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Lipid-Induced Organization of a Primary Amphipathic Peptide: A Coupled AFM-Monolayer Study

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Abstract. To better understand the nature of the mechanism involved in the membrane uptake of a vector peptide, the interactions between dioleoylphosphatidylcholine and a primary amphipathic peptide containing a signal peptide associated with a nuclear localization sequence have been studied by isotherms analysis of mixed monolayers spread at the air-water interface. The peptide and the lipid interact through strong hydrophobic interactions with expansion of the mean molecular area that resulted from a lipid-induced modification of the organization of the peptide at the interface. In addition, a phase separation occurs for peptide molar fraction ranging from about 0.08 to 0.4 Atomic force microscopy observations made on transferred monolayers confirm the existence of phase separation and further reveal that mixed lipid-peptide particles are formed, the size and shape of which depend on the peptide molar fraction. At low peptide contents, round-shaped particles are observed and an increase of the peptide amount, simultaneously to the lipidic phase separation, induces morphological changes from bowls to filamentous particles. Fourier transform infrared spectra (FTIR) obtained on transferred monolayers indicate that the peptide adopts a β-like structure for high peptide molar fractions. Such an approach involving complementary methods allows us to conclude that the lipid and the peptide have a non-ideal miscibility and form mixed particles which phase separate.

Key words: AFM — Mixed monolayers — Langmuir-Blodgett films — Lipid-peptide interactions

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A crucial step for cellular uptake of drugs is the crossing of the membranes separating the intracellular domains from the external environments. Recently, it was shown that synthetic primary amphipathic peptides can act as very efficient drug carriers with a very rapid internalization process (Morris et al., 1997; Vidal et al., 1997). Thus, within less than 5 min incubation with the cells, such synthetic peptides can be mainly localized in the nuclei (Chaloin et al., 1988).

One of the most efficient peptide, P1 in the text, was based on the association of a signal peptide (Briggs & Gierasch, 1986) with a sequence issued from a positively charged nuclear localization motif (Kalderon et al., 1984; Goldfarb et al., 1986) (SP-NLS peptides). P1 has the following sequence: Ac-M-G-L-G-L-H-L-L-V-L^{10}-A-A-A-L-Q-G-A-W-S-Q^{20}-P-K^+-K^+-K^+-R^+-K^+-V-Cya (where Ac and Cya are acetyl and cysteamide groups respectively). To better understand the membrane uptake of this peptide which corresponds to the obligatory first step of the translocation process, measurements of penetration into phospolipid monolayers were previously performed on P1. They showed that the peptide inserts very efficiently into the lipids, either neutral (DOPC) or negatively charged (DOPG). The aim of this paper is to better characterize the nature of the lipid–peptide interactions in monolayers. We describe here the compression isotherm parameters of mixed P1-DOPC films (Subramanian, Seul & McConnell, 1986; Palmer & Thompson, 1989; Maget-Dana & Ptak, 1995; 1997; Huang & Thompson, 1996; Taneva & Keough, 1996), atomic force microscopy observations made on the corresponding transferred films and a FTIR analysis. FTIR gives access to the conformation of the peptide within its environment (Briggs et al., 1986) whereas AFM, which allows the observation of nanometer-sized particles, provides information on the topographical organization of

the transferred monolayer (Edidin, 1997; Zasadzinski et al., 1994; Mou, Czaijkowsky & Shao 1996; ten Grotenhuis et al., 1996).

Materials and Methods

Materials

Dioleoylphosphatidylcholine (DOPC) was purchased from Sigma Chemical (St. Louis, MO); solvents: DMSO, chloroform, and methanol were from Merck (Darmstadt, Germany) and water was tridistilled (once on MnO_4K).

The peptide has the same origin as described in Chaloin et al. (1997) and it was synthesized by solid phase peptide synthesis using the Fmoc strategy with AEDI-Expansin resin on a 9050 Peptidesynthetizer Milligen (Millipore, U.K.) as reported by Vidal et al. (1996). Purification was achieved by semipreparative HPLC using a Nucleosil 300, C8, 5 μm column, 200 × 20, SFCC (Neuilly-Plaisance, France).

Monolayers

Compression isotherms monolayers were recorded using a Langmuir film balance setup with a 657 cm^2 trough. Surface tension was measured with a Prolabo (France) tensiometer using the platinum plate of Wilhelmy method. Isotherms were recorded on a XY Kipp and Zonen model BD 91 (Delft, The Netherlands) apparatus. DOPC solution was prepared in chloroform/methanol solvent (5:1, v/v) and peptide in DMSO/chloroform/methanol (0.03:5:1, v/v/v). Mixtures were achieved to the required compositions by combining volume to volume solutions of pure peptide and pure DOPC at an initial concentration of 0.25 mM. 40 to 100 μl according to the molar ratio of the mixtures were then spread on the water subphase; the solvent was allowed to evaporate for 10 min, then the monolayer was compressed at a rate of 0.15 nm^2/molecule/min. For the subphase unbuffered tridistilled water was used in order to be in the same experimental conditions as these of the transfer (*see below*).

Langmuir-Blodgett Monolayers

The monolayers were achieved in a homemade setup; solutions were spread on a 270 cm^2 trough, after the evaporation of solvent, films were compressed at the rate mentioned above. After waiting for 10 min the films were transferred onto a solid support (germanium crystal for FTIR, or freshly cleaved mica for AFM) by crossing the monolayer from subphase to the air at a rate of 5 mm/min. A feedback system maintained constant surface pressure during the transfer and the transfer pressures were 36 mN/m. To prevent artifacts in the AFM mainly due to the presence of salt crystals, the subphase was unbuffered and salt free.

Spectroscopic Measurements

FTIR spectra were obtained on a Bruker IFS 28 spectrometer equipped with a liquid nitrogen cooled MCT detector. The spectra (5000 scans) were recorded at a spectral resolution of 4 cm^{-1} on monolayers transferred on a germanium crystal; they were analyzed with the OPUS/IR2 program. The complex contour observed for the peptide amide I band was decomposed using second derivative method and curve fitting (Arrondo et al., 1993). Band positions for final curve fitting were determined from second derivative spectra.

AFM Measurements

AFM imaging of LB films was performed using a Nanoscope III atomic force microscope from Digital Instruments (Santa Barbara, CA) under ambient conditions, i.e., in air, using a 14 μm × 14 μm scanner. Topographic images were acquired in constant force mode using silicon nitride tips on integral cantilevers with nominal spring constant of 0.06 N/m (Le Grimellec et al., 1994). Typically, the estimated imaging forces were below 0.5 nN. Images were obtained from at least two different samples prepared on different days and at least five macroscopically separated areas on each sample. Representative images are presented below.

Results

Monolayer Study

The compression isotherms at various peptide molar fractions are shown in Fig. 1. Curve a corresponds to pure DOPC and is in agreement with previously reported results (Beitinger et al., 1989, Vié et al., 1998) while curve b corresponds to the pure peptide. For the sake of clarity not all isotherms measured for the lipid-peptide mixtures are shown.

On curve b (pure P1) of Fig. 1 no collapse can be detected until a pressure of 50 mN/m. In the 10 to 5 nm^2/molecule area range the low value of the surface pressure (<to 1mN/m) reveals the existence of weak electrostatic interactions. For areas below 4 nm^2, the monolayer becomes rigid as observed on the Wilhelmy plate which loses totally its mechanical lateral mobility and the molecules are close-packed at 3 nm^2 per molecule. Concerning the isotherms of lipid-peptide mixtures, in a domain of peptide molar fraction $0.11 < x_p < 0.35$ a plateau of surface pressure is found at a large molecular area (~6 nm^2/molecule). These isotherms are characterized by constant surface pressures that are notably higher than pressures of the pure components in the same area domain. This enhancement of the surface pressure and its high value (about 10 to 12 mN/m) suggests that strong hydrophobic interactions between P1 and DOPC occur at least in this range of P1 molar fractions (Gaines, 1966; Taneva & Keough, 1994). Figure 2 shows the variation of the mean molecular area at a surface pressure of 15 mN/m. An important positive deviation from additivity is observed and this deviation is maximum for $x_p = 0.35$ where the molecular area is double of that expected on the basis of the additivity rule (Crisp, 1949). This expansion is maintained for higher pressures but owing to the lack of precision the data are not shown.

Figure 3 illustrates the variations of the surface pres-

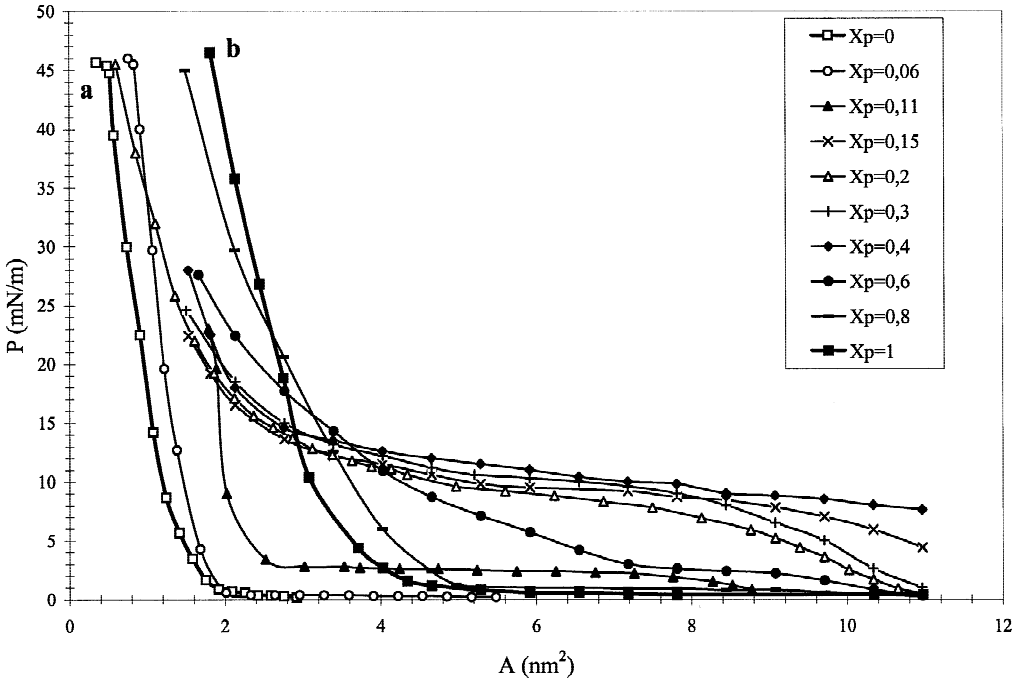

Fig. 1. Variations of the surface pressure with the mean molecular area of mixed DOPC-P1 monolayers at various peptide molar fractions (x_p). Note that when starting the compression at an initial pressure of less than 1 mN/m requiring thus large initial areas (~10 nm^2), our trough design dictates the final areas and pressures.

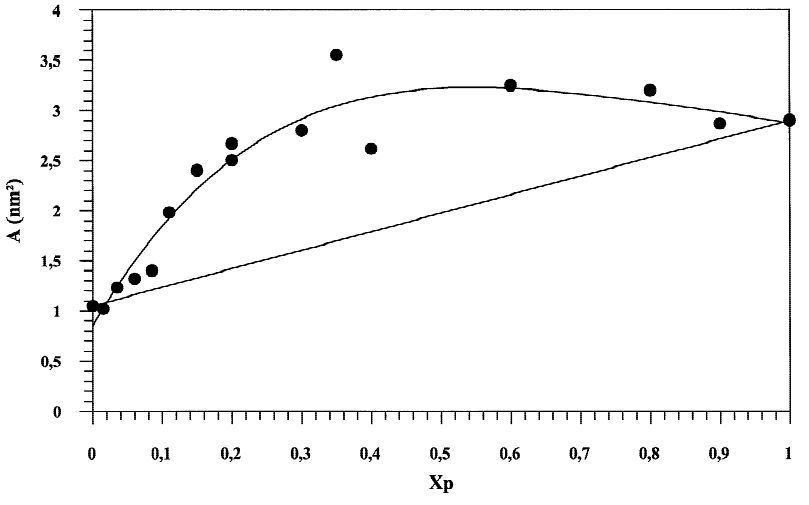

Fig. 2. Variation at 15 mN/m of the mean molecular area of mixed lipid-peptide monolayers as a function of the peptide molar fraction (x_p). The curve was calculated using a 4th order polynomial regression.

sure at the plateau with the peptide molar fraction. A large variation occurs for $0.08 < x_p < 0.15$ suggesting either a conformational change or a drastic rearrangement favoring lipid-peptide interactions and formation of a complex. In the molar fraction peptide domain ranging from 0.11 to about 0.4 a constant value of the surface pressure (10 mN/m) is observed. Above 0.4, the pressure decrease indicates a lowering of the interactions between the components of the monolayer.

Spectroscopy

Figure 4*A* shows the infrared spectra in the Amide I region obtained on Langmuir-Blodgett transferred mixed monolayers at a peptide molar fraction of 0.5 (note that the spectrum corresponding to a molar fraction of 0.25 although less defined for sensitivity reasons is very similar) (Blodgett, 1935). Clearly the spectrum reveals that the dominant conformation corresponds to a β-type

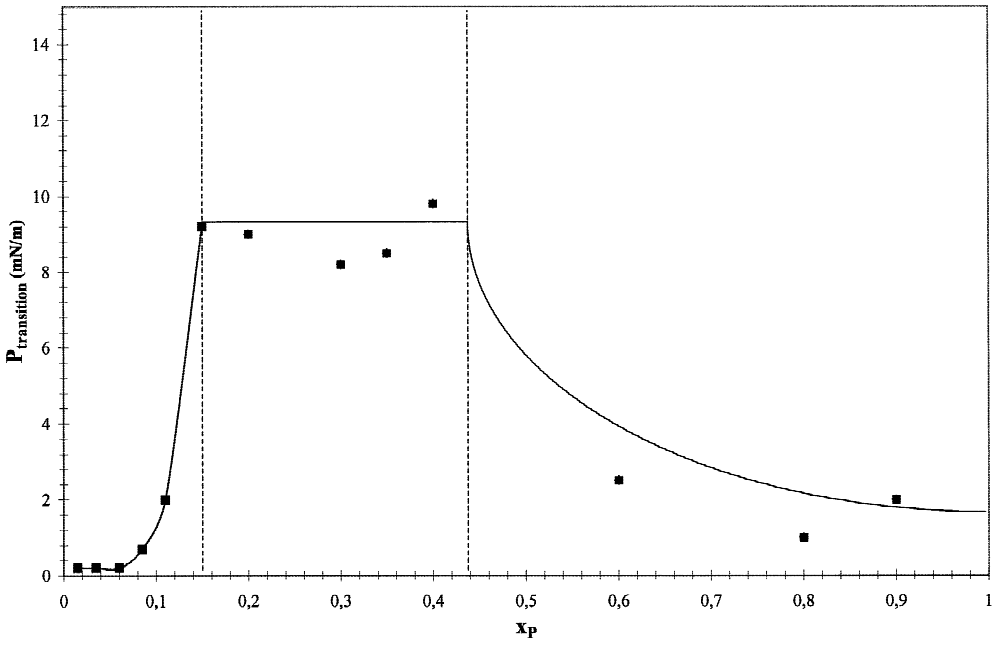

Fig. 3. Variation of the transition surface pressure as a function of the lipid molar fraction (x_p). The dashed vertical bars shows the limits of the phase transitions.

structure which is characterized by a major Amide I component centered around 1625–1630 cm^{-1} (Dong, Huang & Caughey, 1990). Comparison with the spectrum obtained for a pure peptide monolayer indicate that the lipid induces an enhancement of the β-structure (Fig. 4*B*). Owing to the presence of a contribution around 1695–1700 cm^{-1}, it is highly probable that we are dealing with an antiparallel arrangement of the peptide chains. However, close examination of the spectrum reveals that, beside the β-type structure, the presence of small amounts of α-helical structure cannot be ruled out (Amide I band at 1652 cm^{-1}. The 1670 cm^{-1} contribution can be attributed to domains of the peptide which do not adopt any defined conformational state. By analogy with the structure identified in lipid-mimicking media, namely SDS micelles, this latter domain corresponds very probably to the NLS sequence (Chaloin et al., 1997).

AFM Observations

Representative 2 μm × 2 μm scans of mica-supported DOPC/P1 LB films at different peptide molar fractions are shown in Fig. 5. As expected, pure DOPC monolayer transferred at 36 mN/m has an homogeneous and flat aspect on the chosen Z scale of 3 nm (Fig. 5*A*). Whereas the presence of low amount of peptide ($x_p = 0.005$) does not bring significant modification to the DOPC images, round shaped particles protruding from the matrix appeared for $x_p = 0.01$. Most of the particles have an apparent diameter of about 12 nm and emerge from the DOPC matrix by ~1 nm. A few larger particles, 40–60 nm in apparent diameter, some with attached filamentous structure forming a kind of tail are also present (Fig. 5*B*). Raising the peptide concentration to $x_p = 0.05$ induces a dramatic change in the images aspect (Fig. 5*C*): domains of a variety of shape and size are formed. These domains protrude ~1 nm from the matrix and are decorated by round shaped particles of higher height. Besides these domains, the samples are characterized by the presence of long (up to 500 nm), thin (≈11 nm) and frequently branched filaments which often connect small clusters and domains. At $x_p = 0.1$ (Fig. 5*D*) numerous very long and branched filaments coexist with a few small domains. In the images obtained at $x_p = 0.25$ (Fig. 5*E*), filaments cover the surface with no evidence for domains. No well defined particles are observed for $x_p = 0.5$ and the pure peptide monolayers. Identical observations are made on a 0.5 mixed monolayer transferred on another support, namely glass.

500 nm × 500 nm images of the $x_p = 0.01$, 0.05, 0.1 and 0.25 samples (Fig. 6*A*, *B*, *C* and *D*, respectively) provide further details about the structural organization of peptide/DOPC LB films: at the lowest concentration the peptide forms small round-shaped particles (clusters of peptides or mixed peptide/DOPC patches), with a 12 nm mean apparent diameter that show a propensity to associate. When increasing the peptide concentration to $x_p = 0.05$ these particles organize as filaments which are found either under an isolated form, often branched, or at

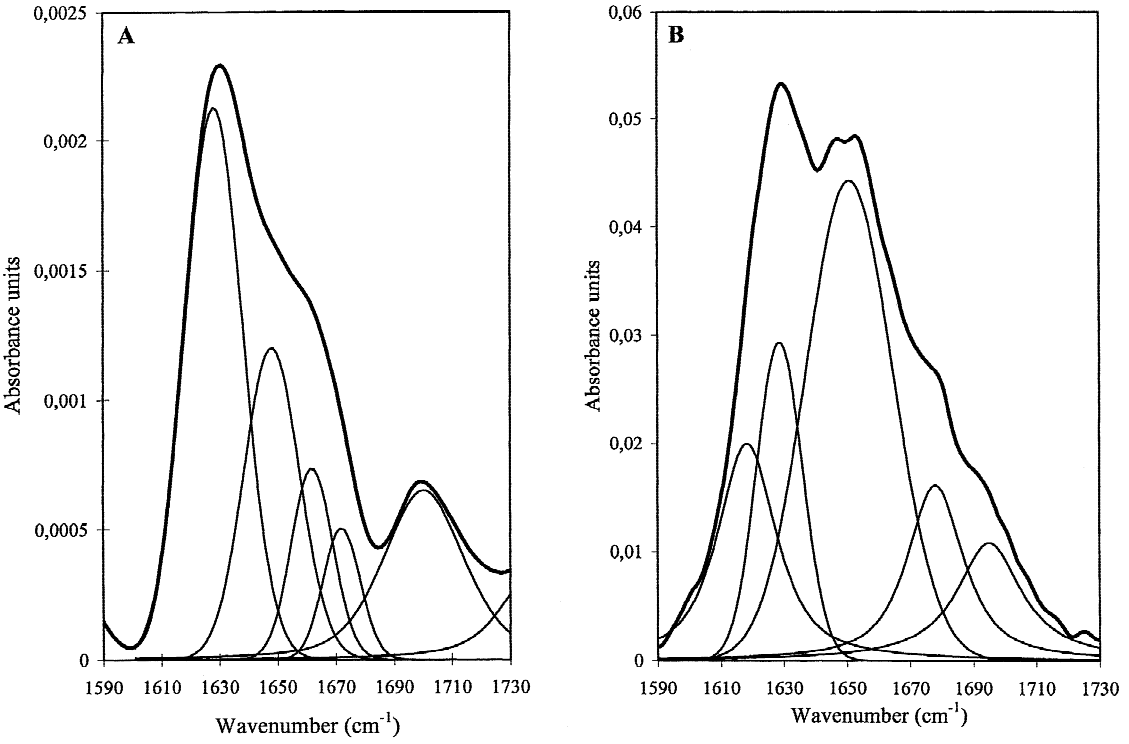

Fig. 4. *A* FTIR spectrum in the Amide I region of a transferred mixed DOPC-peptide monolayer peptide molar fraction (x_p) = 0.5. The positions of the various Amide I band components were determined using the second derivative method (Arrondo et al., 1993). *B* As for *A* but pure peptide.

the boundary of domains which are characteristic for this particular peptide concentration. The length as well as the branching of the filaments, which still appear to be formed from the same small rounded shape units linked by thinner structures, increase with the peptide concentration at the expenses of domains which disappear.

Using 500 nm scans, no well defined structures can be observed for pure P1 (Fig. 7*A*) and this also holds true for $x_p = 0.5$ (Fig. 7*B*). Figure 7*C* which is obtained for $x_p = 0.25$ is provided for comparison purpose to emphasize the organized arrangement.

Discussion and Conclusion

From the compression isotherm obtained for a monolayer containing only P1 the molecular area corresponding to the close-packed situation has been estimated at 3 nm^2. Since the infrared observations indicate that the dominant conformation correspond to an antiparallel β-structure and taking into account the dimensions usually found for this type of structure (0.475 and 0.35 nm for the interchain and interresidue distances, respectively), the measured area corresponds to about 18 residues. Examination of the chemical sequence of the peptide indicates that the N-terminal domain (from Met_1 up to Trp_{18}) which corresponds to the signal peptide sequence is the most hydrophobic part and thus is appropriate for such an interfacial position, while the hydrophilic C-terminal domain points into the water subphase.

Upon mixing with DOPC, the mean molecular area measured at 15 mN/m shows a positive deviation from additivity (Fig. 2). Such behavior can be considered as indicative of strong peptide lipid interactions with an important expansion of the mean molecular area. Taking into account the FTIR data which reveal the presence of β-structure and a lipid-induced increase of this structure, at least for $x_p \geqslant 0.25$, together with the monolayer data such an expansion can have its origin in the peptide induced perturbation of the lipid with an increase of the configurational freedom of the hydrocarbon chains (Chatelain, Defrise-Quertain & Ruysschaert, 1979; Taneva & Keough, 1994) but could also be due to a modification involving the C-terminus of the position of the peptide at the interface induced by the lipid and/or to mixed lipid-peptide particles (Matuo, Motomura & Matuura, 1982). It must be noted that this C-terminus contains four lysine residues and thus 4 × 4 CH_2 groups which, beside the hydrophilic character, will also confer a hydrophobic one to this part of the peptide. Hence, hydrophobic interactions arising between these methylene groups and these of the fatty acids of the lipids can take place. In addition, owing to the cationic character of the lysine side chains electrostatic interactions with

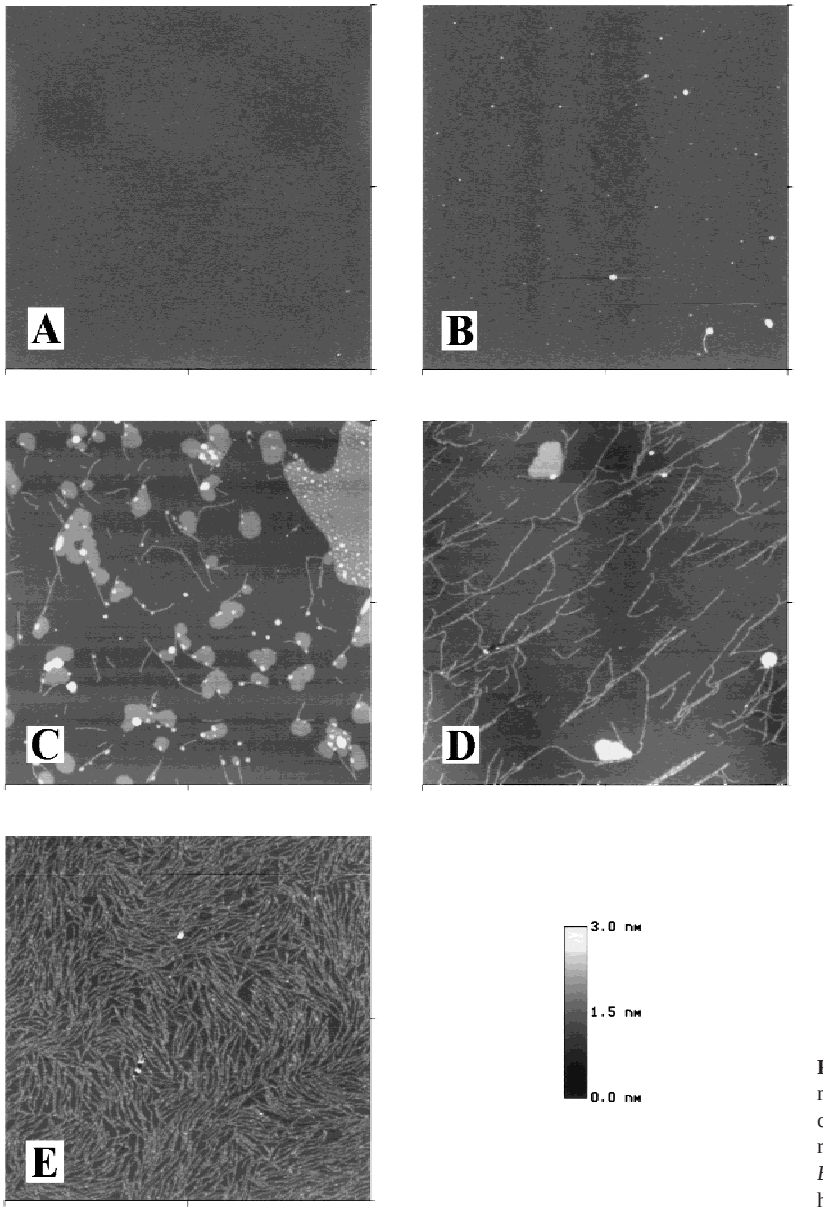

Fig. 5. 2 μm × 2 μm AFM scans of transferred monolayers of DOPC, *A, B, C, D* and *E* correspond to $x_p = 0$, 0.01, 0.05, 0.1 and 0.25, respectively. Scan rates were 3 Hz for *A*, 2 Hz for *B* and *C*, 1.5 Hz for *D* and 2.5 Hz for *E*. The height scale is given in the inset.

the phospholipid headgroups can also induce a modification of the peptide position at the interface. In the case of DOPC, which is zwitterionic, the positively charged part of the molecule is located on the choline moiety and thus could act in a repulsive manner while the negative phosphate groups will act as attractors leading to formation of the mixed particles. The consequence of these interactions is that the lysine side chains will be squeezed out of the bulk and located near the interface. Moreover, examination of Fig. 3 reveals the existence in the molar fraction range $0.08 < x_p < 0.4$ of a transition pressure which remains almost constant at 10 mN/m. Applying the two dimensional phase rule, the freedom degree F is given by the relationship $F = C - P_B - (q - 1)$ where C is the total number of components in the surface (here the lipid and the peptide) and in the bulk, P_B is the number of bulky phases (here the air and the water subphase) and q is the number of surface phases. For our experimental conditions this relationship leads to $F = 3 - q$. From examination of Fig. 3 it can be deduced that for $x_p < 0.08$, $F = 1$ and thus $q = 2$. Above $x_p = 0.08$, $F = 0$ and thus $q = 3$ indication that a phase separation occurs for $x_p = 0.08$. Further examination of Fig. 3 shows that an excess of lipid (low x_p) or of peptide (high x_p) generates low surface pressures. This latter observation suggests that lipid-lipid and peptide-peptide interactions are lower than the lipid-peptide interactions.

For AFM observations the transfer pressure was se-

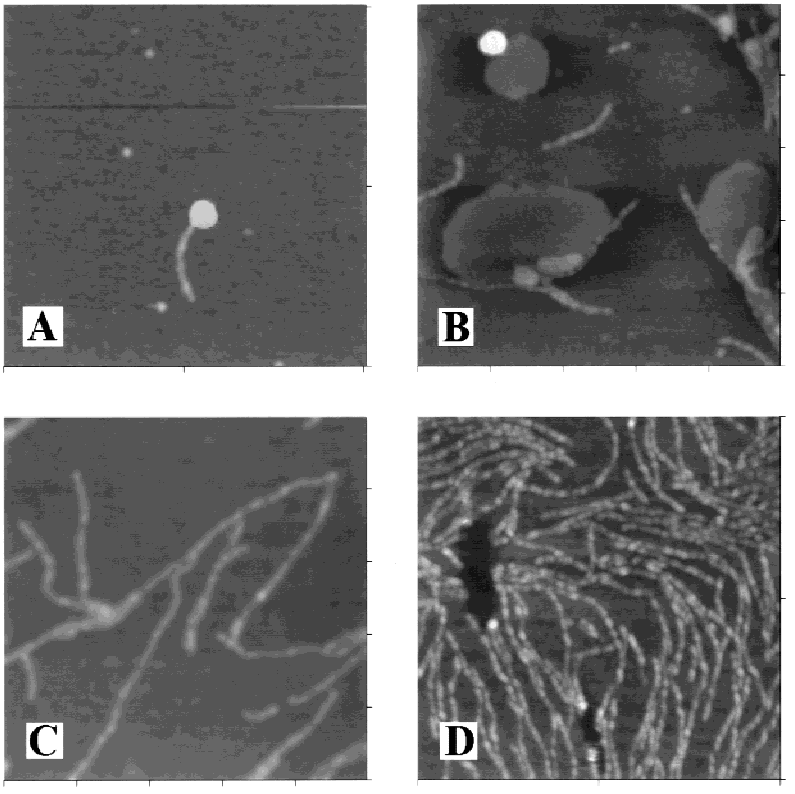

Fig. 6. 500 × 500 nm AFM scans of transferred monolayers of DOPC corresponding to $x_p = 0.01$, 0.05, 0.1 and 0.25 for *A*, *B*, *C*, and *C*, respectively. Same height scale as in Fig. 5. Scan rates were 2 Hz for *A* and *B*, 1.5 Hz for *C* and 4 Hz for *D*.

lected at 36 mN/m since Demel et al. (1975) found that the area per molecule of a membrane corresponds to a surface pressure in a monolayer system of 35 mN/m. Figures 5 and 6 show a strong change with x_p of the shape of the particles that are embedded in the lipid medium. For $x_p = 0.01$, round shaped particles can be observed, of diameter about 12 nm and these particles are built up of clusters of peptides embedded in the lipid matrix. For $x_p = 0.05$ several clear domains indicate a peptide-induced phase separation and for this ratio we observed the beginning of formation of wormlike species which could be lipid to peptide associated particles. Then, when x_p increases we observe a decrease of the surface occupied by the domains for the benefit of filaments which can include lipid and for $x_p = 0.25$ only filaments are detected. These filaments have almost an infinite length but a well-defined apparent width of about 10 nm but the data are not precise enough to decide whether or not a rearrangement of the hydrogen-bonding pattern occurs upon interacting with the lipid. The finding of rosarylike filaments results very probably from the association of the lipid containing bow-like particles. The fact that without lipids no filament can be detected without any major conformational change, indicates that their formation is lipid-induced, a finding that is in line with the above conclusion that the lipids interact with the peptide-forming mixed particles. It is also likely that the presence of lipids in the filaments favors the formation of branches, the linkage occurring mainly through the lipid. The consequences of mixing P1 with DOPC can be schematically represented as shown below.

We can conclude that upon mixing P1 with DOPC the phase transition observed at $x_p = 0.08$ corresponds to a preliminary step for the peptide-lipid complex formation whose stoichiometry would be of two lipids per peptide. Above this molar ratio the peptide remains in a β-sheet structure whatever the organization, well defined or not. As to the bowl-like particles found at low peptide molar fraction, the peptide structure involved in their formation remains to be identified since the peptide amount is too low to allow FTIR investigations on transferred monolayers but a peptide transconformation into an α-helical form cannot be ruled out.

In spite of a slight discrepancy in the concentrations required to identify the various transitions, which lies

Peptide Molar fraction (x_p)	0	0.08	0.4	1
Langmuir isotherms		2 phases	3 phases	2 phases
Conformation of the peptide (FTIR)		?	β-sheet	β-sheet
AFM		Bow-like particles	Filaments + bowls and domains	Nondefined organization

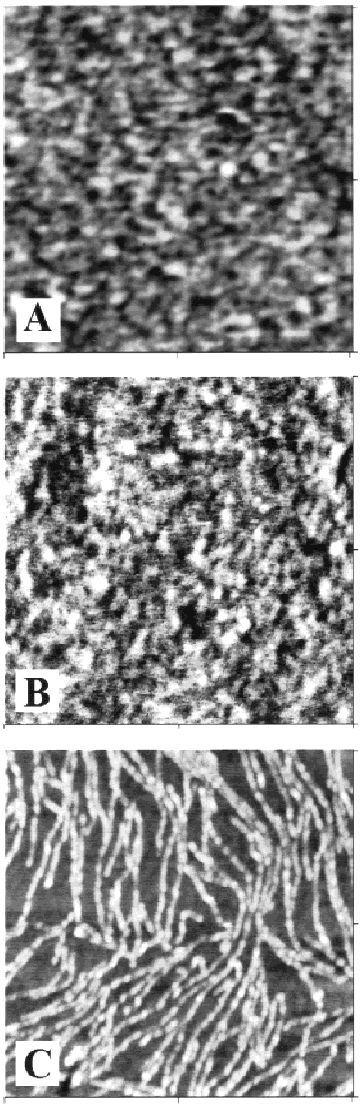

Fig. 7. 500 × 500 nm AFM scan of transferred monolayers corresponding to $x_p = 1$, 0.5 and 0.25 for *A*, *B* and *C*, respectively. The scale of height is 2 nm for *A* and *C* and 0.8 nm for *B*. Scan rates are 2 Hz for *A* and *C* and 5 Hz for *B*.

very probably in the fact that the experimental conditions are different and that the transfer procedure might disturb the system, it must be emphasized that, at the macroscopic level, the conclusions raised from the monolayer study correlate very well with the AFM observations. This concerns especially the finding of ''domains'' and it appears clearly that a complementary combination of surface pressure measurements with atomic force microscopy observations is required for the interpretation of the data (Vié et al., 1998).

On the basis of the results reported in this paper it appears that the membrane uptake of a primary amphipathic peptide mainly in a β-type conformational state is favored by strong lipid-peptide interactions. Atomic force microscopy, which allows observation at the mesoscopic scale reveals that the local structural organization (from bowl-like particles to filaments) strongly depends on the peptide molar fraction and that a lipid-peptide complex is formed. Attempts to improve the understanding of the local organization of such type of peptide in more complex lipid media are currently under way in our laboratories and deal with the influence of the physical state together with the role of headgroup charges of the phospholipids and also in mixtures of lipid types. All these informations are crucial for the design of other peptides where the selectivity for various types of membranes will also be taken into account.

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