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Study in mono and bilayers of the interaction of hepatitis G virus (GBV-C/HGV) synthetic antigen E2(99-118) with cell membrane phospholipids

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Abstract

The interaction of the hepatitis G synthetic peptide E2(99-118) with cell membrane phospholipids of different characteristics such as dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) was studied by Langmuir isotherms. Epifluorescence microscopy and Atomic force microscopy (AFM) was also used to study interactions with DPPC. Compression isotherms of DPPC/E2(99-118) and DPPG/E2(99-118) mixed monolayers showed negative deviation from ideallity consistent with the existence of attractive interactions. The incorporation of the peptide in DPPC monolayer was also confirmed in epifluorescence microscopy and AFM studies. The peptide retarded the formation of DPPC domains and did not let the phospholipid get organized. No important differences in the interactions with DPPC (neutral) or DPPG (anionic) were found, thus suggesting that electrostatics forces do not have a predominant influence in these interactions.

Keywords: Hepatitis G; Monolayers; Dipalmitoylphosphatidylcholine (DPPC); Dipalmitoylphosphatidylglycerol (DPPG); GBV-C/HGV

1. Introduction

GBV-C/HGV is a bloodborne virus, associated worldwide with non-ABCDE hepatitis. It is the most closely-related human virus to the hepatitis C virus (HCV), a major worldwide pathogen [1]. Recently, a stretch of conserved, hydrophobic amino acids within the envelop glycoprotein of HCV has been proposed as the virus fusion peptide [2], however, the mode of entry of GBV-C/HGV into target cells is at present unknown. An approach to get information about the potential antigenicity/immunogenicity [3] of the peptides is the analysis of their physicochemical properties by using model membranes. The comprehension of the interaction of peptides belonging to relevant protein domains with model phospholipid membranes is important

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to gain insight into the infection and proliferation mechanism of the virus.

In this sense, the main aim of the present work is to study how an antigenic synthetic peptide belonging to E2 structural protein, as is E2(99-118) peptide sequence interacts with a zwiterionic and an anionic phospholipid. The fact that these two lipids had different net charge should let us to detect possible electrostatic interactions and they will serve as an excellent model in addressing potential mechanisms of peptide–lipid interactions.

We employed different techniques, like surface pressure–area, epifluorescence microscopy and atomic force microscopy. All this should give us more insights about the interaction events occurring in biological situations between GBV-C/HGV and cell membranes in the infection process.

2. Materials and methods

The synthesis of the peptide, whose structure follows, VSWFASTGGRDSKIDVWSLV (pI= 5.93) was described elsewhere [4].

Crude peptide was purified by preparative high performance liquid chromatography. Purified peptide was characterized by analytical HPLC, amino acid analysis and electrospray mass spectrometry.

DPPC (L-α-Dipalmitoyl phosphatidylcholine) and DPPG (Dipalmitoyl phosphatidylglycerol) were provided by Sigma. The fluorescent probe 1-palmitoyl-2-{12-[7-nitrobenz-2oxa-1,3-diazo-4-yl) amino] dodecanoyl}-*sn*-glycerol-3phosphocholine (NBD-PC) was obtained from Avanti Polar Lipids Inc., AL, USA. Bidistilled water was deionised with a MilliQ system (Millipore Corp.) having a resistivity of 18.2 M Ωcm. Chloroform and methanol were from Merck. Stock solutions of each compound at 1.0 mg/ml in chloroform:methanol (3:1, v/v) were used to prepare the mixtures. These were stored at −20 °C.

2.1. Compression isotherms

Compression isotherms were performed on a Langmuir film balance KSV5000 equipped with a Wilhelmy platinum plate, with a Teflon trough (surface area 17.000 mm^2, volume 1000 ml).

DPPC, DPPG, peptide and lipid/peptide (with and without the fluorescence probe) mixed monolayers were formed by spreading chloroform/methanol solutions. After 15 min period for evaporation and equilibration, the monolayers were compressed at a rate of 60 mm^2/min. Changes in the compression rate did not alter the shape of the isotherms. Stability of the monolayers was assessed by compressing them and stopping the barrier at different pressures, and observing that no pressure decay occurred after 30 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.

2.2. Epifluorescence microscopy

Fluorescence imaging of the adsorbed surface monolayers containing 1% mol NBD-PC was achieved by exciting the probe at 460 nm and observing the fluorescence emission at 534 nm (green). The Langmuir trough rests on a commercial epifluorescence microscope (Axiovert 10, Carl Zeiss, Jena, Germany) equipped with a charge coupled device (CCD) camera (Sony, Japan), attached to an intensifier (Stardan II, Videoscope International, Japan). The light source used is usually a mercury lamp (100 W). The necessary blue filters were used (in-built set up in the microscope) selected for excitation and emission wavelengths.

Monolayers were viewed with a 35× objective lens with a numerical aperture of 0.4 and a 1× ocular lens of a Zeiss epifluorescence microscope equipped with a 100 W mercury lamp. Images of the monolayers were recorded with the Retiga™ digital camera attached to a Stardancer 2 intensifier and analysed using the Northern Eclipse (VI) imaging software.

2.3. Transfer of Langmuir Blodgett films onto mica

Films of DPPC: peptide (98:1, 96:3, 93:6, 89:10, molar ratio) containing 1 mol.% NBD-PC were compressed to pressures at 7 and 10 m Nm^{-1}. Once the desired pressure was obtained, a waiting

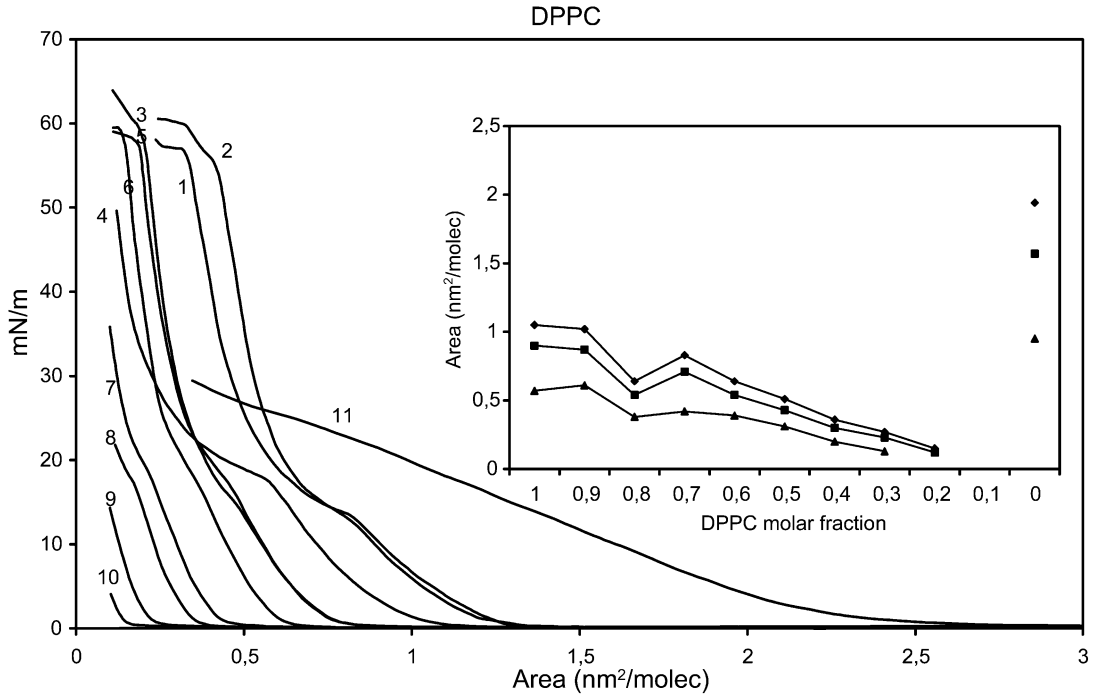


Fig. 1. Surface–pressure compression isotherms for: **1**. DPPC; **2**. DPPC 0.9/E2 (99-118) 0.1; **3**. DPPC 0.8/E2 (99-118) 0.2; **4**. DPPC 0.7/E2 (99-118) 0.3; **5**. DPPC 0.6/E2 (99-118) 0.4; **6.** DPPC 0.5/E2 (99-118) 0.5; **7.** DPPC 0.4/E2 (99-118) 0.6; **8.** DPPC 0.3/E2 (99-118) 0.7; **9**. DPPC 0,2/E2 (99-118) 0.8; **10.** DPPC 0.1/E2 (99-118) 0.9; **11.** E2 (99-118) (Mean area/molecule vs. monolayer composition for DPPC/E2(99-118) mixtures at surface pressure of ♦ 5 mN/m, □ 10 mN/m, □ 20 mN/m).

period of 10 min was introduced before the freshly cleaved mica was raised vertically through the monolayers and out of the trough at a rate of 10 mm min^{-1}. Compressing the film at equal rate compensated the transfer of material onto mica. All deposited monolayers were then visualized using contact mode atomic force microscopy in air within 2 h of deposition [5].

2.4. Atomic force microscopy (AFM)

All Langmuir–Blodgett films were analyzed using contact mode AFM. Images were obtained using a Nanoscope IIIa equipped with a multimode head and an oxide sharpened silicon nitride cantilever with a length of 200 μm and a spring constant of 0.06 N/m. The films were scanned at rates ranging from 0.1 to 2 Hz with the higher scan rates used to scan smaller sizes [6].

3. Results

3.1. Compression isotherms

3.1.1. Mixed monolayers of E2(99-118) and DPPC

Pure peptide E2(99-118) was spread at the air/water interface from a chloroform solution. The isotherm obtained (Isotherm 11 in Fig. 1) is quite expanded and the collapse was not reached through compression. Spreading larger volumes of the peptide solution did not produce important changes in the shape of the isotherm and the collapse was neither reached. Value of area/residue at the lift off was 0.19 nm^2 and extrapolated area at $\pi = 0$ mN/m was 0.12 nm^2. These were slightly lower than those obtained for other peptides and proteins [7], however, our peptide was shorter that those ones. Nevertheless, the results were very similar to the ones we had previously obtained when

Table 1
ΔG_M^{EX} values for DPPC/E2(99-118) and DPPG/E2(99-118) mixed monolayers

Lipid/E2(99-118)	5 mN/m ΔG_M^{EX} (J/mol)		10 mN/m ΔG_M^{EX} (J/mol)		20 mN/m ΔG_M^{EX} (J/mol)	
	DPPC	DPPG	DPPC	DPPG	DPPC	DPPG
0.8/0.2	−694,452	−762,873	−3302,531	−3810,390	−7585,245	−2983,432
0.6/0.4	−544,479	−471,721	−2680,476	−2982,469	−5318,068	−2983,433
0.4/0.6	−260,796	−400,409	−1700,052	−2750,222	−3995,297	−3697,881
0.2/0.8	−132,506	−168,885	−996,083	−3697,881	−884,899	−1426,126

working with hepatitis A synthetic antigens of similar length [8]. All these findings are compatible with unstable monolayers, however, no pressure decay was observed when stopping the barrier for 30 min during the compression. Some authors have studied substances that even though that the isotherms did not have collapse formed stable monolayers [9,10] and other authors have studied mixed monolayers where one of the components did not for stable monolayers [11,12].

Compression isotherms of E2(99-118)/DPPC mixed monolayers are plotted in Fig. 1. Collapse pressures were approximately 50–60 mN/m in mixed films with a low contain of peptide ($X_p <$ 0.6), similar to that of pure DPPC (60 mN/m). At higher peptide concentration mixed films had a tendency to breakdown before collapsing as observed for other peptides [13]. The phase change of DPPC, could be also observed in mixed monolayers till proportions of $X_p < 0.7$, however, the shape of this phase change underwent transformations; varying the pressure where it takes place and becoming smoother. The shape of the isotherms underwent a change and the increase in the proportion of peptide in the monolayer led to its progressive reduction.

The area/molecule in the mixed films was determined from the former curves at different surface pressures and plotted against the lipid molar fraction, being represented in the insert of Fig. 1. Whatever the given pressure, the area values present negative deviations from additivity rule, suggesting associative interactions between the peptide and DPPC molecules. A slight break in the graphics can be observed between X_p 0.8 and 0.7 corresponding with the change in the shape of the isotherms.

The excess free energy of mixing (ΔG_M^{EX}) was calculated from mixed monolayers applying Goodrich and Pagano approach, Eq. (1), [14,15].

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

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 software of the Langmuir balance to another program that calculates the area under the isotherm at a fixed pressure according to the method of Simpson. These results were transferred to a calculus page of Excel2000 (Microsoft Corporation) to undertake the other calculations.

In Table 1 are listed the values of ΔG_M^{EX} calculated. As expected all the values are negative, evidencing that mixed monolayers are thermodynamically stable, although the majority of the ΔG_M^{EX} values are not particularly high, being < RT. The stabilization of the mixed monolayers is due to associative interactions established between the peptide and DPPC.

3.1.2. Mixed monolayers of DPPG and E2(99-118)

In order to find possible electrostatic interactions, in addition to hydrophobic ones, we studied the miscibility of our peptide with DPPG monolayers (at pH 7.4, DPPG has negative net charge).

Compression isotherms showed similar features that those of DPPC mixtures, as can be seen in Fig. 2. Collapse pressures are high and similar to

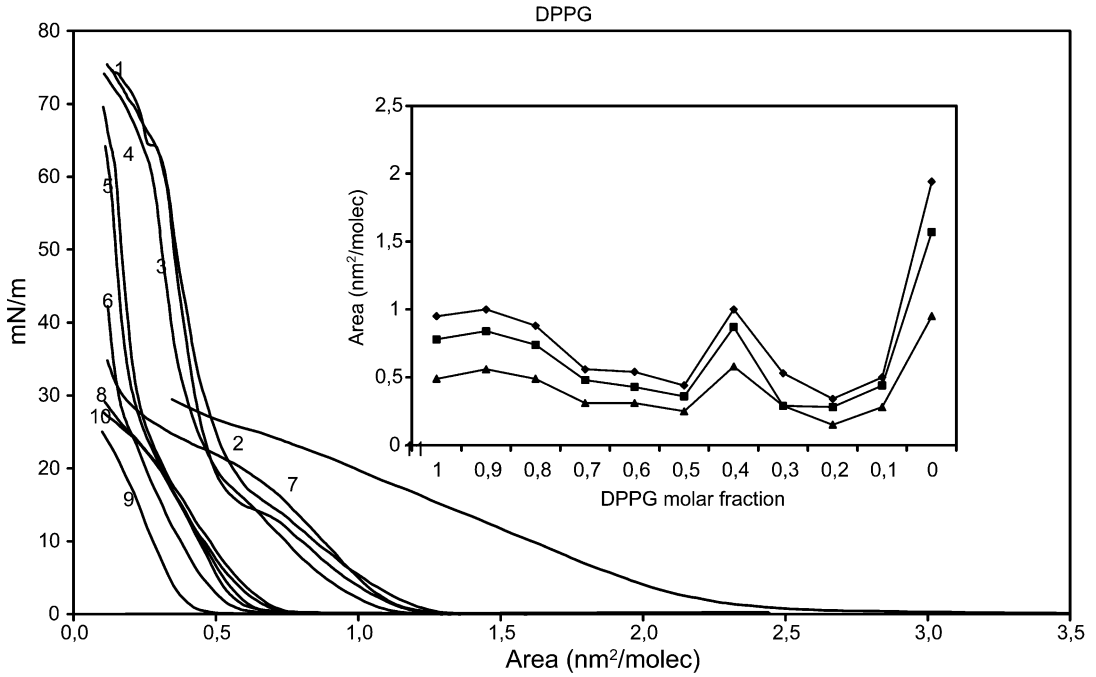


Fig. 2. Surface–pressure compression isotherms for: **1**. DPPG; **2**. DPPG 0.9/E2(99-118) 0.1; **3**. DPPG 0.8/E2 (99-118) 0.2; **4**. DPPG 0.7/E2 (99-118) 0.3; **5**. DPPG 0.6/E2 (99-118) 0.4; **6.** DPPG 0.5/E2 (99-118) 0.5; **7.** DPPG 0,4/E2 (99-118) 0.6; **8.** DPPG 0.3/E2 (99-118) 0.7; **9**. DPPG 0.2/E2 (99-118) 0.8; **10.** DPPG 0.1/E2 (99-118) 0.9; **11.** E2 (99-118) (Mean area/molecule vs. monolayer composition for DPPG/E2(99-118) mixtures at surface pressure of ◆ 5 mN/m, □ 10 mN/m, □ 20 mN/m).

DPPG pure monolayers for mixtures $X_p < 0.4$. DPPG phase change disappears in the mixed monolayers at lower concentration of peptide compared with DPPC mixtures. Between $X_p = 0.5–0.6$, the shape of the isotherms changes dramatically from one to another and collapse was not reached. However, for $X_p > 0.6$ all the isotherms seem to collapse at approximately 27 mN/m.

In the insert of Fig. 2 it can be appreciated that similarly to DPPC mixed monolayers, E2(99-118) is miscible with DPPG and showed negative deviations. These deviations have moderate to high negative values that are shown in Table 1, very similar to those found before for DPPC.

3.2. Fluorescence image analysis

Monolayers of pure peptide, DPPC, DPPG both in the pure and in the mixed form, doped with 1 mol.% of the fluorescence probe, were visualized using epifluorescence microscopy. Pure peptide, when mixed with the probe, did not produce any significant characteristics. The entire monolayer became homogeneous, i.e no change in the formation of organized regions or phase transition was observed. However, pure DPPC showed some characteristic features. It is in accordance with the earlier results [16], where it was found that in the surface pressure range 7–10 mN/m, the phospholipid underwent a phase transition from the liquid expanded (LE) to the liquid condensed (LC) state. DPPC formed kidney bean shaped (two or three such domains got associated) in the mentioned surface pressure region as shown in Fig. 3. Such LC regions were devoid of the fluorescent probe for which they appeared black when observed under the epifluorescence microscope. The relative area of such domains got increased with the increase in surface pressure up to 30 mN/m beyond which the relative area of the probe-

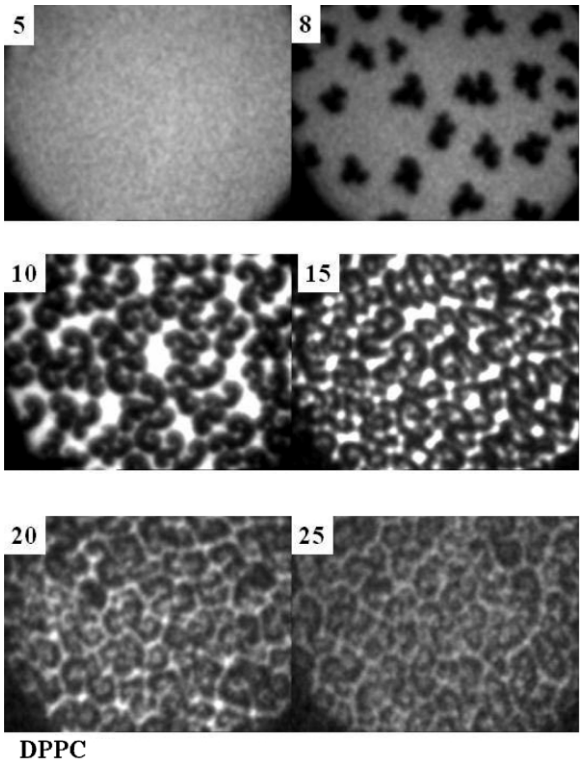


Fig. 3. Fluorescence images of DPPC monolayer, doped with 1 mol.% NBDPC, at different surface pressures. Area of the images 250×250 μm^2. Surface pressures are indicated on the left top corner of the images.

excluded LC regions got decreased. The results are in accordance with the earlier observed reports [17]. The effect of the peptide on the fluorescence behavior of DPPC, doped with the probe was noteworthy. Upon addition of the peptide, the formation of the organized probe-excluded domains got retarded. When a 9:1 DPPC: peptide mixture was compressed, it was found that the domain formation started at higher surface pressure ($\geq$10 m Nm^{-1}) compared to pure DPPC monolayer (shown in Fig. 4). Even at higher surface pressures deformations in the shapes of the organized domains from pure DPPC were observed. Such happenings clearly indicated the interaction of the peptide with DPPC monolayer. Some peptides are known to have the property to diffuse into the DPPC monolayer [18]. Therefore, change in the domain characteristics of DPPC monolayer by the peptide is not unwanted. One thing is also to be noted about the advantage of the epifluorescence microscopy over the surface pressure–area behavior. The effect of peptide on the phase transition of DPPC monolayer could have been

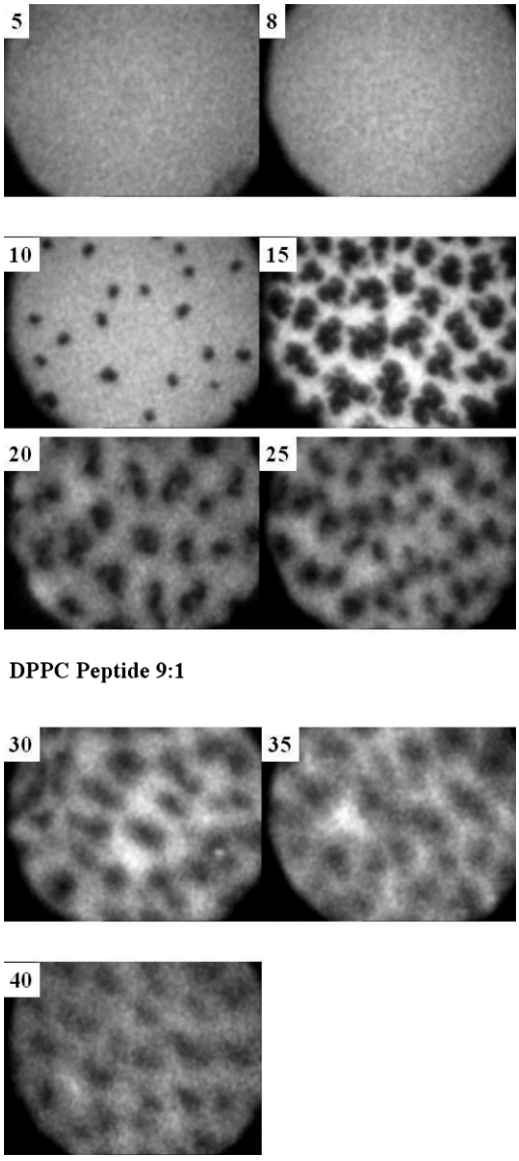


Fig. 4. Fluorescence images of DPPC/peptide (9:1) mixed monolayer, doped with 1 mol.% NBDPC, at different surface pressures. Area of the image 250×250 μm^2. Surface pressures are indicated on the left top corner of the images.

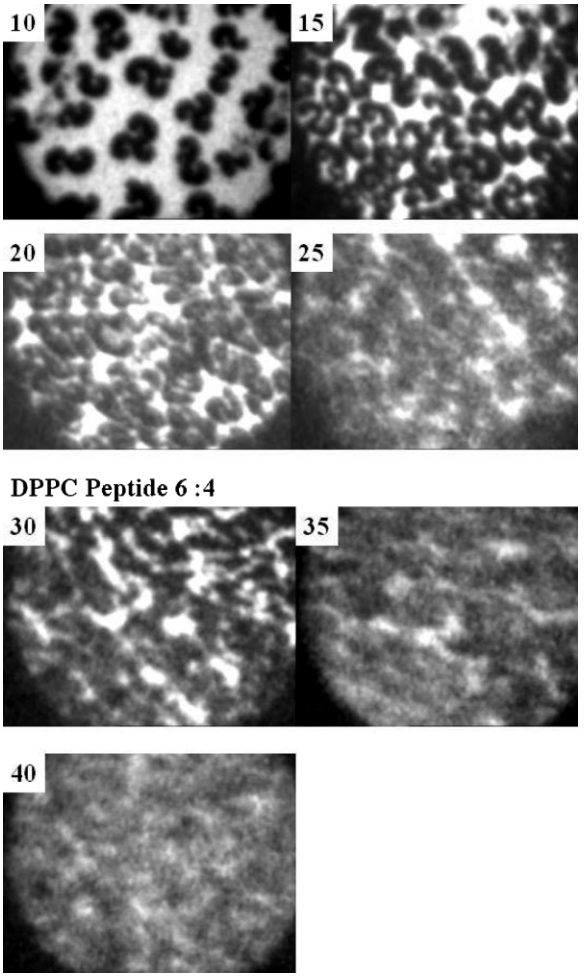


Fig. 5. Fluorescence images of DPPC/peptide (6:4) mixed monolayer, doped with 1 mol.% NBDPC, at different surface pressures. Area of the image 250×250 μm^2. Surface pressures are indicated on the left top corner of the images.

detected only after a significant amount of peptide addition to DPPC (approx. X_{DPPC}, 0.7). But when studied under fluorescence, effect of the peptide could have been noticed even at $X_{DPPC} \sim 0.9$. Therefore, fluorescence technique has been found to provide some additional information regarding the interaction between lipid and peptide. Progressive addition of the peptide to DPPC followed the similar trend as in retarding the domain formation as well as the blurring of the domains. As seen in Fig. 5 for a DPPC: peptide 6:4 mixture, that after 25 mN/m surface pressure the domain characteristic, which was present in pure DPPC, got abolished. Therefore, it could be concluded that the peptide completely diffused into the DPPC monolayer beyond this surface pressure and composition.

A better comparison on the effect of peptide on DPPC monolayer is shown in Fig. 6 where it was observed that the peptide retarded the formation of DPPC LC domains, even the relative area of

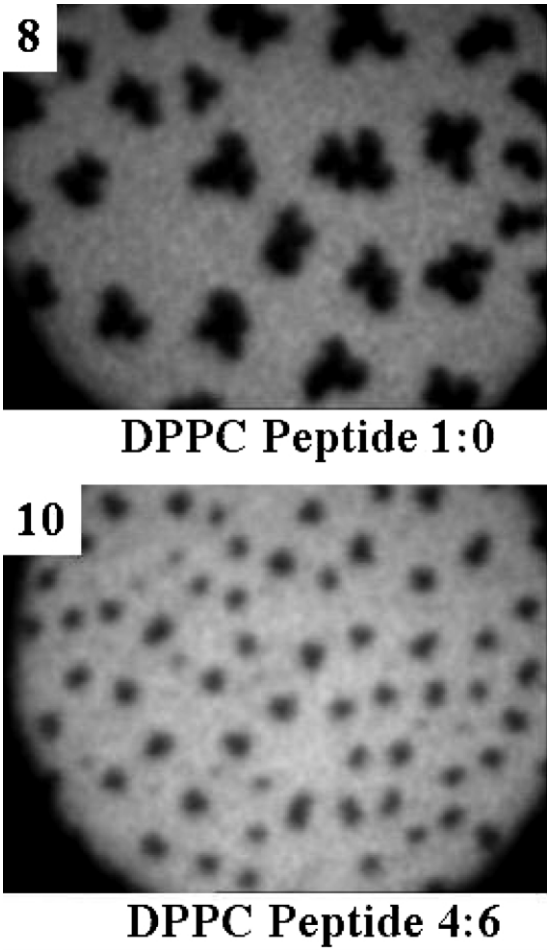


Fig. 6. Fluorescence images of pure DPPC (top) and DPPC/peptide (4:6, bottom) mixed monolayer, doped with 1 mol.% NBDPC, at different surface pressures. Area of the image 250×250 μm^2. Surface pressures are indicated on the left top corner of the images.

coverage of the domains was less in the mixture having peptide.

We also have tried to study the effect of the peptide on the DPPG monolayer, but failed. This might be due to the opposite charges of the probe NBD-PC and DPPG for which the probe could not partition separately in the organized LC and LE regions. When studied under epifluorescence microscope, we could only observe a homogeneous monolayer.

3.3. Atomic force microscopy

The monolayers containing pure DPPC and a mixture of DPPC and peptide, after transferring onto a freshly cleave mica by Langmuir–Blodgett (LB) technique, were also studied under atomic force microscope by contact mode. Representative results of the AFM measurements in height mode are shown in Fig. 7, where the AFM images of the same monolayer, as seen by fluorescence microscope are reported. To the contrary of fluorescence images, here the probe excluded regions appear brighter. The LC regions are more organized and the hydrocarbon tails of DPPC molecules are more straightened towards air, therefore they have higher profiles than the LE regions. Hence the LC regions appeared brighter [6]. The AFM measurements re-established the fluorescence studies, where the DPPC molecules at low surface pressure (approx. 8 mN/m) showed kidney bean shaped LC regions (Fig. 7 top). On addition of the peptide, the organization got interfered; a more diffuse region was observed. Therefore more disordered structures were observed.

4. Discussion

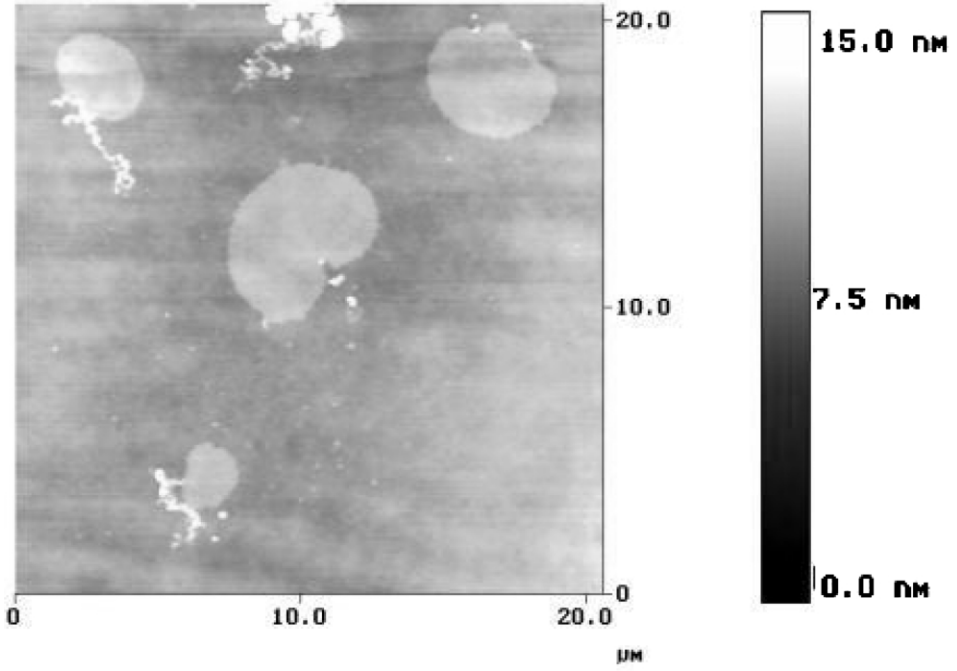
Different techniques were used to study the interaction of synthetic Hepatitis G virus antigen E2(99-118) with DPPC and DPPG monolayers.

Compression isotherms of mixed films of each lipid with the peptide showed a high degree of miscibility. Negative deviations were found in both cases indicative that associative interactions were established and/or that the peptide was able to incorporate into the lipid monolayer. As the quantity of peptide in the monolayers increases, the compression isotherms undergo a change due to the interactions between the two components, some small peaks in the deviations from additivity are produced by the rearrangement of the two components. Epifluorescence and AFM studies confirmed these results. The presence of peptide retarded the formation of DPPC domains and changed its shape, suggesting that E2(99-118) does not let the lipid to get organized. The diffusion of the peptide into DPPC monolayer was also observed, especially at high surface pressures. With progressive addition of peptide, the density of the domains decreased and more irregular shapes were observed indicating that the peptide destroyed the structural organization of DPPC. This destruction can be the responsible of the abrupt changes observed in the shape of the isotherms, especially at high peptide concentrations.

In previous DSC (differential scanning calorimetry) studies with DPPC [19], the peptide also showed a decrease in ΔH and the widening in the peak of transition temperature, indicative that the peptide penetrates into the bilayer at the level of the hydrocarbon chains of the phospholipids. Similar studies conducted for DPPG [20], showed the same behavior. Therefore considering all the observations, it can be deduced that E2(99-118) is able to incorporate both in mono and bilayers of DPPC or DPPG.

Even though, epifluorescence and AFM studies could not be carried out for DPPG, the similar results obtained for both lipids with the other techniques suggest that a similar behavior could be expected.

E2(99-118) has no net charge at pH 7.4 and has a high ratio polar/apolar amino acids (0.25). What is more, observing the sequence of this peptide we can see that the charged amino acids are concentrated in two regions of the peptide, and grouped in pairs of different charge. For this reason, it is quite possible that the electrostatic interactions between them difficult the interactions with outsider molecules. The preponderance of hydrophobic interactions over electrostatic ones, even in the presence of potential electrostatic interactions, has been observed by other authors [7]. Therefore, is it not surprising that the peptide



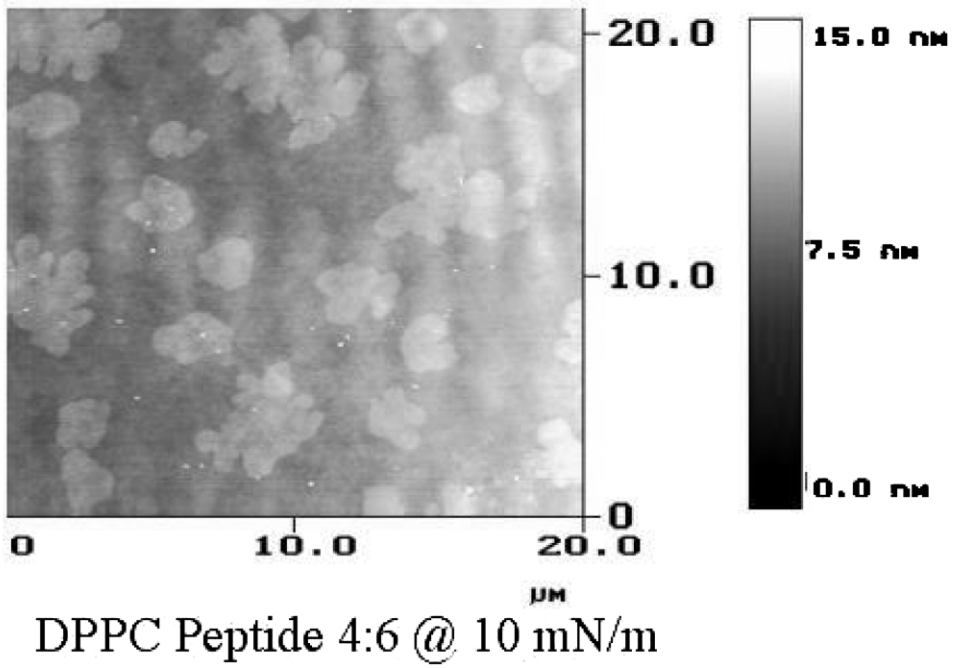


Fig. 7. AFM images of pure DPPC (top) and DPPC/peptide (9:1, bottom) mixed monolayer, doped with 1 mol.% NBDPC in the height mode. Pure DPPC was deposited at 8 m Nm^{-1} while the mixture was deposited at 10 m Nm^{-1}.

is able to incorporate in mono and bilayers in the same extent, which also is independent of the net charge on the lipid. The interactions established are mainly hydrophobic at the level of the hydrocarbon chains of the phospholipids and electrostatic interactions seem to have a very small role. In other studies carried out in our group we have found that above the gel-to-liquid crystalline transition of DPPC it can be clearly observed that the E2(99-118) peptide permeabilized vesicles efficiently [21], thus possibly playing a role in the intermolecular interaction of the E2 structural protein of GB virus C with the lipids of the target membrane.

The observed interaction of E2(99-118) peptide with DPPC vesicles by means of DSC and fluorescence measurements does not induce a significantly more ordered conformation in the peptide. The results obtained by CD [19], indicate that the binding of this structural GBV-C/HGV peptide to lipid membranes occurs without any restructuration. Even though, recent studies have shown that structural amphiphilicity rather than specific structural element is a dominating factor in the fusion process [22].

Therefore, if the peptide is able to adopt a suitable three-dimensional orientation exposing an hydrophobic region, it could participate in membrane destabilizing processes mainly through hydrophobic interactions.

Acknowledgments

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