Miscibility of Hepatitis A Synthetic Antigen Peptides with Lipid Monolayers: Effect of the Amino Acid Sequence Change

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Interaction of hepatitis A virus (HAV) with cells during infection and immunological response is not yet well-known. It seems that hydrophobic and electrostatic interactions with the membrane lipids play an important role as well as the RGD triplet located within the (110−121) linear HAV epitope which belongs to the HAV−VP3 structural protein. To obtain more knowledge about these interactions in the present work we have studied the miscibility of two HAV peptides (where the RGD triplet was replaced by RGE and RKD) with three different lipids (dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, and stearylamine) through compression isotherms. Both peptides [Lys][113] VP3110 and [Glu][114] VP3110 showed deviations from ideality consistent with miscibility, except in some cases at the collapse pressure. In all cases the interactions were higher with [Glu][114] VP3110. However, these interactions were positive in the majority of cases and then not related with the net charge of the lipid that was different in any case. These results seems to point to the fact that hydrophobic interactions play a major role, even though the compressibility of the peptide should also to be taken into account.

Introduction

Acute viral hepatitis is one of the most common infectious diseases, and hepatitis A is the most common form of acute viral hepatitis in the world.[1] The hepatitis A virus (HAV) is a small, nonenveloped, spherical particle composed of four structural proteins: VP1, VP2, VP3, and VP4. The penetration of HAV in the host cell remains ill-defined, the liver being the only target organ of injury. HAV replication occurs exclusively in the cytoplasm of the infected hepatocyte.

As we previously have shown, the highly conserved RGD tripeptide located within the (110−121) linear HAV epitope which belongs to the HAV−VP3 structural protein is an important determinant for the interaction with convalescent sera. In our hands, several synthetic peptide analogues that were obtained by changing the amino acids of the RGD tripeptide failed to react with the sera.[2] On the other hand, our results support the tendency of the VP3(110−121) peptide to adopt a β structure in the presence of model membranes such as liposomes. From our previous results we could also conclude that the interactions between the native peptide and lipids is not only of electrostatic nature. It was more likely to think that some interaction occurs by penetration of the hydrophobic side chains into the hydrocarbon region of the bilayer.

Information about the physicochemical membrane lipids or peptide behavior can be useful to elucidate a wide range of biologically relevant questions. For example, the comprehension of the interaction between antigenic surface-active peptides with model phospholipidic membranes has been the subject of many of our recent studies[3,4] and that of other authors studying other viruses.[5,6] These sorts of studies have been mainly carried out to gain insight into the infection and proliferation mechanism of the hepatitis A virus and also to investigate the use of peptide-loaded liposome formulations for targeting.

The main aim of the present work is to experimentally test the effect on the membrane−peptide interaction of the replacement of the RGD tripeptide by RGE ([Glu][114] VP3110) or RKD ([Lys][113] VP3110) sequences having as result, respectively, a global negatively charged and a neutral sequence. These studies were carried out evaluating the miscibility of both peptides with lipids of different net charge through the performance of compression isotherms.

Experimental Section

Chemicals. The synthesis of the peptides, whose structures are the following, is described elsewhere.[7]

[Glu][114] VP3(110−121): FW**R**G**E**LVF**D**FQV

[Lys][113] VP3(110−121): FW**RKD**LVF**D**FQV

Positively charged amino acids at neutral pH are shown in bold. Those negatively charged are shown in bold and underlined.

DPPC (dipalmitoyl phosphatidylcholine), DPPG (dipalmitoyl phosphatidylglycerol), and SA (stearylamine) were provided by Sigma. Bidistilled water was deionized with a MilliQ system (Millipore Corp.); resistivity 18.2 MΩ cm. Chloroform and methanol were from Merck. DMSO (dimethyl sulfoxide) ACS reagent was from Sigma.

The subphase was phosphate-buffered saline (PBS), pH 7.4 (0.017 M $NaH_2PO_4 \cdot 2\ H_2O$, 0.081 M $Na_2HPO_4 \cdot 12\ H_2O$, 0.05 M NaCl). All the buffer components were provided by Merck.

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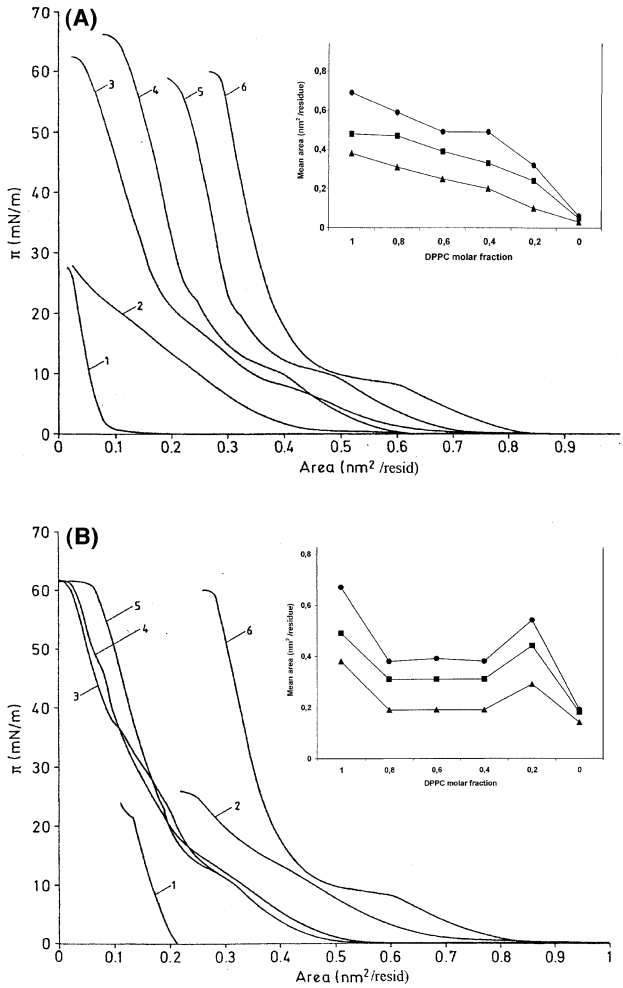

Figure 1. Compression isotherms of mixed monolayers (area in nm^2/residue). (a) DPPC/[Lys]^{113} VP3110. Pure peptide: 2, $X_p = 0.8$; 3, $X_p = 0.6$; 4, $X_p = 0.4$; 5, $X_p = 0.2$; 6, DPPC. (Inset) Mean area/residue versus lipid molar fraction. ● 5 mN/m; ■ 10 mN/m; ▲ 20 mN/m. (b) DPPC/[Glu]^{114} VP3110. Pure peptide: 2, $X_p = 0.8$; 3, $X_p = 0.6$; 4, $X_p = 0.4$; 5, $X_p = 0.2$; 6, DPPC. (Inset) Mean area/residue versus lipid molar fraction: ● 5 mN/m; ■ 10 mN/m; ▲ 20 mN/m.

Methods

Measurements were performed on a Langmuir film balance, KSV5000, equipped with a Wilhelmy platinum plate. Compression isotherms were performed in a Teflon trough (surface area 17 000 mm^2, volume 1000 mL). DPPC and DPPG were dissolved in chloroform (concentration 1.6×10^{-3} M). SA solutions were prepared at the same concentration in a chloroform/methanol mixture (3:1). Both peptides were dissolved in DMSO (concentration 6.2×10^{-4} M). 25 μL of the former solutions and their mixtures were spread onto the clean surface of the PBS subphase using a Hamilton microsyringe. After allowing 15 min for stabilization, films were compressed (symmetrical compression) with an area reduction rate of 60 mm^2/min. Each run was repeated three times, and the accuracy of the measurements was ± 0.01 nm^2/residue.

All the experiments were carried out at a temperature of 294 ± 1 K.

Results

Pressure−Area Isotherms for DPPC Mixed Monolayers. Figure 1 (a−b) shows the pressure−area isotherms for mixed monolayers of DPPC with [Lys]^{113} VP3110 or [Glu]^{114} VP3110. In [Lys]^{113} VP3110 mixtures, it can be clearly seen that when the proportion of peptide increases, the pressure of the phase transition changes and gradually disappears.

In [Glu]^{114} VP3110 mixed monolayers, the behavior is quite different. The addition of a small quantity of peptide (0.2 peptide/0.8 DPPC) produces important changes in the area/residue values and in the phase change pressure. The following mixtures ($X_p = 0.4$ and 0.6) have a very similar shape, followed by another sharp change when $X_p = 0.8$. Here the phase change disappears and the collapse pressure is strongly reduced. However, in both peptides when $X_p = 0.8$ the shape of the isotherm is totally different from the previous ones and more similar to that of the pure peptide. This fact could be due to the squeezing-out of DPPC from the monolayer to the subphase.

The insets in Figure 1 (a−b) show the plot of area/residue versus DPPC molar fraction for both mixtures. They represent nonlinearity. In [Lys]^{113} VP3110 monolayers small positive deviations were observed, implying the establishment of repulsive forces between the peptide and DPPC. [Glu]^{114} VP3110 mixed monolayers present small negative deviations except for the 0.8 peptide/0.2 DPPC mixture that is positive and corresponds with a sharp change in the shape of the isotherm.

These results were also confirmed by the determination of the limit area (A_{lim}). A_{lim} values were determined as the intercept on the *x* axis of the tangent straight line to the isotherm in the most condensed region of the monolayer.[8] Values in Figure 2 for [Lys]^{113} VP3110/DPPC monolayers are larger than the ideal values, especially when the molar fraction of peptide in the monolayers is high. [Glu]^{114} VP3110/DPPC mixtures present a condensing effect when the amount of peptide in the monolayer is low. When $X_p = 0.8$, repulsive interactions are established with A_{lim} values higher than the ideal.

Deviations from ideality were quantified calculating ΔG_M^{EX} and ΔH[9−11] following equations 1, 2, and 3, where $A_{1,2}$ is the mean area per residue in the mixed film, A_1 and A_2 are the areas per residue in the pure films, and π is the surface pressure in mN/m. Numerical data were transferred from the informatic program of the Langmuir balance to another program that calculates the area under the isotherm at a fixed pressure according to the mathematical method of Simpson. These results were transferred to a calculus page of Excel2000 (Microsoft Corporation) to undertake the other calculations.

$$\Delta G_M^{EX} = \int_{\pi\to 0}^{\pi} A_{1,2}\, d\pi - X_1 \int_{\pi\to 0}^{\pi} A_1\, d\pi - X_2 \int_{\pi\to 0}^{\pi} A_2\, d\pi \quad (1)$$

$$\alpha = \frac{\Delta G_M^{EX}}{RT(X_1X_2^2 + X_1^2X_2)} \quad (2)$$

$$\Delta H = \frac{RT\alpha}{Z} \quad (3)$$

To calculate the coordination number (*Z*) the model of Quikenden and Tam was followed,[12] considering that in a closely packed monolayer (collapse), each molecule is surrounded by six neighbors. For lower pressures, eq 4 was applied to calculate the packing fraction (PF). This value was used to obtain the corresponding *Z* value, according to the equivalence given by Quickenden and Tam, where $A_{c,m}$ is the area/residue of the

TABLE 1: Thermodynamic Values for [Lys]^{113} VP3110/DPPC and [Glu]^{114} VP3110/DPPC Mixed Monolayers

DPPC molar fraction	ΔG_M^{EX} (J/mol)			ΔH (J/mol)		
	5 mN/m	10 mN/m	20 mN/m	5 mN/m	10 mN/m	20 mN/m
			[Lys]^{113} VP3110/DPPC			
0.8	58.18	−325.60	189.12	18.18	−1017.50	591.00
0.6	144.60	−77.80	507.10	301.10	−162.10	1056.50
0.4	163.70	428.30	1162.40	341.10	892.40	2421.70
0.2	142.90	236.60	1177.50			
			[Glu]^{114} VP3110/DPPC			
0.8	−9.52	−454.86	−221.29	−29.70	−1424.40	−691.50
0.6	100.83	−62.88	398.24	210.10	−131.00	829.70
0.4	60.59	−37.70	305.85	126.20	−78.50	637.20
0.2	382.34	638.56	11611.39	1194.8	1995.50	2517.80

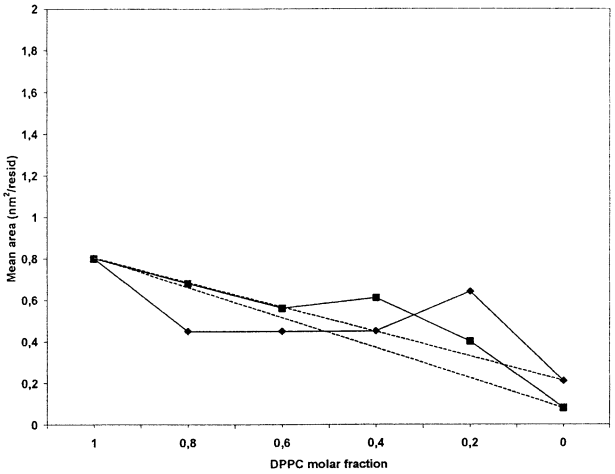

Figure 2. Limiting area versus lipid molar fraction. ■ [Lys]^{113} VP3110, ◆[Glu]^{114} VP3110.

mixture at the collapse point and A_m the area/residue of the mixture at the pressure studied.

$$PF = 0.907 \frac{A_{c,m}}{A_m} \quad (4)$$

In Table 1 we report the thermodynamic values calculated as function of the molar fraction. In general, ΔG_M^{Ex} values found are not particularly high (< RT), implying that even though interactions are established between the peptides and DPPC, they are not very strong. In some mixtures ΔH could not be calculated because the isotherms did not reach the collapse.

Higher ΔG_M^{Ex} were those of a proportion 0.8:0.2 of [Glu]^{114} VP3110/DPPC, as was expected from the results found before. At 10 mN/m (corresponding to the phase change pressure) negative values are obtained for 0.2 and 0.4 peptide molar fraction for both peptides, thus suggesting that some kind of rearrangement in the monolayer takes place. Another approach to determine in binary systems if the components of the mixed monolayer are or not miscible consists of the application of the two-dimensional phase rule[13] of Crisp. It can be observed in Figure 3 that the transition from liquid expanded to condensed liquid varies with the monolayer composition for both mixtures of the two peptides with DPPC. This fact implies that in order to define the system correctly, one variable has to be fixed (composition or surface pressure), then the degree of freedom of the system has to be $L = 1$. Applying Crisp's phase rule, $L = C - F + 1$, where C is the number of components in the interphase (DPPC and peptide), then it results that there have to be two phases in equilibrium, one formed by both components in expanded liquid and another phase consisting of both components in condensed liquid.

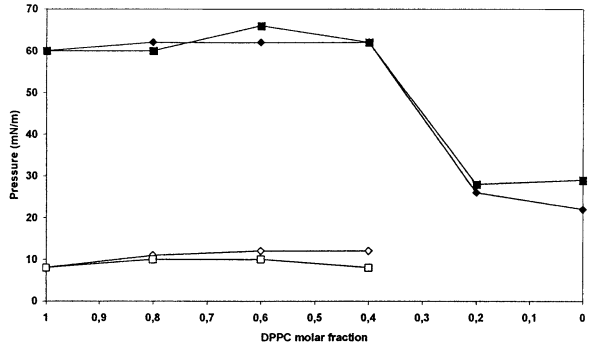

Figure 3. Pressure values at the phase change versus lipid molar fraction (□[Lys]^{113} VP3110, ◇ [Glu]^{114} VP3110) and at the collapse (■ [Lys]^{113} VP3110, ◆ [Glu]^{114} VP3110).

Applying the same rule to the collapse pressure, it can be observed that in [Glu]^{114} VP3110 mixtures this pressure changes with the molar fraction for DPPC < 0.4, thus implying one degree of freedom and then two phases in equilibrium, one composed by the monolayer in liquid condensed and the other where the mixed monolayer is collapsed. When DPPC molar fraction > 0.4, collapse pressure can be considered constant, the degree of freedom being $L = 0$, suggesting the existence of three phases in equilibrium and immiscibles (the mixture peptide−DPPC in condensed liquid state, DPPC in excess in condensed liquid, and the mixture peptide−DPPC collapsed). In the mixtures composed of DPPC and [Lys]^{113} VP3110, collapse pressure changes in all the range of molar fractions, thus implying that $L = 1$ and $F = 2$, so there are two phases in equilibrium, one composed by both components miscible in condensed liquid and the other by the mixed monolayer in the collapsed state.

Pressure−Area Isotherms for DPPG Mixed Monolayers. In Figure 4 a−b the isotherms of mixed monolayers of both peptides with DPPG are represented. [Lys]^{113} VP3110 mixtures (except [Lys]^{113} VP3110/DPPG (0.8:0.2)) present a shape very similar to DPPG, although area/residue ratios are lower and modifications in the phase transition are present. It is particularly interesting the shape of [Lys]^{113} VP3110/DPPG (0.8:0.2) monolayer, which is completely different from the other monolayers, as was observed before with DPPC.

In [Glu]^{114} VP3110/DPPG monolayers the behavior is very erratic. The progressive increase of peptide in the monolayer produces monolayers more expanded and the phase transition of DPPG disappears. However, when $X_p = 0.8$ the area/residue diminishes considerably, which is consistent with a compressing effect. Insets of Figure 4 show the values of area/residue versus lipid molar fraction. In [Lys]^{113} VP3110 mixed monolayers, negative deviations can be observed for monolayers with low peptide molar fraction. When the molar fraction of peptide in the monolayer is greater than 0.4, deviations are positive. It

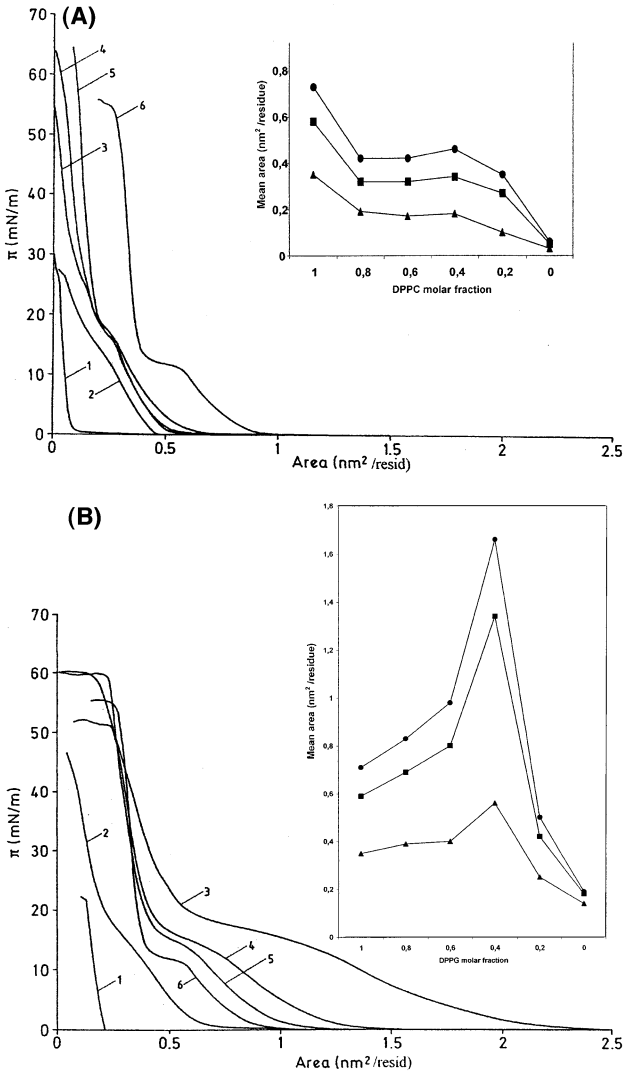

Figure 4. Compression isotherms of mixed monolayers (area in nm^2/residue). (a) DPPG/[Lys][113] VP3110. Pure peptide: 2, $X_p = 0.8$; 3, $X_p = 0.6$; 4, $X_p = 0.4$; 5, $X_p = 0.2$; 6, DPPC. (Inset) Mean area/residue versus lipid molar fraction: ● 5 mN/m; ■ 10 mN/m; ▲ 20 mN/m. (b) DPPG/[Glu][114] VP3110. Pure peptide: 2, $X_p = 0.8$; 3, $X_p = 0.6$; 4, $X_p = 0.4$; 5, $X_p = 0.2$; 6, DPPC. (Inset) Mean area/residue versus lipid molar fraction: ● 5 mN/m, ■ 10 mN/m, ▲ 20 mN/m.

seems that for low peptide concentrations attractive interactions are established between the components of the monolayer; however, when the amount of the peptide increases the interactions become repulsive.

[Glu][114] VP3110/DPPG monolayers show in all cases positive deviations, particularly when the molar fraction of peptide is 0.6. This is not surprising considering that the net charge of the peptide is negative as DPPG.

Values of A_{lim} (Figure 5) show more clearly the behavior of both peptides commented above. Thermodynamic values (Table 2) present differences for both peptides. They are very low for [Lys][113] VP3110/DPPG mixtures compared with [Glu][114] VP3110/DPPG, these last ones being > RT in some cases. In all, it is very shocking that major repulsive interactions are found in [Glu][114] VP3110/DPPG (0.6/0.4) mixtures instead of 0.8 peptide molar fraction. This finding could be due to a sharp reorganization of the monolayer resulting from the high amount of peptide present that could also lead to the expulsion of DPPG molecules from the monolayer through compression.

The application of the phase rule to these mixtures reveals that at the change phase the behavior is quite similar to the one

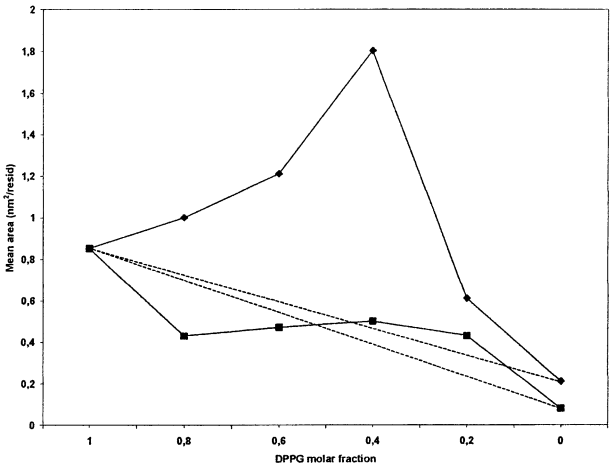

Figure 5. Limiting area versus lipid molar fraction: ■ [Lys][113] VP3110, ◆ [Glu][114] VP3110.

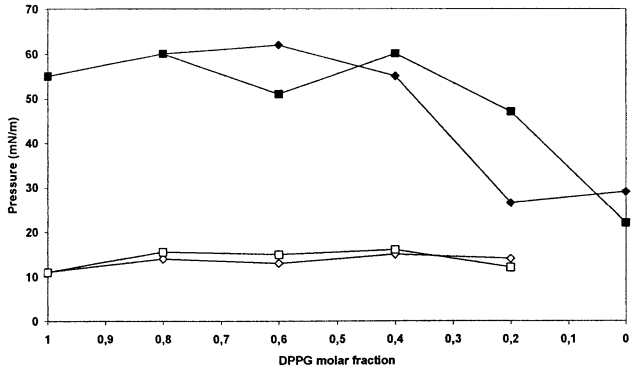

Figure 6. Pressure values at the phase change versus lipid molar fraction (□ [Lys][113] VP3110, ◇ [Glu][114] VP3110) and at the collapse (■ [Lys][113] VP3110, ◆ [Glu][114] VP3110).

TABLE 2: Thermodynamic Values for [Lys][113] VP3110/DPPG and [Glu][114] VP3110/DPPG Mixed Monolayers

DPPG molar fraction	ΔG_M^{EX} (J/mol)			ΔH (J/mol)		
	5 mN/m	10 mN/m	20 mN/m	5 mN/m	10 mN/m	20 mN/m
			[Lys]113 VP3110/DPPG			
0.8	−101.0	−436.3	−1562.4	−315.5	−1363.5	−4882.8
0.6	87.5	−154.6	132.2	182.4	−322.2	275.6
0.4	202.1	379.9	1127.7	421.1	791.5	2349.5
0.2	64.3	134.5	1214.9			
			[Glu][114] VP3110/DPPG			
0.8	168.8	357.5	1478.8	527.8	1117.3	4621.5
0.6	314.8	789.4	3118.5	656.0	1645.3	6497.1
0.4	983.6	2087.4	8578.6	2049.3	4348.9	17872.2
0.2	403.3	601.9	1840.3	1260.3	1881.1	5751.2

observed with DPPC, which correlates with a pattern of miscibility. However, at the collapse region the results are different. As can be observed in Figure 6, the collapse pressure is different when the composition of the monolayer changes. This finding implies that $L = 1$, then there are two phases in equilibrium, one composed of the miscibles from both components in condensed liquid and another formed by the monolayer collapsed.

Pressure−Area Isotherms for SA Mixed Monolayers. As can be observed in Figure 7a, mixtures of [Lys][113] VP3110/SA show, for 0.2−0.6 peptide molar fractions, isotherms with high collapse pressures and phase changes at different levels. The 0.6 peptide molar fraction monolayer especially shows two phase transitions, thus being indicative of reorganizations of the monolayer's components.

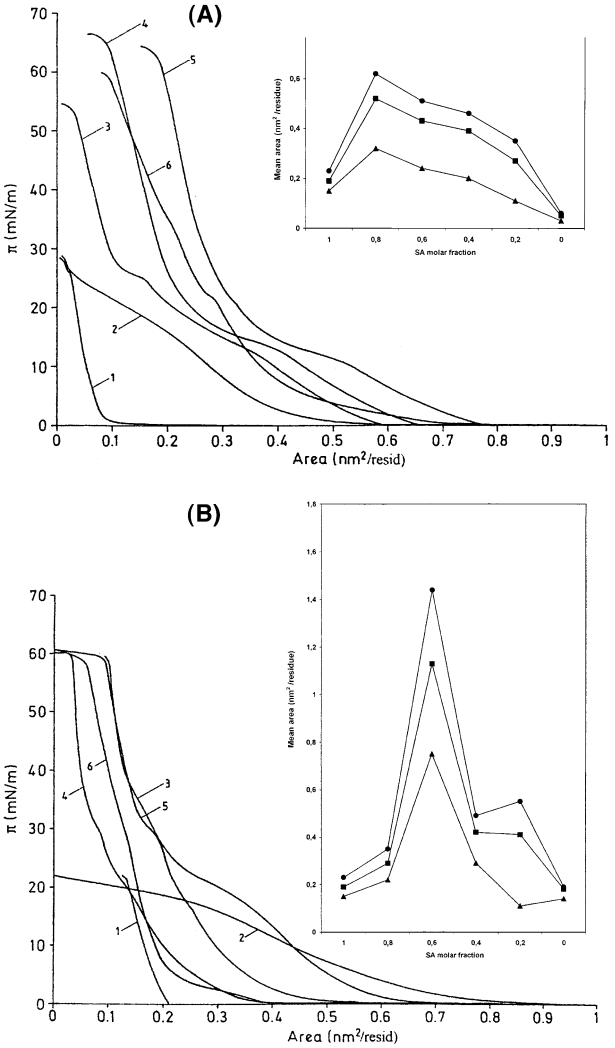

Figure 7. Compression isotherms of mixed monolayers (area in nm^2/residue). (a) SA/[Lys]^{113} VP3110. Pure peptide: 2, $X_p = 0.8$; 3, $X_p = 0.6$; 4, $X_p = 0.4$; 5, $X_p = 0.2$; 6, DPPC. (Inset) Mean area/residue versus lipid molar fraction: ● 5 mN/m, ■ 10 mN/m; ▲ 20 mN/m. (b) SA/[Glu]^{114} VP3110. Pure peptide: 2, $X_p = 0.8$; 3, $X_p = 0.6$; 4, $X_p = 0.4$; 5, $X_p = 0.2$; 6, DPPC. (Inset) Mean area/residue versus lipid molar fraction: ● 5 mN/m, ■ 10 mN/m, ▲ 20 mN/m. (b) SA/[Glu]^{114} VP3110.

Similarly to the other cases described before, the 0.8 peptide molar fraction monolayer has a very different shape. In [Glu]^{114} VP3110/SA monolayers (Figure 7b) the shape of the isotherm changes drastically at X_p of 0.8. The monolayers are very expanded and the collapse was not reached through compression. Positive deviations from additivity are found for all the monolayers (insets Figure 7), with the [Glu]^{114} VP3110/SA (0.4/0.6) monolayer showing the higher values. However, A_{lim} (Figure 8) confirms the findings described for [Lys]^{113} VP3110/SA mixtures but not for [Glu]^{114} VP3110 that at X_p 0.2−0.4 deviations are nil or slightly negative. ΔG_M^{Ex} and ΔH values show important interactions for [Glu]^{114} VP3110/SA monolayers, with values very high and >RT in the majority of cases (Table 3).

When the rule phase was applied to these mixtures, they showed the same results as DPPC. Both components are miscible at the phase transition, but at the collapse region there is a pattern of inmiscibility in [Glu]^{114} VP3110/SA mixtures (Figure 9) when $X_p < 0.6$. Therefore, even though some slight differences are present between the peptides, both of them show a similar pattern of behavior in front of the monolayers assayed; this fact has been summarized in Table 4.

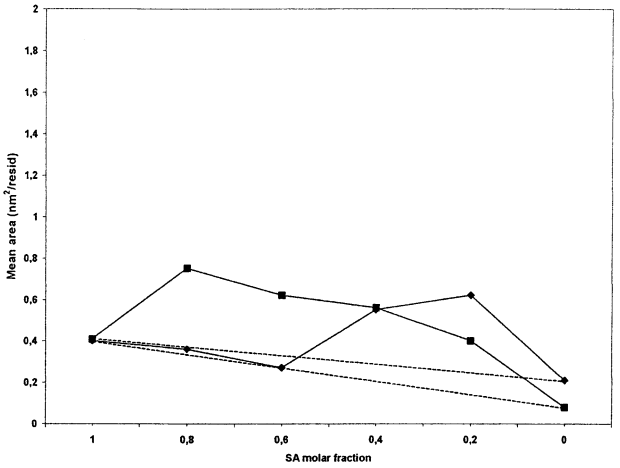

Figure 8. Limiting area versus lipid molar fraction: ■ [Lys]^{113} VP3110, ◆ [Glu]^{114} VP3110.

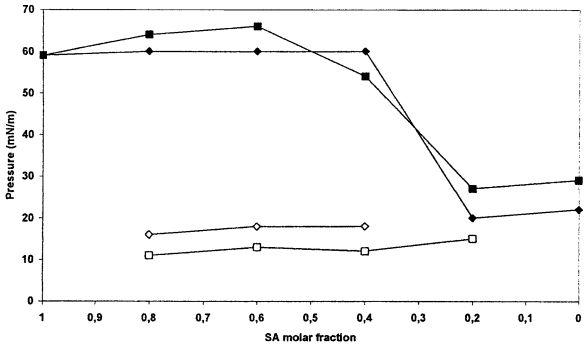

Figure 9. Pressure values at the phase change versus lipid molar fraction ([Lys]^{113} VP3110, ◇[Glu]^{114} VP3110) and at the collapse (■ [Lys]^{113} VP3110, ◆ [Glu]^{114} VP3110).

TABLE 3: Thermodynamic Values for [Lys]^{114} VP3110/SA and [Glu]^{114} VP3110/SA Mixed Monolayers

SA molar fraction	ΔG_M^{EX} (J/mol)			ΔH (J/mol)		
	5 mN/m	10 mN/m	20 mN/m	5 mN/m	10 mN/m	20 mN/m
	[Glu]^{114} VP3110/SA					
0.8	42.9	349.5	1909.5	434.2	587.5	1229.3
0.6	18.7	299.2	1870.6	131.0	1092.4	7307.0
0.4	52.8	282.0	1770.8	110.2	587.5	6829.3
0.2	277.9	577.3	3278.0			
	[Glu]^{114} VP3110/SA					
0.8	47.1	164.0	422.0	141.2	512.7	1319.0
0.6	1270.2	2527.7	5660.1	2646.4	5266.1	11792.0
0.4	201.7	410.8	1395.1	420.4	856.0	2906.6
0.2	503.3	1100.1	3694.3	1579.2	3438.0	3847.9

Discussion

The degree of interaction of [Lys]^{113} VP3110 with the lipids assayed is lower than that of [Glu]^{114} VP3110; its miscibility is better, especially at the collapse pressure. The fact that [Lys]^{113} VP3110 is neutral at pH 7.4 could account for its more regular behavior, probably due to the fact that electrostatic interactions are probably very low.

In some of the series studied, the shape of the isotherm corresponding to $X_p = 0.8$ changes drastically or the area/residue values are quite different, indicating as we stated before that part of the lipid is ejected to the subphase during compression or maybe the presence of high quantities of peptide completely changes the monolayer structure. It can be concluded that the differences between these peptides do not modify the fact that when the amount of peptide in the monolayer is high the

TABLE 4: Comparative Results for [Lys][113] VP3110 or [Glu][114] VP3110 Mixed Monolayers with DPPC, DPPG, or SA

		DPPC	DPPG	SA
deviations from additivity rule	[Lys][113] VP3110	small to moderate positive or negative	small to moderate positive or negative	small to moderate positive
	[Glu][114] VP3110	small to moderate positive or negative	larger positive deviations, especially at peptide/DPPG (0.6/0.4)	Larger positive deviations, epecially at peptide/SA (0.4/0.6)
Crisp's phase rule	[Lys][113] VP3110	miscible	miscible	miscible
	[Glu][114] VP3110	not miscible at the collapse	miscible	miscible

composition is not stable through compression. Moreover, the differences in the lipids do not influence this behavior either, as has also been found with other peptides.[14]

The high interactions of [Glu][114] VP3110 with lipids could be in part explained by the presence of a negative net charge at pH 7.4. With an anionic lipid such as DPPG, hydrophobic interactions could be increased by the existence of repulsive electrostatic forces between them; however, they do not alter the degree of miscibility between both components, even at collapse pressure.

Obviously, electrostatic interactions should be also present in the mixtures formed by of [Glu][114] VP3110 and the cationic lipid SA. Even so, these interactions should be of an attractive character; results obtained are not very different from those of DPPG monolayers, the only difference being located on the miscibility pattern at the collapse. It should be supposed that hydrophobic interactions have a more major role than electrostatic interactions.

In addition to these interactions, it has also to be taken into account that the peptide conformation could play an important role. Previous assays carried out by circular dichroism have shown that both peptides, being very small, do not adopt any conformation in the presence of lipids.[7] However, there is an important difference in their compressibility modulus (C^{SS})[15] ($C^{SS} = 49.2$ mN/m for [Glu][114] VP3110 and $C^{SS} = 24$ mN/m for [Lys][113] VP3110). From these values it can be deduced that [Glu][114] VP3110, being more expanded and rigid, presents more opposition to compression, thereby influencing its ability to change its position through compression and the interaction with the lipidic component of the monolayer.

Then it can be concluded that the interactions established are principally hydrophobic with a small contribution of electrostatic interactions. The simple change of an amino acid in a small structure such as the ones assayed produces some changes in the pattern of their interaction with lipids. These changes are probably due to differences in the established electrostatic interactions, but other factors such as rigidity of the peptide structure can be also responsible for this different behavior. All this should be taken into account when studying the interaction of synthetic peptides with cellular membranes.

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