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Interaction of chitosan and self-assembled distearoylphosphatidic acid molecules at liquid/liquid and air/water interfaces. Effect of temperature†

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The effect of the polyelectrolyte, chitosan (CHI), on distearoylphosphatidic acid (DSPA) films was analyzed by cyclic voltammetry, surface pressure-area isotherm, and Brewster angle microscopy. Distearoylphosphatidic acid films formed at liquid/liquid interface produced a blocking effect to the electrochemical transfer of tetraethylammonium (TEA^+) cation from the aqueous to the organic phase, as can be deduced from the cyclic voltammetry experiments. Monolayers of DSPA were performed at different experimental conditions, temperature, subphase solutions (LiCl or $CaCl_2$), and CHI concentration. Both aqueous electrolytes studied exert strong interaction with the negative-charged polar head groups of phospholipids, which enhance the lateral interactions of the hydrocarbon chains, forming a very structured film. Under these conditions the presence of CHI produces a shift of TEA^+ transfer potential toward more positive values because of the less availability of negative sites of the phospholipids for TEA^+ adsorption, because they are occupied by the positive-charged amine groups in CHI chain.

Surface pressure-molecular area isotherms for DSPA monolayers change in the presence of CHI, demonstrating that this polymer produces an expansion of the DSPA film and modifies the surface elasticity of the film. The effect of the temperature was also studied; an exothermic phase transition was observed in the surface pressure-molecular area isotherms for DSPA-CHI monolayers, which involves the rupture of interactions between them. Images of Brewster angle microscopy evidence an increase in the optical thickness of the DSPA films in presence of CHI that indicates that the polymer interacts with DSPA molecules at all molecular areas. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: air/water interfaces; chitosan; Langmuir isotherms; liquid/liquid interfaces; phospholipids monolayers

INTRODUCTION

Chitosan (CHI) is a linear polysaccharide obtained from deacetylation reaction of chitin (2-acetamido-2-deoxy-β-d-glucan), which is the second most abundant natural polysaccharide after cellulose on the earth. Chitosan is a copolymer composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-d-glucosamine (acetylated unit), with different degrees of deacetylation (between 70% and 95%) and molecular weights (between 10 and 1000 kDa).[1,2] The amino group in CHI has a pKa value of approximately 6.5; thus, it is positively charged and soluble in acidic to neutral solutions with a positive charge density ($-NH_3^+$ groups) dependent on pH and the degree of deacetylation.[3]

Because of its biocompatibility, mucoadhesiveness, good mechanical properties, biodegradability, and its positive charge, CHI has been used in biomedical applications as a bioadhesive, bactericide agent,[4] gene delivery systems[5] or in pharmaceutical formulations, as a controlled release agent in oral preparations.[6] For this reason, many papers focused on the physicochemical properties of this polymer have been published in the literature, and the study concerning to the interaction between CHI and anionic or cationic lipids[7–10] has been of great interest because most of the uses of CHI involve the contact with cell membranes.[11]

Krajewska et al investigated the influence of CHI on the structural and thermodynamic characteristics of dipalmitoylphosphatidylglycerol (DPPG) Langmuir monolayers. For this purpose, the surface pressure-area (π-A) isotherms were measured at 5 temperatures in the range 15°C to 37°C.[12] They demonstrated that CHI interacts with the lipid film not only superficially but also partially inserted into the film, and this effect is favored by increasing the temperature. Furthermore, they demonstrated that the transition of the monolayer sate from the liquid-expanded to the liquid-condensed phase was an endothermic process accompanied by an increase in disorder. In that work, the authors propose that the interaction of CHI with DPPG monolayers is not only due to electrostatic but also due to significant non-electrostatic contributions.

Electrochemical methods applied to an interface formed by two immiscible electrolyte solutions (aqueous phase/organic phase) modified by different films have been used in the last

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decades with the aim of developing new biomimetic membrane models. Therefore, the adsorption of lipid monolayer,[13,14] proteins,[15,16] or polyelectrolyte[17–19] at liquid-liquid interfaces has been studied by cyclic voltammetry, electrochemical impedance spectroscopy, and surface tension measurements. An area of special interest has been the study of the interaction between phospholipid monolayers and different cations,[20] or peptides,[21] as well as between polyelectrolytes with different ions[3,22] and DNA.[23] All references listed above demonstrate that electrochemical techniques applied at the interface between 2 immiscible electrolyte solutions are ideal to follow dynamic changes in the lipid layer compactness and interfacial interactions at a hydrophobic/hydrophilic boundary.

In a previous paper we have studied the effect of CHI on distearoyl phosphatidylglycerol (DSPG) films formed at liquid/liquid or at air/water interfaces.[10] The experiments demonstrated that the presence of CHI in the aqueous phase produces an expansion of the DSPG film, an enhancement of the permeability, and also modifies the surface compression modulus. Moreover, we have shown that the nature of the supporting aqueous electrolyte (LiCl or $CaCl_2$) has an important role, because in the presence of LiCl the permeability of the film increased when CHI was present in the aqueous phase, while in presence of Ca^{2+}, the enhancement of permeability was not observed, probably because of the impediment of CHI to penetrate into the very tightly compacted film of DSPG. Images of Brewster angle microscopy (BAM) evidenced an increase in the optical thickness of the DSPG films in presence of CHI indicating that the polymer interacts with DSPG molecules at low and high molecular areas.

With the aim of better characterizing the nature of the interaction between CHI and phospholipid monolayers, we analyze, in the present paper, the effect of CHI on a more compact monolayer, such as distearoylphosphatidic acid (DSPA) films formed at water/1,2-dichloroethane or at air/water interfaces. Distearoylphosphatidic acid molecules adsorb at the liquid | liquid interface forming highly structured films, which produce a blocking effect on ion transfer, more important than that observed for DSPG monolayers.[14] Therefore, it is interesting to analyze the ability of CHI to interact and penetrate such very compact films. The study is performed using cyclic voltammetry, surface pressure-area isotherm, and BAM to evaluate the permeability and the compactness of the monolayer in the presence and the absence of CHI and different aqueous electrolytes (LiCl or $CaCl_2$). Moreover, with the purpose of elucidating the nature of the process responsible of the phase transition observed in the presence of the polymer, even for DSPG films, the effect of temperature is analyzed. The interesting in such anionic phospholipids molecules is based in the fact that they have been revealed as a major component of the bacterial cell membrane; therefore, the study of the interaction with CHI is important because of the widely extended applications of this polymer in the elaboration of antimicrobial adhesive films.

EXPERIMENTAL

Materials and electrochemical cell

A 4-electrode system using a conventional glass cell of 0.16 cm^2 interfacial area was used for the electrochemical measurements. The reference electrodes were Ag/AgCl, and 2 platinum wires were used as counter electrodes. The reference electrode in contact with the organic phase was immersed in an aqueous solution of 1.0×10^{-2} M tetraphenylarsonium chloride (TPAsCl, Aldrich). All potential values (ΔE) reported in this work are those that include $\Delta\varphi^0_{tr,\ TPAs+} = 0.364$ V for the transfer of $TPAs^+$ as reference ion.

The solutions of base electrolytes were 1.0×10^{-2} M MCl_z ($M^{z+} = Ca^{2+}$, Li^+) (p.a. grade) in ultra pure water and 1.0×10^{-2} M tetraphenyl arsonium dicarbollyl cobaltate (TPAsDCC) in 1,2-dichloroethane (DCE, Dorwill p.a.). Tetraphenyl arsonium dicarbollyl cobaltate was prepared by metathesis of TPAsCl and sodium dicarbollyl cobaltate (Aldrich p.a.). The pH of the aqueous solution was 3.00, adjusted with 2.00% v/v glacial acetic acid (Baker Analyzed). In all experiments 1.00 mL of organic and 4.00 mL of aqueous phase were used to fill the cell.

Chitosan (CHI, Sigma Aldrich, MW: 50-190 KDa, >75% deacetylated) was added to the aqueous phase (w) in a concentration range from 0% to 1.00% w/v.

Distearoylphosphatidic acid was of analytical grade (Sigma-Aldrich). A solution containing 0.80 mg mL^{-1} of DSPA in 1:2 methanol:chloroform was prepared. To be able to form the lipid film, 50 μL of DSPA solution was injected at the liquid/liquid interface after both phases were put in contact in the electrochemical cell, using a Hamilton microsyringe. A stable lipid film was formed after 60 minutes since the injection, after this time the voltammetric response was reproducible. All electrochemical experiments were performed after this equilibration time at room temperature equal to 25°C ± 1°C. Temperature was controlled with a temperature/humidity monitor.

It is important to remark that at pH = 3.00 polar head groups of DSPA molecules at the interface are partially ionized with negative charge while CHI is positively charged.

Cyclic voltammetry experiments

Voltammograms were performed using an aqueous solution of 5.0×10^{-4} M tetraethylammonium chloride (TEACl, Sigma). The cation tetraethylammonium (TEA^+) was used as a probe ion, because it transfers from the aqueous to the organic phase according to a direct reversible diffusion controlled mechanism.[24] With the aim of evaluating the ion permeability of the film, the voltammetric profiles for TEA^+ before and after injection of DSPA, in the absence and presence of CHI dissolved in the aqueous phase, were compared.

For the electrochemical transfer of ions corresponding to a direct reversible diffusion controlled mechanism, the peak current depends on sweep rate and other experimental factors according to the next equation:

$$I_p = 0.4463\, z\ F\ A\ c_X^w \left(D_X^w\right)^{1/2} \left(\frac{zFv}{RT}\right)^{1/2}, \qquad (1)$$

where I_p is the peak current, in amperes, z is the ion charge, A is the interfacial area (cm^2), D is the diffusion coefficient of the ion ($cm^2\ s^{-1}$), c is the ion concentration, and v is the sweep rate ($V\ s^{-1}$).

In this way, the electrochemical cell used was as follows:

		TPAsCl	TPAsDCC	MCl_n		
Ag	AgCl	$1.0x10^{-2}$ M (w′)	$1.0x10^{-2}$ M (o)	$1.0x10^{-2}$M	AgCl	Ag
				CHI		
				0-1.00%w/v		
				TEACl		
				$5.0x10^{-4}$M (w)		

A 4-electrode potentiostat with periodic current interruption for automatic elimination of solution resistance was used for cyclic voltammetry experiments. The voltage potential was changed from 0.200 to 0.750 V with a potential sweep generator (L y P Electrónica, Argentina). Voltammograms were recorded using a 10-bit computer board acquisition card connected to a personal computer. The voltammograms were obtained with typical errors of ±10% in current values.

Surface pressure-molecular area isotherms

A mini-trough II from KSV Instruments Ltd (Helsinki, Finland) was used to obtain the surface pressure-molecular area isotherms. The surface tension was measured using the Wilhelmy plate method with a platinum plate.

The aqueous subphase was 1.0×10^{-2} M MCl_z ($M^{z+}=Ca^{2+}$, Li^+), 2.00% v/v acetic acid, pH = 3.00, with or without CHI at different concentrations using a Teflon trough (364 mm × 75 mm effective film area). In all cases CHI was present in the subphase before the spreading of the phospholipid at the surface.

A volume of 30 μL of DSPA solution in 1:2 methanol:chloroform (0.40 mg mL^{-1}) was carefully spread at the surface with a Hamilton microsyringe, to prepare DSPA monolayers at the air/water interface. Before spreading DSPA solution, the subphase surface was cleaned by sweeping it with the Delrin barriers, and then any surface contaminant was removed by suction from the interface. The cleaning of the surface was checked by recording an isotherm in the absence of DSPA and verifying a surface pressure value lower than 0.20 mN m^{-1}. After spreading, the solvent was allowed to evaporate by 10 minutes, and then the film was compressed with 2 barriers at a compression speed of 5 mm min^{-1} while the automatic measurement of the lateral surface pressure (π) was performed.

Experiments were performed within a temperature range from 10°C ± 1°C to 50°C ± 1°C using a HAAKE G thermostat. The experiments were done, at least, in duplicate for each condition, and results with a typical area and collapse pressure errors of ±2 $Å^2$ and ±1 mN m^{-1}, respectively were obtained.

From the compression isotherm, the surface compression modulus κ (mN m^{-1}) was calculated as

$$\kappa = -A\times\left(\frac{\partial\pi}{\partial A}\right)_T, \qquad (2)$$

where π is the surface pressure in mN m^{-1} and A is the molecular area per molecule. The uncertainty of surface compression modulus was ±10 mN m^{-1}.

Brewster angle microscopy

The BAM experiments were performed using an EP3 Imaging ellipsometer (Acucurion, Goettingen, Germany) with a 20× or a 10× objective. The monolayer was formed in a Langmuir film balance (KSV mini-trough, KSV Instruments, Ltd, Helsinki, Finland) using the same volumes and DSPA solution than those described in Section 2.3. Images were registered after 10 minutes from the injection of DSPA solution, in simultaneous with the surface pressure-molecular area isotherm.

For BAM equipment calibration, 0 reflection was set with a polarized 532 λ laser incident on the bare aqueous surfaces at the experimentally calibrated Brewster angle (~53.1°). The reflected light was collected with a 20× objective.[25] After calibration and monolayer formation, the reflectivity and the optical thickness (h) were calculated from the BAM images. The grey level of each section of the image can then be converted to reflected light intensity (Rp), and h was calculated assuming a smooth but thin interface in which the refractive index varies along the normal to the interface on a distance h, much smaller than the incident light wavelength λ (λ = 532 nm)[26] that leads to

$$h = \frac{\sqrt{Rp}}{\sin(2\theta_B - 90)}\left(\frac{\pi\sqrt{n_1^2+n_2^2}\left(n_1^2-n^2\right)\left(n_2^2-n^2\right)}{\lambda(n_1^2-n_2^2)n^2}\right)^{-1}. \qquad (3)$$

In Equation 3 n_1, n, and n_2 are the air, film, and subphase refractive index, respectively, and θ_B is the Brewster angle.

The refractive index used for DSPA monolayers in the absence of CHI was 1.45, because this is the value reported for condensed films.[27] As detailed below, when CHI was present in the subphase, the DSPA film became more expanded, and then, the refractive index is expected to decrease.[28] Because the refractive index at this condition was unknown, we determined the monolayer thicknesses using 1.42 (index for liquid expanded-phases) and 1.45 (index for liquid-condensed phases),[28] and in this way, the whole range of possible height values could be evaluated. The refractive index for the subphases was calculated for each experiment from the experimental Brewster angle ($n_2 = tg(\theta_B)$, using 1.00 as the refractive index of air); the following values were obtained: 1.336 for the subphase with 1.0×10^{-2} M MCl_z (M = Li^+ or Ca^{2+}) – 2.00% v/v acetic acid in absence and 1.337 in presence of CHI.

RESULTS AND DISCUSSION

Cyclic voltammetry

Effect of CHI on DSPA adsorption

The effect of CHI on DSPA monolayers was studied by cyclic voltammetry adding the polymer to the aqueous phase before the film formation at the liquid/liquid interface.

Figure 1 shows the voltammograms of TEA^+ transfer across the bare interface or in the presence of DSPA monolayer, formed as detailed in Section 2.1, at different CHI concentration in the range from 0% to 1.00% w/v present in the aqueous phase

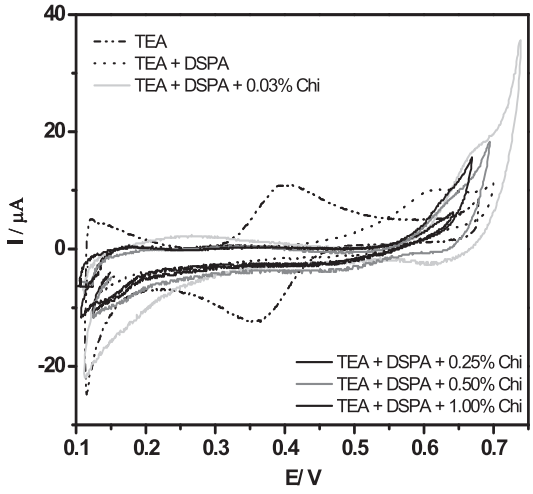
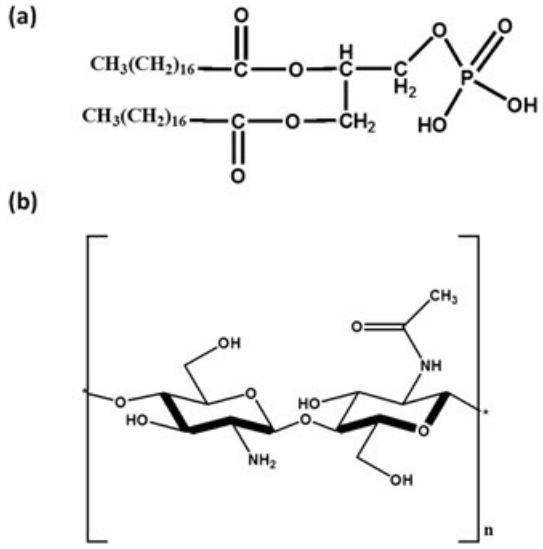


Figure 1. Cyclic voltamograms for TEA^+ transfer through the bare interface (— · —) or 60 min after the injection of 50 μL of 1 mM DSPA in 1:2 methanol:chloroform solution at different concentrations of CHI: (·····) 0%, (—) 0.03%, (—) 0.25%, (—) 0.50% y (—)1.00% w/v. Aqueous phase composition: 1.0×10^{-2} M LiCl. Sweep rate: 0.05 V s^{-1}. TEA indicates tetraethylammonium; DSPA, distearoylphosphatidic acid



Scheme 1. Structures of (A) distearoylphosphatidic acid and (B) chitosan

containing LiCl as supporting electrolyte. Tetraethylammonium transfer process across the bare liquid/liquid interface presents the very well-known response with a reversible diffusion controlled behavior. It can be observed a positive current peak at 0.400 V, corresponding to TEA^+ transfer from the aqueous to the organic phase, and the negative current peak corresponding to the reverse process, with a peak to peak separation equal to 0.060 V. The positive peak current, Ip^+, is linear with square root of sweep rate, $v^{1/2}$, for the whole range of scan rates analyzed (10-200 mV s^{-1}, data not shown). When the interface is covered by DSPA film, an important shift of peak potential toward more positive values and a decrease of the peak current were observed in the absence of CHI in the aqueous phase. In the reverse scan the negative peak current, Ip, corresponding to the transfer of TEA^+ from the organic to the aqueous phase, is almost negligible. These responses are evidencing a blocking effect of the layer to cation transfer. According to previous results,[14] TEA^+ is not able to be transferred to the organic phase, by permeation, across this highly structured DSPA film; instead, it irreversibly adsorbs at the negative polar head group of DSPA molecules. The presence of CHI in the aqueous phase does not improve the permeability of the film. This behavior can be explained taken into consideration that DSPA films are formed by molecules with extremely high molecular cohesion each other, in contrast with the behavior observed with other phospholipids such as DSPG.[10] The small polar head group of DSPA allows strong interactions between the hydrocarbon chains, so the penetration of CHI into this highly organized film is hindered. Similar results were obtained for $CaCl_2$ as aqueous electrolyte (data not shown). Moreover, the shift of TEA^+ transfer potential toward more positive values in the presence of CHI, observed in Figure 1, could be due to the less availability of negative sites of the phospholipids for TEA^+ adsorption, because they are occupied by the positive-charged amine groups in CHI chain.

These results are marking an important difference with the response obtained for DSPG films in our previous work.[10] In that case, CHI produced a decrease in the monolayer structuring and increased its permeability, effects not observed in the present study with DSPA. The explanation for these opposite behaviors is based on the differences in the structure of both films.

Summing up, the results obtained up to this point indicate that DSPA monolayer has a blocking effect on TEA^+ transfer, and the compaction of the film is independent on the cation present in aqueous phase. These phospholipids molecules, with a very small polar head group, allow a strong interaction of the hydrocarbon tails, forming a very structured film. For these reason, the polymer CHI is not able to penetrate and disorganize this very tightly compacted film (Scheme 1).

Langmuir monolayer

Surface pressure-molecular area isotherms

Figure 2A shows the surface pressure-area isotherms obtained for the DSPA monolayer adsorbed at the air/water interface, at 25°C, in the absence and in the presence of different concentrations of CHI using LiCl as the electrolyte in the subphase (similar results were obtained using $CaCl_2$, not shown). The isotherms obtained in the absence of CHI exhibit a collapse pressure of 42.4 or 45.9 mN m^{-1} with mean molecular area of 38.1 and 35.8 $Å^2$ per molecule for LiCl or $CaCl_2$ subphases, respectively. Important changes in the isotherm are evident in this figure when CHI is present in the subphase: a shift toward large areas per molecule, more pronounced at low pressures, can be observed. This behavior indicates that CHI can penetrate the DSPA monolayer at low pressure values.[29–31] On the other hand, at pressure values higher than 40 mN m^{-1}, the

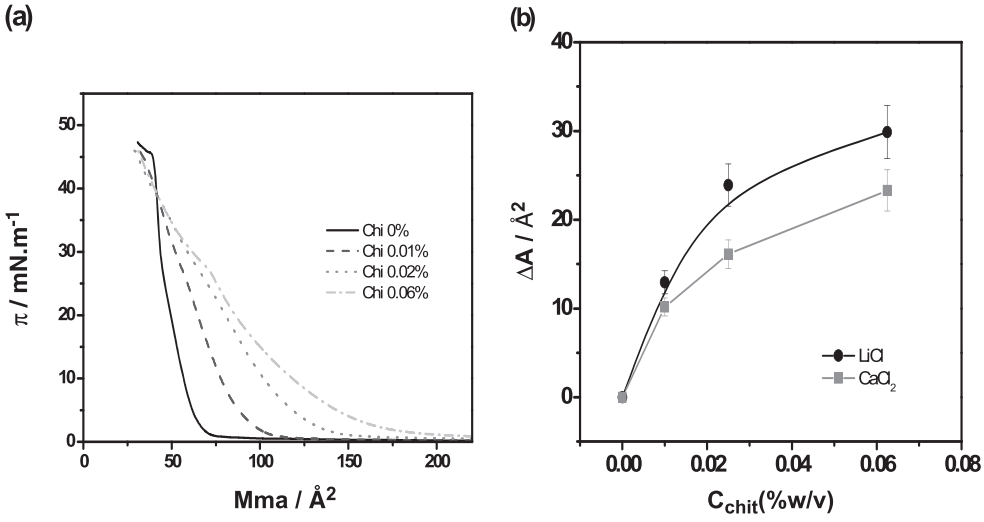
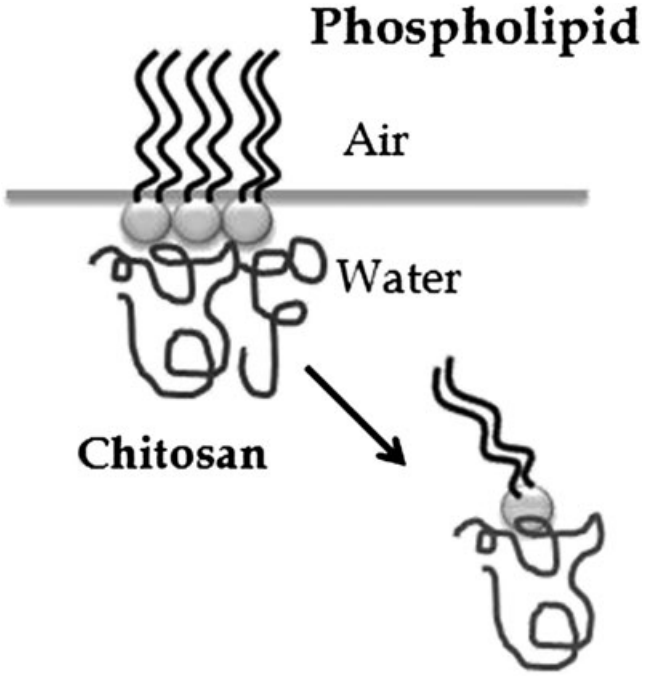


Figure 2. (A) Surface pressure (π) as function of the mean molecular area for DSPA monolayer at the air-water interface. Subphase composition: 2.00% v/v acetic acid, CHI: (———) 0%, (— —) 0.01%, (·····) 0.02%, and (— · —) 0.06% w/v in 1.0×10^{-2} M LiCl pH = 3.00. (B) Difference in areas per molecule obtained from the isotherms in the absence and the presence of different concentrations of CHI. $\pi = 25$ mN m^{-1}. Subphase composition: (●) 1×10^{-2} M LiCl or (■) 1×10^{-2} M $CaCl_2$, 2.00% v/v acetic acid, pH = 3.00



Scheme 2. Schematic model for distearoylphosphatidic acid–chitosan interactions at the air/water interface

Table 1. Surface compression modulus, κ, for DSPA monolayer formed on subphases containing 1.0×10^{-2} M LiCl or 1.0×10^{-2} M $CaCl_2$ in the absence or in the presence of different CHI concentrations (T: 25°C)

C_{chit}/% w/v	κ/mN m^{-1}	
	LiCl	$CaCl_2$
0	300 ± 10	200 ± 10
0.01	84 ± 8	90 ± 9
0.02	68 ± 7	77 ± 8
0.06	63 ± 10	60 ± 6

Abbreviations: CHI, chitosan; DSPA, distearoylphosphatidic acid.
The values of *k* were calculated at $\pi = 30$ mN m^{-1}.

molecular area in presence of CHI is lower than those for pure DSPA monolayer; this result can be explained considering that, at this point, CHI is expelled from the interface and, because of the strong coulombic interaction between the polymer and the phospholipids, a little amount of phospholipid molecules is also expelled from the interface to the subphase linked to the polymer (Scheme 2). Another explanation could be based on the assumption of reorganization on the molecular packing of the hydrophobic chains, giving rise to a film with low molecular area.

Table 1 shows the values of the compression modulus κ, calculated according to Equation 2, from the isotherms in Figure 2, at constant pressure equal to 30 mN m^{-1}, for subphases of LiCl or $CaCl_2$ and different concentrations of CHI. The surface compression modulus allows to classify the state of the monolayer as liquid-expanded (κ = 10-100 mN m^{-1}), liquid-condensed (κ = 100-250 mN m^{-1}), and condense ($\kappa > 250$ mN m^{-1}).[32] The κ values in the absence of CHI, equal to 300 mN m^{-1}, for LiCl, or 200 mN m^{-1} for $CaCl_2$, correspond to the DSPA pure monolayer in a condensed or a liquid-condensed state. As CHI concentration increases in the subphase, κ decreases, reaching typical values for liquid-expanded monolayer. This behavior confirms the presence of a new component at the interface, which produces changes in the state of the monolayer from condensed or liquid-condensed to liquid-expanded phase. Pavinatto et al attributed the change of the compression modulus at high surface pressures to the interaction between the phospholipids polar head groups and CHI, which makes the film more compressible.[31]

Figure 2B shows the change in the area per phospholipid molecule ($\Delta A = A^{\text{in presence of CHI}} - A^{\text{in absence of CHI}}$), produced by CHI, at 25 mN m^{-1}. As it can be noticed, the increase in ΔA is more evident for LiCl than for $CaCl_2$ as aqueous electrolyte; this enhancement is due to the progressive penetration of CHI into the monolayer, exercising hydrophobic interactions (with the carbon chains) and coulombic interactions (with negative-charged polar head groups). The effect on monolayer condensation produced by different cations has also been shown by electrochemical experiments for different phospholipids.[24,33] These studies have led to the conclusion that Ca^{2+} cations, because of their higher charge/size ratio compared to Li^+, interact with negative polar head groups of phospholipids with higher association constants, producing more structured monolayers. For this reason, the penetration of CHI into the monolayer results more efficient when LiCl is present as aqueous electrolyte, and therefore, the increase in ΔA observed for LiCl in Figure 2B is higher than that corresponding to $CaCl_2$.

Temperature effect on DSPA and DSPA-chitosan monolayers.

Surface pressure-area isotherm of DSPA films in the presence and the absence of CHI at different temperatures values was performed. Figure 3 shows the curves obtained at 10°C, 25°C, 40°C, and 50°C; the inset in the figure corresponds to the monolayer in absence of the polymer.

As can be seen, in the absence of CHI (Figure 3 inset), DSPA isotherms show slight changes as the temperature of the sub-

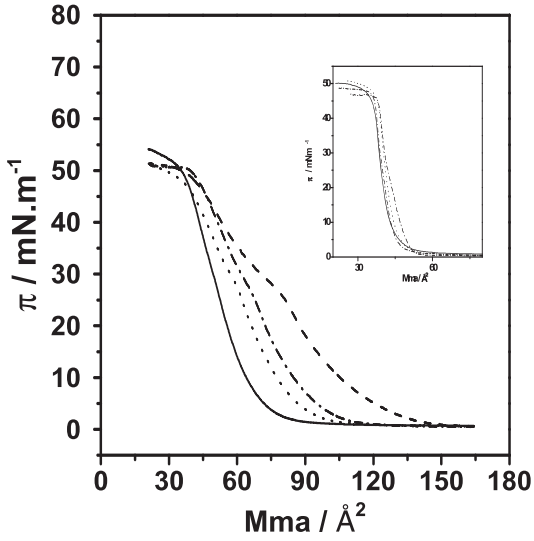


Figure 3. Surface pressure (π) as a function of the mean molecular area at different temperature values for distearoylphosphatidic acid monolayer with subphases containing: 1×10^{-2} M LiCl and 0.06% w/v chitosan. Temperature/°C: (——)10, (·····) 25, (– · –) 40, and (– – –) 50. Inset: Surface pressure (π) as a function of the mean molecular area at different temperature values for distearoylphosphatidic acid monolayer, in the absence of chitosan. Subphase containing 1×10^{-2} M LiCl

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Table 2. Surface compression modulus, κ, for DSPA monolayer formed on subphases containing 1.0×10^{-2} M $CaCl_2$ and 0.06% w/v CHI at different temperatures

Temperature °C	κ/mN m^{-1}	
	π 25 mN m^{-1} (State 1)	π 35 mN m^{-1} (State 2)
10	76.16	57.32
25	63.13	57.00
35	57.41	52.21
50	40.33	31.16

Abbreviations: CHI, chitosan; DSPA, distearoylphosphatidic acid. The values of κ were calculated at $\pi = 25$ mN m^{-1} and 35 mN m^{-1}.

phase increases: the monolayer slightly expands with a minor increase in the collapse area. More important changes are observed in the presence of CHI, where an evident shift toward higher areas is observed as temperature increases. These changes are better highlighted by the surface compression modulus (Table 2): it diminishes around 30 mN m^{-1} when temperature increases from 10°C to 50°C. This indicates that high temperatures lead to a less stable monolayer with low molecular cohesion. To evaluate the expansion of the monolayer in the absence and the presence of CHI, the lift-off area as a function of the temperature was analyzed (Figure 4). It can be observed that, for the whole temperature range studied, the lift-off area values are higher for the monolayer in presence of the polymer than those for pure DSPA. The variation of temperature does not produce significant changes on the lift-off area values corresponding to pure DSPA monolayer, while an important increase is more evident in the case of DSPA-CHI monolayers, reaching values of 134 or 113 Å^2 per molecule for LiCl or $CaCl_2$, respectively, at 50°C.

Also, from these results it is possible to conclude that the presence of Li^+ and Ca^{2+} in the subphases has different effects on the expansion process produced by CHI at different temperatures, because of the different interaction forces of the polar head group of phospholipids with these cations. Taking into account the difference between lift-off area, $\Delta A_{\text{lift off}} = A_{\text{lift off}}^{\text{CHI-DSPA}} - A_{\text{lift off}}^{\text{DSPA}}$, in the presence and in the absence of CHI, the values obtained were 81.2 Å^2 per molecule, when LiCl was used as the subphase electrolyte, and 54.5 Å^2 per molecule, when $CaCl_2$ was used, at 50°C. This confirms that Li^+ present in the subphase allows the polymer to penetrate more efficiently than in the presence of Ca^{2+}.

The effect of the temperature on the DSPA-CHI monolayers structuration can be explained considering the following points:

- First, the temperature increase produces an enhancement of molecular movement of DSPA and CHI. This is the reason for the less structured monolayer of DSPA (slight expansion),[34,35] and the stretching of the polymer, with higher flexibility,[36] which allows CHI penetration into the film.
- On the other hand, the increase of temperature produces a change in the charge density of the molecules, favoring the dissociation of the ionizable groups, promoting the negative charge of phosphate group on DSPA molecules, and leading to a more expanded film, because of electrostatic repulsion. On the contrary, in the case of CHI molecules, the increase of temperature favors the amine group dissociation, leading to diminished positive charges on the polymer, so the contraction of the polymer occurs.[12]

Taking into account the isotherms (Figure 3 and its inset), it is possible to conclude that temperature effect on DSPA-CHI monolayer is more important than in pure DSPA monolayer. The most important effect that can be occurring is the flexibilization and stretching of CHI chains that allows it to easily penetrate between the DSPA molecules in the monolayer, impelling the fluidization of the film.

Another distinctive characteristic of DSPA-CHI film is the presence of a transition state between 25 and 30 mN m^{-1}, independently on the cation present in the subphase (Li^+ or Ca^{2+}), being this transition more pronounced at higher temperatures.

Figure 5A shows the variation of the transition pressure (π_t) as function of the temperature for the DSPA-CHI monolayer. It can be observed that the pressure at which the transition occurs decreases with the temperature; this behavior was also observed by Krajewska et al[12] for DPPG-CHI monolayers. This transition shows a linear dependence with temperature, for both suphase solutions, LiCl and $CaCl_2$, with a negative slopes of −0.0077 and −0.073 mN (m.K)$^{-1}$, respectively. This behavior is characteristic of expanded monolayers, whose components establish bonds (ie, hydrogen bond) with the subphase component, so that as the film is compressed, these bonds are broken, as a consequence of the reorientation of the molecules.[37]

In this case, the interaction between DSPA and CHI occurs through coulombic attraction by the ionizable group and probably by the formation of hydrogen bonds. An increase of the surface pressure in DSPA-CHI system produces the rupture of the interactions above mentioned, so the monolayer changes from a state of low

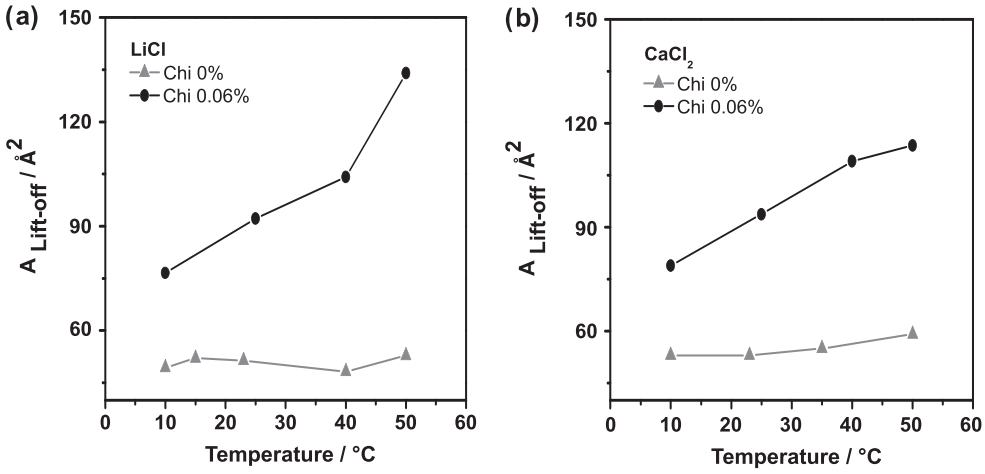

Figure 4. Lift-off area (Å^2 per molecule) as function of the temperature (°C) for distearoylphosphatidic acid monolayers, in the absence and the presence of chitosan and different aqueous electrolytes: (A) LiCl and (B) $CaCl_2$. Chitosan concentration: (▲) 0% and (●) 0.06% w/v

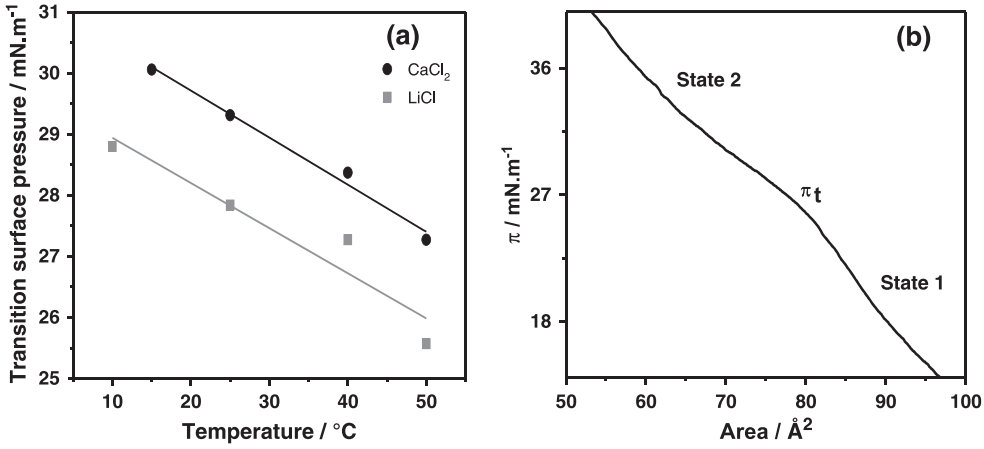

Figure 5. (A) Surface pressure transition (π_t/mN m^{-1}) as function of the temperature (°C) for distearoylphosphatidic acid–chitosan monolayers. Subphase composition: 0.063% w/v chitosan + 2.00% v/v acetic acid + (■) 1×10^{-2} M LiCl or (●) 1×10^{-2} M $CaCl_2$. (B) Surface pressure-molecular area isotherm for the distearoylphosphatidic acid–chitosan monolayer. Subphase composition: 0.06% w/v chitosan + 2.00% v/v acetic acid + 1×10^{-2} M LiCl, pH 3.00, T: 50°C

compressibility (as a consequence of more organized interactions), characterized by higher compressibility coefficients, state 1 (Figure 5B), toward a more compressible state (less ordered), state 2, in which the interactions with the subphase are not strong enough, so the monolayer is more flexible. This state is characterized by lower compressibility coefficients.

Figure 6A shows the variation of the surface compression modulus with the surface pressure for DSPA monolayer in the absence and in the presence of CHI in LiCl subphases at 23°C and 50°C. It is possible to distinguish 2 different behaviors, depending on the presence of CHI in the system. The κ coefficient values of DSPA monolayer in the absence of CHI increase with the surface pressure, which corresponds to a change from a lower molecular cohesion, at low pressure values (higher molecular areas), to another state with higher molecular cohesion, at high pressure values. At the same time, if 2 temperature values are compared, for all surface pressure values studied, the compressibility coefficient is higher at lower temperature, in accordance with a more rigidity of the film.

The behavior observed in the presence of CHI is quite different; at low pressure values, an increase of κ is observed (state 1) up to π values around 20 to 25 mN m^{-1}, after which κ starts to diminish (the transition occurs at these π values) and then, the κ coefficient increases again up to at another maximum value, lower than the first (state 2). These changes correspond to the transition from a film with a higher resistance to compression, in which the interactions DSPA-CHI are strong (state 1) to a film with less resistance to compression (state 2) as a consequence of the partial rupture of the DSPA-CHI interactions.

The linear behavior observed between the transition pressure and the temperature (Figure 5A) for DSPA-CHI films allows to relate the slope of this graph with the heat involved in the transition, ΔH_t, through the Clayperon equation in 2 dimensions, (Equation 4)

$$d\pi_t / dT = \Delta H_t / T(Ae - Ac), \quad (4)$$

where *Ae* is the molecular area where the transition begins and *Ac* the area where it finished. From this relationship the heat involved in the transition was calculated at the different temperature values; the result obtained is plotted in Figure 6B. As can be seen, the enthalpy values are negative, indicating that the change from state 2 to 1 is an exothermic process.[38] On the other hand, the change from state 1 to 2 occurs with absorption of heat, because it is necessary to break the interactions between DSPA and CHI, at the same time the system changes from a more ordered to a less ordered state. The heat value calculated for this system, −1.2 kJ mol^{-1}, is lower than the values obtained for transition states in pure phospholipids monolayers that correspond to the change from liquid-expanded to liquid-condensed state; for example, heat transition values equal to 40 kJ mol^{-1} have been determined for DSPG monolayers at 40°C.[12] The ΔH_t value obtained in the present study confirms that the change observed involves weak interactions.

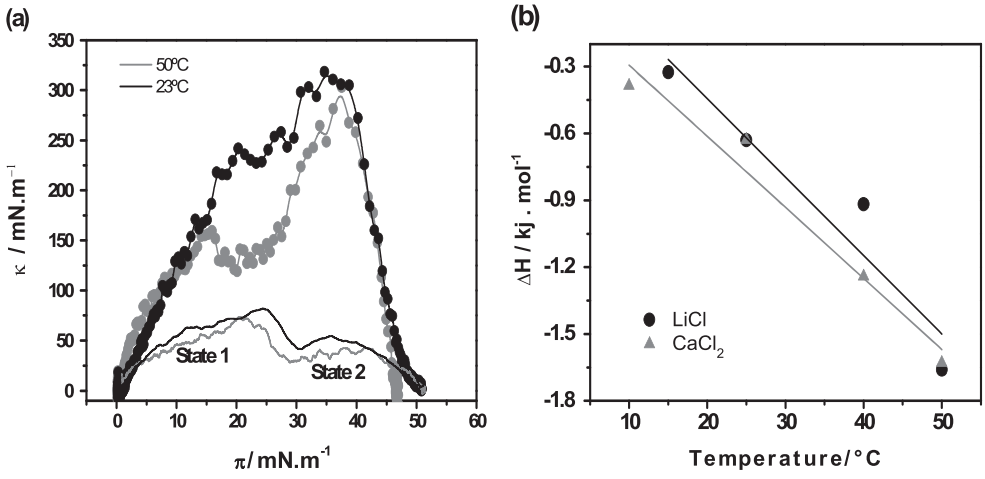

Figure 6. (A) Compression modulus as function of surface pressure for distearoylphosphatidic acid monolayer at different temperature: 23°C (●) and 50°C (●). Subphase composition: 1.0×10^{-2} M LiCl, 2.00% v/v acetic acid, in the absence (●,●) and the presence of 0.06% w/v chitosan (solid lines). (B) Enthalpy change (KJ mol^{-1}) for the transition as function of the temperature for distearoylphosphatidic acid monolayer in presence of 0.06% w/v chitosan. Aqueous phase composition: (●) 1.0×10^{-2} M LiCl or (▲) 1.0×10^{-2} M $CaCl_2$, 2.00% v/v acetic acid

Another important effect was observed for the DSPA-CHI monolayer at 25°C, when the isotherm was obtained in a discontinued way, namely, interrupting the compression during a time and

then continuing the compression at the same rate (data not shown). Under these conditions the isotherm obtained did not exhibit the transition shown in Figure 3. These differences can be explained taking into account the reorganization of the DSPA-CHI interactions that depend on time. When the isotherm is obtained in a continuum mode, the interactions are reorganized abruptly (transition state). On the contrary, when the isotherm is obtained in a discontinued way, the system goes through intermediate equilibrium, and in the periods during the compression stopped, the molecules of DSPA and CHI are rearranged, and consequently their interaction are modified.[37,39,40]

As partial conclusion, the transition observed on surface pressure-molecular area isotherms for DSPA-CHI monolayers is an exothermic process, which involves the rupture of interaction between them, in that way the film goes from a less compressible (low pressures) to a more compressible state (high pressures).

Brewster angle microscopy experiments

With the aim to confirm the presence of CHI at the interface and its interaction with DSPA monolayer, Brewster angle microscopy was carried on. Figure 7A shows micrographs obtained by BAM at different lateral pressures, for DSPA films in the absence (i) and in the presence (ii) of 0.06% w/v CHI solution in 1.0×10^{-2} M LiCl and 2.00% v/v acetic acid. As can be observed, there is a clear difference in gray level between the micrograph for DSPA and DSPA-CHI films. For the first case, the BAM images show 2 different zones, a first region at $\pi = 0\ \mathrm{mN\,m^{-1}}$ with circular domains that correspond to the transition phase from gas to liquid expanded, and the presence of homogeneous blocks, given by molecules of DSPA in condensed state. As the monolayer is compressed, expanded domains disappear (at π higher than $1\ \mathrm{mN\,m^{-1}}$) and a homogenous film of DSPA can be observed ($\pi = 12\ \mathrm{mN\,m^{-1}}$ and $45\ \mathrm{mN\,m^{-1}}$). In the presence of CHI the images obtained are different even at low pressures. We attribute these differences to the fact that CHI interacts with DSPA molecules, both at low and high pressures, producing an increase in the surface thickness. At $\pi = 0\ \mathrm{mN\,m^{-1}}$ 2 domains are also observed in the presence of CHI, but the region in expanded state is more bright and inhomogeneous. This behavior was also observed using $CaCl_2$ as aqueous electrolyte (micrograph not shown). As the monolayer is compressed, the domains grow up, appearing more bright and more inhomogeneous than in the absence of CHI. At the collapse pressure, bright small domains can be observed over the DSPA-CHI film, probably because of the formation of multilayer or aggregation of CHI on film defects.[29,41,42]

From this photographs and taking into account Equation 3, it was possible to calculate the optical thickness as indicated in Section 2. A comparison of the thickness values obtained for DSPA and for DSPA-CHI films at different pressure, in presence of 2 different aqueous electrolytes, is shown in Figure 7B. For both electrolytes (LiCl or $CaCl_2$) the monolayer thickness in absence of CHI was around 20 Å. In the presence of CHI,

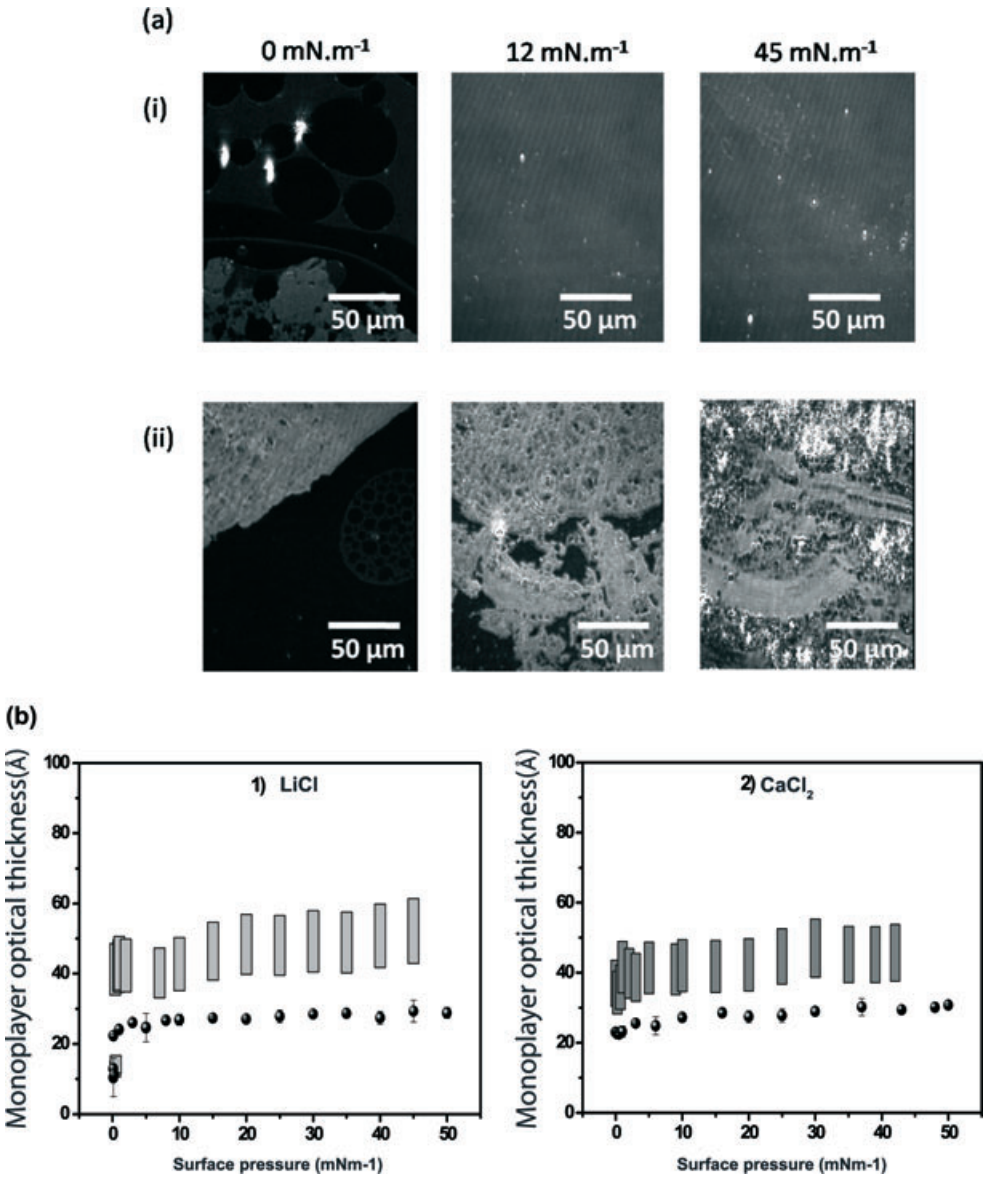

Figure 7. (A) Brewster angle microscopy images for monolayers of distearoylphosphatidic acid at subphases of 1×10^{-2} M LiCl, 2.00% v/v acetic acid, without (i) and with (ii) 0.06% w/v chitosan. (B) Monolayer optical thickness for subphases containing 2.00% v/v acetic acid and (1) 1×10^{-2} M LiCl or (2) 1×10^{-2} M $CaCl_2$ in absence (●) and in presence (▯) of chitosan (whole range of possible values)

the thickness values increase up to 30 and 50 Å, getting over the values obtained for pure DSPA monolayer within the whole range of surface pressure measured. These results are evidencing that the polyelectrolyte is present at the interface, interacting with DSPA molecules, even at high lateral pressure values.

CONCLUSIONS

Taking into account the results obtained in the present paper, we propose the model shown in Scheme 2 for the interaction between DSPA and CHI. In this model 2 stages for the interaction of CHI with DSPA can be established: (a) at low pressures (large molecular areas), the interaction is driven by Van der Waals forces between the DSPA hydrocarbon tails and the hydrophobic zones of CHI. This interaction is facilitated by CHI penetration into the monolayer in gaseous state. Beside the hydrophobic interaction, coulombic attraction between the phosphate groups of DSPA and the positive-charged amino groups of CHI can also be established.

(b) At high pressures, the strong electrostatic interaction between DSPA and CHI leads to a partial amount of the complex CHI-DSPA be expelled from the interface to the bulk of the subphase.

In surface pressure-molecular area isotherms, a transition at 30 mN m^{-1} is observed. With the aim of better characterizing the nature of this transition and, therefore, the nature of the interaction between CHI and phospholipid monolayers, the strategy of analyzing the effect of temperature has been explored. In this way, as an important contribution of this paper, we could attribute this transition to a reorganization process of DSPA/CHI film. The results obtained from the analysis of temperature variation indicate that this transition is an exothermic process with loss of order in the monolayer (diminution of the compressibility coefficient). This transition arises as a consequence of the reorganization in phospholipids and CHI interactions.

Moreover, BAM experiments demonstrate the presence of CHI at the interface interacting with the phospholipids, at low and high molecular area conditions. This resulted in an increase of the thickness of the film from 20 to 50 Å for the film formed by DSPA molecules and the polymer.

Previous results concerning to the effect of CHI on DSPG,[10] demonstrated that the presence of CHI in the aqueous phase produces an expansion of the DSPG film and an enhancement of the permeability when LiCl was present as aqueous electrolyte. This effect was not observed in the case of $CaCl_2$ because of the impediment of CHI to penetrate into the very tightly compacted film of DSPG in the presence of Ca^{2+} cations. From comparison of these results with those obtained in the present paper, it can be concluded that, because of highly structured films formed by DSPA molecules at the liquid | liquid interface, the blocking effect on ion transfer produced by DSPA is not reversed by the presence of CHI, regardless of the cation present in the aqueous electrolyte, unlike what has been observed DSPG. This behavior could be explained considering the smaller polar head group of DSPA, compared to DSPG, which allows strong interactions between the hydrocarbon chains, and so the penetration of CHI into this highly organized film is hindered.

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