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Surface and hysteresis properties of lipid interphases composed by head group substituted phosphatidylethanolamines

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ABSTRACT

This work analyzes the surface properties of PE-containing membranes modified at the head group region by the addition of methyl and ethyl residues at or near the amine group. These residues alter the lipid–lipid and lipid–water interactions by changes in the hydrogen bonding capability and the charge density of the amine group thus affecting the electrostatic interaction.

The results obtained by measuring the dipole potential, the zeta potential, the area per lipid and the compressibility properties allow to conclude that the H-bonding capability prevails in the lipid–lipid interaction. The non polar groups attached to the C_2-carbon of the ethanolamine chain introduces a steric hindrance against compression and increases the dipole potential. The analysis of areas suggests that lipids with methylated head groups have a much larger compressibility at expense of the elimination of hydration water, which is congruent with the broader extent of the hysteresis loop.

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 [1]. 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 expectative that they may have a key functional role [2,3]. The hydration of solid dimyristoylphosphatidylethanolamine (DMPE) produces a negligible shift in the asymmetric stretching frequency of the phosphate groups in contrast to that in dimyristoylphosphatidylcholine (DMPC). This accounts for the fact that the strong lateral interactions between the phosphate (PO_4) and amine (NH_3) groups, present in the solid PEs, still remain when the lipids are fully hydrated [4]. The lower mobility of the head group is reflected in a higher energy to translocate the phosphoethanolamine (P—N) dipoles in an electrical field, which decreases in the presence of increasing ratios of PCs of saturated chains in phosphoethanolamine monolayers [5]. In addition, it has been proposed that in PC – PE mixtures the propensity of the membrane to abandon the bilayer structure is determined by changes in the hydration of the polar head group [6,7]. This suggests that the interaction of the amine group with adjacent phosphate groups is hindered by the presence of methyl groups of the PCs.

On the other hand, subtle changes in the thermotropic behavior of substituted PEs were found when methyl groups were covalently attached to the amine group of PEs [8,9]. The addition of only one methyl group to the amine of PE results in thermotropic properties similar to those found in fully methylated amines such as in PCs. That is, transition temperatures shift in the order DMPE (52 °C), *N*-monomethyl DMPE (42 °C), *N, N*-dimethyl DMPE (26 °C) and DMPC (24 °C). In addition, methylation completely eliminates the hysteresis between the heating and the cooling thermograms observed in DMPE. This lipid does not show pretransition, but it appears with one methylation at 20 °C, with two methylations at 8 °C and with three methylations at 16 °C. The enthalpy of the pretransition, which is associated with hydration, also increases in the order *N*-methyl DMPE, *N,N* -dimethyl DMPE, DMPC (Frías et al., to be published). These results are in agreement with those reported for *N*-methylated DPPE's [27].

Abbreviations: DMPE, dimyristoylphosphatidylethanolamine; N-methyl DMPE, *N*-mono methyl dimyristoylphosphatidylethanolamine; C_2 ethyl DMPE, C_2 ethyl dimyristoylphosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine.

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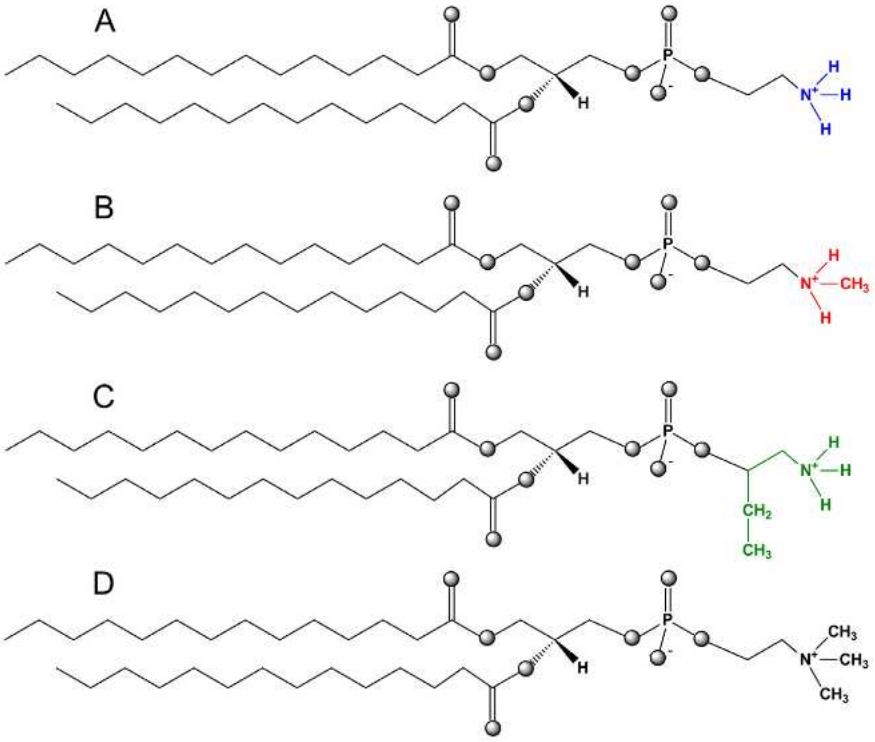

Fig. 1. Schematic description of A) dimyristoylphosphatidylethanolamine (DMPE) (T_c: 52 °C), B) *N*-mono methyl dimyristoylphosphatidylethanolamine. (*N*-methyl DMPE) (T_c: 45 °C), C) C_2 ethyl dimyristoylphosphatidylethanolamine (C_2 ethyl PE) (T_c: 35 °C) and D) dimyristoylphosphatidylcholine, (DMPC) (T_c: 24 °C).

A simple explanation of those results is that the presence of one, two or three methyl groups affects the head to head interaction and thus the phase behavior. However, there are several properties that may change concomitantly due to the presence of those voluminous groups, such as: the area per lipid, the polarization of water at the exposed methyl groups to water, the H-bonding network between adjacent PEs and the electrostatic interactions between PO_4 and NH_3 groups.

Considering that the size of the polar head group could be related to the amount of water immobilized around it. As PE hydrates less in a bulk phase than PC [7,28], the interaction with water would be different for PE than for PC due to the higher positive charge density of ethanolamine in comparison to choline [10]. Water and polar head group arrangements resulting from the lateral interaction determines the free energy of the interphase necessary for the adsorption of additives present in the aqueous environment. In this regard, differences in the insertion of aminoacids have been found when methyl groups are covalently bounded to the amine group of phosphatidylethanolamines [11].

However, it is not clear how the strong lateral interactions of PO_4 and NH_3 groups of adjacent molecules is governed by net hydrogen bonds or by electrostatic interactions, both contributing to the lateral cohesion forces.

Systematic information on the surface properties of head group substituted phosphatidylethanolamine regarding the surface properties and the intermolecular forces is not available. For this reason, this work analyzes the surface properties of PE-containing membranes in terms of the lipid–lipid and lipid–water interactions of different phosphoethanolamines and their modifications by substitution in the ethanolamine group. The structural changes in the polar head group of the PE by the insertion of methyl and ethyl groups at or near the amine group may alter the hydrogen bonding capability and change the charge density of the amine group thus affecting the electrostatic interaction. The methyl group blocks the ability to form hydrogen bonds, while the introduction of a bulky group in the C-chain of the ethanolamine should introduce a steric hindrance for lateral packing without affecting hydrogen-bonding groups. To enhance the steric hindrance an ethyl group was introduced in the C-chain of the ethanolamine leaving the NH groups of the PE free to interact by hydrogen bonding.

With this aim, the dipole potential, zeta potential, area per lipid and the compressibility properties of the lipids schematically described in Fig. 1 were measured in monolayers and bilayers.

2. Materials and methods

2.1. Lipids and solutions

The sources of commercially available chemicals, solvents, and chromatographic adsorbents were ACS grade and were redistilled before use [12].

1,2-dimyristoylphosphatidylcholine (DMPC) and 1,2- dimyristoylphosphatidylethanolamine (DMPE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Purity of the lipids was found to be >99% by thin layer chromatography and used without further purification.

N-monomethyl DMPE and C_2 ethyl DMPE were synthesized from their respective phosphatidylcholines by transphosphatidylation using savoy cabbage phospholipase D [13] and purified by silicic acid column chromatography [12–16].

2.2. Liposome preparation

Multilamellar liposomes (MLVs) were prepared dispersing the lipids by vortexing in 1 mM KCl at temperatures higher than that of the phase transition, for 60 min. Large unilamellar vesicles (LUVs) were prepared by extruding the liposome dispersions through a polycarbonate membrane (pore size 1000 nm) above the transition temperature of the lipids. After several tests, this size of the unilamellar vesicles was chosen in order to ensure optical visibility

to determine the diameter and the electrophoretic mobility in the available instrument. Details of these determinations are given below. After extrusion, vesicle samples were cooled down to the working temperature. Unless otherwise stated, working temperature was 18 ± 2 or at 22 ± 2° C.

2.3. Zeta potential

The zeta potentials (z) and electrophoretic mobilities (n) of DMPC, *N*-methyl DMPE, C_2 ethyl DMPE and DMPE liposomes and 1000 nm radius LUVs were determined in Zeta-Meter System 3.0 (Zeta Meter, Inc, Staunton, VA, USA) equipment, at 18 ± 2 or at 22 ± 2° C. The voltage was fixed at 75 V. In this method, individual vesicles are visualized under the microscope and the mobility is determined automatically vesicle by vesicle. For this reason, 1000 nm LUVs prepared by extrusion through polycarbonate membranes of the corresponding pore diameter were used. The size and number of vesicles in each sample were determined using the software provided in an Olympus CKX41 inverted fluorescence microscope with a magnification of 40×. The total lipid concentration in all cases was 36 mM.

A total of 20 measurements were carried out chosen a given vesicle under the optical field. Different vesicles of a sample were chosen to achieve a mean value statistically significant (standard deviation lower than the experimental error). Data reported are the average of the measurements done for each condition with, at least, three different batches of vesicles with their respective standard deviations.

2.4. Dipole potential

Dipole potential (Ψ_D) is defined as

$$\psi_d = \frac{\mu}{A \cdot \varepsilon_0 \varepsilon}$$

where μ is the average component of the lipid molecular dipole moment, including membrane-associated water molecules perpendicular to the plane of the membrane, A is the area available for the lipids, ε_0 is the permittivity of free space, and ε is the local dielectric constant.

The area per lipid was determined in monolayers formed by spreading chloroform solutions of the different lipids on an air–water interface of an aqueous subphase (KCl 1 mM) at constant area (A) and temperature (18 ± 2 or at 22 ± 2° C).

Briefly, measured aliquots of the lipid solution in the organic solvent were spread on an air–water surface. Subsequent aliquots were added, after allowing solvent evaporation, until a surface pressure of 47.8 mN/m for DMPC and 45.0 mN/m for DMPE were obtained, respectively, at 22 °C. At this temperature, both lipids are in the liquid condensed state. In these conditions, the monolayer self-assembled at the air/water surface is saturated and the resulting area per lipid corresponds to those reported in literature for each lipid state as described before [11,17].

The values of the interfacial potential were determined through a circuit of high impedance, connecting a vibrating electrode above the monolayer and a reference Ag/AgCl electrode in the aqueous subphase. Zeroing of the potential was performed with the aqueous solution without lipids after extensive cleaning by vacuum.

Lipids were added in carefully measured aliquots of a solution of known concentration of a chloroform solution. After each addition the potential was allowed to stabilize. The dipole potential reached a saturation value after subsequent additions.

The number of lipid molecules at each stabilized potential was used to plot surface potential vs area per lipid as shown in Fig. 2. The values of areas with this method were obtained following a procedure described above [17] and contrasted with the area determined in the surface pressure curves detailed in the next session.

Table 1
Electrophoretic mobilities and zeta potentials of (LUVs) of substituted PEs.

Lipid	Zeta potential (mV)	Electrophoretic mobility (cm/s volt)
DMPC	−20.8 ± 4.2	−1.7 ± 0.9
N-methyl DMPE	−16.2 ± 1.6	−1.2 ± 0.2
DMPE	−47.9 ± 10.2	−3.4 ± 0.7
C_2 ethyl DMPE	−37.5 ± 5.1	−2.5 ± 0.3

Errors represent the standard deviation from an average value taken for an $n = 20$ in, at least, three independent assays with different vesicles batches in the same conditions of temperature (18 °C).

2.5. Surface pressure vs. area curves

Langmuir monolayers were prepared on a Langmuir trough (KSV Minitrough model from KSV Nima, Biolin Scientific, Finland) housed in a clean thermostatized room, equipped with a Wilhelmy plate made of platinum which makes a contact with the water subphase of 30 mm length. Chloroform solutions of the lipids were spread on tridistilled water as aqueous subphase. After equilibration before measurements the pH of the solutions was 4.5 in all cases. Surface pressure–area (π–A) isotherms were obtained with a monolayer compression rate of 5 mm min^{-1}. For these experiments, temperature was set at 18 ± 2 or at 22 ± 2 °C. At these temperatures all lipids used in this study are in the liquid condensed state in monolayer. Results of surface pressure were expressed in mN/m.

2.6. Hysteresis

The hysteresis in compression-expansion cycles was measured in the KSV trough described above. A target value was chosen to assure that the expansion process initiated at area values below the collapse point. As a criterion of comparison, the hysteresis properties consisted in measuring the difference in area corresponding to a similar surface pressure (5 mN/m) for the compression and the expansion. All measurements were done at temperatures below the phase transition of the lipids.

3. Results

The zeta potentials and electrophoretic mobilities of DMPC, *N*-methyl DMPE, DMPE, and C_2 ethyl DMPE unilamellar vesicles measured in 1 mM KCl, (pH 4.5) are shown in Table 1. It is observed that surface potential of similar sized vesicles varies with the substitution of methyl and ethyl groups in the head group of PEs. When a methyl group is attached to the amine group of DMPE, the value of around −48 mV corresponding to DMPE decreases to −16 mV, a value comparable to DMPC vesicles. In contrast, when an ethyl group is added in the C_2 of the PE ethanolamine group (see Fig. 1) without affecting the NH bonds of the amine group of PE, the zeta potential is slightly lower than that of DMPE, although the non polar residue is more voluminous than a methyl group. No significant differences were found in the zeta potential when the same measurements were done using of multilamellar liposomes.

In another series of experiments, the dipole potential was determined as a function of the area per lipid in DMPE, *N*-methyl DMPE, C_2 ethyl DMPE and DMPC monolayers (Fig. 2). It is observed in Fig. 2A that the dipole potential of DMPC and *N*-methyl DMPE reaches different saturation values at similar number of lipids at constant area in the air-water surface. A similar behavior is observed between them when DMPE and C_2 ethyl DMPE are compared (Fig. 2B).

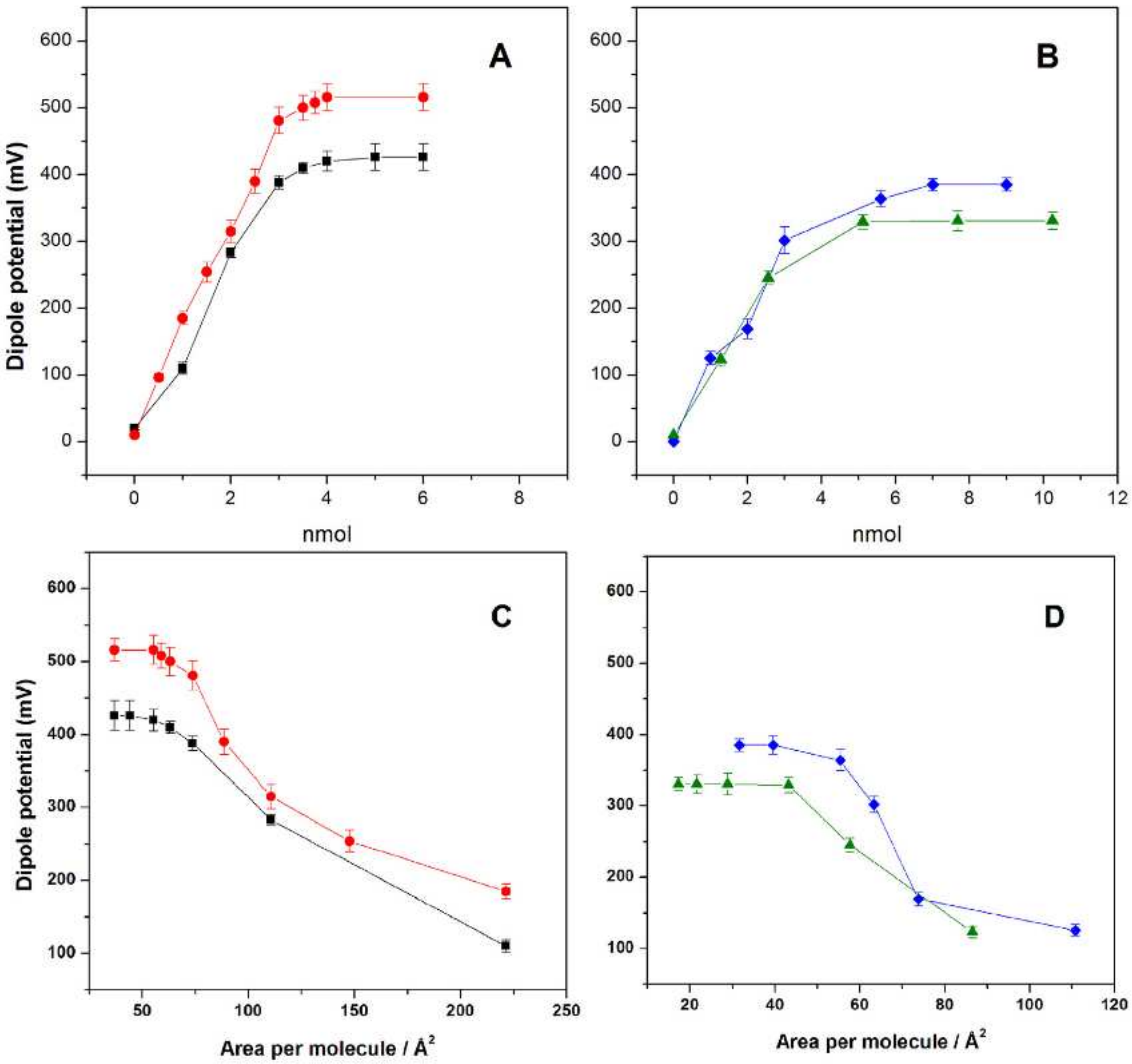

Fig. 2. Dipole potential in function of the number of lipid molecules added to the air-water interface (A and B) and area per lipid (C and D). DMPC (□), *N*- methyl PE (⋆), DMPE (○), C_2 ethyl PE (N). Error bars represent the standard deviation from an average value taken from, at least, five different assays for each lipid in the same conditions of temperature (18 °C).

A plot of the dipole potential as a function of the area per lipid shows that the blocking of H bonding groups with one or more methyl groups give similar areas and different dipole potentials, being the dipole potential of the mono substituted DMPE c.a. 100 mV higher than DMPC (Fig. 2C). In the case of DMPE and C_2 ethyl DMPE (Fig. 2D), the saturation areas are much lower than the lipids in part C, and the differences in dipole potentials between the unsubstituted and the substituted DMPE is less than 40 mV. The area change when the H bonding groups are not blocked is only 5%, in spite that a voluminous non-polar group is added to the C_2 chain of the ethanolamine (Fig. 2D).

Table 2
Molecular areas and dipole potentials of substituted dimyristoylphosphatidylethanolamine at 22.5 °C.

Lipid	Saturation Area (Å² mol⁻¹)	Dipole potential (mV)
DMPC	63.3 ± 4.0	410 ± 10
N-methyl DMPE	59.1 ± 5.0	508 ± 17
DMPE	38.1 ± 2.0	394.9 ± 15.0
C_2 ethyl DMPE	43.3 ± 3.0	339.3 ± 11.0

Errors represent the standard deviation from an average value taken for, at least, three independent assays in the same conditions of temperature.

A resume of the values is shown in Table 2. DMPC and *N*-methyl DMPE have comparable areas larger than those of DMPE and C_2 ethyl DMPE, which are quite similar between them. In addition, the dipole potential of DMPC is slightly lower than that of *N*-methyl DMPE and that of DMPE lower than for C_2 ethyl DMPE in spite of comparable areas.

The surface pressure- area isotherms for the four lipids (Fig. 3) show that the collapse areas of DMPE and C_2 ethyl DMPE are similar, although reached at different pressures. In addition, the collapse area of DMPC and *N*-methyl DMPE are higher than those of the first two lipids and the surface pressure reached are quite different.

It is interesting to note that the surface pressures at the collapse are similar for C_2 ethyl DMPE and *N*-methyl DMPE although at different areas. This denotes a similar effect on the water surface pressure.

When lipids were compressed to reach a similar surface pressure of 32 mN/m, the area is comparable for the unblocked H-bond groups. The recovery of DMPE and C_2 ethyl DMPE by expansion shows a hysteresis twice smaller than the DMPC and *N*-methyl DMPE lipids (Fig. 4, Table 3).

The collapse areas and the areas at the solid region of lipids with unblocked H bonding groups are consistently lower than those of methylated DMPE groups. This is also observed in the areas at saturation shown in Table 2.

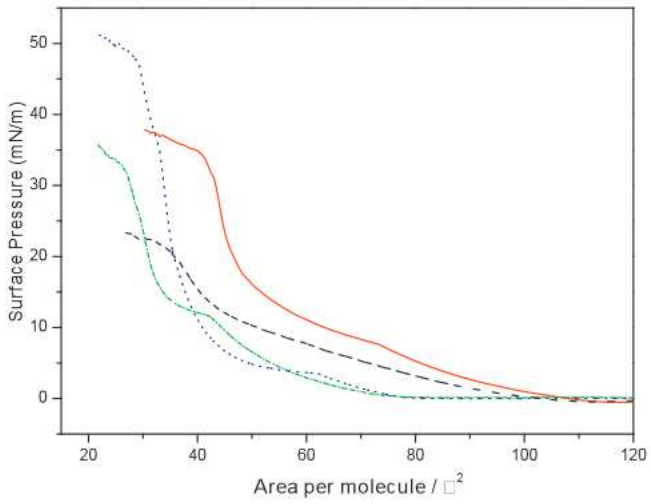

Fig. 3. Surface pressure-area isotherms of DMPE (-----, black), *N*-methyl-DMPE (——, red), C_2 ethyl DMPE (– – – –, red) and DMPC (— — —, blue) monolayers. Subphase: Water pH: 4.5, Temperature = 18–20 °C. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

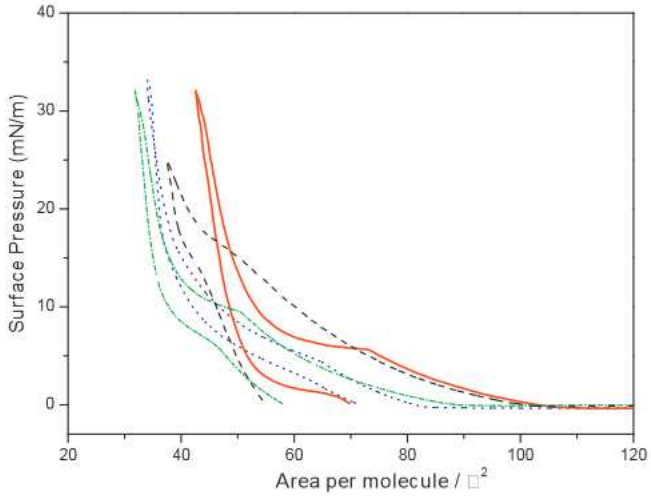

Fig. 4. Compression–expansion isotherms of DMPE (-----, black), *N*-methyl-DMPE (——, red), C_2 ethyl DMPE (– – – –, red) and DMPC (— — —, blue) monolayers. Subphase: Water pH: 4.5, Temperature = 18–20 °C. (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

4. Discussion

The introduction of a methyl group at the NH of the PE affects the H-bonding capability and the charge density of the positively charged residue. In contrast, when the apolar residue is introduced in the C_2 chain, the charge density also reduces according to the final volume of the group but the H-bonding capability remains unchanged (see Fig. 1).

The results of this work denotes that the substitution of apolar residues produces different and important changes in the surface and mechanical behavior of the lipid interface if they are made in the PE group blocking the NH bonds or at the C_2 chain of the ethanolamine leaving the NH group H-bonding capability unaltered. This conclusion is reached due to a similar behavior of *N*-methyl DMPE and DMPC on one hand and DMPE and C_2 ethyl DMPE on the other.

The addition of the nonpolar residues to the DMPE head group has relevant consequences on surface potentials. When the methyl groups block the H bonding residues, the dipole potential is consistently higher and the zeta potentials less negative than those lipids in which the NH groups remain unblocked.

As the dipole potential introduces a positive image charge inside the membrane [18,19], the large dipole potentials of DMPC and *N*-methyl DMPE would shield the negative charges, similar in all lipids due to the phosphate groups, giving as a result a less negative zeta potential in comparison to DMPE and C_2 ethyl DMPE, which has lower dipole potentials.

The higher dipole potentials of DMPC and *N*-methyl DMPE in comparison to the PEs unblocked NH lipids can be explained due to the exposure of non polar residues to water. The larger areas of these lipids should decrease the dipole potential (see Table 2). However, the monolayer average dipole potential is due to the apparent partial dipole moments resulting from water polarization, orientation of the lipid polar head groups and the CH_3 bonds of the lipid aliphatic chains [20]. As the lipid acyl chains are similar, assuming that the changes at the polar head group do not affect packing and chain organization, the variations in the dipole potential could be ascribed to the water polarized on them [21]. The reason for which DMPC and *N*-methyl DMPE have higher dipole potentials in comparison to DMPE and C_2 ethyl DMPE may be ascribed to the possibility that water can be polarized at the non polar residues of the head group attached to the amine.

This implies a larger contact of the methyl-substituted residues with water. The two lipids that show a non-polar group at the head group level (*N*-methyl DMPE and C_2 ethyl DMPE) have similar collapse pressures, indicating similar interactions with water. However, dipole potential of C_2 ethyl lipid is slightly lower than that of *N* -methyl DMPE, indicating less polarization capability. In addition, the area of C_2 ethyl DMPE is larger than DMPE in spite of having the three NH unblocked. This could be sustained by the fact that the lateral attractions between PO_4 and NH_3 groups are hindered by the ethyl group, which is partially exposed to water although less than the methyl in the *N*-methyl DMPE.

The hysteresis curves indicate that the lipids can be grouped in two families according to the substitution is made at the NH groups or at the C-chain of the ethanolamine. This denotes that unblocked hydrogen bonding groups are important for the surface potentials in spite of significant changes in the volume of the head group residue introduced by the ethyl group.

As discussed before, the larger areas of the NH substituted lipids (DMPC and *N*-methyl DMPE) in comparison to DMPE and C_2 ethyl lipid suggest a higher hydration of the lipids and this would explain the higher dipole potentials of the first two lipids Fig. 3.

The area at saturation (Table 2) reports the limit area reached by the lipids with its hydration shell. In contrast, the collapse area

Table 3
Monolayer parameters for different substituted (DMPEs).

Lipid	DMPC	*N*-methyl DMPE	DMPE	C_2 Ethyl DMPE
Area per molecule at collapse pressure (Å²)	32.6 ± 3.3	40.6 ± 0.8	29.3 ± 0.4	26.1 ± 2.1
Area per molecule at solid phase (Å²)	54.6 ± 2.7	52 ± 2.0	40.0 ± 0.1	37.7 ± 1.0
Hysteresis 5 mN/m	22.8 ± 2.7	23.2 ± 2.0	8.1 ± 0.1	12.8 ± 1.0

Error represents the standard deviation from an average value taken for, at least, three independent assays in the same conditions of temperature (18 °C).

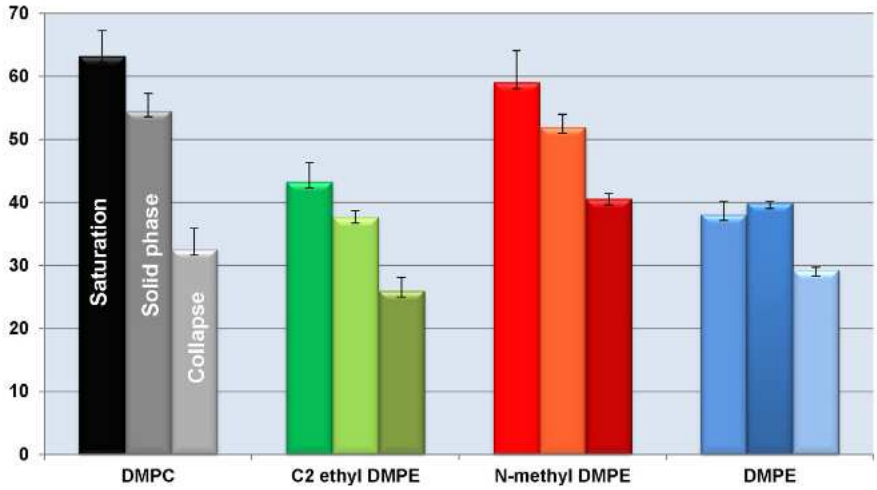

Fig. 5. Comparison of the saturation, collapse and solid state areas of DMPE (—, black), *N*-methyl-DMPE (▬▬), C_2 ethyl DMPE (▪ ▪ ▪ ▪, red) and DMPC (▬ ▬ ▬, blue) monolayers. For definitions see text. Error bars represent the standard deviation from an average value taken from, at least, three different assays for the each lipid in the same conditions of temperature (18 °C). (For interpretation of the color information in this figure legend, the reader is referred to the web version of the article.).

(Table 3) is that at which water in the external hydration shell is displaced by the compression. Thus, the differences between the areas at saturation and the collapse area give the total water that can be extruded from the lipid head group region. The comparison of these data in Fig. 5 denotes that the displaced area is in the order DMPC (30.7 $Å^2 \pm 4.0$), *N*-methyl DMPE (18.5 $Å^2 \pm 2.0$), C_2 ethyl DMPE (17.2 $\pm$ 3.2 $Å^2$) and DMPE (8.8 $\pm$ 2.0 $Å^2$). Similar conclusion can be reached taking as a reference the area obtained at the solid condensed branch of the compression curves in Figure 3.

Thus, the presence of a nonpolar residue in the head group region increases the water that may be displaced by the compression. This is congruent with the higher hydration due to water at the head group region. It is interesting that the difference observed between C_2 ethyl DMPE and *N*-methyl DMPE are comparable, which is congruent with the exposure of non-polar groups inferred from the similar surface pressure at the collapse.

Hydration is strongly different in PEs and in PCs. [22]. Part of the mechanical properties and thermodynamic response of lipid bilayers has been ascribed to water activity at the interphase [23,24]. If the insertion is produced at the NH groups, the H bonding in the surface network is disrupted and that expansion is occupied by water [25].

In addition, the electrostatic forces produced by the net charges on the surface (mainly phosphates) do not affect lateral interactions [26]. Moreover, negative zeta potential it is decreased by the presence of polarized water.

5. Conclusions

The addition of a nonpolar residue to the NH group of phosphoethanolamines increases the dipole potential and the amount of water that may be displaced by compression.

When the nonpolar residue is added to a C-chain of the ethanolamine group without affecting the H-bonding groups, the dipole potential and the compressibility properties remains almost unchanged in comparison with unaltered PE. This suggests that lateral interactions are similar in spite of the introduction of a voluminous group that changes the charge density on the amine moiety. Thus, the main lateral forces acting on PEs are the H bonding between PO_4 and NH_3 groups. This interaction is slightly affected by the steric hindrance of the ethyl group, which in turn, exposes to water giving some similarity, in a much less extent, with the behavior of methyl substituted PEs at the NH group.

The hysteresis in compression–expansion cycles is related with the water that can be displaced from the hydration shell of the lipids, which is higher when the H bonding lateral interactions are reduced.

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