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ORIGINAL CONTRIBUTION

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Thermodynamic and spectroscopic properties of mixtures of β-lactoglobulin and dioleylphosphatidylcholine

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Abstract The behavior of the single spreading monolayers of β-lactoglobulin (β-LG) and dioleylphosphatidylcholine (DOPC), as well as their mixtures, has been studied on subphases containing Na^+ or Ca^{++} ions. The results show an influence of temperature and subphase on the studied systems. The behavior of the areas as a function of the weight fraction of the two components shows significative and prevalently positive deviations from the additivity and their bidimensional miscibility. The variation of ΔG^{ex}, ΔH^{ex} and ΔS^{ex} calculated for the DOPC–β-LG mixture having maximum deviation on two different supports allows to deduce that the interactions are prevalently repulsive.

FTIR–ATR spectra of transferred plurilayers show that DOPC has a surface orientation which can originate the miscibility between the protein and DOPC.

Key words Monolayers of β-lactoglobulin and dioleylphosphatidylcholine

Introduction

It is well known that mixed monolayers of proteic and lipidic components are extensively studied taking into account also their importance as suitable models of natural membranes [1]. Several papers has been published on this topic, in which the proteic component is represented by the β-LG [2].

Recently, we reported on mixed monolayers of a globular protein β-lactoglobulin (β-LG) and two phospholipids with different lengths of the hydrocarbon chains, distearoylphosphatidylcholine (DSPC) and dimirystoylphosphatidylcholine (DMPC): in these cases the different surface orientation of the protein as regards the two phospholipids can be considered as the preminent reason for their bidimensional incompatibility [3].

In the present paper we study the mixtures of β-LG protein with dioleylphosphatidylcholine (DOPC). β-LG, whey protein, was widely used in the dispersed bidimensional systems in the food field [4] and was chosen since this protein, not normally membrane associated, could represent the protein component in the mixed systems and given appropriate conditions, on the contrary, of the major part of proteins, insoluble mono and plurilayers, which are well characterized for thermodynamic and molecular properties.

DOPC is a phospholipid having hydrocarbon chains of the same length of DSPC but with a double bond, so that the surface orientation of DOPC is more compatible with that of β-LG and, therefore, the reciprocal miscibility was expected to be verified [5].

In fact the bidimensional miscibility between different components allows to obtain homogeneous monolayers, which could be quantitatively transferred, to produce LB plurilayers for sovramolecular-oriented systems.

To reach this goal, in the present note we report the spreading isotherms of the DOPC and of their mixtures at

different temperatures and on different subphases containing Ca^{++} or Na^{+} ions. The FTIR–ATR spectra of mono and plurilayers of DOPC and one of their mixture transferred on suitable supports by LB method are also studied.

Experimental

β-lactoglobulin (β-LG), a globular whey protein from bovine milk with molecular weight = 18 000, with 160 aminoacid residues, from Sigma (Lot 0130) and stored at 4 °C was used. Dioleylphosphatidylcholine (DOPC), $C_{44}H_{84}NO_8P$, supplied from Sigma (Lot P4578 C), was 99% pure and stored as chloroform solution at 4 °C.

Bidistilled water purified by Milli-Q apparatus was used to obtain subphase solutions. NaCl and $CaCl_2 \cdot 2H_2O$ salts were used to prepare subphase solutions with ionic strength equal to 0.1 and pH = 5.6. The spreading solvent was a chloroform–methanol mixture with a small quantity of HCl as previously reported (3, 6). This spreading solution was used for single component as well as for mixtures.

The surface pressure was determined by Wilhelmy method using a KSV 3000 balance. The accuracy of π was 0.05 mN/m and 0.01 m^2/mg for area. Surface balance was worked in a continuous compression mode, moving the Teflon barrier with a speed of 3 mm/min. The compression rate was fixed on that level, giving the repeating shape of experimental isotherms, that corresponded to the equilibrium state, and was kept constant for all measurements. Before each experiment, the surface was cleaned until the value of surface pressure remained about zero on reducing surface area. Ten minutes after spreading the solution the π/A isotherms were recorded. The subphase temperature was controlled by a Haake FK thermostat with an accuracy of 0.1 °C.

The plurilayers on Germanium ATR plates were prepared and transferred [3] by the LB method by means of a KSV balance. The transfer ratios change from 0.8 to 1. The extraction and immersion speed were, respectively, 1 mm/min and 3 mm/min. The π extraction was 23 mN/m for DOPC, 17 mN/m for β-LG and 15 mN/m for their mixture, so that all the values were lower than collapse pressure (π_c) and in the range of liquid-condensed film. The FTIR spectra were obtained with a Bruker IFS120HR FTIR spectrometer, equipped with a MCT detector by using a ATR accessory. For each spectrum 500 interferograms were collected at a nominal 2 cm^{-1} resolution.

The solid-state spectra of DOPC has been acquired from the solutions for evaporation of $CHCl_3$.

The deconvolution spectra have been obtained with a band-fitting program using a Gaussian band shape.

Results and discussion

The spreading isotherms of β-LG, DOPC and their mixtures on both subphases and at two different temperatures are shown in Figs. 1a and b.

The spreading isotherms are determined at 15, 20, 25 and 30 °C, but in Fig. 1 only those obtained at 15 and 30 °C are reported, which correspond to the minimum and the maximum temperatures examined, to better distinguish the single isotherms. The areas are reported as m^2/mg for β-LG, since in the case of macromolecular compounds, and especially, in the case of proteins, the molecular weight is not determined with sufficient accuracy [7]. For homogeneity, the same measurement units are used in the case of lipid.

One-component monolayers

β-lactoglobulin

The behavior of β-LG isotherms is consistent with that previously reported [3]. Figures 1a, b shows that β-LG monolayers are temperature and subphase dependent. Temperature variation shows regular thermal behavior of monomolecular films. The limiting area values (A_0) obtained by extrapolating the linear portion at the higher surface pressure of the spreading isotherms π–A increase at higher temperatures and the monolayer reaches a more expanded phase. The observation was confirmed by the values of the surface compressional modulus:

$$C_s^{-1} = A \cdot \left(\frac{\delta\pi}{\delta A}\right) T ,$$

where A is the molecular area in the film, which decreases with the increase of temperature, and all C_s^{-1} values are in the range of liquid-expanded phase [8] (see Table 1). The collapse pressure of β-LG decrease with the increase of temperature, as usually reported in the literature, either for high or low molecular weight substances [9].

The comparison of the parameters found for monolayers on both subphases allows us to draw the following conclusion: monolayers on the subphase containing Ca^{++} ions are sensitive to the condensing effect of these ions, as it is evident from the low limiting area and C_s^{-1} values which are slightly higher (Table 1).

The prevalent form of β-LG, obtained from $CHCl_3$ solution, which is a well-known solvent inducing the helix form, at W/A interface appears to be the α-helix form, in agreement with that reported by Loeb [10]; on the contrary, the β-sheet form is predominant in aqueous solution.

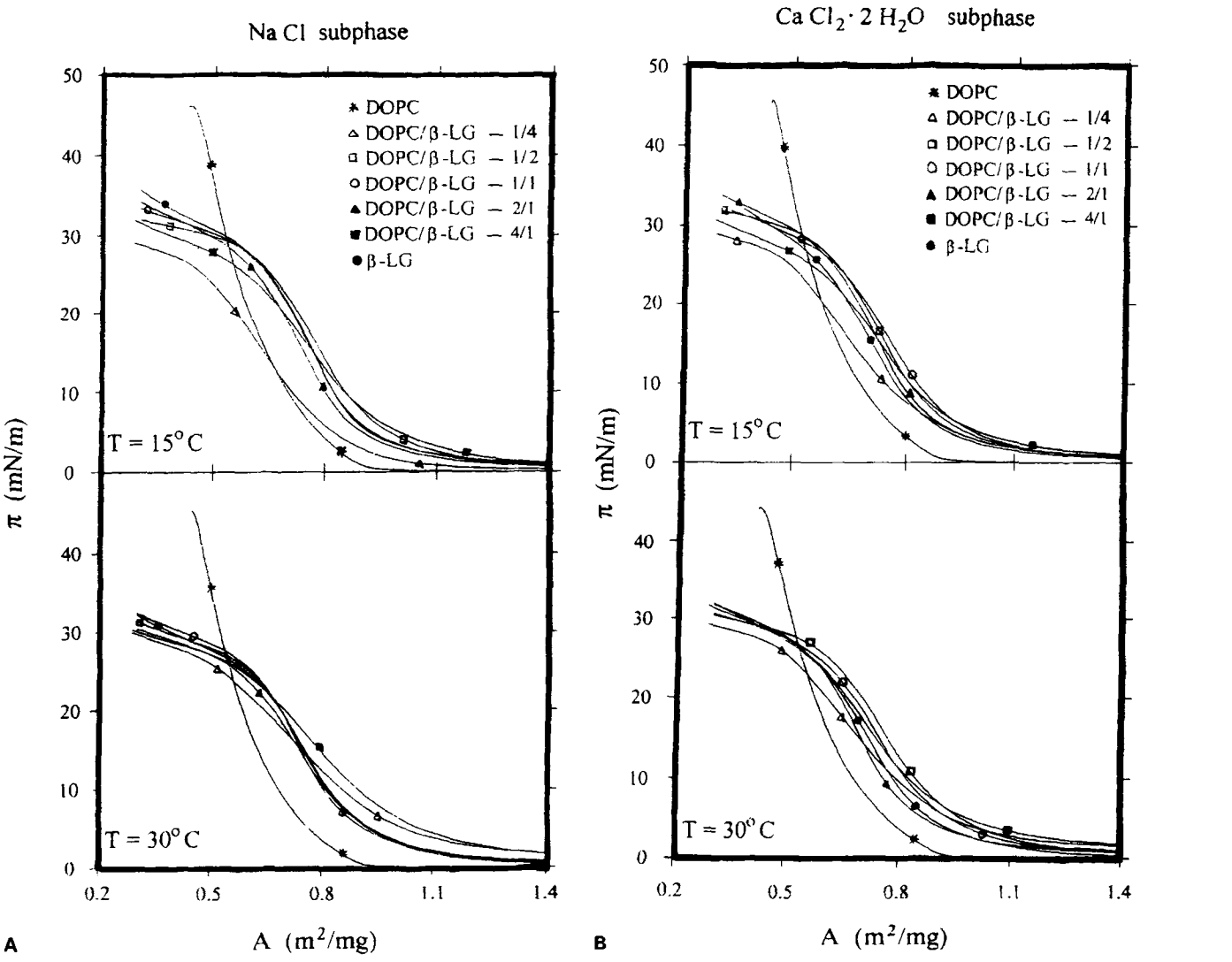

Fig. 1A Spreading isotherms of DOPC/β-LG system on 0.1 M NaCl (pH = 5.6) subphase at 15° and 30 °C. **B** Spreading isotherms of DOPC/β-LG system on 0.03 M $CaCl_2 \cdot 2H_2O$ (pH = 5.6) subphase at 15° and 30 °C

Table 1 Surface parameters of the two components

Subphase ions	DOPC		β-LG	
	Na^+	Ca^{++}	Na^+	Ca^{++}
A_0^*	91.0 ± 0.5	91.0 ± 0.5	18.0 ± 0.5	16.0 ± 0.5
C_s^{-1}**	98 ± 1	97 ± 1	35 ± 1	42 ± 1

* Values of DOPC are in A^2/molecule and of β-LG are in A^2/molecular residues.
** All values are expressed in mN/m.

This is proved by the two following aspects:

a) the form of the collapse of β-LG, which is represented by a plateau in the isotherm π–A, indicates, as previously reported [3, 11], that the prevalent form at the surface is the α-helix;

b) the spectroscopic analysis of FTIR-ATR spectra previously reported and discussed [3] are also in agreement with results reported by Loeb [10].

DOPC

The isotherms of DOPC monolayers are in satisfactory agreement with those previously reported [12, 13] and correspond to films in expanded liquid phase. The values of the limiting areas and of C_s^{-1} are reported in Table 1. The values of limiting areas on both subphases show a interface orientation of hydrocarbon chains different

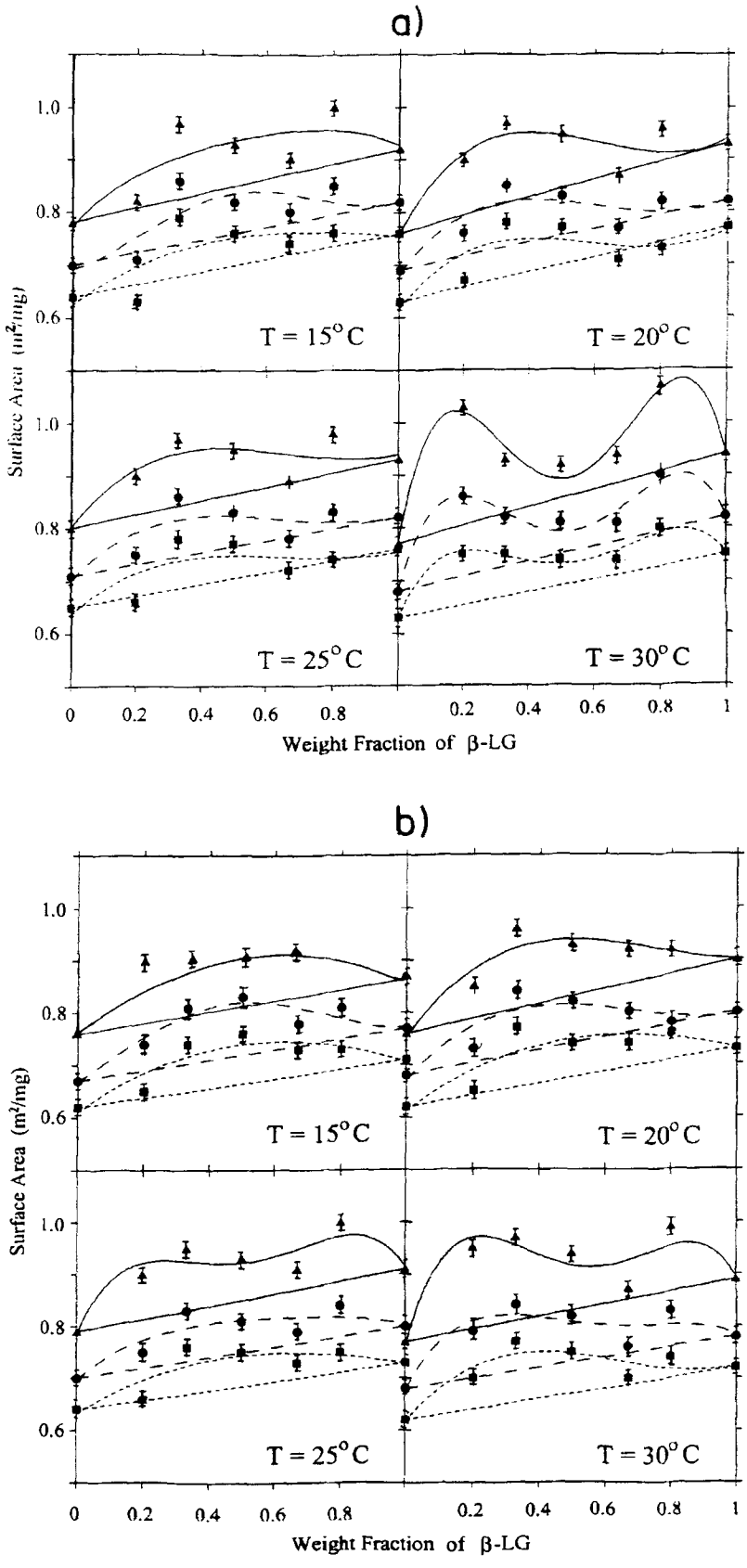

Fig. 2a Surface area vs β-LG weight fraction for the DOPC/β-LG system on 0.1 M NaCl subphase at different temperatures for: $\pi = 5$ mN/m (◆), $\pi = 10$ mN/m (●) and $\pi = 15$ mN/m (■). **b** Surface area vs β-LG weight fraction for the DOPC/β-LG system on 0.03 M $CaCl_2 \cdot 2H_2O$ subphase at different temperatures for: $\pi = 5$ mN/m (◆), $\pi = 10$ mN/m (●) and $\pi = 15$ mN/m (■)

from that previously found in the case of phospholipids without double bonds [14]. The π_c decreases when the temperature increases like in the case of β-LG.

Mixed monolayers

The π–A isotherms of mixed DOPC/β-LG monolayers at different temperatures 15, 20, 25, 30 °C were determined for different weight fractions. All these curves show a change with temperature and subphase.

In Figs. 2a and b the surface areas are reported as a function of the mixture composition at constant surface pressure for various temperatures and for both subphases. The mixed monolayers of β-LG and DOPC show nonideal behavior of the subphases studied. The deviation from ideality is indicative of bidimensional miscibility of the two components at W/A interface. In addition, the miscibility was confirmed by collapse-pressure values, which vary with the composition of mixtures [9]. Generally, the mixtures of DOPC and β-LG show positive deviations from additivity. Figures 2a and b show the behavior of area in m^2/mg as a function of composition in weight fractions, so these diagrams should be considered as representative, because the measurement units used are consistent with each other, as indicated by other authors [7]. To confirm the behavior of the mixtures, the study has been performed at 20 °C, for the spectroscopic and thermodynamic measurements. In Figs. 3a and b the surface moduli of compressibility (C_s^{-1}) in mN/m, for the pure components and their mixtures, are reported.

As it is clearly shown, the behavior is not additive and the values are of the same order of magnitude of the more expanded phase for all mixtures.

Therefore, the miscibility of the two components is confirmed and the prevalent interaction appears to be repulsive. To validate this result, we choose the mixture that presented the maximum positive deviation of the areas as a function of the composition: this mixture corresponds to the molar ratio 0.777 β-LG/0.223 DOPC (corresponding to about 1 β-LG 2 DOPC mixture in weight fraction). To obtain this previous ratio in moles and the areas in molar areas, we considered the mean molar mass of the residues in the case of β-LG, as it was suggested by Barnes [7] and as it was used previously for this protein [6].

The free surface energy (ΔG_s) values, for the single components and the mixture, obtained from graphic integration of the isotherm π–A (at 20 °C) between $\pi \to 0$ and $\pi = 20$, are reported in Table 2, together with the values of surface thermodynamic mixing function, ΔG_{mix}, ΔH_{mix} and ΔS_{mix}, calculated by using the methods reported by Bacon and Barnes [15]. The mixing surface energy (ΔG_{mix}) is positive at every temperature and on both subphases; this is indicative of the lower thermodynamic stability of the

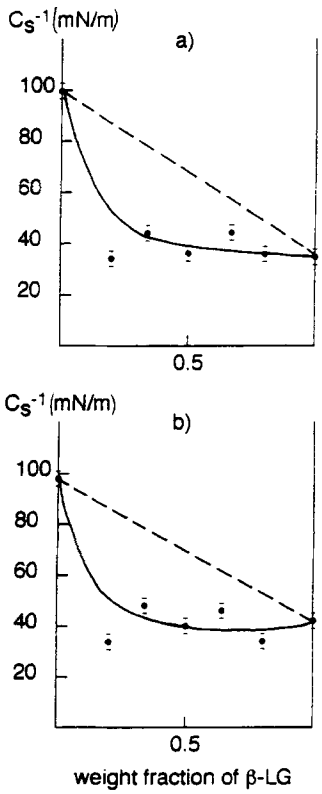

Fig. 3a Behaviour of C_s^{-1} (surface compressional modulus = $A(d\pi/dA)T$) in function of composition on 0.1 M NaCl subphase **b** Behaviour of C_s^{-1} (surface compressional modulus = $A(d\pi/dA)T$) in function of composition on 0.03 M $CaCl_2 \cdot 2H_2O$ subphase

Table 2 Surface thermodynamic functions

Subphase with Na^+			
T (°K)	288	293	298
ΔG_s (DOPC)	11140	11140	11140
ΔG_s (β-LG)	1980	1980	1980
ΔG_s (mixture)	4695	4756	4870
ΔG_{mix}	+ 675	+ 728	+ 770
ΔS_{mix}	—	− 6.2	—
ΔH_{mix}	—	− 1088	—
Subphase with Ca^{++}			
T (°K)	288	293	298
ΔG_s (DOPC)	10840	10840	10990
ΔG_s (β-LG)	1806	1926	1926
ΔG_s (mixture)	4395	4515	4515
ΔG_{mix}	+ 575	+ 590	+ 570
ΔS_{mix}	—	+ 3	—
ΔH_{mix}	—	+ 1470	—

Note: All values of ΔG_s and ΔH are expressed in J/mol.
All values of ΔS_{mix} are expressed in J/mol 0 °K.
All values reported are approximated at 2%.

mixture with respect to the pure components and of the presence of repulsive energies between the components. In the case of the subphase containing Na^+ ions, the values of ΔG_{mix} increase with temperature and, thus, the mixing entropy is negative, demonstrating that in this case the miscibility is entropically unfavorable. Moreover, the ΔH_{mix} results are negative, indicating the miscibilty is favored by enthalpic factor.

Taking into account that β-LG with phospholipids without double bond is insoluble [2, 3], it is possible to correlate the miscibility with the more favorable conformation of the oleic chain compared to the idrophobic chain of the protein. In case of the subphase containing Ca^{++} ions, the variation of ΔG_{mix} with the temperature is negligible and so the value of ΔS_{mix} reported in Table 2 should be correlated only with the positive entropic contribution of the H_2O molecule on the surface. Since the ΔH_{mix} is positive and unfavorable to the miscibility, this different behavior is probably related to the presence of the Ca^{++} ions in the bidimensional state.

FTIR ATR spectra of pure components

a) *β-Lactoglobulin* β-LG spectra has been reported in a previous paper [3].

b) *DOPC* DOPC plurilayers obtained for deposition on Ge plates from a subphase containing NaCl are reported in Fig. 4. A worse transfer of DOPC monolayers was achieved in the case of $CaCl_2$ subphase. The spectra of monolayer and plurilayers show similar shape.

If we compare these spectra with those previously reported for DMPC, DSPC and DPPC monolayers, we can see significant differences [3, 17, 20]. The decrease in intensity of the C=O stretching band found in previous cases passing from the solid state to the LB film, is no more detected in the spectra of DOPC. In fact, for this compound the intensities of the CH_2 stretching bands at 2850 and 2925 cm^{-1} are lower than that of the C=O stretching band at 1728 cm^{-1}.

In the case of DPPC LB film, the hydrocarbon chains are oriented perpendicularly to the germanium surface with all trans conformation [16]. As a consequence, we can suppose that the orientation of DOPC at the surface is nearly horizontal, compared to DPPC, DMPC and DSPC. Other authors have demonstrated, in the case of oleic acid and DPPC, that the LB depositing process retains the monolayer structure, which is present at the air/water interface [17–19]. Hence, we can conclude that the orientation of DOPC at air/water interface is different from the other lipidic molecules.

By comparing the DOPC spectra of the solid state (Fig. 4) with the LB film spectra (Fig. 5), the presence in the second case of a new band at 1594 cm^{-1} is evident, which cannot be easily assigned [17]. One possibility is that the C=C stretching band that in the solid state is very weak and at higher frequency (1665 cm^{-1}), as is detected also in the case of oleic acid [20], could be lowered in frequency

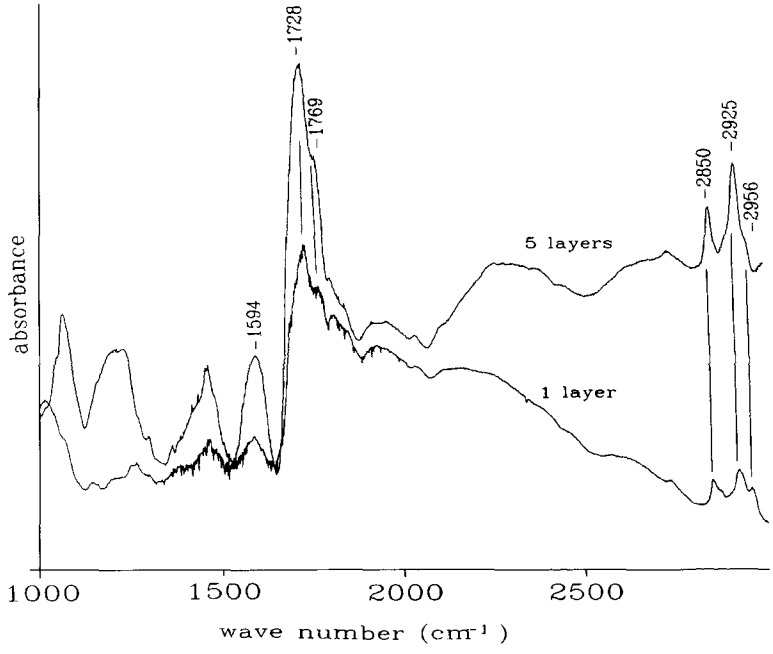

Fig. 4 Monolayers and plurilayers ATR-FTIR spectra of DOPC

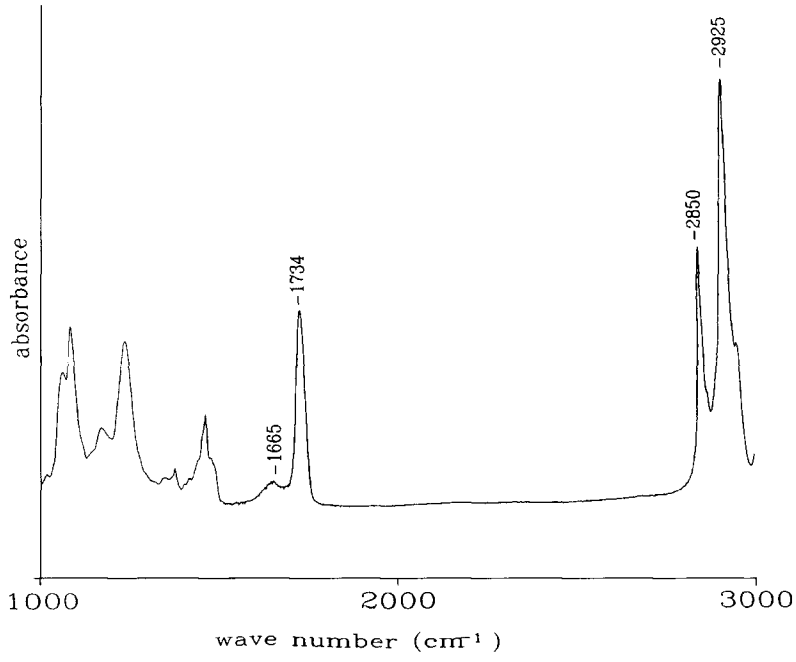

Fig. 5 Solid state spectrum of DOPC

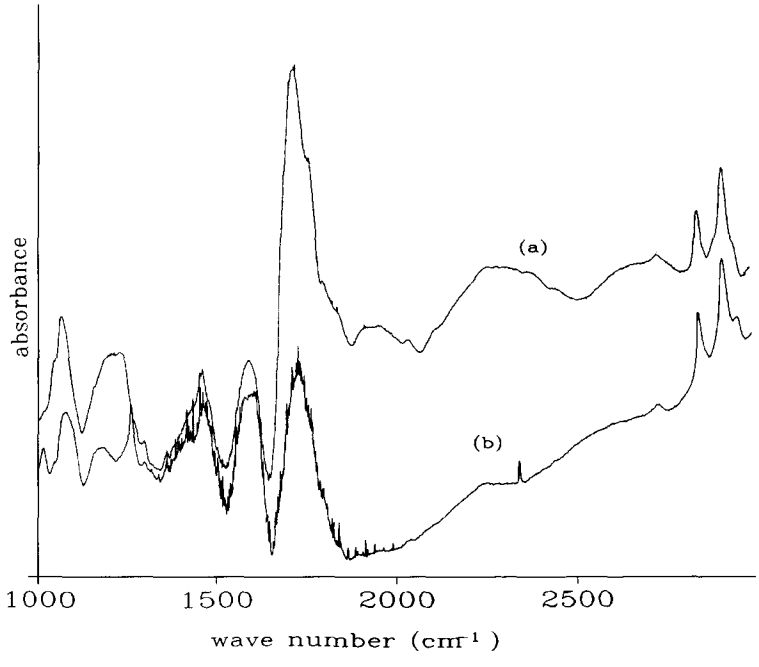

Fig. 6 ATR–FTIR spectra: **a** 5 LB layers of DOPC; **b** 5 LB layers of mixture

and increased in intensity in the LB films due to the interaction with the germanium surface.

c) *Mixture* In Fig. 6 the spectrum of DOPC and of the mixture LB films is reported for comparison [21, 22]. The mixture monolayers have been transferred on Ge plates from a subphase of $CaCl_2$, (poorly reliable results have been obtained by using NaCl subphase).

The spectra a and b of Fig. 6 are quite different and this could be indicative that not only the DOPC has been

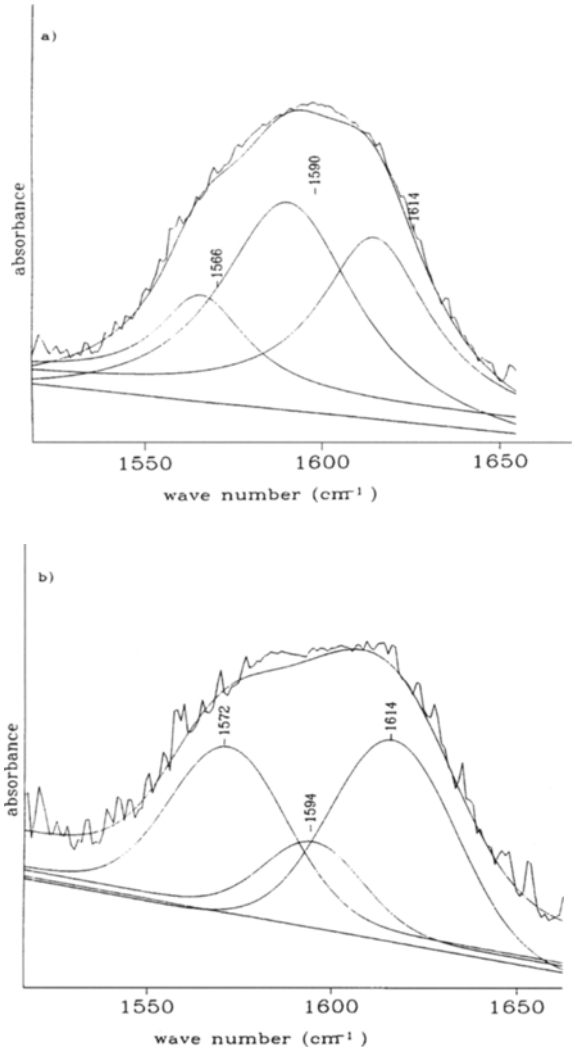

Fig. 7 Deconvolution spectra: **A** band at 1594 cm^{-1} of DOPC LB layers; **B** band at 1594 cm^{-1} of the mixture LB layers

transferred on the surface, but both components, according to bidimensional miscibility as discussed previously.

To better resolve the structure of the large band at 1594 cm^{-1}, the deconvolution technique has been applied, like it has been reported from many authors in the case of protein spectra [23] (Figs. 7a and b). By comparing the spectra a and b in the case of the mixture spectrum, an increase in intensity of the lower-frequency component is shown, probably due to the presence of the amide II band of β-LG for which a frequency of 1530 cm^{-1} has been reported in a previous paper [3].

Conclusions

The previous results allow to conclude the following:

1) The different miscibility of β-LG with phospholipids is clearly demonstrated. β-LG is miscible at the W/A interface with DOPC, whereas it is immiscible with DSPC and DMPC. Its miscibility with prevalently repulsive interactions is clearly demonstrated by the behavior of its surface areas and the values of π_c as functions of composition.

2) ΔG_{mix} results are positive for the more representative mixture, in subphase containing either Ca^{++} or Na^{+} ions, and the enthalpic and entropic contributions are dependent on the ions present in the subphase.

3) The results of the spectroscopic experiments indicate a DOPC disposition at the interface almost horizontal and therefore favorable for miscibility with the protein.

Thus, the previous results appear useful to prepare organized mixed systems as LB sovramolecular structures constituted by lipids and proteins.

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