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Fengycin interaction with lipid monolayers at the air–aqueous interface—implications for the effect of fengycin on biological membranes

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Abstract

In this study, we investigated the interaction of fengycin, a lipopeptide produced by *Bacillus subtilis*, with lipid monolayers using the Langmuir trough technique in combination with Brewster angle microscopy. Thermodynamic analyses were performed to get further information about the mixing behavior and the molecular interactions between the two components. The effect of fengycin on the structural and morphological characteristics of DPPC monolayers, as a simple model of biological membranes, depends on the fengycin molar ratio. With a small proportion of fengycin ($X_f \leqslant 0.1$), the compressibility of the monolayer is modified but the morphological characteristics of the DPPC are not significantly affected. At an intermediate molar ratio ($0.1 < X_f \leqslant 0.5$), fengycin has a fluidizing effect on the DPPC monolayer by interacting partially with DPPC molecules. At higher molar ratio ($X_f = 0.66$), fengycin totally dissolves the ordered phase of the lipid. These results highlight the capacity of fengycin to perturb the DPPC organization and are discussed in relation to fengycin capacity to affect biological membranes.

Keywords: Lipopeptide; DPPC; Isotherm; Thermodynamic analysis; BAM

1. Introduction

Fengycin is a lipopeptide produced by *Bacillus subtilis*. Its structure is composed of a β-hydroxy fatty acid linked to a peptide part comprising 10 amino acids, including 8 in a cycle (Fig. 1). It has been shown that it has antifungal activity against filamentous fungi and that its hemolytic activity is 40-fold less than that of surfactin [1,2], another lipopeptide produced by *Bacillus subtilis*. The mechanism of its activity is not yet understood, although it is likely that fengycin acts at the membrane level of the sensitive cells.

Hence, investigating the molecular interactions between fengycin and biological membranes should be of great interest to understand its biological properties.

In this study, we have investigated the molecular interaction of fengycin with a dipalmitoylphosphatidylcholine (DPPC) monolayer taken as a model membrane.

Although monolayers do not reflect the complexity of membrane structure, they are considered useful models to learn about interactions at interfaces [3]. A number of parameters including the nature and the packing of the lipid molecules, the composition of the aqueous phase, and the temperature can be varied readily and in a well-defined way [4]. Phosphatidylcholines are a major component of cell membranes, and monolayers of DPPC are well characterized both with respect to pressure–molecular area isotherm behavior and domain shape [5–12]. DPPC is also the major lipid in lung surfactants [13].

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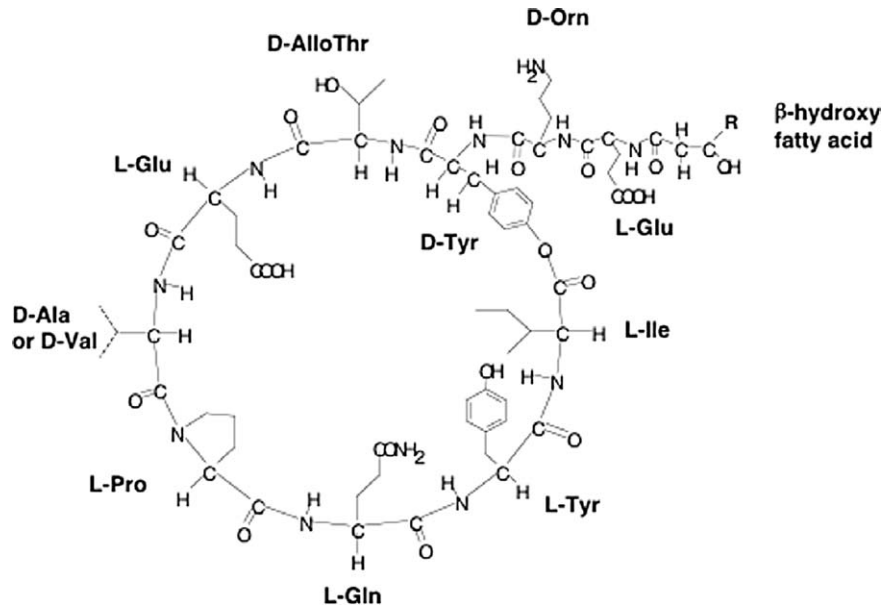

Fig. 1. Primary structure of fengycin.

The effect of fengycin on the structural and morphological characteristics of DPPC monolayer is studied by using the Langmuir trough technique in combination with the Brewster angle microscopy.

The mixing behavior (miscibility and stability) and the molecular interactions between DPPC and fengycin are discussed based on a simple thermodynamic analysis.

2. Experimental

2.1. Materials

Fengycin was produced by fermentation of the *Bacillus subtilis* strain S499 in a optimized culture media as described by Jacques et al. [14] and extracted in a semi-preparative scale from the culture medium by solid-phase extraction on Bond Elut C18 (50 g, Varian CA), as previously described [15].

The crude extract was applied to a silica gel 60 column (30×2.5 cm, 45 g, 250–325 mesh, Merck, Darmstadt, Germany) for separating fengycin from surfactin and iturin A by flash chromatography. Fengycin was eluted with chloroform/methanol/water/ethanol (7/3/1.5/3.5, by volume) after elution of surfactin and iturin A. The identification and the verification of the purity were made by IR spectroscopy, amino acid analysis, analytical RP–HPLC, and MALDI–TOF spectrometry. Fengycin is composed of two isoform compounds (isoform A with D-Ala and isoform B with D-Val; see Fig. 1), each containing homologous molecules.

Dipalmitoylphosphatidylcholine (DPPC, +99%) obtained from Sigma (St. Louis, USA) was used without further purification. HPLC chloroform (sds, Peypin, France) and methanol (Merck, Germany) of analytical grade were used as spreading solvent. The subphase was 10 mM Tris (Sigma, St. Louis, USA), 150 mM NaCl (Sigma, St. Louis, USA) buffer solution adjusted to pH 7.4 with HCl (Merck, Germany) and prepared with Millipore water.

2.2. Method

An automatically controlled Langmuir trough (KSV Minitrough, KSV Instruments Ltd., Helsinki, Finland), equipped with a platinum Wilhelmy plate and placed on a vibration-isolated table, was used to obtain the surface pressure–area (Π–A) isotherms of monolayers at the air/water interface. The temperature was maintained at $30 \pm 0.1\,^{\circ}\mathrm{C}$ by external water bath circulation. The cleanliness of the surface was ensured by closing the barriers, followed by aspiration of the subphase surface, before each experiment. When the fluctuation of the surface pressure was less than 0.1 mN/m during the compression cycle, an experiment was started.

DPPC, fengycin, and a mixture of DPPC and fengycin were spread from a 1 mM chloroform/methanol (2/1, v/v) solution on the subphase. At least 10 min was allowed for solvent evaporation from the interface. The air/water interface is then compressed or expanded symmetrically with two Delrin barriers at a rate of 5.8 Å^2 molecule^{-1} min^{-1}. The reproducibility of the area values remained within ±7%. The accuracy on surface pressure is within 0.1 mN/m.

The imaging of the monolayer was performed with a Brewster angle microscope (Optrel Multiskop BAM, Berlin, Germany) mounted on the trough apparatus.

Each experiment was replicated at least two times.

2.3. Thermodynamic analysis

From the Π–A isotherms of the mixed monolayers, the excess free energy of mixing, ΔG^{ex}, and the free energy

of mixing, ΔG^{M}, were calculated according to the equations [4,16,17]

$$\Delta G^{\mathrm{ex}} = \int_0^{\Pi} A_{12}\,\mathrm{d}\Pi - X_1 \int_0^{\Pi} A_1\,\mathrm{d}\Pi - X_2 \int_0^{\Pi} A_2\,\mathrm{d}\Pi, \qquad (1)$$

where A is the mean molecular area, X is the molar fraction, and subscripts 1 and 2 refer, respectively, to pure components 1 and 2 and 12 to their mixtures;

$$\Delta G^{\mathrm{M}} = \Delta G^{\mathrm{ex}} + \Delta G^{\mathrm{id}}, \qquad (2)$$

where ΔG^{id} is the free energy for ideal mixing and can be calculated from the equation

$$\Delta G^{\mathrm{id}} = RT(X_1 \ln X_1 + X_2 \ln X_2), \qquad (3)$$

where R is the universal gas constant and T the absolute temperature.

3. Results and discussion

3.1. Pure monolayers

The pure DPPC isotherm (Fig. 2) shows a characteristic inflection point at $\Pi = 15.4$ mN/m, signifying the onset of the phase transition between the so-called liquid expanded (LE) and liquid condensed (LC) phases, and a collapse pressure of 52.0 mN/m, which are in accordance with the literature [9,18]. The BAM images agree with those obtained by other authors [9,11]. Characteristic domains of the condensed phase (Fig. 3b) appear in the expanded phase at the beginning of the LE → LC coexistence region. In the phase transition, the domains increase in size and adopt an irregular shape with small protrusions bordering their edges (Fig. 3c). Upon further compression, the domains are packed tightly and the borders less defined (Fig. 3d). Finally, at very high surface pressure, they coalesce into a fully condensed state (Fig. 3e).

The pure isotherm of fengycin presents a progressive slope decreasing around 25 mN/m (Fig. 2). The absence of particular features in the BAM images (Figs. 4a and 4b) suggests a uniform film. Furthermore, the BAM images become brighter during the course of the compression (Figs. 4a and 4b). This indicates a progressive increase of the layer thickness as the relative reflectivity is proportional in the square of the thickness [11]. Fengycin seems then unable, under employed conditions, to form two-dimensional aggregates with a long-range orientation order. This implies poor molecular attractive interactions between the molecules [19]. As discussed by Maget-Dana and Ptak [20] for surfactin at high pH, we expect the cyclic peptide ring of fengycin to orient parallel to the interface. This gives a large area per molecule of the peptide ring. In addition, the fatty acid chain is expected to orient in such a way that it interacts intramolecularly with the hydrophobic amino acids, as

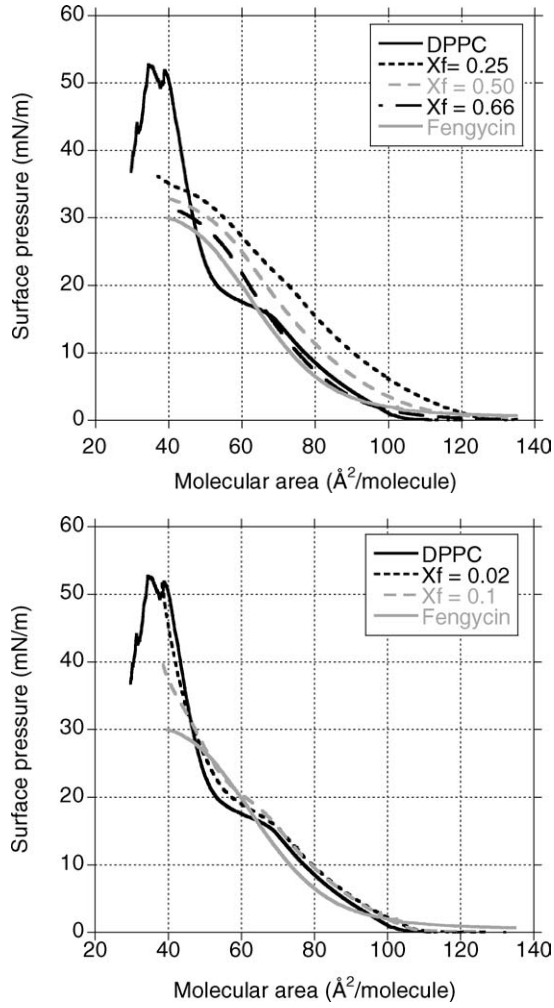

Fig. 2. Surface pressure–molecular area isotherms of DPPC, of fengycin, and of their mixtures with different fengycin molar fraction (X_f) on a subphase of 10 mM Tris, 150 mM NaCl at pH 7.4 and 30 °C. The figure was divided in two for clarity.

shown for surfactin [21]. The hydrophobic intermolecular interactions between the fatty acid chains of adjacent molecules, and consequently, the formation of ordered aggregates are therefore unlikely. Moreover, electrostatic repulsions between fengycin molecules counteract domains formation, as the net charge of fengycin is expected to be negative at pH 7.4 (one negative charge on each of the two glutamic acid residues ($\mathrm{p}K = 4.07$ [22]) and one positive charge on the ornithin residue ($\mathrm{p}K = 10.76$ [23])). The absence of condensed phase is confirmed by the fact that no sharp increase of the pressure is obtained when a five-fold larger amount is spread and compressed at the interface (data not shown).

Decompression of fengycin monolayer after compression gives rise to a hysteresis (Fig. 5a) that can be attributed to a transfer of molecules into the subphase during compression or to a formation of small aggregates and/or a reorganization of the molecules at the interface [24]. The partition of fengycin between the spread monolayer and the subphase can be evaluated by the thermodynamic analysis of Schwartz

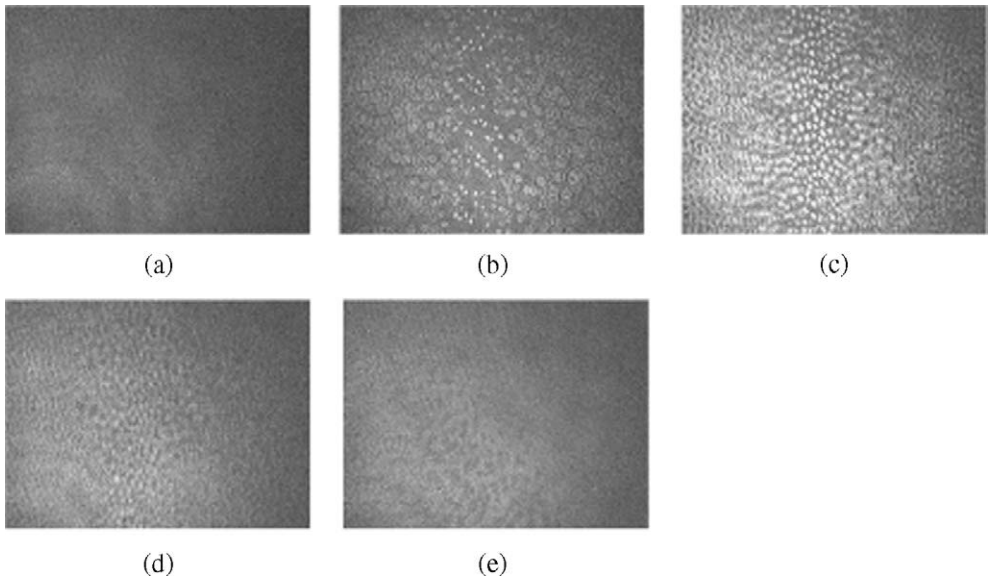

Fig. 3. BAM images of DPPC monolayer: (a) 0.04, (b) 16.39, (c) 19.50, (d) 29.14, and (e) 40.70 mN/m. All images have the same scale of 570 × 760 μm.

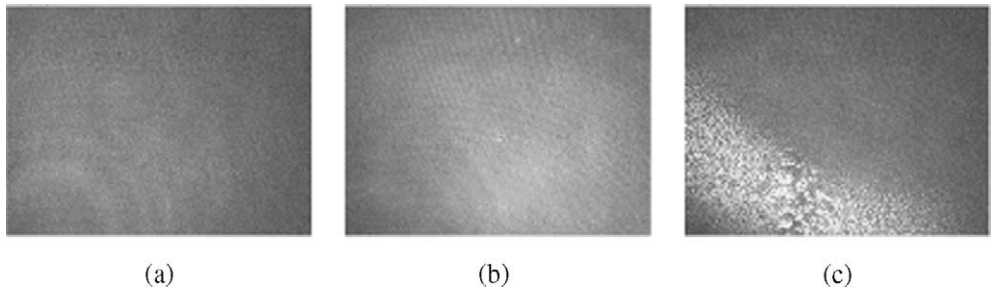

Fig. 4. BAM images of fengycin monolayer: (a) 0.70, (b) 24.80, (c) 30.50 mN/m. All images have the same scale of 570 × 760 μm.

and Taylor [25]. Because of mass conservation, the following relation should be obeyed,

$$n = \Gamma A + VC,$$

where n is a given total amount of the spread molecule, Γ is the surface concentration of the molecule, A is the trough area, which is gradually decreasing, V is the volume of the subphase, which is constant, and C is the molecule concentration in the subphase. Under equilibrium conditions Γ, as well as C, is invariant at fixed Π. Thus, n is expected to be a linear function of A, taken from different isotherms at the same surface pressure, with a slope equal to Γ and an intercept equal to $V \times C$ (Fig. 5b). For all the surface pressures, even those close to the collapse of the film ($\Pi_c \approx 30$ mN/m), C is negative but not far from zero (-0.0044, -0.0256, and -0.0186 nmol/cm^3 for $\Pi = 3$, 18, and 26 mN/m, respectively). This shows that fengycins do not desorb from the spread monolayer. The hysteresis observed during the compression/decompression cycle (Fig. 5a) can thus be attributed to a reorganization and/or formation of small aggregates of the molecules at the interface. These smaller aggregates are not visible by BAM at lower pressure.

At higher surface pressures the bright large clusters observed (Fig. 4c) suggest aggregation or multilayer formation at the interface at high Π (>28 mN/m). Accordingly the change of slope in the fengycin isotherm can be attributed to collapse of the monolayer by aggregation or multilayer formation. The process is not well defined, probably due to the chain length polydispersity of the fengycin fatty acid chains. A similar behavior was observed for an α-helical isopeptide by Maget-Dana et al. [26].

3.2. Mixed monolayers

When fengycin is mixed with DPPC, the isotherms differ from those of pure DPPC and pure fengycin (Fig. 2). At very low fengycin molar fraction ($X_f = 0.02$ and $X_f = 0.1$), the characteristics of the DPPC phase transition and condensed phase are visible but less pronounced. This suggests that fengycin exerts an effect on the compressibility of DPPC monolayer. The compressibility increase of the condensed phase is a first evidence of the lower monolayer stability. At smaller fengycin molar fractions, the presence of fengycin in the DPPC monolayer does not affect the DPPC domains, as apparent from the BAM images (Figs. 6a and 6b). However, during the course of the compression, we can observe locally very large aggregates with a high reflectivity (Figs. 6c–6e). These aggregates can be supposed to be fengycin multilayers

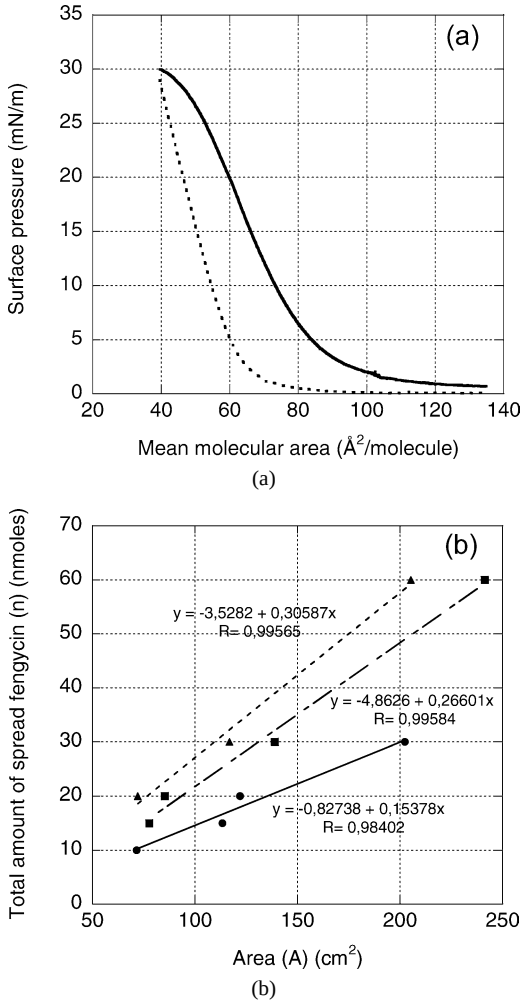

Fig. 5. (a) Fengycin compression–decompression isotherm. (b) Partition of fengycin between the monolayer and the subphase at different surface pressures: (●) 3, (■) 18, and (▲) 26 mN/m.

formed in zones of local high surface pressure as they look similar to those observed in the case of pure fengycin at high surface pressure. They are indicative of a phase separation in the film.

At $X_f = 0.25$, the characteristic plateau of the DPPC phase transition is no longer observed, although a slight inflection can be observed around 20 mN/m. For $X_f \geqslant 0.50$, the shape of the isotherm is similar to that of pure fengycin and no longer presents the characteristics of the DPPC isotherm. The BAM images of $X_f = 0.25$ and $X_f = 0.50$ monolayers feature domains (Figs. 6g–6m) that lack the characteristics of pure DPPC isotherm. The smaller size of the domains and the absence of a continuous condensed phase suggest that fengycin interacts partially with DPPC, and hence modify the interactions between DPPC molecules. At higher X_f ($X_f = 0.66$), the BAM analysis shows the existence of a homogeneous film without domain (data not shown) as it was observed for pure fengycin monolayer. This suggests a complete miscibility of the two components and/or possibly that DPPC is partially squeezed out from the monolayer.

We conclude that at a fengycin molar fraction higher than 0.25 significant changes in the DPPC organization at the interface occur.

To get further information about the mixing behavior and the molecular interactions between DPPC and fengycin, thermodynamic analyses were performed as described by Maget-Dana [4] and Fang et al. [17].

The plot of the mean molecular area versus the fengycin molar fraction (Fig. 7a) at different surface pressures reveals a significant positive deviation from the additivity rule for $X_f = 0.25$ and $X_f = 0.50$. The additivity rule corresponds to total immiscibility or ideal miscibility of the two components [4]. Any deviation can be attributed to specific interaction between the two components [4]. Therefore we can conclude that DPPC and fengycin are partially miscible and form nonideal mixed monolayers at the studied compositions and surface pressures. At $X_f \leqslant 0.1$, the smaller positive deviation at the three surface pressures denotes more ideal behavior and since the BAM images show phase separation, results indicate a total immiscibility. Another proof of immiscibility [27] is given by the fact that the inflection of the isotherm occurs at nearly the same surface pressure ($\sim$16 mN/m) for $X_f \leqslant 0.1$. At $X_f = 0.66$, the deviation from the additivity rule is small. This result taken together with the BAM images showing a uniform film gives evidence of a complete miscibility of the two components.

Once the partial miscibility has been established, a more detailed examination of the thermodynamics of the system can provide further information on the energetics of the miscibility process and upon the possible specific interactions between the two components [4].

Positive values of ΔG^{ex} (Fig. 7b) mean that mutual interactions between the two components are weaker than interactions between the pure molecules themselves. The large value of ΔG^{ex} for $X_f = 0.25$ and $X_f = 0.5$ indicates the formation of bidimensional aggregates. According to the BAM images, these aggregates are composed of DPPC or of a mixture of DPPC and fengycin molecules but not of fengycin molecules themselves only. As mentioned above and if we assumed that the conformation of fengycin is not influenced by the surrounding lipids, the orientation of the alkyl chain of fengycin within the molecule is not likely to the interaction with the hydrocarbon chains of other molecules. Moreover, local electrostatic repulsions between the negative phosphate of DPPC (DPPC is zwitterionic at pH 7.4 [28]) and the two negative carboxylic groups of the fengycin and between the positive charged choline group of the DPPC and the positive ornithin residue of fengycin might occur, although these interactions are expected to be screened by the salt in the subphase. The existence of electrostatic repulsions is supported by the fact that the values of ΔG^{ex} becomes progressively more positive as surface pressure increases, i.e., as intermolecular distances become

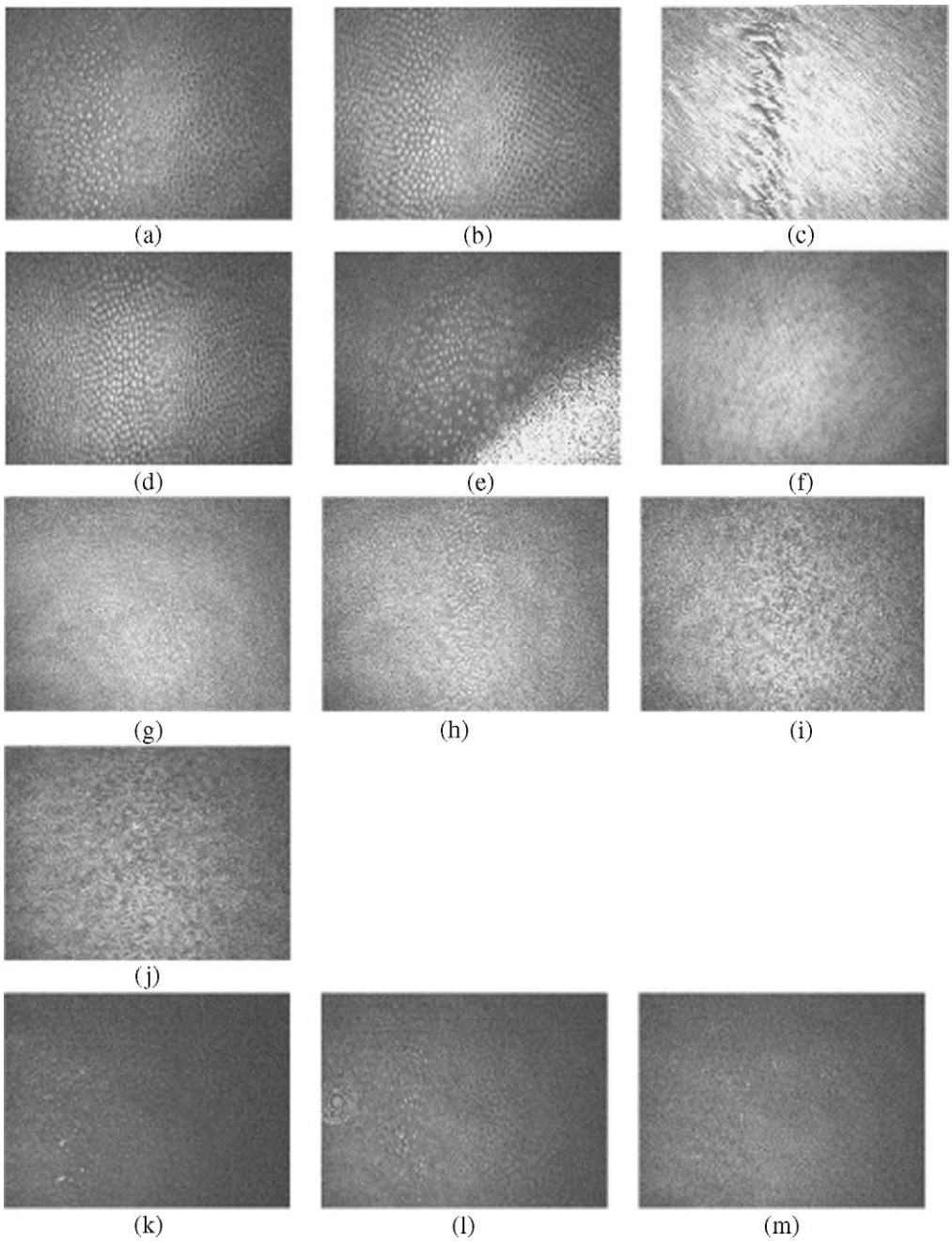

Fig. 6. BAM images of $X_f = 0.1$ mixed monolayer: (a) 18.57, (b) 20.78, (c) 21.65, (d) 23.19, (e) 25.73, and (f) 37.23 mN/m; of $X_f = 0.25$ mixed monolayer: (g) 22.22, (h) 26.08, (i) 32.48, and (j) 36.16 mN/m, and of $X_f = 0.5$ mixed monolayer: (k) 23.33, (l) 32.87, and (m) 33.71 mN/m. All images have the same scale of 570 × 760 μm.

shorter. This makes the molecular interactions between the mixed components less attractive.

From the value of ΔG^M (Fig. 7c), the stability of the mixed DPPC/fengycin monolayer can be understood. The sign of the ΔG^M depends on both the composition of the monolayer and the surface pressure. ΔG^M is positive when $X_f = 0.25$ and $\Pi > 20$ mN/m, implying that the mixing behavior of DPPC and fengycin is thermodynamically unfavorable at higher surface pressure and intermediate fengycin molar fraction. At this composition the monolayer becomes unstable. ΔG^M increases with the surface pressure, indicating that the thermodynamic stability of the mixed monolayer becomes worse when the layer is more compressed. This can be attributed to repulsion between the molecules. This phenomena has been observed by other authors for phosphatidylcholine and arachidic acid mixed monolayer [17].

From the thermodynamic analysis, we can then conclude that the organization of the DPPC monolayer is strongly affected by the lipopeptide when it is present at a molar fraction higher than 0.25.

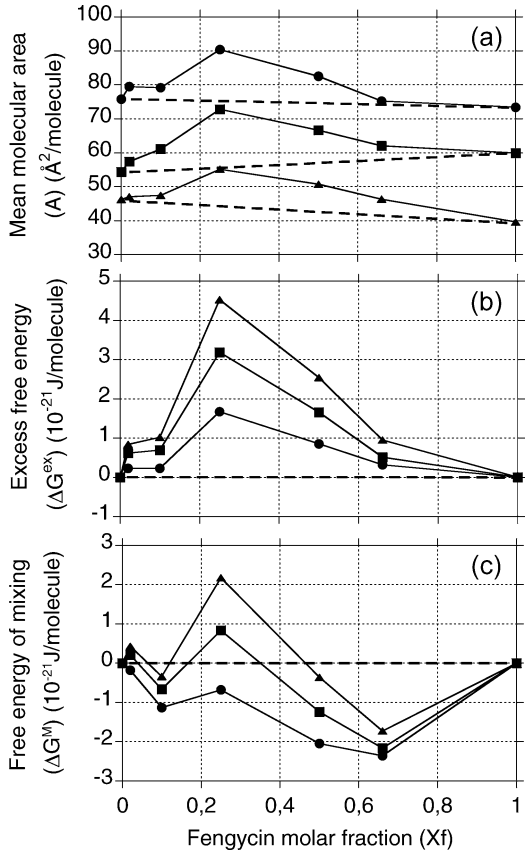

Fig. 7. Mean molecular area (a), excess free energy (ΔG^{ex}) (b), and free energy of mixing (ΔG^{M}) (c) as a function of the fengycin molar ratio at various surface pressures: (●) 10, (■) 20, and (▲) 30 mN/m. The dashed line in (a) corresponds to the additivity rule.

4. General discussion and conclusion

The compression isotherm analysis in combination with the Brewster angle microscopy of DPPC, fengycin, and mixed systems gives evidence of the perturbing effect of fengycin on the organization of DPPC monolayer taken as a model of biological membrane. The effect is fengycin concentration-dependent.

When fengycin is present in a small proportion ($X_f \leqslant 0.1$), it mainly disturbs the compressibility properties of the DPPC monolayer by locally forming large aggregates without having a significant influence on the DPPC morphological characteristics.

When the fengycin concentration increases ($0.1 < X_f \leqslant 0.5$), a stronger effect on the DPPC organization was observed. Fengycin is partially miscible with the DPPC monolayer and consequently reduces the formation of DPPC condensed domains, which are supposed to play an important structural role in cell membranes. In other words, fengycin interferes with the tight packing of the phospholipid molecules that is required for gel formation, having a similar fluidizing effect as has been shown for cholesterol [18].

At a higher fengycin molar fraction ($X_f = 0.66$), the DPPC layer is completely disorganized. Fengycin totally dissolves the ordered phase of the lipid, as has previously been reported for fluorinated surfactants [29].

Results obtained on monolayers cannot be irrefutably transposed to biological systems because of difference in structure complexity and discrepancy in the concentration ratio active molecule-membrane lipid in the two systems. However, it can be suggested that the antifungal activity of fengycin is related to its effects on the structural properties (packing) of the membrane. Two mechanisms of action depending on the fengycin concentration can be proposed. At low concentration, fengycin aggregates to form pores leading to permeability changes of the membrane.

At high concentration, fengycin acts as a detergent by solubilizing the membrane as it has been reported for surfactin [30,31].

These results give some interesting insights about the molecular mechanisms underlying the antimicrobial activity of fengycin. However additional studies are required. Work is at the moment in progress to investigate the penetration properties (binding and insertion) of fengycin in model membranes. Moreover, study of dilatational rheology of mixed monolayers would also be interesting to have valuable additional information about the effect of fengycin on the mechanical properties of lipid monolayer. Such data could also be useful for design of novel antimicrobial agents.

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