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Thermodynamic and structural study of the main phospholipid components comprising the mitochondrial inner membrane

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Abstract

Cardiolipin (CL) is a phospholipid found in the energy-transducing membranes of bacteria and mitochondria and it is thought to be involved in relevant biological processes as apoptosis. In this work, the mixing properties of CL and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocoline (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) at the air–water interface, have been examined using the thermodynamic framework analysis of compression isotherms. Accordingly, the values of the Gibbs energy of mixing, the more stable monolayers assayed were: POPC:CL (0.6:0.4, mol:mol) and POPE:CL (0.8:0.2, mol:mol). The results reflect that attractive forces are the greatest contributors to the total interaction in these compositions. Supported planar bilayers (SPBs) with such compositions were examined using atomic force microscopy (AFM) at different temperatures. With the POPC:CL mixture, rounded and featureless SPBs were obtained at 4 °C and 24 °C. In contrast, the extension of the POPE:CL mixture revealed the existence of different lipid domains at 24 °C and 37 °C. Three lipid domains coexisted which can be distinguished by measuring the step height difference between the uncovered mica and the bilayer. While the low and intermediate domains were temperature dependent, the high domain was composition dependent. When cytochrome *c* (cyt *c*) was injected into the fluid cell, the protein showed a preferential adsorption onto the high domain of the POPC:CL. These results suggest that the high domain is mainly formed by CL.

Keywords: Monolayers; Supported planar bilayers; Atomic force microscopy

1. Introduction

The three major components comprising the mitochondrial inner membrane of eukaryotic cells are phosphatidylcholine (PC, 40% in weight), phosphatidylethanolamine (PE, 40%), and cardiolipin (CL, 20%) [1]. Naturally occurring phospholipids include mixed acyl chains, one saturated (at the *sn*-1 position) and the other unsaturated (at the *sn*-2 position) linked to the glycerol backbone, which is normally in a fluid state under physiological conditions [2]. Such is the case with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), whose mixing properties at the interface have been previously reported [3]. Our interest in these particular species stems from our finding that transmembrane protein reconstitution in proteoliposomes was highly dependent upon the presence of phospholipids with low gel-to-liquid crystalline phase transition temperatures (T_m) [4,5]. Moreover, the physiological activity of many transmembrane proteins is dependent upon the ability of phospholipids, such as PE, to establish intermolecular hydrogen bonding [6,7]. While neutral phospholipid matrices (i.e., POPC) are widely used in biophysical studies [8,9], mixtures with POPE or anionic phospholipids [10] that establish hydrogen bonds at the protein–membrane interface should be used to mimic standard physiological conditions. For these purposes, one effective phospholipid is CL, which is a doubly negatively-charged four-tailed phospholipid, and the most unsaturated

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lipid found not only in mitochondria [11], and in bacterial membranes, for which few studies on its interactions with other phospholipid species are available [12]. On the other hand, the proportion of CL in the inner membrane of mitochondria is quite variable, ranging from 3 to 9 mol%, depending on the evolution stage [13]. Significantly, CL is essential for fundamental processes that occur at the inner mitochondrial membranes, such as ATP synthesis, ADP/ATP translocation, or electron transport. Furthermore, many inner membrane proteins and transporters (i.e., cytochrome *c* (cyt *c*)), interact selectively with CL as their activity depends on its presence [14,15]. In particular, it has been reported that electron transport through cytochrome *c* oxidase requires at least 3 or 4 molecules of CL to be available within the neighboring phospholipid matrix [16].

The loss of CL from the inner membrane of mitochondria has been related to several pathologic conditions such as ischemia [17,18] and aging [19,20]. Moreover, it has been suggested that the release of cyt *c* from the inner mitochondrial membrane during apoptosis may be related to or dependent upon levels of CL [21]. Thus, although several studies [22,23] have established the crucial role of mitochondrial lipids during cell death, it remains unclear why CL appears to be so fundamental to the overall process. Hence, an investigation of the mixing properties of CL with other major components of the inner mitochondrial membrane (PE and PC) will provide valuable information on the nature of their interactions and their possible implications for processes of biological interest.

Here, we examine the mixing properties of two binary systems, POPC:CL and POPE:CL, at the air–water interface using a thermodynamic framework analysis of compression isotherms. Moreover, we have studied the bilayer structure by atomic force microscopy (AFM), which is an effective technique for obtaining information on phospholipid domains [24,25] under biomimetic conditions. The occurrence of laterally segregated lipid regions is important, considering that lipid domains might play a role in the insertion of proteins [10,26]. Hence, to examine them in our own phospholipid matrices, we used AFM to characterize supported planar bilayers (SPBs) of those POPC:CL and POPE:CL compositions that were judged, based on a thermodynamic study, to be the most stable.

2. Materials and methods

2.1. Langmuir trough

2.1.1. Preparation of lipid monolayers

POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine), and CL (Cardiolipin), specified as 99% pure, were purchased from Avanti Polar Lipids (Alabaster, AL, USA) and used without further purification. Horse heart cytochrome *c* was purchased from Sigma Chemical Co. (Madrid, Spain). The buffer beneath the monolayers was 50 mM Tris–HCl buffer (pH 7.40) containing 150 mM NaCl, prepared in Ultrapure water (Milli Q® reverse osmosis system, 18.3 MΩ·cm resistivity) and filtered with a Kitasato system (450 nm pore diameter) before use. The preparation of the monolayers was performed in a 312 DMC Langmuir-Blodgett trough manufactured by NIMA Technology Ltd. (Coventry, England). The trough (total area: 137 cm^2) was placed on a vibration-isolated table (Newport, Irvine, CA, USA) and enclosed in an environmental chamber. The resolution of surface pressure measurement was ±0.1 mN m^{-1}. In all experiments the temperature was maintained at 24.0±0.2 °C via an external circulating water bath. Before each experiment, the trough was washed with chloroform and rinsed thoroughly with purified water. The cleanliness of the trough and subphase was ensured before each run by cycling the full range of the

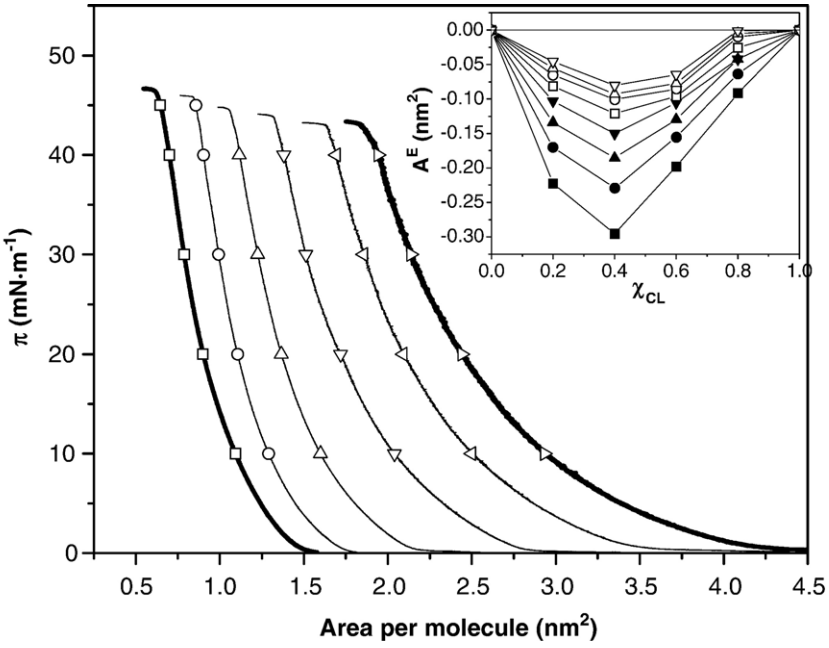

Fig. 1. Surface pressure versus area per molecule isotherms of (□) POPC, (○) χ_{CL}=0.2, (△) χ_{CL}=0.4, (▽) χ_{CL}=0.6, (◁) χ_{CL}=0.8, and (▷) CL. Inset: Excess area per molecule as a function of composition for POPC:CL mixed monolayers at various surfaces pressures: (■) 5, (●) 10, (▲) 15, (▼) 20, (□) 25, (○) 30, (△) 35, (▽) 40 mN·m^{-1}.

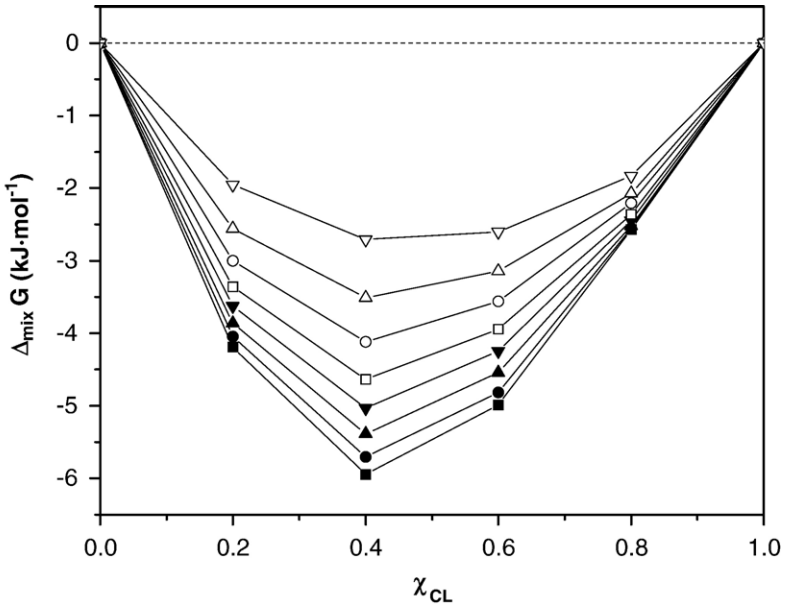

Fig. 2. Variation of the Gibbs energy of mixing for the POPC:Cl system with the mole fraction of CL at different surface pressures: (■) 5, (●) 10, (▲) 15, (▼) 20, (□) 25, (○) 30, (△) 35, (▽) 40 mN·m^{-1}.

trough area and aspirating the air–water surface, while at the minimal surface area, to zero surface pressure.

The lipids were dissolved in chloroform–methanol (3:1, v/v) to a final concentration of 1 mg·mL^{-1}. The corresponding aliquot of lipid was spread onto the surface of the subphase solution with a Hamilton microsyringe. A 15-min period was required to allow the solvent to evaporate before the experiment was begun. The compression barrier speed, calculated to the final surface pressure, was 5 cm^2·min^{-1}. Every π-A isotherm was repeated three times minimum, with the isotherms showing satisfactory reproducibility.

2.1.2. Isotherm analysis

The area per molecule of an ideal mixed monolayer of two components can be calculated as follows:

$$A^{id} = \chi_1 A_1 + \chi_2 A_2 \quad (1)$$

where A^{id} is the area per molecule of the mixed monolayer and χ_1, A_1 and χ_2, A_2 are the molar fractions and the area per molecule of the pure component 1 and 2, respectively.

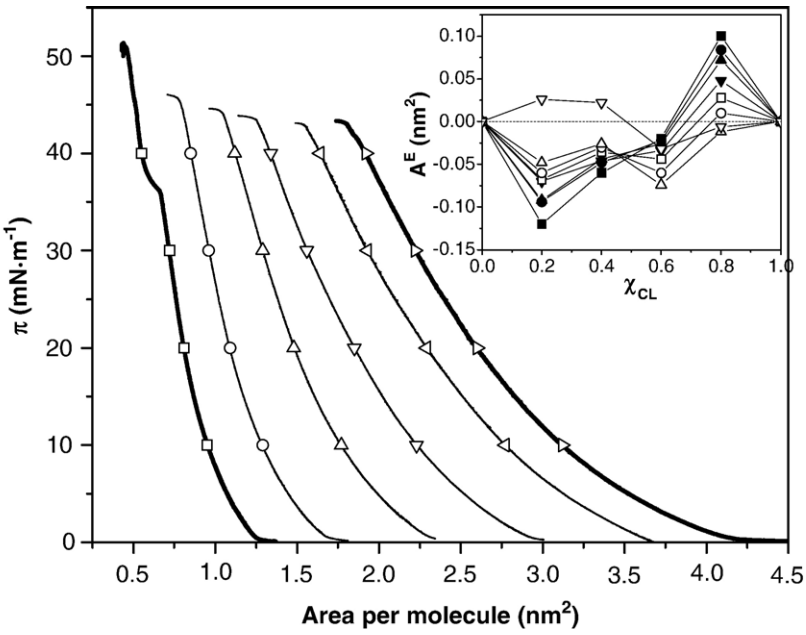

Fig. 3. Surface pressure versus area per molecule isotherms of (□) POPE, (○) χ_{CL}=0.2, (△) χ_{CL}=0.4, (▽) χ_{CL}=0.6, (◁) χ_{CL}=0.8, and (▷) CL. Inset: excess area per molecule as a function of composition for POPE:CL mixed monolayers at various surfaces pressures: (■) 5, (●) 10, (▲) 15, (▼) 20, (□) 25, (○) 30, (△) 35, (▽) 40 mN·m^{-1}.

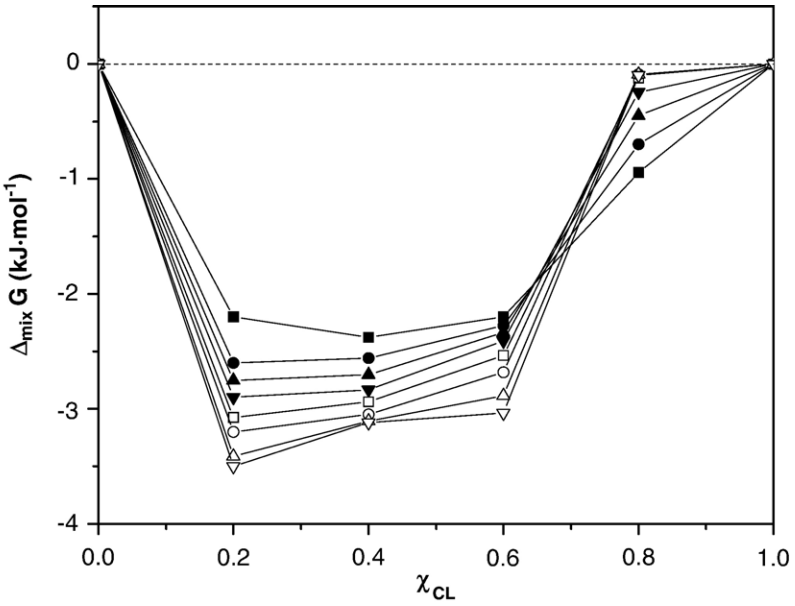

Fig. 4. Variation of the Gibbs energy of mixing for the POPE:CL system with the mole fraction of CL at different surface pressures: (■) 5, (●) 10, (▲) 15, (▼) 20, (□) 25, (○) 30, (△) 35, (▽) 40 mN·m^{-1}.

The excess area, A^E, for a binary monolayer can be expressed as follows:

$$A^2 = A_{12} - (\chi_1 A_1 + \chi_2 A_2) \quad (2)$$

where A_{12} is the area per molecule of the mixed monolayer.

The interaction between two phospholipid components in a mixed monolayer, at a constant surface pressure π and temperature, can be evaluated from the calculation of the excess Gibbs energy (G^E), which is given by

$$G^E = \int_0^\pi [A_{12} - (\chi_1 A_1 + \chi_2 A_2)]\mathrm{d}\pi \quad (3)$$

The Gibbs energy of mixing is given by

$$\Delta_{mix} G = \Delta_{mix} G^{id} + G^E \quad (4)$$

where the first term, the ideal Gibbs energy of mixing (Δ_{mix} G^{id}), can be calculated from the equation

$$\Delta_{mix} G^{id} = RT(\chi_1 \mathrm{Ln}\chi_1 + \chi_2 \mathrm{Ln}\chi_2) \quad (5)$$

where R is the universal gas constant and T is the temperature.

2.2. Supported bilayer studies

2.2.1. Preparation of liposomes

The method for obtaining liposomes has been previously described [25]. Briefly, lipids were dissolved in chloroform:methanol (3:1, v/v) and mixed to obtain the desired composition in a conical tube. The solvent was evaporated under a stream of nitrogen. The lipid film was maintained under reduced pressure for at least 2 h. The resulting film was hydrated in 50 mM Tris–HCl, 150 mM NaCl, pH 7.40 to a final concentration of 250 μM. To obtain unilamellar vesicles, the hydrated lipids were extruded 10 times through two polycarbonate filters (400 nm pore size, Nucleopore, CA, USA) with an extruder device obtained from Lipex Biomembranes (Vancouver, BC, Canada). The size and polydispersity of each preparation were monitored systematically by quasi-elastic light scattering using an Autosizer IIc photon correlation spectrophotometer (Malvern Instruments, U.K.). A dominant peak was typically observed indicating the presence of ~400 nm sized particles.

2.2.2. Atomic force microscopy

Images were generated with a commercial AFM (Nanoscope IV, Digital Instruments, CA, USA) and Si_3N_4 cantilevers (Olympus, Tokyo, Japan) with a nominal spring constant of 0.08 N m^{-1}. The instrument was equipped with a "J" scanner (120 μm) and the Tapping mode fluid cell was extensively washed with ethanol and water before each experiment. Mica squares (0.4 cm^2, from Asheville-Schoonmaker Mica Co., VA, USA) were glued to a Teflon disc mounted on a steal disc. Subsequently, 50 μl of liposomes were spread on the mica. For in situ AFM measurements, cyt *c* was added into the fluid cell at a final concentration of 5 μM. The height difference between the heating element on the piezo-scanner and the surface of the sample is around 3–5 mm. It has been seen experimentally that the difference between the temperature of the heating element and the mica surface can reach up to a 25%, so the real temperature of the sample was measured with a thermocouple (Cole Palmer thermocouple thermometer EW-91100-20 DigiSense, Resolution: 0.1 °C, Accuracy: ±0.25% provided with Omega Precision Fine Wire Thermocouples). A dwell time of

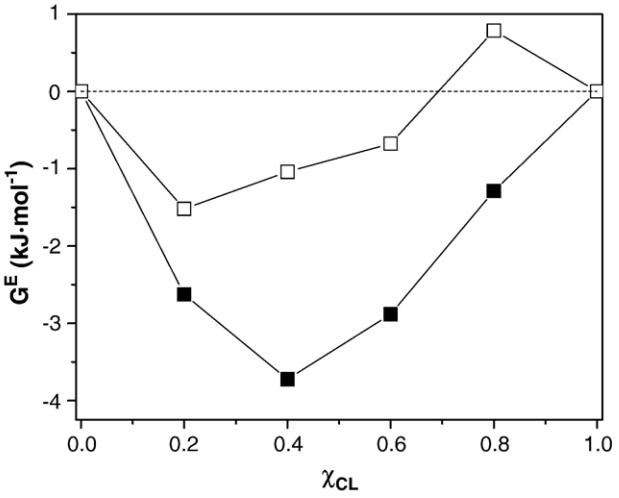

Fig. 5. Excess Gibbs energy of mixing versus composition for POPC:CL (■) and POPE:CL (□) monolayers at 30 mN·m^{-1}.

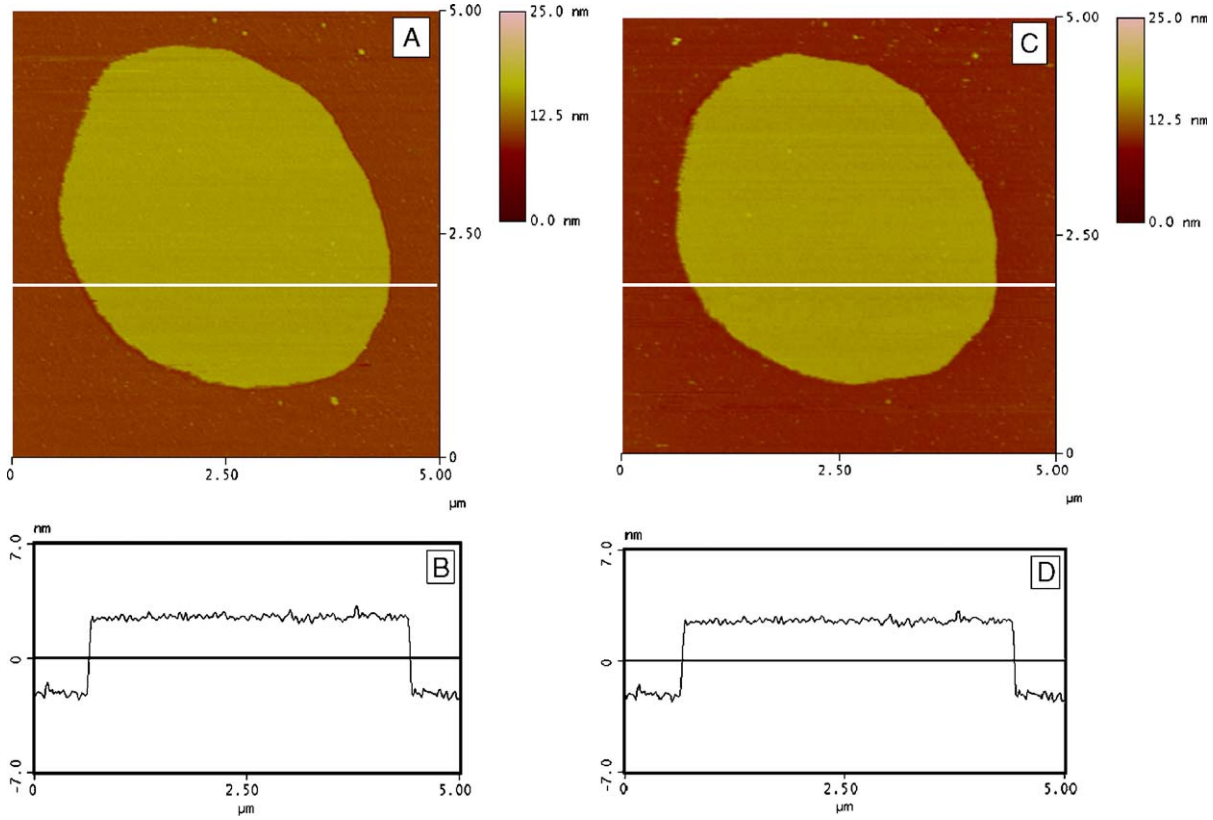

Fig. 6. (A, C) Topographic image of an SPB of POPC:CL (0.6:0.4, mol:mol) at 4 °C and 24 °C, respectively. Buffer (pH 7.40), 50 mM Tris–HCl and 150 mM NaCl. (B, D) Height profile analyses along the white line shown in A and C, respectively.

20 min is waited for the sample to reach thermal equilibrium with the heating element after increasing the temperature. Images were recorded in a constant-force mode and in liquid. The set point was continuously adjusted during the imaging to minimize the force applied. Prior to imaging the sample, the tip-sample pair was thermally stabilized. All the images were processed using Digital Instruments software.

3. Results and discussion

3.1. Langmuir trough

Lipid monolayers formed at the air–water interface constitute a versatile membrane model to investigate the interactions and physicochemical properties of their components. Thus, the mixing properties of CL with the two major phospholipids components of the inner mitochondrial membrane (POPC and POPE) may be related to, or influence the interaction and activity of, some relevant proteins (i.e., cyt *c*).

The surface pressure-area (π-A) isotherms of the pure phospholipids and POPC:CL mixed monolayers are shown in Fig. 1. At the temperature of these experiments (24.0±0.2 °C), all monolayers exhibited a liquid-expanded (LE) phase.[1] Collapse pressure values (π_c) of the POPC:CL mixed monolayers fell within the values of the pure components, 46 mN m^{-1} and 43.1 mN m^{-1} for POPC and CL respectively, following a linear relationship. These results are consistent with those reported elsewhere [12] and can be interpreted according to the Gibbs phase rule as an indication of complete miscibility between POPC and CL at the air–water interface.

The miscibility of POPC and CL, however, can be further evaluated by using Eq. (1) and calculating the excess area per molecule (A^E). As can be seen in the inset of Fig. 1, the values of A^E were negative for all compositions and surface pressures. The minimum negative values were observed at $\chi_{CL}=0.4$. These results reflect the existence of attractive forces between POPC and CL, whose exact nature is difficult to ascertain based only on thermodynamic analysis. Indeed, if A^E is evaluated at high proportions of CL ($\chi_{CL}\geq 0.8$), its values approach zero. Thus, behavior, at this limit, would reflect the relative increase of the repulsive forces due to increasing encounters of CL molecules at the interface. However, proportions of POPC as low as 20% would reduce the frequency of such encounters, allowing the monolayer to become stable. For a more accurate interpretation, the values of $\Delta_{mix}G$, calculated according to Eq. (4) and associated with changes in χ_{CL}, are shown in Fig. 2. As can be seen, $\Delta_{mix}G$ values were all negative, suggesting that no phase separation occurs in the mixed monolayers. In concordance with the values given in the inset of Fig. 1, the minimum value occurred at $\chi_{CL}=0.4$ for all surface pressures studied. Hence, we can conclude that this monolayer is, in comparison with the others, the more stable. It is worth noting, however, that this molar ratio was higher in CL than the naturally occurring molar

[1] The features of the pure POPE and POPC monolayers and the interaction in mixed monolayers have been discussed in a previous paper [3].

ratio found in the mitochondria inner membrane ($\chi_{CL}=0.2$) [1]. Our results are similar to those found for the egg PC:CL system [12], which underlines the fact that neutral species have a definite influence on CL mixing properties, distribution, and ultimately on the potential formation of domains. Obviously, the presence of other phospholipids such as PE will result in substantial changes in the PC:CL molar ratio. To evaluate such an influence, the binary mixture of PE and CL was also investigated.

The surface pressure-area (π-A) isotherms of POPE:CL mixed monolayers are shown in Fig. 3 at 24±0.2 °C for both phospholipids and their mixtures. The features of the POPE monolayer, mainly its collapse at 50.7 mN m^{-1} and the nature of the typical LC–LC′ phase transition [27], have been previously discussed [3]. Of note, the POPE monolayer aside, surface collapse pressure values decreased gradually by increasing the molar fraction of CL. According to the Gibbs phase rule, this indicates that the mixed monolayers are miscible at $\chi_{CL}>0.2$. Beginning with this lowest proportion of CL, the LC–LC′ phase transition becomes abolished (Fig. 3).

The values of A^E versus the molar fraction of CL at different surface pressures are shown in the inset of Fig. 3. As can be seen at $\chi_{CL}\geq 0.8$, the values of A^E were positive and decreased when surface pressure increased. The excess areas were negative for $\chi_{CL}<0.8$ and up to $\pi=30$ mN m^{-1}, suggesting that attractive forces are the greatest contributor to the total interaction. Indeed, this is quite conceivable based on the tendency of POPE to form hydrogen bonds [28]. On the other hand, repulsive forces would predominate at high mole fractions of CL (>80%), as indicated by the positive values of A^E obtained. This behavior, also observed at low mole fractions of CL (30–40%) at 40 mN m^{-1}, most likely results from the negative charge carried by CL. It is worth noting that the surface charge density increases with increasing surface pressure, which results in increased repulsion between CL molecules. Remarkably, except for the values calculated at 40 mN m^{-1}, A^E displayed opposite behavioral tendencies. As can be inferred from the $dA^E/d\pi$ values, repulsive forces would predominate as the surface pressure increased, while attractive forces would predominate as surface pressures decreased.

Fig. 4 shows the Gibbs energy resulting from mixing the POPE:CL binary system at different surface pressures. As can be seen, at high CL molar fractions ($\chi_{CL}\geq 0.8$) the monolayers become less stable and repulsive forces arise. As discussed above for A^E values, this behavior would result from the negative electric charge carried by CL molecules. The more thermodynamically stable composition occurs at $\chi_{CL}=0.2$ over the range of surface pressures studied. At such a composition, the attractive forces in the system would be higher than in the other compositions. This POPE:CL molar ratio coincides with the proportion of those components found in the mitochondrial inner membrane.

For a quantitative justification of the interaction between PC, PE and CL, G^E values can be calculated from Eq. (4). Taking for instance the monolayer at 30 mN m^{-1} (Fig. 5), it becomes clear that the POPC:CL system is more stable than the POPE:CL system. It is also evident that the replacement of the PC by PE group changes the character of the interaction from attractive to repulsive at $\chi_{CL}\geq 0.8$.

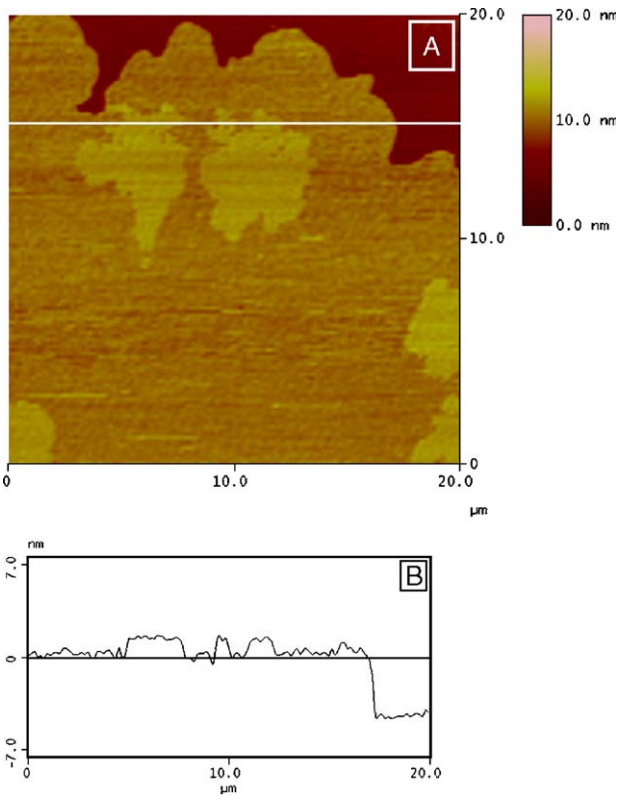

Fig. 7. (A) Topographic image of an SPB of POPE:CL (0.8:0.2, mol:mol) at 24 °C. Buffer (pH 7.40): 50 mM Tris–HCl and 150 mM NaCl. (B) Height profile analysis along the white line shown in panel A.

3.2. AFM study of supported planar bilayers (SPBs)

It is well known that the phospholipid composition of certain biological membranes, contain high proportions of PE and CL. SPBs, formed from simple extensions of liposomes onto

Table 1
Height and roughness (R_a) at two temperatures of the SPBs of POPC:CL (0.6:0.4, mol:mol) presented in Fig. 5A and C

Lipid domain height	T=4 °C		T=24 °C	
	h (nm)	R_a (nm)	h (nm)	R_a (nm)
No lipid domains	5.60±0.18	0.15	4.52±0.16	0.17

Table 2
Height and roughness (R_a) at the two temperatures of the lipid domains in the SPBs of POPE:CL (0.8:0.2, mol:mol) presented in Fig. 7A and B

Lipid domain height	T=24 °C		T=37 °C	
	h (nm)	R_a (nm)	h (nm)	R_a (nm)
Low	4.7±0.2	0.14	4.2±0.2	0.17
Intermediate	5.1±0.2	0.16	4.7±0.1	0.19
High	6.6±0.2	0.13	6.4±0.2	0.23

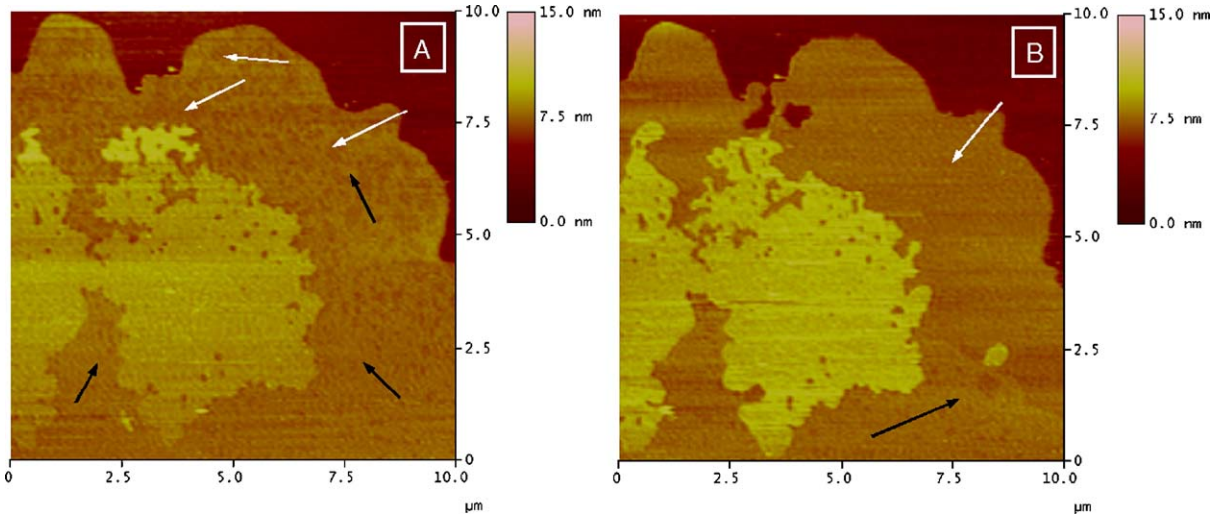

Fig. 8. Higher resolution topographic image of the same SPB presented in Fig. 6 at A 24 °C and B 37 °C. The white and black arrows correspond to the low and intermediate domains.

convenient surfaces [29] have been accepted as suitable models for studying the topographic characteristics of bilayers. Although comparisons of monolayers and bilayers can be contentious, it is accepted that the surface pressure of the biological membranes is about 30 mN m^{-1} [30]. Thus, the most stable monolayers at this pressure (Fig. 5) were taken to form SPBs with the same composition.

Characterization of the SPBs formed from POPC:CL liposomes was carried out at 4 °C and 24 °C, right and above the phase transition temperature (T_m) of the mixture in solution. On the other hand the characterization of the SPBs formed from POPE:CL liposomes was carried out at 24 °C and 37 °C, above the T_m.[2] Nevertheless, the use of T_m here is merely indicative, because it has been shown that the supporting material has a definite effect on the phase transition of each phospholipid mixture [31].

By spreading liposomes formed with the POPC:CL (0.6:0.4, mol:mol) phospholipid matrix onto mica, rounded and featureless SPBs were obtained, at 4 °C (Fig. 6A) and 24 °C (Fig. 6C). As suggested by the thermodynamic analysis performed above, lateral separations were not observed. The bilayers' thicknesses, calculated from cross-sectional analysis (Fig. 6B and D, respectively), decreased as the visualization temperature increased, while the roughness (R_a) values were very similar (Table 1).

A topographic image of an SPB formed with the POPE:CL (0.8:0.2, mol:mol) phospholipid matrix above the T_m of the mixture is shown in Fig. 7. The POPE:CL (0.8:0.2, mol:mol) system covers more substrate than the POPC:CL (0.6:0.4, mol:mol) for the same extension time. The differences in the spreading behavior between POPE:CL and POPC:CL preparations could be related with: (i) the propensity of POPE and CL to form non-lamellar phases [32]; and (ii) the different hydration degrees of PC and PE [33,34].

The thickness, obtained by measuring the step height in Fig. 7B between the uncovered mica (darkest region in Fig. 7A) and the layer, was established in 5.1 ± 0.2 nm (n = 50) with a mean R_a value of 0.16 nm (n = 50) (Table 2). Interestingly, lipid domains appeared, which can be distinguished by differences in the color scale. This observation is in concordance with CL domains revealed in natural membranes using fluorescent probes [35] and recent AFM observations [36]. From Fig. 7B, two lipid domains were visualized with a difference in height of 1.5 ± 0.2 nm (n = 50). Similar values have been reported for interdomain differences with other phospholipids systems [24,37]. Interestingly, close inspection of high-resolution images in the same area revealed the existence of two different lipid subdomains (Fig. 8A) in the lower domain of Fig. 7A. At this time, three lipid

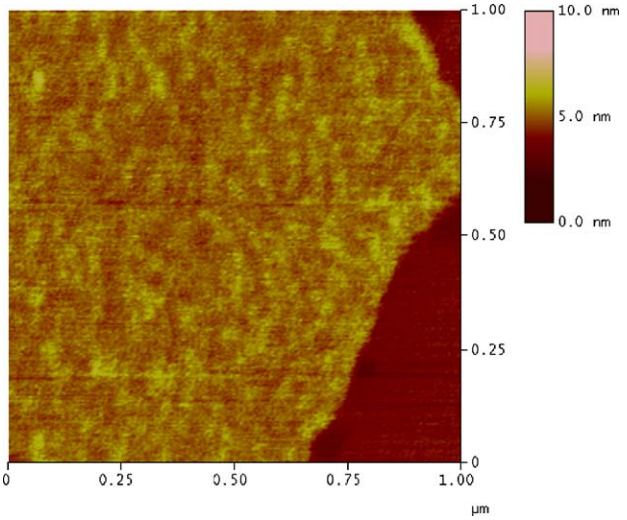

Fig. 9. Topographic image of an SPB of POPE:CL (0.8:0.2, mol:mol) at 24 °C. Buffer (pH 7.40): 50 mM Tris–HCl and 150 mM NaCl after in situ injection of cyt c (1 μM).

[2] T_m for POPC, CL and POPE are −5 °C, 19 °C and 25.5 °C, respectively; the T_m for the POPC:CL and POPE:CL mixtures used in the AFM experiments were established at 4 °C and 22 °C (unpublished TMA-DPH and DPH steady-state anisotropy experiments).

domains coexisted: low, intermediate and high (Table 2). Although the differences in height between the low and intermediate domains were small, they were always reproducible and much lower than the high domain. The differences between the low and the intermediate domains could result from a difference in tilt of the species, asymmetric distribution of the species, or alternatively, because of the formation of non-lamellar phases [38]. To investigate the nature of each lipid domain, the temperature of the sample was changed. AFM images taken at 37 °C (Fig. 8B) show that the low domain (white arrow) spreads, occupying a larger area than at 24 °C (Fig. 8A), while the intermediate domain (black arrow) is restricted to a minor area. Strikingly, while the low and intermediate domains were temperature-dependent the higher domain remained unaltered in height and extension when the observation temperature was increased. This behavior could result because of CL is present in just one leaflet in these regions, which could in turn be related with the uncoupling transition of top and distal monolayers of the SPBs [31].

These observations suggest that CL might be laterally segregated from POPE. At this point, we presume that the highest lipid domain could be mainly enriched in CL, based in the fact that its extension is 17% of the total area of the SPB and is consistent with the nominal composition of the liposomes used to form these SPBs. In agreement with this interpretation, we have observed (data not shown) that the area increases as the proportion of CL in the binary mixture increases. On the other hand, this behavior compares well with the domains observed in SPBs of POPE:POPG [10]. Caution, however, should be taken with this interpretation taking into account the impossibility of assigning these domains to a particular phospholipid species. On the other hand, thermodynamic analysis of POPE:CL monolayers suggests that some sort of miscibility exists between both phospholipids. Hence, it is possible that the low and intermediate domains could result from a different POPE enrichment in CL than in the high domain.

To explore the surface charge exposed by each domain, cyt *c* (positively charged at this pH) [39] was injected into the fluid cell where SPBs similar to that shown in Fig. 7A were previously formed. In agreement with other works [40], protein molecules were not topographically visible onto the SPBs interface (Fig. 9). Importantly, there was a remarkable increase, from 0.13 (Table 2) to 0.29, in the R_a value of the high domain while the low domain remain unaffected. These observations strongly suggest that the high domain is mainly formed by CL [12]. This observation is in agreement with results elsewhere published [41] where it has been shown that cyt *c* adsorbs preferentially at anionic bilayer-buffer interfaces.

It is well known that CL is functionally relevant for the energy transduction process based on evidence that it facilitates the binding of cyt *c* to cyt *c* oxidase. There are currently three proposed models to explain the effects of CL on cyt *c* oxidase [42]. One of these proposed models is based on the fact that CL may increase the surface concentration of cyt *c* close to the respiratory chain. Thus, the interaction of cyt *c* with the SPBs showed here will be of relevance for a fuller understanding of electron-transfer processes, as well as for greater insight into apoptosis when the matrix where the proteins are located is modified. Work is currently underway in our laboratory to demonstrate these points.

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