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Effects of high electrolyte concentration on DPPC-multilayers: an ESR and DSC investigation

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Abstract: The effects of salinity on the lateral headgroup interactions of dipalmitoylphosphatidylcholine (DPPC) molecules in fully hydrated multilayers have been investigated by spin label electron spin resonance (ESR) spectroscopy and differential scanning calorimetry (DSC).

By increasing the NaCl concentration from 0 to 3 M in the multilayers' dispersion medium, the ESR measurements performed with the 5-stearic acid spin label and di-tert-butyl-nitroxide show an increase in the orientational degree of order of the lipid molecules, mainly in the gel phase, and a decrease of the membrane permeability. An upward shift from 31.5° to 36.5 °C and from 40.5° to 41.9 °C of the pre- and main DPPC phase transition temperatures, respectively, is observed with 5-SASL, while slightly higher values are detected with DTBN. Small effects are evident on the properties of the liquid crystalline phase of the DPPC multilayers.

The DSC measurements also reveal an upward shift of the pre- and main transition temperatures. The shifts, however, are more marked if compared to the ones observed with the ESR technique.

The findings suggest an increase in the packing density of the DPPC molecules in the multilayers in presence of high salt concentration. Dehydration of the DPPC interfacial region with a variation of the lateral electrostatic interactions between phospholipid polar heads trigger the phenomena observed.

Key words: Ionic strength – DPPC – multilayer – ESR –DSC

Introduction

The role of ions in determining the structure and function of biological membranes is well ascertained. Owing to this, the effects of ions on natural membranes as well as on their related model systems have been extensively investigated [1–8]. However, all studies performed with fluorescence, DSC, ESR, NMR, and x-ray diffraction techniques have been concerned with mono-, bi- and trivalent salts up to 1 M concentration in the dispersion medium. In addition, in these investigations membrane model systems made with lipids carrying one or more charges per polar head giving rise to a net charge density on the membrane surface were used. The results of these investigations are quite well understood in the limit of the Gouy–Chapman–Stern lipid double layer theory [9]. On the other hand, very few works have appeared on the effects of salt concentrations higher than 1 M and on the lateral headgroup interaction of neutral phospholipid molecules such as phosphatidylcholine and phosphatidyl-ethanolamine [6, 10–19].

In this respect, we have investigated the effects on the orientational degree of order and fluidity of DPPC multilayers when the NaCl concentration in the dispersion medium is increased up to 3 M.

The upward shift of the pre- and main phase transition temperatures of DPPC multilayers observed either with the ESR spectroscopy or with the DSC technique suggests an overall increase in the packing density of the phosphocholine molecules forming the multilayers. Such an increase is related to variations of the hydration state of the neutral polar head of the DPPC molecules and to reduction of the electrostatic interactions occurring by raising the salt concentration in the multilayers dispersion medium.

Materials and methods

Experimental

Synthetic 1,2-dipalmitoyl, sn-glycero-3-phosphocholine (DPPC) was used as obtained by Fluka. The spin labels 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5-SASL) and di-tert-butyl-nitroxide (DTBN) were Aldrich products stored at 4 °C in ethanol solution. NaCl of reagent grade was from C. Erba. Distilled water was used throughout.

5-SASL containing DPPC multilayers was prepared as follows with a spin label/DPPC concentration molar ratio of 1:100. The spin label was added to DPPC in ethanol and, after evaporating off the solvent with a stream of dry nitrogen, the lipid film was placed for 12 h under vacuum. The dried sample was then fully hydrated with a 10 mM phosphate buffer solution (PBS) at pH 8.0 containing 0, 1, 2 or 3 M NaCl to obtain a final DPPC concentration of 73 mg/ml (0.1 M). The dispersion was incubated for 1 h at 50 °C and vortexed [20]. The multilayer samples were sealed off in a glass capillary and incubated at 4 °C for 12 h before running ESR spectra [21].

The multilayers used for DTBN spin label investigations were prepared as described above. The hydration of the dried DPPC film was obtained with a 5×10^{-4} M solution of DTBN in PBS saline solution at pH 8.0.

The ESR spectra were recorded with a Bruker ER 200D-SRC spectrometer operating at 10 GHz equipped with the ER 4111 VT temperature control unit (accuracy ± 0.3 °C), the ESP 1600 Data System and the TE_{102} standard cavity.

All spectra were recorded with the following experimental set-up: 10 mW microwave power, 1 G_{pp} field modulation amplitude for 5-SASL containing DPPC multilayers and 0.25 G_{pp} for the DTBN ones, and displayed as first derivative in-phase absorption signal. 100 kHz modulation frequency was used for phase-sensitive detection.

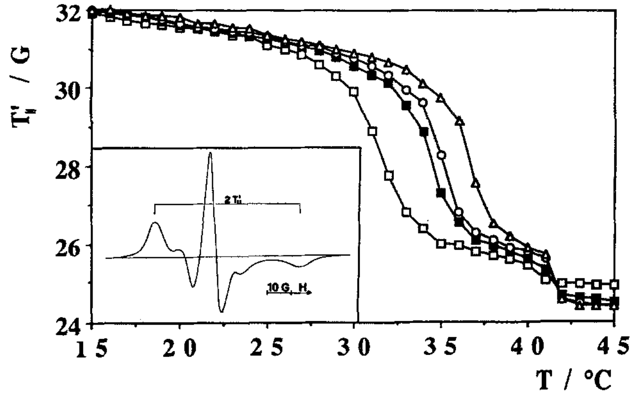

Fig. 1. $T'_{\parallel}$ values vs. temperature of the spin label 5-SASL in DPPC-multilayers dispersed in solutions with different salinity at pH 8.0: –□–, 0 M NaCl; –■–, 1 M NaCl; –○–, 2 M NaCl; –△–, 3 M NaCl. In the inset is shown a typical ESR spectrum of 5-SASL in the DPPC gel phase; the separation between the outer resonance lines is also indicated. The error on $T'_{\parallel}$ is less than the dimension of the symbols

DSC studies were performed on samples prepared as for the ESR measurements, but without spin labels with the help of a Setaram DSC-92 instrument, which uses indium as calibrant for temperature and energy. Samples (20–30 mg) were introduced into steel-cells and then sealed and heated to the initial temperature for 1 h.

Thermograms were digitized with an IBM PS2-60 computer which allowed the determination of the phase transition temperatures and enthalpies. These thermodynamic parameters were studied at the heating rates of 0.3 °C min^{-1}.

ESR measurements

The ESR spectrum of randomly oriented 5-SASL is reported in the inset of Fig. 1. From the spectrum the motionally averaged $T'_{\parallel}$ component of the nitrogen hyperfine T-tensor, related to the segmental lipids dynamics is evaluated. It gives a relative measure of the local order of the lipid bilayer: large $T'_{\parallel}$ values correspond to an ordered spin label environment while small values correspond to a more disordered one [22–24].

The spectrum in the inset of Fig. 2, instead, comes from the overlap of ESR spectra of DTBN executing rapid isotropic motion ($\tau_c < 10^{-9}$ s) either in PBS or in the fluid hydrophobic region of

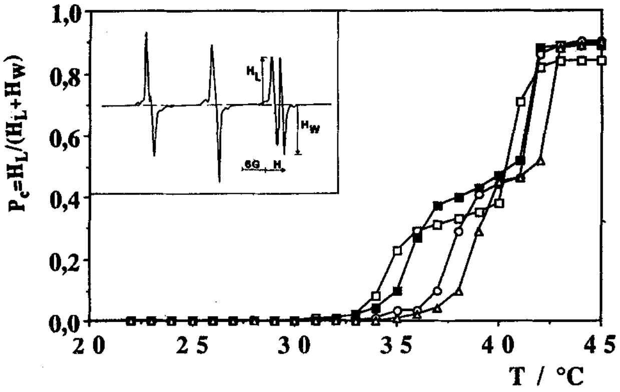

Fig. 2. P_c values vs. temperature of the spin probe DTBN in DPPC multilayers dispersed in solutions with salinity and pH as in Fig. 1. In the inset a typical ESR spectrum showing the partition of the spin label in the bulk solution and in the fluid phase of the multilayers is shown. The resonance line heights H_L and H_W are also indicated

DPPC multilayers. The measure of the label signal in the aqueous phase, H_W, and in the fluid lipid one, H_L, allows an estimation of the partition coefficient, $P_c = H_L/(H_L + H_W)$ (fraction of DTBN dissolved in the fluid hydrophobic region of the bilayer), which gives a measure of the membrane fluidity [24].

Transition cooperativity

According to the model of cooperative transitions in lipid bilayers [25, 26] it is assumed that the lipid molecules can essentially take up three states during the transition, namely, the ordered state s, the fluid state f, and the interfacial region between the ordered and fluid phases i. A measure of the mean size of the fluid regions at the center of the transition, i.e., when the same number of molecules is in the ordered and fluid state, expressed as the mean number of molecules in the fluid state per molecule in the interfacial one, can be given by:

$$\langle \nu_f \rangle_{T_m} = 1 + 1/\sqrt{\sigma}\,, \tag{1}$$

where $1/\sqrt{\sigma}$ is referred to as the size of the cooperative unit and σ, the cooperativity parameter, is the statistical weight of a molecule in the intermediate state with respect to a molecule in the fluid one:

$$\sigma = \exp(-F_i/RT)\,, \tag{2}$$

with F_i the additional free energy of a molecule in that state with respect to one in the fluid state; R and T have the usual meaning.

Cooperativity arises because F_i tends to decrease the length of the mismatch interfacial regions; in fact, it is energetically favorable for a molecule to be surrounded by molecules in the same state as itself [26].

The cooperativity parameter is an index of the cooperativity of the transition. The smaller σ, the greater is the degree of cooperativity, which can be expressed in terms of the transition temperature T_m, the transition width ΔT_m and of the molar enthalpy, ΔH_m, so that $\langle \nu_f \rangle_{T_m}$ in Eq. (1) takes the expression [26]:

$$\langle \nu_f \rangle_{T_m} = 1 + 2R(T_m)^2/\Delta T_m|\Delta H_m| \tag{3}$$

Results

From the ESR spectra of 5-SASL located into the DPPC multilayers the separation between the outer resonance extreme, $2T'_{\parallel}$ (inset of Fig. 1), is measured while from the DTBN ones (inset of Fig. 2) the partition coefficient, P_c, is calculated.

The plots of $T'_{\parallel}$-values vs. temperature in the presence of different salt content in the dispersion medium are given in Fig. 1. As can be seen, in absence of NaCl the pre- and main phase transitions of DPPC, as determined with the spin label 5-SASL, occur at $T_p = 31.5\,^\circ\mathrm{C}$ and $T_m = 40.5\,^\circ\mathrm{C}$, respectively. The pre-transition temperature of the DPPC multilayers shifts to 36.5 °C and the main one to about 41.9 °C in the presence of up to 3 M NaCl (Table 1). The transition width, ΔT_m, changes from 3.9 °C in absence of salt to 2.45 °C in the presence of 3 M NaCl while, the transition height does not significantly change with salinity.

A similar behavior is observed using the spin probe DTBN. In fact, following the partition of this label in the bilayers, an increase of the transition temperatures is also observed (Fig. 2 and Table 1). The shift is from 34.3° to 38.5 °C and from 40.6° to 42.5 °C for the pre- and main phase transition, respectively. A very small increase in partition is observed in the liquid crystalline phase of DPPC multilayers with salt concentration increase.

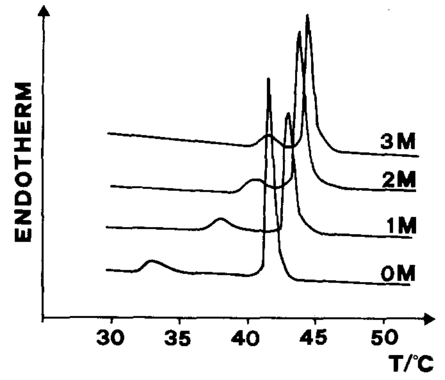

Fig. 3. DSC thermograms of DPPC multilayers dispersed at pH 8.0 in solutions with different salinity

DSC experiments show an increase of both transition temperatures, too (Fig. 3 and Table 1). Moreover, the pre-transition results shifted more upward than the main one. In fact, with 3 M NaCl in the multilayers dispersion medium the pre-transition reaches the high value of 41.5 °C. The shape of both transitions remains quite unchanged as well as their enthalpies with NaCl concentration increase.

Calculation of the main number of fluid molecules per interfacial molecule at the mid-point of the main phase transition, using the calorimetric and the DTBN partition data, shows that the mean size of the cooperative unit increases from about 109 fluid molecules per interfacial molecule to 149 when the salt concentration is changed from 0 to 3 M, respectively (Table 1).

Discussion

The ESR spectroscopy with the spin probe DTBN and the spin label 5-SASL together with the DSC technique have been used to investigate the effects of very high ionic strength on the structure and fluidity of DPPC multilamellae.

The partition of DTBN allows to get insight on the formation of the fluid phase with temperature and salinity increase, while the spin label 5-SASL gives information on the orientational degree of order of the DPPC molecules in the bilayers. Differential scanning calorimetry gives information on the temperature, enthalpy, and cooperativity of the phase transitions occurring in the DPPC multilayers.

In absence of the 1:1 electrolyte the pre-, $L_{\beta'} \rightarrow P_{\beta'}$, and main-, $P_{\beta'} \rightarrow L_{\alpha}$, DPPC phase transitions temperatures found either with ESR spectroscopy or the DSC method (Table 1) are in agreement with literature data on the same mesophase and experimental methods of investigation used. In fact, the T_m-values found by us with both ESR methods compare fairly well with the value of 40.17 °C reported in literature [6, 9, 20, 28] as well as our DSC T_m-value with that of 41.3 °C given in refs. [11, 12, 28].

When the NaCl concentration in the dispersion medium is raised to 3 M, the plots of $T'_{\parallel}$ vs. temperature in Fig. 1 and the T_p- and T_m-values in Table 1 suggest that salinity induces a more tightly packed multilayer molecular structure. In fact, at each temperature in the gel phases higher $T'_{\parallel}$ values, indicating a more anisotropic motion of 5-SASL, are found together with a slight

Table 1. Pre- and main phase transition temperatures deduced from ESR and DSC measurements for DPPC multilayers dispersed in media containing NaCl concentration together with the pre- and main phase transition enthalpies and cooperativity parameter, ν

	ESR				DSC				
[NaCl]	5-SASL		DTBN						
Mol.	T_p^*)	T_m	T_p	T_m	T_p	T_m	ΔH_p	ΔH_m	ν
0	31.5	40.5	34.3	40.6	33.6	41.4	1.8	7.8	109
1	34.4	41.5	35.4	41.4	38.0	43.0	1.7	7.8	130
2	34.8	41.5	37.6	41.6	40.5	43.8	1.8	7.9	139
3	36.5	41.9	38.5	42.5	41.4	44.5	1.6	7.9	149

*) T_p and T_m are in °C, whereas the enthalpies in kcal/mol. The accuracy on T_p and T_m is ± 0.1 °C, on both enthalpy values of ± 0.2 kcal/mol and on the cooperativity parameter of ± 5

increase of the cooperativity of the pre-transition. A small upward shift of T_m is observed, too.

Similar effects are sensed by DTBN (Fig. 2). The partition of this spin probe, which measures the formation of the fluid phase of DPPC, reduces with salinity and the T_p and T_m temperatures result increased.

Also, the DSC thermograms in Fig. 3 show an upward shift of T_p and T_m. However, the effects of salinity, as detected by this technique, are more marked: the pretransition temperature results upward shifted by 7.8 °C and the main one by 3.1 °C in presence of 3 M NaCl if compared to (Table 1). At the present there is not an explanation of why the different methods used to determine T_p and T_m give slightly different results. It could likely depend on the fact that the three techniques look at different phenomena, i.e., orientational order, partition and rotational isomerisms formation along the phosphocholine fragment of the DPPC molecules.

In every case, the ESR and DSC data clearly suggest that the packing density of the DPPC molecules in the multilayers increases in presence of salt. In other words, by increasing the salt concentration in the medium a reduction of the free volumes existing either in the polar zone or in the hydrophobic region of the gel phases of the DPPC multilayers in absence of salt takes place.

Two mechanisms could be put forward to explain the observed packing density increase: first, ions induce dehydration of the interfacial region of the bilayers, i.e., dehydration of the P^-N^+ dipole on the polar head of the DPPC molecules; the dehydration increases with ionic strength increase. Second, ions modify to some extent the structure of water around the P^-N^+ dipoles and screen the coulombic interactions between them. Chapman and co-workers [11] have reported the effects of varying water contents on the transition parameters of DPPC multilayers. They found that with the increase of the weight fraction of lipids, c = wt. lipid/(wt. lipid + wt. water), the transition temperature of both endotherms shifts upward and for $c > 0.8$ the two endotherms start to merge. Cunningham et al. have already reported the increase of T_p and T_m in presence of 1 M NaCl in solution [12]. In the present case, working at the highest concentration of 3 M salt, we have not observed the endotherms merging process seen by the former authors so that the dehydration of the DPPC polar heads should be excluded. Our findings agree with those of Cunningham as both have been obtained under conditions of full hydration of the DPPC multilayers. The reduction of both the angular cone of the wobbling motion of 5-SASL and of the DTBN partition, hence the packing density increase of the lipid matrix, is related to the second kind of interaction occurring at the lipid bilayer-bulk solution interface. The Cl^- ions are effective in screening the positive charge on the $-N^+(CH_3)_3$ groups exposed to water and in modifying to some extent the water structure around them. In this way, the polar character of the interfacial zone is reduced, giving rise to an increase of hydrophobic attractive van der Waals forces between phosphocholines so that the free volume between the DPPC polar heads decreases and, consequently, the lateral molecular separation decreases, too. The hydrated Na^+ ions cannot easily reach the $-PO^-$ zone to modify to some extent the existing hydrogen bonds network as they do when the DPPC molecules form vesicles, i.e., a mesophase with quite different radius of curvature compared to multilamellae ($R \cong 100$ Å and $R = \infty$, respectively) [8]. In this case, in fact, opposite effects have been observed by us with increasing the NaCl concentration up to 3 M: the $L_{\beta'} \rightarrow P_{\beta'}$ transition disappears and the temperature of the main one is downward shifted.

The new equilibrium between the van der Waals, electrostatic and hydration forces in presence of salt has the final effect to increase the packing density of the DPPC molecules in the multilayers.

The above picture is also supported from the calculated values of the mean number of molecules of the cooperative unit at the mid-point of the transition obtained using the enthalpy of the $P_{\beta'} \rightarrow L_\alpha$ main phase transition, the T_m and ΔT_m values determined by the DTBN partition in the fluid phase of lipids [25]. In fact, that the size of the cooperative unit increases from 109 (this value results lower than that reported in ref. [25] is likely due to a different purity of the DPPC used [27]) to 149 molecules for interfacial molecule with salt concentration is related to the increased energetic stability of the fluid phase. Indeed, from Eqs. (1) and (2) it follows that:

$$\langle v_f \rangle_{T_m} = 1 + \exp(F_i/2RT)\,, \tag{4}$$

where, as already mentioned, F_i is the lowering in the mean free energy of a molecule in passing from the interfacial to the fluid state. Thus, the increase in the cooperativity unit is indicative of higher intermolecular bindings, giving a negative contribution to the mean free energy per molecule.

That the enthalpy of the main phase transition, i.e., of the melting of the phospholipids acyl chains, remains quite constant within the DSC experimental error also supports the hypotheses that the ionic strength-induced modifications essentially concerns the interfacial region of the DPPC multilayers. In fact, the increase in F_i, i.e., in the stabilization of the fluid phase, with salt concentration, comes from the interfacial zone likely due to the increase of the attractive van der Waals interaction occurring therein.

Conclusions

The data reported above suggest that the presence of NaCl up to 3 M in the dispersion medium causes an increase in packing of the DPPC molecules in the multilayers. The increased degree of molecular order, the reduced bilayer permeability and the enhanced main phase transition cooperativity support this conclusion.

The increased packing density of phospholipids is not due to dehydration of the interfacial region of the bilayers, but rather essentially to screening effects by Cl^- ions of the $-N^+(CH_3)_3$ groups, with consequent reduction of the electrostatic repulsion between polar heads and enhancement of the attractive van der Waals interaction in the interfacial zone. This picture is also supported by the increase of interfacial energy, F_i, as deduced from the statistical analysis of the cooperativity data.

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