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Structural and dynamical surface properties of phosphatidylethanolamine containing membranes

A.M. Bouchet^a, M.A. Frías^a, F. Lairion^a, F. Martini^a, H. Almaleck^a, G. Gordillo^b, E.A. Disalvo^{a,*}

^a Laboratorio de Fisicoquímica de Membranas Lipídicas, Cátedra de Química General e Inorgánica, Departamento de Química Analítica y Fisicoquímica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 2° piso (1113), Buenos Aires, Argentina

^b Departamento de Química Inorgánica, Analítica y Química Física, Facultad de Ciencias Exactas y Naturales, INQUIMAE, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2 (1428), Buenos Aires, Argentina

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ABSTRACT

The hydration of solid dimyristoylphosphatidylethanolamine (DMPE) produces a negligible shift in the asymmetric stretching frequency of the phosphate groups in contrast to dimyristoylphosphatidylcholine (DMPC). This suggests that the hydration of DMPE is not a consequence of the disruption of the solid lattice of the phosphate groups as occurs in DMPC. The strong lateral interactions between NH_3 and PO_2^- groups present in the solid PEs remain when the lipids are fully hydrated and seem to be a limiting factor for the hydration of the phosphate group hindering the reorientation of the polar heads. The lower mobility is reflected in a higher energy to translocate the phosphoethanolamine (P–N) dipoles in an electrical field. This energy is decreased in the presence of increasing ratios of PCs of saturated chains in phosphoethanolamine monolayer. The association of PC and PE in the membrane affecting the reorientation of the P–N groups is dependent of the chain–chain interaction. The dipole potentials of PCs and PEs mixtures show different behaviors according to the saturation of the acyl chain. This was correlated with the area in monolayers and the hydration of the P–N groups. In spite of the low hydration, DMPE is still able to adsorb fully hydrated proteins, although in a lower rate than DMPC at the same surface pressure. This indicates that PE interfaces possess an excess of surface free energy to drive protein interaction. The relation of this free energy with the low water content is discussed.

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1. Introduction

Phosphatidylethanolamine (PE) is one of the most abundant lipids in eukaryotic cell membranes unevenly distributed between the inner and the outer leaflets of the bilayer. The higher ratio of PEs in the membrane leaflet facing the inner media in comparison to the external one has called the attention to the topological properties of those surfaces with the expectation that they may be a key to functional roles of this lipid [1–6]. Studies with pure natural and synthetic lipids have shown a complex, nonlamellar lipid polymorphism in unsaturated phosphatidylethanolamines [7–13]. ^{31}P -NMR, EM, AFM and X-ray diffractions have provided information on the topological properties of pure PE and in mixtures with phosphatidylcholine (PC) and cholesterol [14–16]. Unsaturated PEs such as dioleoylphosphatidylethanolamine (DOPE) has received considerable interest by its preference to form non bilayer aggregates, adopting the inverse hexagonal phase (H_{II}) [7]. The stabilization of PE in the hexagonal phase has been related to the conical shape of the molecule, in contrast with the cylindrical one of the PCs, which stabilizes in bilayers [7–11]. These conformational changes could reflect the

occurrence of considerable structural reorientations within the polar head group of the molecules. This may be important to interpret mechanisms of the membrane permeability, fusion behavior and in particular the interaction of proteins present in the water media [17,18]. In this regard, a number of intrinsic and extrinsic factors may play a role in the stabilization of PEs in water, among them temperature and water. In this direction, FTIR spectroscopy studies of PEs have provided information about the hydration of the polar groups facing the water phase. As a result, strong variations in the spectral parameters were found when hydrated and dehydrated lipids were compared [8]. The frequency of the asymmetric PO_2^- stretching vibration depends on the nature of the polar head group. It varies from 1230 cm^{-1} in fully hydrated DMPC to 1217 cm^{-1} in DMPE. The low frequency observed in DMPE has been explained by intra and intermolecular hydrogen bonding of NH_3 with the PO_2^- [19,20].

Considering that the size of the polar head group could be related to the amount of water immobilized around it, the interaction with water would be different for PE than for PC due to the higher positive charge density of ethanolamine in comparison to cholines [7,9,11].

Hydration features of DOPE that stabilize in the hexagonal H_{II} phase have been contrasted with the behavior of saturated PEs, which stabilizes in lamellar forms. Although both lipids absorb much less water than PCs, DOPE is somewhat higher hydrated than DPPE [19].

* Corresponding author.

E-mail address: eadisal@yahoo.com.ar (E.A. Disalvo).

The L_{α} phases of PE acyl and alkyl derivatives have limiting hydrations at temperatures 5 °C above the gel–fluid transition, of 28.5 and 17.4 wt.%, corresponding respectively to 12 and 9.5 water per lipid. This contrasts with the far greater hydration of 23–29 water per lipid observed for the phosphatidylcholines in the fluid bilayer phase [21,22]. For egg phosphatidylethanolamine, the limiting hydrations in the L_{α} phase, obtained by X-ray diffraction, is estimated in around 40% [9,23]. The maximum hydration in the inverted hexagonal H_{II} phase is 33.8 and 28 wt.% for acyl and alkyl PEs corresponding respectively to 15.5 and 17.5 water per lipid. This denotes that changes in the carbonyl regions do not affect significantly the hydration of the lipid molecules.

Membranes enriched in unsaturated PEs tend to abandon the bilayer conformation [24]. The possible mechanism requires an understanding of the interaction of the PE molecule with its neighbors in the membrane phase and with water molecules at the interphase. In this regard, it has been proposed that in mixtures with PCs, PE determines the propensity of the membrane to abandon the bilayer structure [10]. This indicates that the dilution of PEs affects the lateral interaction. The triggering of the lamellar–non lamellar transition has been explained as a consequence of the lateral migration of PE molecules due to the strong lateral interactions. These PE properties were ascribed to the strong formation of H-bonds between the phosphate group and the amine group [8,20,23]. Thus, different lateral interactions should be expected if pure PE is in the lamellar gel or fluid phase in comparison to the hexagonal and when it is mixed with other lipids such as PCs. However, details of the interaction of PE–PE and PE–water at the head group level are scarce or not completely analyzed.

The presence of PE would affect the packing, the curvature and the polarization of the surface. More importantly, water and polar head group arrangements derived by the lateral interaction would determine the free energy of the interphase necessary for the adsorption of additives present in the aqueous environment. Among these, it is of particular interest to understand the behavior of aqueous soluble proteins such as proteases previously studied in inverted micelles and monolayers [25–27].

Generally speaking protein interaction can be followed by the increase of the surface pressure of a monolayer spread at the air–water interface. The influence of the degree of hydration of the lipid interphase as a driving force has not been yet reported. As PEs are hard to hydrate the head–head group interaction would hinder the protein interaction.

For this reason, this work attempts to analyze the surface properties of PE-containing membranes in terms of the lipid–lipid and lipid–water interactions of different phosphoethanolamines and their modifications in mixtures with phosphocholine. The structural studies in the phosphate region were done by FTIR spectrometry. The PO asymmetric stretching (ν_a PO) mode has been taken as an indicator for hydration and dehydration of PC dispersions [28,29]. The analysis of the PO frequencies can provide information of the H-bond of the group between lipids or between lipids and water. The infrared information was correlated with the water–lipid ratio, the area per lipid, the dipole potential and the dynamical properties for different PC–PE mixtures subjected to an electrical field. This information was used to interpret the interaction of a soluble protein with DMPE and DMPC monolayers at different surface pressures.

2. Materials and methods

2.1. Lipids and chemicals

Dimyristoylphosphatidylcholine (DMPC), 1,2-di-*O*-tetradecyl-*sn*-glycero-3-phosphocholine [D(ether)PC], dimyristoylphosphatidylethanolamine (DMPE), 1,2-di-*O*-tetradecyl-*sn*-glycero-3-phosphoethanolamine[D(ether)PE], phosphatidylethanolamine and phosphatidylcholine derived from egg yolk (EggPE and EggPC), 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE), 1,2-Dio-

leoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL) and used as received. The purity of lipids was checked by thin layer chromatography using chloroform:methanol:acetic acid:water mixture as running solvent, by FTIR spectra of lyophilized samples and by differential scanning calorimetry.

2.2. Water per lipid molecule in inverted micelles

Egg phosphatidylcholine (EggPC) and egg phosphatidylethanolamine (EggPE) in different molar fractions were dissolved in chloroform and titrated with water at 25 °C. After each addition a brief sonication was imposed to achieve a transparent solution. The final point of the titration was taken as that in which the addition of an excess of water promoted the appearance of turbidity in the sample, which remains with further sonication. These final points indicate the water molecules needed, for each lipid or the mixtures, to achieve reversed micelles [30].

2.3. FTIR measurements

FTIR Nicolet™ 380 spectrophotometer, provided with a DTGS detector, was used. The resolution was 1 cm^{-1} . Values corresponding to non hydrated lipids were measured in KBr at a relative humidity (RH) of 20% in the ambient. The water content of the lipid films was estimated by means of the spectral parameter defined as the ratio of the integral absorbance of the $\nu_{1,3}$ OH band of water centered near 3400 cm^{-1} and of the integral absorbance of the C–H stretching region (3000–2750 cm^{-1}) after baseline correction [31]. The value obtained in this condition was coincident with that reported by Pohle et al. [32] and was taken as the value of reference for the measurements made in fully hydrated lipids.

Lipids equilibrated at this RH were then dispersed in H₂O and sealed in a cell with AgCl windows at the temperature above the corresponding phase transition indicated in each case. A total of 64 scans were done in each condition and the spectra were analyzed using the mathematical software provided by the instrument. A number of different samples (no less than three) were processed in order to obtain a standard deviation below the resolution of the equipment.

2.4. Surface properties of lipid monolayers

Monolayers were formed on an air–water interface by spreading chloroform solutions of the different pure lipids and its mixtures on an aqueous subphase (KCl 1 mM) [33,34]. The formation of monolayers on the surface of aqueous solutions was monitored by measurements of the surface pressure of the different lipid monolayers in a Kibron μ Trough S equipment, at constant area and temperature. Aliquots of a chloroform solution of lipids were spread on a clean surface of water and left to reach constant surface pressures, until no changes were observed with further additions of lipids (saturation). In this condition, lipids in excess form aggregates in the subphase and the thermodynamic and interfacial properties are comparable with those of a bilayer [33,34]. With this procedure, the lipids are stabilized spontaneously according to the aqueous solution properties and temperature, without forcing the lipids by the lateral pressure. The areas per lipid were calculated from curves of monolayer surface pressure, expressed in mN/m vs. nmoles of lipid added to a trough of known constant area. The plateau of saturation was the best straight line obtained with, at least, three points. For details in the procedure see Lairion et al. [35].

2.5. Dipole potential

Dipole potential (Ψ_D) was determined when the surface pressure of the monolayers reached the saturation point described above, c.a. a

Table 1

Shift in the asymmetric stretching mode of the phosphate groups and of trapped water in mixtures of PC and PE.

Lipid	$\nu_a \text{PO}_2^- / \text{cm}^{-1}$	Water per lipid
EggPC	1228.7	14
EggPC/EggPE [1:1]	1223.5	7–8
EggPE	1218.6	2–4

surface pressure between 44 to 49 mN/m, depending on the lipids used.

The values of the interfacial potential were determined through a circuit of high impedance, connecting an ionizing electrode above the monolayer and a reference electrode in the aqueous subphase, using the following expression:

$$V_{\text{surf}} = V_{\text{Ag/AgCl}} - V_{\text{grd}}$$

where V_{surf} is the potential of the clean aqueous surface, measured as the potential difference between an Ag/AgCl reference electrode, immersed in the solution underneath the surface ($V_{\text{Ag/AgCl}}$) and the grid displaced c.a. 2 mm above the surface (V_{grd}). This grid is the sensor of the ionizing electrode that emits alpha particles in order to achieve the electrical connection across the air. The dipole potential of the monolayer (ψ_D) was evaluated as:

$$\psi_D = V_{\text{lip}} - V_{\text{surf}}$$

where V_{surf} is the potential of the clean surface (without lipids) described above and V_{lip} is the potential measured with the same set-up after the lipid monolayer was formed on the air–water interphase. The values of monolayer potentials were taken within an experimental error of ± 20 mV. Temperature was set at the values indicated in each assay (mostly 18 and 28 °C) and measured with a calibrated thermocouple immersed in the subphase and maintained within ± 0.5 °C.

2.6. Cyclic voltammetry

Monolayers of different PC/PE ratios formed on an air–water interface of a buffer solutions $\text{KH}_2\text{PO}_4 / \text{K}_2\text{HPO}_4$ (pH 7.2) at the saturation pressure described above, were transferred to the surface of an Hg drop by passing the drop across the lipid monolayer. For details see Almaleck et al., [36] and references therein.

Potential cyclic linear sweeps between +0.1 V and –1.5 V against Ag|AgCl, placed in the solution, were applied to interfaces Hg|phospholipid|solution at a sweep speed of 1 V/s. All the experiments were carried out at temperatures below the phase transition.

2.7. Surface pressure changes induced by protein adsorption

The interaction of an aqueous protease, Rennet from *Mucor miehei*, with different lipid monolayers has been studied as a model system considering that the activity and stability of aqueous soluble enzymes, such as this protease and others like trypsin and α -chymotrypsin, increase drastically with the decrease in the total water content of micelles lumen [26,27]. The changes of the surface pressure of monolayers induced by Rennet protease were measured in a Kibron μ Trough S equipment, at constant temperature (25 ± 0.5 °C). The surface of an aqueous solution contained in a Teflon trough of fixed area was exhaustively cleaned. Then, a chloroform or chloroform:methanol (9:1) solution of phospholipids was spread on this surface, to reach surface pressures between 16 and 42 mN/m. In this range, the surface pressure–area isotherms of DMPC and DMPE show that the lipids are forming monolayers [33,34,37]. At each chosen surface pressure, a protein solution volume was injected in the subphase to accomplish a concentration of 1.57 μ M and the changes on the surface pressure were followed during time to reach a constant value. This

constant value was taken as the value for the protein equilibrated in the interphase and its difference with the basal value without protein was plotted as a function of the initial surface pressure. The same procedure was followed for all monolayer compositions. Surface pressure and increases of surface pressure at constant surface area were automatically recorded. The spreading of this fixed quantity of protein on a clean aqueous surface, as well as the injection of it into water, results in a surface pressure of 13 mN/m, which is below the surface pressure of the studied lipid monolayers.

3. Results

The titration with water of chloroform solutions of different EggPC/PE ratios produces the entrapment of different amounts of water in reversed micelles (see Materials and methods). Data in Table 1 indicate that pure PC incorporates up to 14 water molecules per lipid, while pure PE incorporates between 2 and 4. FTIR measurements to determine the hydration of the lipids following the shift of the asymmetric stretching band of the PO groups denote a decrease in the frequency for lower numbers of water molecules per lipid with the increase of PE. This is contrary to the observation in PCs in which the decrease in the frequency corresponds to an increase in hydration of the PO_2^- group as a consequence of the weakening of the PO bond due to H bond formation with water [28,29].

According to previous results, EggPE gel– L_α transition is, at pH 7.0, at 10–11 °C and the L_α – H_{II} transition at 28–31 °C, as measured by differential scanning calorimetry and ESR [38,39]. In addition, the gel– L_α transition in DMPE is around 50 °C [40–42]. With these data we analyze FTIR results of the lipids at 18 °C in different phases (Table 2). The difference in frequency between the solid and the hydrated state for DMPE is negligible in comparison to the difference found in DMPCs. In addition, the value for non hydrated DMPE is considerably lower than that corresponding to DMPC dehydrated in vacuum, as reported results, and also of the fully hydrated DMPC in the gel state. The absence of carbonyl groups in the alkyl PE does not affect the frequency shift. In contrast, the absence of carbonyl groups in PCs, produces a larger area per lipid with a concomitant shift of the asymmetric stretching phosphate band to lower frequencies [35,43,44].

In conclusion, the interaction with water does not change significantly the phosphate frequency in the gel PEs. However, higher differences are observed for the non hydrated and hydrated forms of unsaturated PEs (EggPE) and between them for lipids stabilized in hexagonal form (DOPE). In the same table (Table 2), data of PO frequency for the different lipids are compared with the area per lipid reported in literature in different phases. The data of frequency shifts does not follow a correlation with the area reported in literature for the different phase states of the PEs. Congruently, DMPE in the gel state at 18 °C shows the smaller shift for a smaller area. The frequency shift for EggPE in the fluid lamellar phase is lower than for the gel state for a higher area. However, the higher value of PO_2^- vibration shift corresponds to DOPE in the hexagonal phase (H_{II}) in which the area is around 47.2 \AA^2 . The reason for this could be that the phosphate shifts,

Table 2

Changes in $\nu_a \text{PO}_2^-$ band of PEs and PCs upon hydration of the solid and the corresponding area per lipid in aqueous dispersions.

	Solid/ cm^{-1}	Fully hydrated/ cm^{-1}	$\Delta\nu_a \text{PO}_2^- / \text{cm}^{-1}$	Area/lipid (\AA^2)
DMPC	1251.4	1229.3 (gel)	22.1	56.0 \pm 4.7
DOPE	1234.8	1223.0 (H_{II})	11.8	47.2*
EggPE	1227.0	1218.6 (fluid)	8.4	74.1 \pm 4.4
DMPE	1224.8	1222.7 (gel)	2.1	40.7–52 **
D(ether)PE	1221.1	1218.7 (gel)	2.4	ND

* Taken from refs. [15] and [59].

** Taken from refs. [8] and [16].

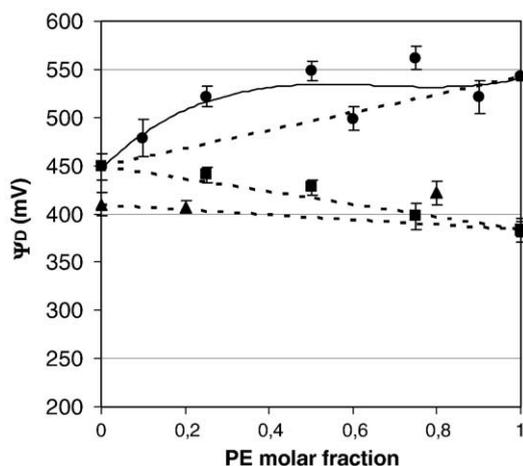


Fig. 1. Dipole potential as a function of the molar ratio of different phosphatidylcholine and phosphoethanolamine mixtures. DMPC: DMPE (●); DMPC: EggPE (■); EggPC: EggPE (▲). Determinations were done in monolayers formed on 1 mM KCl at 22 °C as described in Materials and methods. Lines are drawn to indicate the ideal behavior. The errors of the determinations are those described in Materials and methods.

taken as a criterion of hydration, and its correlation with area values are valid for similar topological states of the different PEs.

A drastic difference in the topology of the lipid interphase is found when bilayers and monolayers are compared. In the first system, corrugated and local curvatures structures are present which has not been shown in monolayers. Thus parallel to FTIR determinations, the properties of PE interfaces were determined in monolayers and compared with the behavior of PCs, in similar conditions.

The area values obtained in monolayers are coincident for the values reported in literature except that corresponding to DMPE in the liquid condensed state. In this case, the values of monolayer are 56.4 Å², which is considerably higher than that found in bilayers. It appears that topological arrangements in PE such as the presence of corrugated phases in the gel state, would determine different area values as it was also observed between EggPE and DOPE which show different topological arrangements.

The analysis of the dipole potential in monolayers mixtures of PCs and PEs is shown in Fig. 1. The dipole potential of the monolayers increases with the ratio of DMPE when both lipids are maintained in the liquid condensed state at 18 °C. The value of pure DMPE is much higher than that for DMPC. In contrast, the addition of fluid PE promotes a decrease in the dipole potential of DMPC at the same temperature to reach a value for pure EggPE lower than that for DMPC.

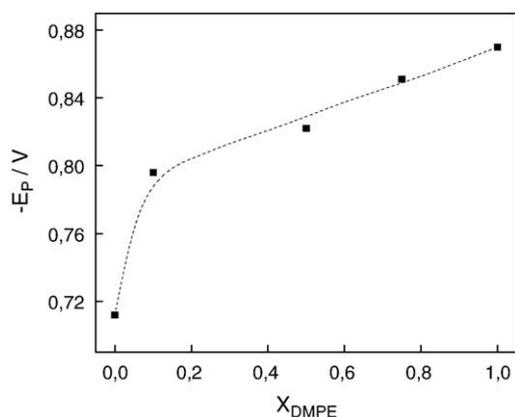


Fig. 2. Potential peak variation with the DMPC/DMPE mixtures. The potential peaks were obtained from voltammograms performed on monolayers spread on a Hg drop at 22 °C at a surface pressure of c.a. 47 mN/m.

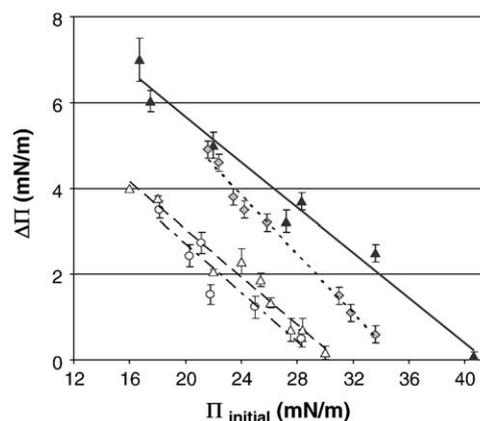


Fig. 3. Changes in the surface pressure as a function of the initial surface pressure of different monolayers: DMPC (▲); DMPE (△); D(ether)PE (○) and DMPE:DMPC 1:1 (●). The increments of surface pressure are equilibrium values obtained in monolayers spread at differential surface pressure on 1 mM KCl at 25 °C.

Fluid PE has the same dipole potential than fluid PC and no changes are observed in the mixtures.

The analysis by cyclic voltammetry gives another insight of the particular properties of the DMPC/DMPE monolayers. The presence of saturated PE in saturated PC mixtures has a noticeable effect on the dynamical properties of the membrane interphase groups when an electrical field is applied across a monolayer. The central peak in the voltammogram is displaced to more negative values with the increase of the fraction of DMPE in DMPC (Fig. 2). The addition of increasing ratio of DMPE to DMPC (in the liquid condensed state) increases the energy required to reorganize the monolayer. In this condition, the mixtures of DMPE with DMPC show also a positive deviation of the potential peak from the ideal behavior as observed in dipole potential (Fig. 1).

The different dynamical properties of the lipid mixtures observed by cyclic voltammetry can be related to the interfacial free energy at different surface pressures. The energy involved in these transitions is related to the surface free energy. As it is known, this energy is related with the surface tension that can be reduced by the concentration excess produced by adsorbates at the interface, according to the Gibbs adsorption isotherm. A decrease in the surface tension can be obtained by the adsorption of lipids or the adsorption of protein onto the interface. The difference in the surface tension of the water interphase with and without lipids is denoted by the surface pressure at a given area value. The lower the surface tension, the lower is the free energy excess triggering the interaction of adsorbable solutes. Congruent with this picture, the surface pressure changes of PC and PE monolayers due to the interaction of an aspartyl protease decrease for higher surface pressures. As the purpose is to put into relevance the participation of water in the different arrangements conferred by the lipids, this protein was chosen because it is not a membrane protein and has been reported as a fully aqueous soluble one [25]. As observed in Fig. 3 and Table 3, the cut off for DMPE is lower than that

Table 3

Dipole potential, area per lipid and surface pressure at saturation for PC and PE and 1:1 mixtures in monolayers in the condensed state.

	DMPC gel	DMPC/DMPE	DMPE gel
n_w	14	7	2
Area (Å ²)	56.3 ± 4.7	44.1 ± 4.7	56.1 ± 4.7
Saturation surface pressure (mN/m)	47.8	49.0	43.6
Dipole potential (mV)	450	540	540
Cut off surface pressure (mN/m)	41.5*	35.0	31.0
ν_a PO ₂ ⁻ /cm ⁻¹	1229.3	1228.4	1222.7

* Martini M.F., Disalvo E. A.; ref. [25].

for DMPC monolayers. However, in a 1:1 mixture the cut off decreases to a value intermediate to the pure lipids. This curve tends to that corresponding to PCs at low surface pressures. In the same figure (Fig. 3), data corresponding to D(ether)PE indicates that the surface pressure is not affected by the presence of carbonyl groups. Therefore, penetration of the protein is decreased but not hindered in monolayers with PEs.

4. Discussion

In the function of the membrane in its interaction with proteins, the presence of specific lipids such as PEs is required. In this case, membrane can provide a hydrophobic environment or specific sites at the surface. However, molecular mechanisms of the interaction of fully aqueous soluble proteins with lipid interfaces are not known due to a lack of knowledge of the physicochemical properties of the interphase region. Apparently, such a function may depend in a great extent on the hydrogen bonding ability of the head groups [18].

The different hydration of PEs in comparison to PCs could provide, in addition to the topological changes, different physicochemical characteristics of the interface in the lamellar phase related to the number of water molecules per lipid content and its thermodynamical activity. These features determine dynamic and structural properties such the area per lipid, the interfacial tension, the relaxation of the groups under an electrical field and the dipole potential.

In this context, the properties of PEs with different acyl chains were analyzed in comparison with PCs. The addition of water to DMPC promotes a higher change in the phosphate frequency in comparison to DMPE (Table 2). As described in Materials and methods, we obtain a value of 1251.4 cm^{-1} for non hydrated DMPC, which is comparable to those reported previously [19,31,32]. This suggests that in fully hydrated samples the phosphate in the PC gains additional degrees of freedom interacting with water. DMPE samples processed in the same way show a much lower difference between the solid and the fully hydrated lipids. The comparison of the phosphate band frequency of solid samples with fully hydrated DMPE and D(ether)PE (Table 2) suggests that the interaction of the phosphate is similar in both conditions. In this case, the value for anhydrous PE reported in literature is $1231\text{--}1234\text{ cm}^{-1}$ [32]. The solid samples of PE equilibrated with 20% RH are similar to the values in the anhydrous sample and show negligible changes in the phosphate frequency when it is fully hydrated (Table 2). This suggests that the excess of water does not affect the degrees of freedom of the head group as it was the case in PCs. This is congruent with the possibility that the P–N interaction still remains in fully hydrated PEs, hindering the hydration. This can be understood by assuming that the interaction of the phosphate group in the non-hydrated PE remains when it is hydrated or, that beyond the first water of adsorption, no more water can be intercalated. This behavior is congruent with the existence of a dense hydrogen bonding network formed between the phosphate and the NH_3^+ unities of neighbor PE molecules in the solid that water is not able to disrupt.

It is important to notice that this effect occurs when saturated PCs and PEs are compared. The shifts in frequency are more pronounced in the case of PE with unsaturated chains, suggesting that the hydration in the head group moiety depends on the packing of the acyl chains. The inspection of data in Table 2 shows that the frequency shift is higher for larger areas when lipids in the lamellar phase are considered (e.g. DMPE and EggPE at the same temperature). This correlation is similar to that found for PCs, and therefore the correspondence of lower frequency values for the asymmetric vibration of phosphate with the area increase is valid as a criterion of hydration for lamellar phase, in PCs and in PEs as well. However, the correspondence of frequency and area falls apart from those found for lamellar phase when a topological change to hexagonal phase occurs

as it is the case for DOPE. In this case, the PO_2^- vibration shift is the higher value for an intermediate area of around 47.2 \AA^2 . This behavior indicates that the stabilization of the interphase is not a consequence of the hydration of the polar head group but that other interactions of the chains could also be involved.

The frequency values for the asymmetric vibrational mode of the phosphate group are much lower for EggPE in comparison to EggPC in the same state (Table 1). In addition, the value for a 1:1 mixture of EggPC/PE is intermediate with that found for pure lipids. However, the frequency decreases with the decrease in hydration. Thus, the increase of the frequency by the presence of PCs can be understood by a weakening of the P–N interaction. This means that the interactions between PEs at the polar region can be attenuated by the presence of PCs. A result in this direction is clearly observed in the behavior of the dipole potential of PE monolayers mixed with saturated and unsaturated PCs (Fig. 1). The weakening of the interaction can be ascribed either to a dilution of the PEs or by an additional interaction between PEs and PCs. It is known that the P–N group esterified to the glycerol behaves as an electrometer [45]. Thus, the energy input to reorient the P–N dipole should be higher when the membrane is enriched in PEs (Fig. 3). The voltammetric response of DMPC/DMPE monolayers adsorbed on mercury shows a similar trend to that found for dipole potential of monolayers with the same composition spread on an air–solution interface. It is clear that the shift to negative potentials indicates that the energy cost is increased when the DMPE is included in DMPC monolayers in the condensed state. In other words, the dilution of DMPE with DMPC weakens the lateral interactions. The energy input shows a positive deviation suggesting an affinity of PE and PC.

One consequence in the organization of the lipid head groups can result in the average value of the monolayer dipole potential. The inspection of the dipole potential of DMPC and DMPE mixtures in monolayers at $18\text{ }^\circ\text{C}$ denotes a positive deviation with respect to the ideal mixture (Fig. 2). At the temperature of measure of the dipole potential, the mixtures DMPC/DMPE are all in the gel state according to the phase diagrams in lipid suspensions and therefore it reflects phase homogeneous properties [41,42]. However, the departure observed at 0.2 molar ratio of DMPE, for the dipole potential in Fig. 2 indicates that this mixture is not ideal in their interphase properties. This is consistent with the fact that head group interactions are enhanced probably due to the condensing effect promoted by the lateral interactions of the similar acyl chain length and saturation. This condensing effect would promote a different arrangement of the head groups manifested in the dipole potential values.

A different picture is observed when DMPC is diluted by EggPEs. In this case, the dipole potential decreases with the increase in PE molar ratio. Moreover, when PCs and PEs, both unsaturated, are mixed, no changes in the dipole potential were observed within the experimental error denoting an ideal mixing behavior. These observations suggest that the mobility of the acyl chain in the PEs would modulate the lateral headgroup PE/PE interactions. The presence of unsaturated bonds would hinder the lateral interactions decreasing the dipole potential. This is also reflected in the value of the PO frequency in the mixtures of DMPC (gel) and DMPE, and of EggPC and EggPE which are intermediate to that of pure lipids (Tables 1 and 3).

One of the major contributions to the dipole potential is water polarized by the carbonyl and phosphate groups [46–50]. In this regard, the lower hydration of PEs would suggest a lower dipole potential. However, the higher dipole potential of DMPE in comparison to DMPC suggests that it would be mainly determined by constitutive groups' components oriented normal to the interphase and not by the water dipoles. This arrangement can be modulated by the lipid chain saturation and the presence of PCs.

The phosphate frequencies for DMPC, DMPC/DMPE and pure DMPE are consistent with the dipole potential values. An increase in the dipole potential by enrichment with PE is parallel by the decrease

in frequency denoting stronger P–N interactions. This is congruent with the singular lower area and the higher surface pressure for the mixture in comparison to the pure lipid (Table 3). The strong decrease of the area per lipid in the 1:1 mixture in comparison to pure PEs and PCs (Table 3), suggests a strong interaction between the two components of the mixture giving place to a different geometry in the organization of the lipid head groups.

The determination of the dipole potential has been done following the methodology proposed by Simon et al. [49,50]. An air–water interphase is saturated with lipids until a constant value is reached. In this condition, an equilibrium between lipids in the monolayer and lipids stabilized as liposomes in the subphase has been proposed. In these conditions the area per lipid is coincident for all the lipids tested following other methods both in monolayers and in bilayers, but is somehow higher for DMPE in comparison to that reported. Discrepancies in the area of others saturated PEs has also been reported [51]. Considering our data of area for DMPE in Table 2 and those previously reported [35,44] the corresponding dipole moment of the component perpendicular to the membrane plane is around 712 mD. This is higher than that calculated by Brockman [52] from π vs A curves which amounts 619 mD for an area of 48.7 \AA^2 . Taken the onset of the area change in the lowest value considering the error of the method our value would be around 51.4 \AA^2 . The difference is 2.7 \AA^2 , which is much less than the transversal area of a water molecule.

The high dipole potential of DMPE monolayers prepared as described above suggests a larger contribution of component of the P–N dipole normal to the membrane surface. The average P–N vector would deviate from the normal due to an increase in the number of intermolecular hydrogen bonds between the NH_3 and PO_2^- group between the adjacent PEs [51]. In contrast, in PC, the P–N vector would be pointing to the hydrophobic phase due to the presence of the methylene groups [45]. If this is the case, the lateral interaction would result in an alignment causing the dipole potential increase in comparison to DMPC as observed in Fig. 1. The lateral interaction is reinforced by the presence of saturated chains as it is derived from the lower values of the dipole potential of EggPE.

As the lateral interaction of the lipids at the head group region determines the packing, the polarization and the surface free energy of the interphase, the presence of PE may modulate the surface properties of lipid membranes in its interaction with proteins. In this regard, the lateral interactions affect the dynamic conformation of lipids as a function of the packing change of the polar head groups produced by the condensation in the gel state of the lipid chains. Differences in promoting fusion of vesicles containing PE in comparison to that made with PC can be related to the surface [53]. The low values of hydration for DMPE would give the expectation that proteins, as a surface active elements, would not affect the surface pressure of PE monolayers. However, although decreased, the effect of the aqueous soluble protease is not null in DMPE in the condensed state.

Among the physicochemical properties of the lipid interface is the excess free surface energy reflected in the surface tension. A decrease of the surface tension means a relaxation of organized arrangements at the interphase, especially in water, of the intermolecular interactions by H bonds. Proteins and lipids decrease the surface tension when added by separated onto the surface of an air–water interface. However, after a monolayer is formed further additions of solute to the aqueous media can produce an additional decrease, denoted by an increase in the surface pressure. In the case of proteins, the surface pressure increase, related to an interfacial tension decrease, is generally interpreted as a consequence of protein penetration intercalated between the lipids. Without any restriction for the type of lipids, the changes in the surface pressure (Π) by a protein is ascribed to a lipid–protein interaction. However, the increase does not imply a specific interaction of the protein with a site of the lipids.

According to the model of Defay and Prigogine [54,55], the surface pressure can be related to the water activity (a_w) in the surface by

$$\Pi = -n_w RT \ln a_w \quad (1)$$

where n_w is the number of water molecule per area at the interface. From this, it is immediate to realize that changes in the water activity of the lipids due to competition with adsorbable solutes can affect the surface pressure.

The analysis of the cut off in the curves of Fig. 3 indicates that the protein becomes inactive at surface pressures much higher in DMPC than in DMPE monolayers. For a given surface pressure, the effect of aqueous soluble proteins is much lower on PE monolayers in comparison to PCs for similar hydrocarbon chains, which would correlate with the lower hydration level.

The cut off values denote that the interfacial changes are energetically more difficult in PE than in PC containing monolayers. Rewriting the Defay–Prigogine equation as

$$\gamma = \gamma' + n_w RT \ln a_w \quad (2)$$

where γ is the interfacial surface tension of the monolayer in the presence of a protein, γ' is the surface pressure corresponding to the monolayer without protein and it is related to the initial surface pressure (Π). The decrease in surface tension will be lower for lower hydration. In the absence of protein interaction the water at the interface is not perturbed. Hence $a_w = 1$ and consequently $\gamma = \gamma'$.

However, it must be noticed that at low surface pressures the PC:PE curve derives asymptotically to that for pure PC. Such behavior can be understood as a consequence of the association of PC/PE that is affected in a different way by the lateral pressure in comparison with the pure components. This deviation of the ideality in DMPE/DMPC membranes was also put into relevance by the cyclic voltammetry assays of Fig. 2.

The cut off surface pressure is lower than that corresponding to saturation of the surface with lipids both in DMPC and in DMPE monolayers. Considering the isotherm published by Brockman et al. [52] the cut off value of DMPE corresponds to an area of 40 \AA^2 and that for DMPC to $42\text{--}45 \text{ \AA}^2$. Thus, the difference between the effective values of the area for the two lipids is less one water molecule. This means that at similar distances, i.e. similar n_w according to equation (1), the free energy of the surface of DMPE and DMPC are different in their thermodynamic responses. Hence the surface pressure is a function of a_w and not of n_w and the area.

The present results give new insights on the hydration properties of lipid membranes containing PEs, especially those in the lamellar phase, that usually are underestimated. They put mainly into relevance that the possible contributions that may affect the dynamical behavior of a lipid interphase are the lateral lipid–lipid polar head interactions between adjacent molecules, the interaction with water and the interaction of the polar heads modulating the surface energy triggering the adsorption of proteins.

The effects of varying chain length and type of chain linkage show considerable differences at the interphase that could be ascribed to water accommodation and intercalation. In ordered chains, such as DMPC and DMPE, the gel phase becomes favored and a higher departure of ideal mixing is noticeable in comparison with structures formed with chains containing unsaturated lipid in the diacylphosphatidylethanolamines. It may be possible that for unsaturated chains the packing geometry becomes more difficult to satisfy the relative packing of the head groups, giving a different surface organization.

The principal message of this paper is to call the attention that membranes containing PEs have peculiar properties at the interphase region even when it is in the lamellar (gel or fluid) state. That is, PE is not only important because its propensity to stabilize in the hexagonal phase [24] (which have received extensive attention in literature). Perhaps, specific conditions in the lamellar phase might trigger the transition to H_{II} phase. The transition between the

lamellar and the inverted hexagonal (H_{II}) membrane structure is of importance for several biological processes. Among them, vesicle fusion starts with the adhesion of the corresponding membrane surfaces followed by the formation of spherical inverted micelles within cusp-like interbilayer attachment sites. This structural organization is considered to be a transient intermediate in the process of membrane fusion but also in the lamellar-to-hexagonal phase transition [56–59]. However, the surface free energy excess involved in the structures prepared to promote that triggering is far of having a clear understanding. Only few papers have dealt with the pre-state of the interface [24,58]. The present results denote that: membranes with PE have different surface (dipole) potentials according with its mixture with saturated and unsaturated PCs; the group's dynamic changes according to the PC/PE ratio, and the adsorptive properties of the membrane for proteins is also modulated by PEs content.

In summary, the much lower hydration of phosphatidylethanolamine, in comparison to phosphatidylcholine, produces a strong P–N interaction between lateral lipids, similar to that present in the non-hydrated lattice. This strong interaction between the PO_2^- and NH_3^+ of adjacent PEs is modulated by the lateral interactions in PCs, when present, and by the nature of the acyl hydrocarbon chains. On the other hand, these results call the attention to the fact that shifts in the asymmetric phosphate band is not always a straightforward indication of the hydration process in PEs.

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