-II is located deeper in the membrane than HPD [12]. In this study we employed a spectroscopic technique to measure the binding constants of HPD and PF-II to PC liposomes. We also report on the difference in the vertical distribution of HPD and PF-II in the membrane and on the effect of cholesterol, DMPC and DPPC on this distribution.

Small unilamellar egg phosphatidylcholine (Sigma) vesicles containing cholesterol, DMPC or DPPC (Sigma) were prepared by sonication, according to the method described previously [12]. For the binding measurements, small aliquots from a 5 mg/ml liposomes' stock suspension were added to an aqueous solution (pH = 6.8) of either HPD (prepared by the method of Dougherty et al. [1]) or PF-II (obtained from Photomedica, Raritan, NJ), allowing 5 min incubation prior to recording the fluorescence intensity. For the fluorescence quenching study, aliquots from a 10 mM ethanolic stock solution of stearic acid labeled with TEMPO spin-label (Sigma) were added to an aqueous suspension of liposomes equilibrated with the porphyrin, reaching up to 5% (v/v) ethanol in the sample. The sample was let to incubate for 10 min with the stearic acid [13] before measuring the fluorescence intensity. The emission of HPD and PF-II was measured at 632 nm and excited at 570 nm (on a Perkin-Elmer MPF-66 fluorimeter) where the absorption of both HPD and PF-II is low enough to establish a linear dependence of the fluorescence intensity on the fluorophore concentration. In

Abbreviations: HPD, hematoporphyrin derivative; PF-II, photofrin-II; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; *n*-NS, *n*-nitroxide stearate; Chol, cholesterol.

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addition, under these conditions the measured fluorescence intensity was almost fully contributed by the membrane-associated species, with less than 5% contribution from the fluorescence band of the aqueously dissolved population of porphyrins, which shows up at 613 nm [11,12].

We manipulated the fluorescence binding results according to Bashford and Smith [14]

$$\varepsilon - 1 = (\varepsilon_b - 1) - K_d(\varepsilon - 1)/m \quad (1)$$

where ε is the relative spectroscopic parameter which changes as a result of the dye's binding and which in our case is defined as

$$\varepsilon = F_{obs}/F_{init} \quad (2)$$

where F_{obs} is the measured fluorescence intensity after lipid addition and F_{init} is the fluorescence intensity before lipid addition. ε_b is the characteristic enhancement of that parameter, i.e. ε at infinite membrane concentration. K_d is the dissociation constant which is the inverse of the binding constant for the dye–membrane interaction. m is the lipid concentration.

A plot of $\varepsilon - 1$ vs. $(\varepsilon - 1)/m$ yields a straight line of slope $-K_d$, and an ordinate intercept of $\varepsilon_b - 1$. Fig. 1A shows the experimental data and the linear least-squares fits for the binding of HPD to liposomes. The upper line is the fit for the binding of HPD to PC liposomes, yielding $K_b = 12.2 \pm 0.3$ $(mg/ml)^{-1}$, and the lower line is the fit for HPD binding to PC liposomes containing 50% cholesterol (w/w), giving $K_b = 6.7 \pm 0.9$ $(mg/ml)^{-1}$. This 45% decrease in the binding constant upon adding 50% cholesterol to the membrane agrees nicely with our previous results where we obtained about 60% decrease [11]. The present technique is obviously more accurate since it involves a linear fit while the former was based on resolving two fluorescence bands by a non-linear fit of Lorentzian band shapes. We did not observe an effect of the incubation time [15] after about 15 min.

Fig. 1B shows the similar plots for PF-II association, with the resulting $K_b = 9.2 \pm 0.8$ $(mg/ml)^{-1}$ for PC liposomes, and $K_b = 8.0 \pm 0.8$ $(mg/ml)^{-1}$ for the mixed PC/Chol liposomes. This 13% decrease is somewhat smaller than that which we had found earlier [12]. It should be reminded that in a situation of non specific dye–membrane interaction, i.e. partition, and at high membrane/dye ratio, the partition coefficient in its usual definition is obtained from the abovementioned binding constant by multiplying it by the density of the membrane phase. It can be noticed that the ordinate intercepts of the lines in Fig. 1 for each dye are different. This means that introducing cholesterol into the bilayer changes the spectroscopic parameters of the two porphyrins immersed in the bilayer either by changing

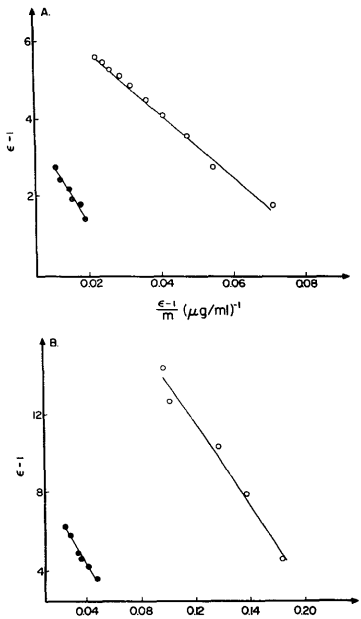

Fig. 1. Fluorescence titration plots of: (A) 10 μg/ml HPD by PC liposomes (○); 2.5 μg/ml HPD by PC/Chol (50%, w/w) liposomes (●). (B) 5 μg/ml PF-II by PC liposomes (○); 2.5 μg/ml PF-II by PC/Chol (50%, w/w) liposomes (●). The fluorescence intensity measured at 633 nm was excited at 570 nm. The lines are least-squares linear fits to Eqn. 1.

the fluorescence quantum yield or the absorption coefficient or both. It should be emphasized at this point that since both HPD and PF-II are mixtures of porphyrins, the binding constant obtained by our, or any other, method should be considered as a fluorescence-averaged, effective binding constant for the corresponding mixtures.

Next we probed the vertical distribution of HPD and PF-II in the bilayer of PC liposomes. To this end we used stearic acid spin-labeled with TEMPO attached to either carbon -5, -12 or -16 in the acyl chain, to quench the fluorescence of the porphyrin molecules which are

present inside the bilayer [13]. The quenching process is expected to follow the Stern-Volmer relationship for collisional quenching. Since only the membrane-embedded quencher molecules participate in the quenching process, we determined the lipid-water partition coefficient of the quencher by the method of Lakowicz et al. [16].

The quencher concentration in the membrane is given by

$$[Q]_m = P[Q]_t/(P\alpha_m + (1-\alpha_m)) \quad (3)$$

where $P = [Q]_m/[Q]_w$ is the partition coefficient, $[Q]_t$ is the total quencher concentration and $\alpha_m = V_m/V_t$ is the volume fraction of the membrane phase. Introducing the expression for $[Q]_m$ into the Stern-Volmer equation

$$F_0/F = 1 + K_Q[Q]_m \quad (4)$$

one obtains

$$F_0/F = 1 + K_Q P[Q]_t/(P\alpha_m + (1-\alpha_m)) = 1 + K_{app}[Q]_t \quad (5)$$

where K_Q is the bimolecular dynamic quenching constant for the membrane-associated porphyrin and F_0 is the fluorescence intensity of that porphyrin in the absence of quencher. The apparent quenching constant is given by:

$$1/K_{app} = \alpha_m(1/K_Q - 1/K_Q P) + 1/K_Q P \quad (6)$$

A plot of $1/K_{app}$ vs. α_m at various membrane concentrations yields a straight line of which the slope $= (1/K_Q - 1/K_Q P)$ and the ordinate intercept equals $1/K_Q P$, thus P can be evaluated. In Fig. 2 we plot the results for K_{app} for three different membrane concentrations obtaining a partition coefficient for the quencher of 32 700. This high coefficient assures that in our experiments more than 99% of the spin probe molecules are in the membrane phase.

In Fig. 3B we show the results of the quenching experiment of the membrane-associated PF-II fluorescence by 5-, 12- and 16-NS according to the Stern-Volmer equation (Eqn. 4) with the corresponding linear least-squares fitted lines. As can be seen the largest quenching constant was obtained for 16-NS, 2.820 $(mg/ml)^{-1}$, while the lowest was obtained for 5-NS, 1.303 $(mg/ml)^{-1}$. In the case of HPD (Fig. 3A) the highest quenching constant was obtained for 12-NS, 2.109 $(mg/ml)^{-1}$, while that for 16-NS, 2.079 $(mg/ml)^{-1}$, is very close to it. As was pointed out before, the spin-labeled stearic acid molecules are alligned preferentially parallel to the acyl chains of the membranes' phospholipids, but the location of the n-th carbon atom of the stearic acid resides actually closer to the membrane's surface than the corresponding n-th acyl carbon of the phospholipid molecules [17,18]. Thus the abovementioned positions 5, 12 and 16 should be

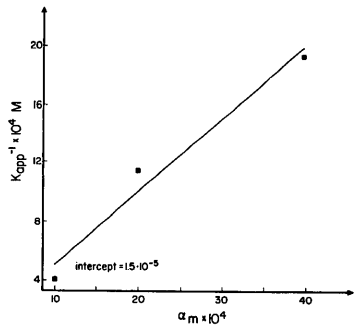

Fig. 2. Observed apparent quenching constant of the fluorescence of 5 μg/ml HPD in PC/Chol (40%, w/w) liposomes by 16-NS as a function of the lipid volume fraction. The line is a linear fit to Eqn. 6 from which the partition coefficient of the quencher was evaluated. The fluorescence was measured as in Fig. 1.

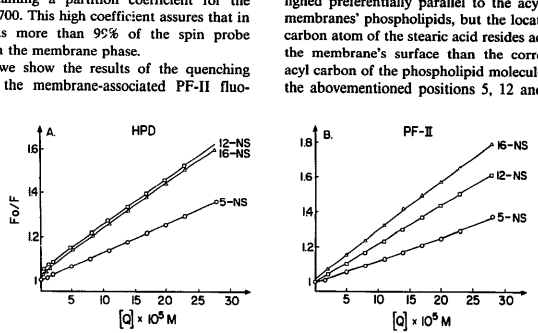

Fig. 3. Stern-Volmer plots for the average dynamic quenching of 5 μg/ml of HPD (A) and PF-II (B), in 2 mg/ml PC liposomes by 5-, 12- and 16-NS. The lines are linear fits to Eqn. 4. The fluorescence was measured as in Fig. 1.

considered as relative measures. We can therefore suggest that the concentration profile of the porphyrins along the vertical axis in the membrane is not homogeneous. In Table I we give the ratio of the quenching constant for 5-NS and 12-NS to that of 16-NS. As can be seen, in PC liposomes (first row) PF-II establishes a monotonous concentration gradient along the vertical to the membrane's plane, starting near the 5th acyl carbon and reaching its highest concentration near the 16th carbon position in the acyl chain which is close to the interlayer area. The HPD distribution profile is practically constant between the 12th and the 16th carbon positions of the stearic acid and, like for PF-II, decreases towards the 5th carbon position although less steeply than PF-II. Another reservation which we want to bring up is related to the fact that HPD and PF-II are mixtures of compounds. Therefore, K_Q, the spin probe quenching constant, is an effective parameter which is an average of the individual quenching efficiencies of the components of HPD or PF-II, each weighted by its relative fluorescence intensity under the experimental conditions. It was reported before [19] that the fluorescence quantum yield of the HPLC fraction which constitutes the high molecular weight species is lower than those of the other components of HPD, which in turn are very close to each other when bound in a low-polarity environment. Thus, the concentration profiles which we obtain for HPD and PF-II are weighted by the fluorescence intensities, yet differences between the two sensitizers and between liposomes of different compositions reflect genuine differences in vertical displacements. Our findings establish quantitatively our suggestion in an earlier study [12], where we found that PF-II is less accessible to aqueously dissolved iodide ions than HPD.

Introduction of cholesterol into the PC membrane changes the concentration distribution profile of the two dyes in a similar manner although to a different extent (Table I). In 20% cholesterol (w/w), both dyes have their highest concentration region around the 12th carbon position, however for PF-II this results from a shift of the profile peak from the 16th to the 12th position, while in the case of HPD it is merely an increase of the relative peak magnitude. At 40% cholesterol the two dyes further redistribute themselves, with HPD exhibiting a high constant profile between the 5th to the 12th position with almost 50% drop by the 16th position, while in the case of PF-II the concentration profile is practically constant over the whole measured range.

These findings can be interpreted on the basis of the difference in polarities of the two dyes. PF-II seems to be more hydrophobic than most of the other components in the HPD mixture, being the last to be extracted in HPLC with an eluent gradient of increasing hydrophobicity [20]. Thus, for thermodynamic reasons, PF-II penetrates into the most hydrophobic region in the membrane which is at the most distant position from the membrane/water interface [21]. The vertical membrane distribution of HPD components, which posses a wide range of polarities [20], is also determined by their polarities and by the profile of the membranes' hydrophobicity as reflected in the dielectric constant [21]. Thus in PC liposomes the distribution of PF-II is skewed toward the 16th position while HPD is evenly spread in the 12th to 16th carbon region.

Introducing cholesterol into the membrane causes a broadening of the low dielectric, hydrophobic, region from the interlayer zone to the peripherals along the vertical direction, by excluding water molecules which penetrate into the bilayer [21] and extruding the dye, as is commonly found with other membrane-bound molecules [22,23]. This interpretation can account for the lower binding constant of the two dyes to PC/Chol liposomes as compared to PC liposomes, as well as to the change in their distribution.

The effect of DPPC and DMPC on the distribution of these porphyrins is generally similar to that of

TABLE I

Average dynamic quenching constants of HPD and PF-II fluorescence by spin-labeled stearic acids normalized to those of 16-NS, in PC liposomes with different additives

The concentration of HPD and PF-II was 5 μg/ml; the concentration of the lipid was 2 mg/ml.

Additive		5-NS		12-NS		16-NS	
		HPD	PF-II	HPD	PF-II	HPD	PF-II
None		0.62±0.02	0.46±0.01	1.01±0.05	0.78±0.01	1.00±0.03	1.00±0.01
20%	Chol	0.92±0.05	0.76±0.04	1.40±0.07	1.29±0.02	1.00±0.03	1.00±0.01
	DMPC	0.67±0.04	0.57±0.03	1.02±0.05	0.81±0.02	1.00±0.03	1.00±0.02
	DPPC	0.54±0.03	0.52±0.03	0.96±0.05	1.00±0.05	1.00±0.03	1.00±0.03
40%	Chol	1.98±0.04	0.97±0.06	1.94±0.04	0.94±0.02	1.00±0.02	1.00±0.01
	DMPC	0.47±0.02	0.44±0.02	1.02±0.04	0.95±0.02	1.00±0.02	1.00±0.01
	DPPC	1.10±0.07	0.62±0.03	1.09±0.08	0.94±0.05	1.00±0.05	1.00±0.03

cholesterol (with the exception of 40% DMPC), although less pronounced, nevertheless the same explanation can be applied here.

In summary, although PF-II has a somewhat smaller binding constant to the membrane than HPD, it intercalates deeper in the membrane than HPD, and in PC liposomes it is more localized in the membrane than HPD. Cholesterol lowers the overall binding constant of the two dyes to the membrane and it also has a strong modulating effect on the vertical membrane distribution of these two dyes.

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